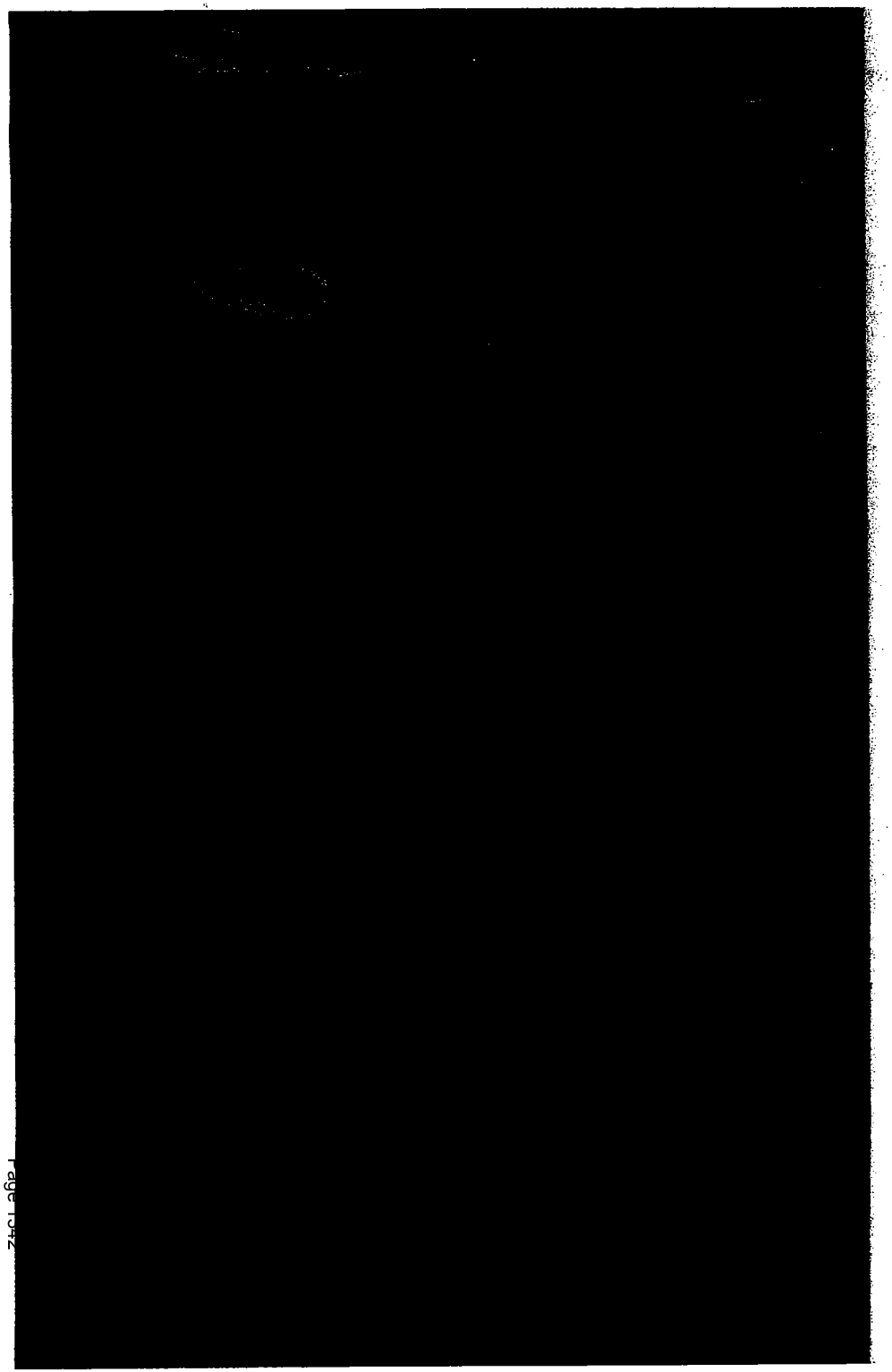
MICRO-ORGANISMS IN FOODS 1

their significance and methods of enumeration

second edition

ICMSF

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The International Commission on Microbiological Specifications for Foods regularly re-evaluates its programs to meet the changing international needs of food microbiology and to keep up with scientific developments. This new edition incorporates the results of recent research into the mode of action of *Bacillus cereus*, *Vibrio parahaemolyticus*, *V. cholerae*, *Clostridium perfringens*, and other organisms in causing food-borne illness. In addition, the Commission has made considerable progress in methods development and evaluation, particularly in *Salmonella* detection. The scope has also been expanded to include sections on zoonotics other than *Salmonella*, and on parasitic protozoa and helminths, hemolytic streptococci, microbial toxins, food spoilage organisms, injury to microbial cells, and food-borne viruses.

The book, doubled in size from the first edition, is in three parts. Part I describes the significance of the presence of various species and groups. Part II provides methods of analysis for microorganisms of importance in food hygiene and food-borne disease. Part III gives formulations of media and reagents used in analysis.

This is a fundamental reference work for those concerned with national food control, national and international public health, and food manufacturing, as well as with teaching and research in food microbiology.



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1

Their significance and methods of enumeration

Second edition

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Preface to the First Edition

More and more countries are appreciating the need to assess the safety and quality of foods, because of wider recognition of the role of foods in spreading disease. This development has led to a rapidly growing diversity of microbiological methods for examining foods. These are applied mainly in official laboratories responsible for the safety or for the standards of quality of foods, but progressive food-processing firms often use the same or similar methods. A cause for concern is the fact that many food-processing establishments still sell products without knowledge of their microbial content.

Surveillance has become more necessary with the increase in international trade in foods and the hazards which could stem from the introduction of new techniques for mass production, rapid and widespread distribution, and introduction into commerce of foods from areas with endemic enteric diseases.[1] For example, the international nature of the salmonellosis problem is well documented.[2]

Manufacturers and control agencies need authoritative guidance on two problems long enveloped in uncertainty: (1) the significance of particular species or groups of microorganisms, when found in

1 See F.S. Thatcher, 'The microbiology of specific frozen foods in relation to public health: Report of an international committee,' *J. Appl. Bacteriol. 26* (1963), 266.

2 See K.W. Newell, 'The investigation and control of salmonellosis,' *Bull. World Health Org. 21* (1959), 279; L.W. Slanetz, C.O. Chichester, A.R. Gaufin, and Z.J. Ordal (eds.), *Microbiological Quality of Foods*, Proc. Conf., Franconia, N.H., 27-29 Aug. 1962 (New York: Academic Press, 1963) E.J. Bowmer, 'The challenge of salmonellosis: Major public health problem,' *Am J. Med. Sci. 274* (1964), 467; U.S. Department of Health, Education, and Welfare, Proc. Natl. Conf. on Salmonellosis, 1964, Atlanta, Georgia (U.S. Public Health Service Publ. 1262, 1965); E. van Oye (ed.), *The World Problem of Salmonellosis* (Monographiae Biologicae, Vol. 13 [The Hague, the Netherlands: Dr W. Junk Publishers, 1964]); B.C. Hobbs, 'Contamination of meat supplies,' *Mon. Bull. Minist. Health Lab. Serv. 24* (1965), 123, 145; E. Vernon, 'Food poisoning in England and Wales,' *Mon. Bull. Minist. Health and Public Health Lab. Serv. 25* (1965), 194.

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foods, and (2) microbiological specifications or standards. Statements related to either problem would have little meaning unless based on the use of effective methods for detection and enumeration of the pertinent microorganisms. Various microbiological specifications for foods, and a plethora of methods, have been proposed. It is clearly desirable to try to establish internationally acceptable microbiological criteria and to reach agreement on the essential supporting methods.

The International Committee on Microbiological Specifications for Foods (ICMSF), a standing committee of the International Association of Microbiological Societies (IAMS), was formed in 1962 for this and related purposes. The 'Agreed Programme' of the Committee is attached as Appendix I. The Committee seeks to aid in providing comparable standards of judgment among countries with sophisticated facilities for food control, and to offer useful procedures to the developing countries; to foster safe international movement of foods; and to dissipate difficulties caused by disparate standards and opinions about the significance of their microbial content. Fulfilment of such objectives should be of value to food industries, to the expansion of international trade in foods, to national control agencies, to the international agencies more concerned with the humanitarian aspects of food distribution, and, eventually, to the health of the consuming public.

The Committee membership includes those associated with government departments of health and agriculture, with industry, and with universities, and having combined interests in research, official regulatory control, industrial control and product development, and education (see Appendix II, Membership). Nineteen different laboratories from 11 countries are represented.

The present document reports part of the outcome of deliberations by the ICMSF at meetings held in 1965 at Cambridge, England, and Vienna, Austria, in 1966 at Moscow, Russia, and in 1967 at London, England. Its purpose is threefold: (1) to describe the occurrence and significance of particular organisms in foods; (2) to offer recommendations on methods for the detection and enumeration of specific groups of food-borne microorganisms and (where feasible) for the detection of toxins causing food-poisoning; and (3) to describe the composition and methods of preparation of the recommended media, and to specify the related diagnostic reagents and tests.

The Committee recognizes two types of methods for different purposes: (a) precise methods of high sensitivity and reproducibility, to provide data for use in legal judgments or in arbitration related to public health or trade regulations; and (b) simpler, cheaper methods

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for routine surveillance, screening, and control. The Committee eventually intends to make recommendations for both types of methods, but this report relates only to the first, precise, type.

The intent has been to avoid proposing another set of 'official' methods. Instead, methods judged effective in different parts of the world have been assembled and their relative merits appraised in the light of the total experience of the Committee members.

The Committee wishes to emphasize that its proposals must be regarded as interim recommendations, and that interlaboratory testing is desirable.

A comprehensive literature review would be an undertaking beyond the scope of this presentation. Only salient references will be cited with emphasis towards those of a review nature.

The terms used to express particular connotations for specific numbers of microorganisms in foods are adapted from definitions by the Food Protection Committee.[3] These definitions follow:

1 A *microbiological specification* is the maximum acceptable number of microorganisms or of specific types of microorganisms, as determined by prescribed methods, in a food being purchased by a firm or agency for its own use.

2 A *recommended microbiological limit* is the suggested maximum acceptable number of microorganisms or of specific types of microorganisms, as determined by prescribed methods, in a food.

3 A *microbiological standard* is that part of a law or administrative regulation designating in a food the maximum acceptable number of microorganisms or of specific types of microorganisms, as determined by prescribed methods.

The methods reported in this document have been adopted, with minor amendments to relate to irradiated foods, by the Expert Panel on Microbiological Specifications and Testing Methods for Irradiated Foods, which met in Vienna, Austria, in June 1965 and November 1967, under the joint sponsorship of the Food and Agriculture Organization and the International Atomic Energy Agency, and which included several members of ICMSF.

January, 1968

F.S.T.
D.S.C.

3 See Food Protection Committee, U.S. National Academy of Sciences–National Research Council, *An Evaluation of Public Health Hazards from Microbiological Contamination of Foods: A Report* (N.A.S.-N.R.C., Washington, D.C., Publ. 1195, 1964).

Preface to the Second Edition

The International Commission on Microbiological Specifications for Foods (ICMSF) regularly re-evaluates its programs to meet the scientific development and the changing international needs of food microbiology. Current efforts are directed towards: (1) revising the Commission's publications, as needed; (2) continuing to evaluate laboratory methods by international comparative and collaborative studies; (3) acting as consultant to developing countries and international agencies such as WHO, FAO, and UNICEF; (4) maintaining liaison with allied national and international groups, such as Codex Alimentarius, the Association of Official Analytical Chemists, and the International Standards Organization; (5) conducting symposia in food microbiology, primarily for the benefit of public health workers in developing countries; and (6) establishing a computerized food microbiology data bank for international use. In addition the Commission is in process of preparing the manuscript for a book on general food microbiology.

Since the first edition of this book appeared, there have been an expansion and several changes in Commission membership. Also, subcommissions for the Balkan-Danubian region and for the Middle East-North African regions have been formed, patterned after a Latin American Subcommission which has been operating for several years. Additional subcommissions may be formed in future to meet regional needs elsewhere in the world.

The rapid progress of science and the work of the Commission have made it necessary to revise the first edition. Several areas of research have recently yielded important results: for example, the mode of action of *Bacillus cereus*, *Vibrio parahaemolyticus*, *V. cholerae*, *Clostridium perfringens*, and other organisms in causing foodborne illness. In addition, the Commission has made considerable progress in methods development and evaluation, particularly for *Salmonella*. Also work is completed or nearly completed on comparative and collaborative studies of methods for *Staphylococcus*, the coliform group, and Enterobacteriaceae.

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The Commission's comprehensive book on sampling for microbiological analysis (ICMSF, 1974) has outdated the small section on sampling that appeared in the first edition of the present volume. This section is, therefore, omitted. The scope of the second edition has, however, been expanded to include sections on zoonotics other than *Salmonella*, parasitic protozoa and helminths, hemolytic streptococci, microbial toxins, food spoilage organisms, injury to microbial cells, and food-borne viruses.

The book is in three parts. Part I describes the significance of the presence and numerical level of various species and groups; Part II proposes methods of analysis for the majority of microorganisms of importance in food-borne disease; and Part III deals with media and reagents used in analysis. The book does not include or recommend methods for a number of bacteria that are seldom reported to cause food-borne disease, for parasitic protozoa and helminths, or for viruses. However, for these reference is made to laboratory methods published elsewhere.

In recent years, basic as well as applied research in food microbiology has yielded results of great importance for the solution of the severe problem of feeding the world's growing population, e.g. by reducing spoilage, increasing safety, and improving economy. Nevertheless, there seem to be areas in which research would be especially fruitful in the future. The Commission wants to emphasize the pressing need for simpler, more sensitive, rapid, and better standardized methods to isolate and identify microorganisms and toxins in all kinds in foods. Improved methods to identify parasites, protozoa, and helminths are particularly needed in some parts of the world. Of concern also is the role played by viruses, fungi, and a number of poorly studied bacteria in human food-poisoning. Finally, improvement of food preservation techniques should receive high priority. Current and future research in these and other fields will, no doubt, make this revised edition obsolete in a few years.

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PART I

Significance of microorganisms and their toxins in foods

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Indicator microorganisms

The presence of microorganisms in foods is not necessarily an indicator of hazard to the consumer or of inferior quality. Except for a few sterilized products, every mouthful of food contains some innocuous yeasts, molds, bacteria, or other microflora. Most foods become potentially hazardous to the consumer only when the principles of sanitation and hygiene are violated. If food has been subjected to conditions that could allow entry and/or growth of infectious or toxigenic agents, it may become the vehicle for transmission of diseases such as salmonellosis or staphylococcal food-poisoning. Detection of these hazards depends upon examination of food samples for the causative agents or for indicators of objectionable contamination.

Routine examination of foods for a multiplicity of pathogenic microorganisms and their toxic products is impractical in most laboratories; however, routine tests for selected pathogens or toxins, such as salmonellae or staphylococcal enterotoxins, are necessary whenever epidemiological or other evidence suggests the occurrence of a specific agent in a particular type of food. For some food-borne diseases, such as infectious hepatitis, reliable methods for detecting the causative agents are not available to the food microbiologist. For other food-borne infections, such as shigellosis, the methods may be unreliable, especially when the pathogens are sparsely or unevenly distributed in foods heavily contaminated with other organisms. Even when sensitive methods are available, some laboratories may not have the technical capabilities required to perform these tests. Such difficulties have led to the widespread use of groups (or species) which are more readily enumerated, and whose presence in foods (within numerical limits) indicates exposure to conditions that might introduce hazardous organisms and/or allow proliferation of infectious or toxigenic species. The groups or species so used are called 'indicator' organisms, and have value in assessing both the microbiological safety and quality of foods.

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Indicator organisms have been used for various purposes. The basis for their use, and an assessment of their significance in various foods, will be considered briefly. The more widely used indicators are discussed first, but the order of presentation does not necessarily reflect their relative value.

The main objective of using bacteria as indicators of insanitary practices is to reveal conditions of treatment which imply a potential hazard, a hazard that is not necessarily present in the particular sample examined but is likely to be present in parallel samples. The methodology for examining foods to detect enteropathogenic and indicator bacteria was reviewed by Lewis and Angelotti (1964) to assist the ICMSF in preparing the first edition of this book. Since then several procedures for the estimation of indicator organisms have been published (e.g., American Public Health Association, 1966; USDA, 1974; Association of Official Analytical Chemists, 1975; NAS-NRC, 1971; and United States Food and Drug Administration, 1972). Although considerable similarity is evident among the methods recommended by these organizations, significant differences in procedural details are also obvious. Progress is being made toward automation of such tests, which, if successful, will reduce the cost, improve reproducibility, and thus encourage the adoption of uniform procedures for microbiological examination of foods.

Detailed consideration of the microscopic analyses of food is outside the scope of this book; however, such analyses are widely used to detect filth, foreign objects, and microorganisms that may indicate exposure of the product to insanitary conditions. In other words, the presence of dirt particles, insect parts, hair, rodent excreta, or large numbers of microorganisms is often regarded as presumptive evidence that the food may also contain infectious or toxic contaminants. Methods for microscopic examination of milk, eggs, cereal products, and other foods have been described by Hausler (1972), AOAC (1975), Harris and Reynolds (1960), and others.

PLATE COUNTS OF BACTERIA

Counts of viable bacteria are commonly based on the number of colonies that develop in nutrient agar plates which have been inoculated with known amounts of diluted food and then incubated under prescribed environmental conditions. Such counts are sometimes erroneously called total plate counts, when in fact only those bacteria which will grow under the particular environmental conditions selected can be counted. A wide variety of conditions can be obtained

by changing the composition of the agar medium, the gaseous environment, and the time and temperature of incubation. For example, incubation at temperatures between 0° and 7°C favors the growth of psychrotrophic bacteria. Many of these organisms cannot grow at 30–37°C, which are the temperatures most suitable for incubation of mesophilic organisms including both the pathogenic and saprophytic species. Incubation at still higher temperatures (50–60°C) allows the development of thermophilic organisms but inhibits the mesophilic and psychrotrophic species. Other groups can be selected for counting by adding selective inhibitors, such as sodium chloride, surface-active agents, or dyes, to the agar medium or by modifying the composition of the atmosphere in the incubator, such as by excluding oxygen. Each type of viable count is potentially useful for special purposes, but the aerobic mesophilic count is most commonly employed to indicate the sanitary quality of foods.

Aerobic, mesophilic, plate counts

Most processed foods (except, for example, fermented products) should be regarded as unwholesome when they have a large population of microorganisms, even if the organisms are not known to be pathogens and they have not altered the character of the food noticeably. For this there are several reasons.

1. High counts in shelf-stable foods often indicate contaminated raw materials or unsatisfactory processing from a sanitary point of view, whereas in perishable products they may also indicate unsuitable time/temperature conditions during storage. The presence of numerous mesophilic bacteria, which grow readily at or near body temperature, means that conditions may have existed which would favor the multiplication of pathogens of human or animal origin.

2. Some strains of common mesophilic bacteria, not usually regarded as causing food-borne disease (e.g., *Proteus* sp., enterococci, and mesophilic pseudomonads), have been reported to cause illness when excessive numbers of living cells were present in food. However, the evidence regarding their enteropathogenicity is conflicting (see other sections of this book for details). Nevertheless, it seems wise to prevent the development of high plate counts in unfermented processed foods.

3. All of the recognized food-borne pathogenic bacteria are mesophilic and in some instances they contribute to the detected plate count.

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4. When food spoilage is due to the growth of microorganisms, high counts are to be expected. The population levels required to cause organoleptically evident changes vary greatly depending on the kind of food and particularly the type of microorganism. By the time decomposition can be detected by odor, taste, or appearance, most foods contain more than 10^6 microorganisms per gram (reviewed by Elliott and Michener, 1961). Some foods may become unacceptable when they contain 10^7 bacteria per gram, but a few others may still be eaten when the bacterial population reaches 10^8 per gram. Fermented products, such as cheese, normally attain microbial populations of 10^9 per gram, but this level of contamination in other foods is usually associated with gross spoilage.

Spoilage of refrigerated foods is often caused by bacteria that cannot grow at 30°C or above (Elliott and Michener, 1965). Thus aerobic plates made from foods spoiled in the refrigerator may yield colony counts one or more log cycles higher when incubated at 5–28°C than when incubated at 35–37°C.

With respect to 1 and 2, the aerobic mesophilic bacteria as a group (i.e., those that grow in agar plates at 30–37°C) generally can be regarded as indicator organisms, though they are a much less precise and reliable measure of the hazard of food-poisoning than are the more closely related indicators which will be considered below. High plate counts of mesophilic bacteria, for example, when applied to raw products, often consist of the normal microflora, or perhaps indicate incipient spoilage, rather than any potential health hazard.

Anaerobic counts

The practice of using counts of aerobic instead of anaerobic bacteria probably developed because it was much easier to incubate under aerobic conditions. Recently developed systems have facilitated and improved anaerobic counting by the use of anaerobic work-chambers made of clear plastic, pre-reduced agar plates, and deep agar in impermeable plastic bags (see, for example, Bladel and Greenberg, 1965; Aranki et al., 1969; Ellner et al., 1973). Anaerobic procedures usually measure not only obligate anaerobes, but also facultatively anaerobic organisms among the Enterobacteriaceae, fecal streptococci, and staphylococci unless selective media are used. Anaerobic counts have some advantages, although they will probably never replace aerobic counts for routine examination of foods. The anaerobic mesophilic count serves as an indicator of conditions favorable to multiplication of anaerobic food-poisoning organisms such as *Clostridium perfringens*, especially in meat products, and it is necessary when examining foods for this species in food-poisoning investigations.

Advantages and limitations of mesophilic counts

In international trade, the food importer often does not have information on the conditions of sanitation or the time and temperature of food production and transportation. He should be encouraged to obtain the proper information, but in its absence an aerobic mesophilic count can be a helpful guide. If it is high, or if it varies widely among samples from different lots or within a lot, microbiological control in processing or transport was probably inadequate. The manufacturer, on his part, can use such counts to test sanitation along his processing line. For this purpose, samples are taken of the ingredients as they are added, of the product before and after those processing steps which may add or destroy microbes, and of the product before, during, and after periods of delay which could permit growth to occur on or in the product. The results may show that one or two particular steps, out of several, are mostly responsible for the contamination of the finished product. This information allows a concentration of efforts to improve cleanliness and disinfection on the steps mainly responsible, thus avoiding a waste of time, effort, and money on other less important steps.

There are important limitations to the value of any such count:

1. In particular types of foods (e.g., fermented sausages, sauerkraut, cheese, and certain other dairy products) a large multiplication of bacteria with a concurrent 'fermentation' or 'ripening' of the food is desired. In these products, a high count, as such, has practically no significance because objectionable organisms are usually not differentiated from the normal microflora (L.E. Barber and Deibel, 1972).

2. The viable population of heat-processed foods may be very low, but a microscopic examination can sometimes reveal dead organisms whose numbers show the raw food to have had unacceptably high levels.

3. Similarly, there is invariably a decrease in the number of viable bacteria when foods are dried or frozen. Thus a plate count may not indicate the microbiological condition before processing, and direct microscopic examination is required to detect whether high counts occurred initially.

4. A mesophilic count is of little value in predicting the life of a food in chill storage, because many mesophilic microorganisms fail to

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grow below 5°C (Michener and Elliott, 1964). For this purpose, a so-called psychrotrophic viable count is preferred, usually at an incubation temperature in the range 0–5°C (Ingram, 1966) or at 7°C for 10 days (Vanderzant and Johns, 1972). Some mesophilic bacteria do grow in the range of 5–15°C, but they can be detected more quickly by incubating plates at a higher temperature.

ENTERIC INDICATOR BACTERIA

Escherichia coli, the coliforms (coli-aerogenes group), and the 'Enterobacteriaceae'

The native habitat for *Escherichia coli* is the enteric tract of man and animals; thus its presence in foods generally indicates direct or indirect pollution of fecal origin. Hence, *E. coli* is the classical indicator of the possible presence of enteric pathogens in water, shellfish, dairy products, and other foods. Enumeration of *E. coli* in water provides a measure of the extent of pollution, whereas the numbers detected in foods may be influenced by other factors, such as multiplication, dying-off, or adhesion of the organisms to food particles. Nevertheless, substantial numbers of *E. coli* in food suggest a general lack of cleanliness in handling and improper storage. The presence of *E. coli* in a food does not connote directly the presence of a pathogen, but only implies a certain risk that it may be present. In other words, the presence of *E. coli* in foods is not always closely correlated with the occurrence of salmonellae or other pathogenic microorganisms.

Much has been published about the relative merits of *E. coli*, the coliforms, or the whole family Enterobacteriaceae as fecal indicators (Buttiaux and Mossel, 1961; APHA et al., 1976, 1966; Hall, 1964; Hausler, 1972; G.S. Wilson and Miles, 1975; Geldreich, 1966; Mossel, 1967, 1975). A common practice is to use tests for coliforms, including *E. coli*, for 'screening' or preliminary tests. If there is reason to determine the likelihood of fecal contamination, the coliforms or other Enterobacteriaceae are subjected to further tests to establish whether any of them are *E. coli*.

The customary term *coliform* includes *E. coli* and several species from other genera of the Enterobacteriaceae. Practically speaking, 'coliforms' are the microorganisms that are detected by 'coliform tests,' such as those described in Part II of this book. Table 1 summarizes the genera detected or not detected with coliform tests, their fecal or non-fecal habitats, and their potential enteropathogenicity for man.

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TABLE 1
Differences among genera of the Enterobacteriaceae[a] in regard to fecal origin, detection, and enteropathogenicity for man

	Genus	Predominantly of fecal origin	Usually detected by 'coliform tests'	Typically enteropathogenic for man
I	*Escherichia*	Yes	Yes	No[f]
II	*Edwardsiella*	Yes	No	No[f]
III	*Citrobacter*	No[b]	Yes[c]	No
IV	*Salmonella*	Yes	No	Yes
V	*Shigella*	Yes	No	Yes
VI	*Klebsiella*	No[b]	Yes	No[f]
VII	*Enterobacter*	No[b]	Yes	No
VIII	*Hafnia*	No[b]	No[d]	No
IX	*Serratia*	No	No	No
X	*Proteus*	No[b]	No	No[f]
XI	*Yersinia*	Yes	No	No[f]
XII	*Erwinia*	No	No[e]	No

a Based on *Bergey's manual of determinative bacteriology*, 8th ed. (Buchanan and Gibbons, 1974; see also Mossel 1975).
b Some strains inhabit the intestinal tract, but they also proliferate in a variety of other natural environments.
c Except slow lactose-fermenting strains.
d Except occasional strains.
e Except strains that are adapted to rapid growth near 37°C.
f Some serotypes contain enteropathogenic strains.

The term *fecal coliforms* has arisen from attempts to find rapid, dependable methods for establishing the presence of *E. coli* and closely related variants without the need to purify cultures obtained in tests for coliforms or to apply the relatively costly confirmatory tests (APHA et al., 1976). The 'fecal coliforms' comprise a group of organisms selected by incubating inocula derived from a coliform enrichment broth at temperatures higher than normal (44–45.5°C, depending on the method; see Part II, p. 132). Such enrichment cultures usually contain a high proportion of *E. coli* types I and II and are thus useful for indicating a probable fecal source of contamination (Fishbein, 1961; Fishbein and Surkiewicz, 1964; Geldreich, 1966; Guinée and Mossel, 1963).

Several laboratories throughout the world now examine foods, which have been processed for safety, by a test that estimates the entire Enterobacteriaceae family (i.e., lactose + and lactose - types). This test is used for the following reasons:

1. The coli-aerogenes or 'coliform' bacteria are, taxonomically, a rather ill-defined group. Hence the coliform count may include a

variety of different bacteria, depending on the specimen, the medium, the incubation temperature, and criteria for reading results. This variability can result in discrepancies among data obtained in different laboratories.

2. A test only for lactose-positive organisms can lead to falsely reassuring results in situations where lactose-negative organisms predominate. This applies not only to the predominantly lactose-negative *Salmonella*, but also to the other pathogenic Enterobacteriaceae. A good illustration is the recent outbreak of gastroenteritis caused by French soft-ripened cheese, contaminated by the enteropathogenic strain of *E. coli*, type O 124 (Marier et al., 1973). This mutant ferments lactose slowly. Coli-aerogenes counts on the incriminated cheese did not exceed 10^3 per gram, which might be in the intermediate acceptance region; however, the Enterobacteriaceae count was about 10^7 per gram, a value which certainly would lead to rejection.

3. *Salmonella* may be more resistant to detrimental influences in foods than *E. coli* or other coliforms. Again, absence of the latter organisms may lead to falsely reassuring results.

In raw foods and on food-processing equipment, several types of Enterobacteriaceae persist longer than *E. coli*. Species of *Erwinia* and *Serratia*, which are included in counts of Enterobacteriaceae and to some extent in coliform enumerations, are associated with plants and do not indicate fecal contamination. Hence *E. coli* is the only valid index organism for the monitoring of fresh vegetable foods. In fresh foods of animal origin, most Enterobacteriaceae stem from fecal contamination, and their occurrence in high numbers may indicate insanitary handling and/or inadequate storage (Hechelmann et al., 1973; Hunyady et al., 1973; Cox et al., 1975). In many instances, the numbers of Enterobacteriaceae may bear no relation to the extent of the original contamination from fecal sources, because Enterobacteriaceae can multiply in some foods whereas they tend to decrease in other commodities and in water.

In foods processed for safety, the presence of considerable numbers of Enterobacteriaceae or coliforms indicates: (1) inadequate processing, and/or post-process recontamination - most frequently from raw materials, dirty equipment, or insanitary handling; (2) microbial proliferation, which could have allowed multiplication of a wide range of pathogenic and toxigenic organisms. However valuable such information may be, it should never be interpreted as indicating with certainty that fecal contamination of such foods has occurred (Mossel and Vincentie, 1969).

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An always pressing question is whether a negative outcome of any of the tests mentioned ensures absence of enteric pathogens. This obviously depends on parameters like: (1) the number and size of aliquots examined; (2) the sensitivity of the method; (3) the numbers of Enterobacteriaceae, coliforms, or *E. coli* and pathogens. Several computations on the reliability of testing for Enterobacteriaceae to assess the risk of *Salmonella* contamination have been published (Drion and Mossel, 1972; Mossel, 1975; Gabis and Silliker, 1976).

The enterococci

Many studies have been published on the suitability of the enterococci, and the Lancefield group D streptococci generally, as fecal indicators. Group D includes, besides the enterococci (*Strep. faecalis* and *Strep. faecium*), less heat-resistant streptococci such as *Strep. bovis* and *Strep. equinus* (Sherman, 1937; Deibel, 1964). The whole group is loosely called 'fecal streptococci.'

Although normally present in mammalian feces, these cocci also occur so widely elsewhere in the environment that their significance as indicators of fecal contamination is severely restricted (Mundt, 1963; Martin and Mundt, 1972). Their use as indicators should be limited to situations where they are known to be manifestations of fecal pollution, e.g., in swimming-pool water. In processed foods that are heated, cured, frozen, dried, or otherwise treated so that the original microflora dies gradually, the correlation between the occurrence of enterococci and of *E. coli*, coliforms, or Enterobacteriaceae is poor (APHA, 1958; Niven, 1963; Hartman et al., 1965; W.S. Clark and Reinbold, 1966). In unprocessed raw foods, in contrast, the correlation between the levels of enterococci and coliforms may be better. The association between Group D streptococci and coliforms in certain raw foods has been noted by Buttiaux (1959) and by Buttiaux and Mossel (1961).

Despite the limitations and uncertainties noted above, the occurrence of large numbers of enterococci in foods, except those fermented by specific strains of these organisms, implies either inadequate sanitary practices or exposure of the food to conditions that would permit extensive multiplication of undesirable bacteria.

The enterococci may have a distinctive role as indicators of poor factory sanitation, owing to their relatively high resistance to drying, high and low temperatures, detergents, or disinfectants. Because of their resistance to freezing, the enterococci are preferred indicators of poor sanitation in frozen-food plants (Niven, 1963); and because

Important considerations for the analyst

LABORATORY QUALITY ASSURANCE

Laboratory quality assurance (LQA) is a program to identify significant sources of variation and to minimize their effects on the reliability of analytical laboratory results. Its goals are: (1) to standardize laboratory methods and practices, (2) to achieve comparability of performance, not only among individual bench-workers within a laboratory, but among different laboratories that perform the same tests and examine the same kinds of food, and (3) to prevent incorrect or unreliable laboratory reports from being used to decide whether a product complies with applicable industry specifications or legal requirements.

Achievement of these goals in food microbiology laboratories requires a much broader program than the traditional approach of using so-called standard or recommended methods and performing some control tests. It involves consideration of all sources of variation (see Table 5) associated with samples, analyses, and reports. Success depends heavily on the conscientious efforts of every sample collector and analyst, as well as on the continuing support of a planned program by the laboratory administrators.

Among the factors that affect the precision of an analytical method are the variations that occur in results from different analysts working in the same laboratory and from analysts in different laboratories. Internal and interlaboratory quality assurance programs can limit these variations.

Internal laboratory quality assurance

The individual analyst is basically responsible for the quality of work done on every specimen submitted to him for examination. He must be fully informed about the appropriate methods, must use these methods exactly as prescribed, including necessary replication and controls, and must report all pertinent facts about his work that may

affect interpretation of the results. To be effective, he needs a favorable work environment, which includes adequate laboratory facilities, necessary equipment, properly prepared media and reagents, and reliable supporting services. An important feature of laboratory quality assurance is the routine monitoring of the work environment at critical points by performance tests, audio-visual checks, and temperature records.

The supervisor and the laboratory director have the responsibility not only of providing a productive environment but of planning and assigning the analytical work, requiring analyses of check samples and standard specimens as needed, and reviewing critically the analyst's data before reporting them officially. Clearly internal LQA is a responsibility of the professional laboratory staff and not of an administrative assistant or of a consultant who provides standards or split samples. However, utilizing such assistance and participating in interlaboratory evaluations are important parts of this responsibility.

Interlaboratory quality assurance

Interlaboratory quality assurance programs depend, first, on the interest and integrity of the participating laboratories and, second, on the leadership and support of a competent organization that can administer the programs impartially. The sponsoring organization must perform several functions, including those listed below:

1. Plan and schedule laboratory evaluations with input from the participants.
2. Determine precisely the methods and foods to be used in making comparative analyses.
3. Prepare and distribute microbiologically stable split samples and standards of known quality.
4. Provide a form for reporting laboratory results.
5. Receive and evaluate the raw data from individual analysts in all laboratories that examine a particular series of split samples.
6. Submit a summary report of findings and recommendations to all participants, in which the results are coded so that each laboratory can identify only its own data.
7. Request feedback report from participants.
8. Provide consultation and training, especially for participants who obtain unsatisfactory results from split samples.
9. Make periodic visits to all laboratories to evaluate the facilities, equipment, performance of standard methods, records, etc.

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TABLE 5
Causes and control of variation in analytical laboratory results

Sources of variation	Control measures	
	Within a laboratory	Among laboratory units
Product heterogeneity	Examine representative samples of statistically adequate number and size for the level of control required	Adhere to uniform plans for sample collection and preparation
Methods	Employ standard method for type of specimen being tested Validate procedure by replicate analysis of positive and negative controls	Participate regularly in interlaboratory collaborative studies involving analysis of reference standards, split samples, and field samples distributed from a common source Issue manuals of standard methods
Analysts	Assign work to properly trained individuals Adhere to procedural details of standard methods	Consult with specialists Exchange information and personnel Compare performance by different analysts when examining comparable samples Sponsor group training
Analytical instruments, special devices, and support facilities	Conduct a rigorous program of preventive maintenance and repair Calibrate instruments frequently against authentic standards Establish the limits of sensitivity, specificity, and reproducibility for each system and work within these limits	Use identical instruments Demonstrate comparability of performance by analysis of standards and split or check samples Follow standard operating procedures for maintenance and use of instruments
Records and reports	Make a permanent record of raw data and explanatory notes at time of observation Check closely for errors arising from computation or transcription of data to final report	Adopt uniform methods for recording, computing, and reporting results Submit reports, including data on controls and standards, to a central unit for comparative evaluation
Management	Provide supervision and incentive Maintain the quality of essential services such as dish-washing, animal care, stock-room, clerical support, and safety devices Supply an adequate laboratory environment with respect to necessary utilities and working conditions (e.g., illumination, ventilation, and general cleanliness)	Establish a continuing review of each laboratory's performance by a 'nonpartisan' organization Specify the minimum quality assurance needed for each kind of laboratory analysis Support laboratory quality assurance as an integral part of every compliance program that involves analysis of samples Foster interlaboratory conferences and related educational activities
Supplies and equipment	Select items having qualities appropriate for intended use Hold under environmental conditions that minimize deterioration	Purchase critical items centrally according to specifications Distribute items from the same lot among all participating laboratories Avoid prolonged storage, especially of 'perishable' items such as enzymes, antisera
Chemical reagents, culture media, and other materials prepared in the laboratory	Follow prescribed directions meticulously Compare performance of new batches with others of known quality Include positive and negative controls each time the material is used When material performs atypically, discard the batch and reject the results obtained	Obtain ingredients having appropriate specifications from a common source Employ a common set of standard operating procedures for preparation and use Exchange information about stability of reagents and difficulties encountered in their use

10. Certify analysts and approve laboratories that demonstrate satisfactory competence to perform specified microbiological tests.

Although interlaboratory quality assurance is costly in terms of time and money, the loss of a single large lot of food, because of analytical errors, may be even more expensive and wasteful. Only a few of the above-mentioned items will be discussed in detail.

An interlaboratory program reveals both analyst and laboratory differences when the samples and methods used by all participants are identical. The sponsoring organization may furnish naturally contaminated or artificially inoculated (spiked) samples. In their day-to-day work, analysts routinely encounter naturally contaminated samples, and therefore it would seem logical to use such samples for interlaboratory studies. However, the characteristics of the organisms in natural contamination are largely unknown – i.e., their relative persistence or viability, or the presence among them of stressed or injured cells; furthermore, a comprehensive interlaboratory study requires a large volume of food material contaminated at various levels, a requirement that is hard to fulfill with naturally contaminated food. Naturally contaminated samples may be adequate to check a specific laboratory, or a small number of local laboratories, when the time interval between first examination and check analysis is short.

The large numbers of samples needed for regional or national multiple-laboratory programs require artificially contaminated material. The sample mass should be virtually free of bacteria prior to inoculation with known cultures. Samples may be liquid, dry, or semiliquid. Frozen samples should not be used because effects on survival are not sufficiently predictable. In any event, sufficient pretesting under various conditions of storage will ascertain the persistence of various population levels of the added cultures. Microbial reactions on media of various brands, or even on media of various batches of the same brand, have frequently shown wide variation (Read and Reyes, 1968). To eliminate this variable from consideration, the sponsoring organization should furnish media from a single batch for the interlaboratory study. Once the study has shown the value and limitations of the method, each laboratory using the method must establish routine media control procedures to ensure its continuing acceptability.

Implementation of laboratory quality assurance activities

Food microbiologists, like other professional people, take pride in the quality of their work, and they continually seek assurance that

their analytical results are reliable and comparable to data obtained by their co-workers. In other words, they tend to undertake laboratory quality assurance activities to satisfy their own interests, which may not always coincide with those of the organization for which they work. Such activities are generally useful, but their uncoordinated proliferation can seriously disrupt the productive work of the analysts involved without necessarily meeting the organization's primary needs. Centralized planning and management are therefore desirable, especially when multiple laboratory units are involved.

The laboratory manager, although not directly involved in sample collection, has a professional obligation to determine whether the samples received are suitable for the analyses requested. Misleading data, resulting from defective samples, can jeopardize the credibility of the most competent laboratory; therefore samples should not be accepted for examination if they are known to be grossly defective in quality or quantity.

The continuing development of microbiological criteria by local, national, and international organizations has stimulated not only the need for properly representative samples but also the need for interlaboratory studies of methods. Both regulatory agencies and the food industry should carry on such studies. Every manager or regulatory official who makes decisions about the acceptability of food on the basis of microbiological criteria should regard such programs as necessary for avoiding costly errors.

The important health and economic benefits to be derived from improving the quality of work in laboratories concerned with environmental analyses, including food microbiology, have not received recognition comparable to that afforded clinical laboratories (Ellis, 1974). For example, the Codex Alimentarius Commission (Joint FAO/WHO, 1973) has developed many food standards for regional or worldwide adoption, but has seldom included microbiology in its proposals. In addition to proposing sampling plans (ICMSF, 1974) and laboratory methods for international use, the ICMSF has sponsored collaborative and comparative studies of these methods (Silliker and Gabis, 1973, 1974; Gabis and Silliker, 1974a, b; Idziak et al., 1974; Erdman, 1974). It is also developing plans for international exchange of analytical data that will facilitate interlaboratory quality assurance activities.

Table 5 lists the sources of variation that may affect the results of laboratory analyses, together with some control measures that can help keep the variation within tolerable limits. This table has been excerpted from an unpublished report prepared for the United States Food and Drug Administration.

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Only research and experience will tell us what controls are critical for a given laboratory examining a given food by a given method to solve a given problem.

THE MICROFLORA OF FOODS

Microorganisms in wide variety generally contaminate foods on the farm and during transport to the processing plant. Whether these organisms grow, survive, or die depends on the nature of the food, the environment, and the processing procedure. Since these factors are unique for each type of food, the microorganisms present on each food are likewise unique to that food. In varying degree, microbiologists have developed enough data on these factors to ascertain what is normal and what is abnormal for various food classes. This knowledge represents the science of food microbiology.

A food is either perishable or shelf-stable depending on whether microorganisms normally grow on it during storage. It follows, then, that microbial spoilage is the normal fate of a perishable food. In contrast, spoilage of a reputedly shelf-stable food is abnormal. A consumer judges a food to be spoiled when bacteria, yeasts, or molds have changed the odor, flavor, appearance, or texture to an unacceptable degree: soured fresh milk is one example. However, he would accept the same change in another product, such as in buttermilk. A particular organism is thus not necessarily a spoilage agent per se, for it sometimes may be so classified only in particular foods.

A major aim of processing is to make a perishable food shelf-stable. Examples are heat sterilization in hermetically sealed containers, treatment with chemical preservatives, fermentation to lower the pH or to produce alcohol, and reduction of the water activity (a_w) by removing water or by adding a solute. Frequently, stability depends on more than one of these factors. Thus shelf-stable foods are not always sterile; those that are not sterile harbor a selected flora. A canned food is commercially sterile, which means that microorganisms surviving the process are incapable of growing in the food under normal circumstances. Obviously organisms surviving an inadequate heat process, or entering the can through a hole punched in its side, may cause spoilage. Less obviously, highly heat-resistant spores, incapable of growing in the canned food at normal storage temperatures, may grow and spoil it at abnormally high storage temperatures.

A dried food is usually not sterile; nevertheless, because of its low a_w, it remains microbiologically stable. If it becomes moist, it will spoil.

The shelf-life of a perishable food is the storage time during which it remains acceptable to the consumer. The nature and size of the initial microbial population, the type of food, and the storage conditions all affect the shelf-life. A microbial level of 10^6 to 10^8 per gram generally signals the end of shelf-life. An adequately long shelf-life is a primary goal of good manufacturing practice (GMP) and the science of food technology.

Traditionally, food microbiologists have concluded that food-spoilage organisms are harmless to the consumer, and that food-poisoning agents, even in large numbers, do not produce recognizable spoilage. Whereas these principles generally hold true, there are exceptions. For example, spoilage molds may produce mycotoxins, *Staphylococcus aureus* can sour milk, and *Clostridium botulinum* frequently produces putrid odors. Perhaps research and experience will cast further doubt on the complete harmlessness of normally spoiled foods. For these reasons, microbiologists continue to object to high populations in perishable foods, particularly in precooked foods, whose microbial content should be low.

In rapidly perishable foods – i.e., those with high moisture content and neutral pH – bacteria grow more rapidly than yeasts or molds and therefore predominate in spoilage. In foods less favorable to microbial growth, bacteria fail to grow or grow very slowly and the yeasts or molds predominate. Thus at temperatures between -5°C and -10°C, where fluid water is limited, molds predominate. Likewise, in foods whose pH is below 5, or whose a_w is below 0.90, yeasts or molds may grow. Thus microbial growth in such foods could produce a mycotoxin but not a bacterial toxin.

The fate of pathogenic and toxigenic organisms in the contaminating flora of a perishable food depends upon both the physical and biochemical environment and the nature of the competing flora – the biological environment. Staphylococci, in small numbers, are relatively frequent contaminants of foods, but they compete poorly with the spoilage flora of most foods of high a_w. However, in foods which contain concentrations of salts or sugar that inhibit development of the competing microflora, the numbers of staphylococci may rise to dangerous levels.

The influence of a process upon the relation and ratio between harmful contaminants and the normal flora of a food is insufficiently recognized. New packaging technology may succeed in extending shelf-life by reducing the growth rate of the existing flora or by selecting a different, slower-growing flora. In either situation, the competition provided by the spoilage flora which usually inhibits

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pathogenic contaminants may be reduced to an ineffective level. With shelf-stable canned foods, the extrapolation of an established technique to a new set of circumstances can have unacceptable consequences. Typical examples are the application to large containers of thermal processes calculated for much smaller ones, and the use of thermal processes calculated from convection heating for products which heat by conduction. In all instances where a process is changed, there is an obligation to ensure that any advantages in economy, quality, or shelf-life are not achieved at the expense of the safety of the product.

There is a special concern, from both spoilage and public health standpoints, for foods described as 'semipreserved.' These foods depend for stability upon the interaction of a number of processing and environmental factors, none of which used alone can confer stability and several of which (temperature or redox potential, for example) may be under poor control. Much more knowledge is required about the microbiology of such foods.

Certain intestinal pathogens, such as *Salmonella*, *Shigella*, and the cholera vibrios, should not be tolerated in ready-to-eat foods, even in small numbers. Other pathogens cause illness only if they reproduce to very high levels in the food. This is true of *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens*, *Vibrio parahaemolyticus*, and *Bacillus cereus*. The organisms in the latter group are harmless in the small numbers unavoidably present in many raw foods.

Most food-spoilage bacteria grow readily at temperatures below the minimal temperatures for growth of the food-poisoning organisms. A low-temperature aerobic plate count (APC), which measures such growth, therefore measures the shelf-life of a food. An APC at or near 35°C eliminates many of the strains of spoilage organisms and permits the aerobic flora of the animal or human body to grow. Some of these latter organisms are pathogens. Thus a high APC at 35°C casts doubt on the safety of the food. There is therefore virtue in restricting the microbial population of foods to control both quality and safety. Microbiological criteria and codes of GMP are two of the tools to accomplish these goals.

The normal spoilage flora frequently inhibits the growth of pathogens. For this reason, one should not reduce the spoilage flora to the point where pathogens can grow readily, for some foods might become a health hazard before they spoil. Consumers usually discard manifestly spoiled foods.

Standardization of methods to make them applicable to all foods is a desirable aim; however, it is frequently necessary to modify

a method for use with certain foods, often because of the nature of the organisms in the background flora. For example, some media are, by design, more inhibitory to the background flora than to the organism sought. This makes it possible to isolate small numbers of pathogens or fecal indicators in the presence of masses of spoilage organisms.

Usually it takes fewer pathogens to make a food hazardous than it takes food-spoilage bacteria to make it stink. Furthermore, the pathogens usually fall within a genus or even within a species, whereas food-spoilage bacteria may comprise many genera or even families, whose only common characteristic is their preference for a common ecological niche. On the one hand, spoilage bacteria cause food losses far in excess of losses from condemnation for the presence of pathogens. On the other hand, they contribute importantly to the human diet in fermentation and similar processes. The Commission will consider food spoilage in a future publication.

INJURY AND RESUSCITATION

At the beginning of this century bacteria were considered 'dead' when they were unable to multiply and viable when they would proliferate. However, the early work of Sherman (1916) and Wright (1917) showed that the numbers of organisms that were apparently damaged by heat or disinfection were greatly influenced by the plating medium. In other words, bacteria which were considered 'dead' on one medium could be shown to be 'alive' on another one. Somewhat earlier Eijkman (1908) and Coplans (1909) demonstrated that exposure to heat or disinfecting agents may prolong the lag phase of surviving organisms. Coplans attributed this effect to some kind of injury. Using *Escherichia coli* in peptone water he related the degree of injury to the length of lag time. The lag increased with severity of injury until the cells were injured to such a degree that 'the power to multiply' was entirely lost. Later Penfold (1914), Chesney (1916), and others also considered recovery from injury to be one of the causes of increased lag times. The well-known phenomenon that cells in the early lag phase, the stationary phase, and the phase of decline show a longer lag time after incubation in a fresh medium than cells in the log phase might be explained by the assumption that such cells are injured to some degree: results of Hurst et al. (1974) support this assumption.

It is therefore possible that in almost every population of microorganisms some cells may be in a damaged state. In addition to the

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'non-intentional' physiological occurrences as described above, injury may be caused by environmental influences like heating, freezing, drying, irradiation, acidification, preservation or disinfection by chemical agents, and chilling. It is not known whether these different detrimental conditions cause different cellular lesions. It is likely that the type of microorganisms and the substrate will have an effect. However, there is evidence that loss of cellular membrane integrity and/or degradation of ribosomal ribonucleic acid (RRNA) may always be involved. In addition to the extension of the lag phase, damaged cells also show in many cases more exacting nutritional requirements. If prolonged lag and increased dependence on certain nutrients were the only characteristics of injury, the consequences for practical analytical food microbiology would not be too serious. However, around the middle of the century Gunderson and Rose (1948), Hartsell (1951), and Labots (1959) demonstrated that several bacterial species in frozen or heated foods were unable to grow in selective media. Injured organisms have sensitivity to antimicrobial compounds such as sodium chloride, bile salts, and other surface-active agents and dyes, and this increased sensitivity was later found to be a major characteristic of injury. This phenomenon has compelled food microbiologists to include in detection and enumeration procedures a so-called recovery of resuscitation treatment (Allen et al., 1952).

The resuscitation treatments used depend on the type of injury incurred and the proportion of stressed cells in a population. These, in turn, are related to (1) the type of organism (e.g., spores, Gram-negative rods, Gram-positive catalase-negative cocci, or Gram-positive catalase-positive cocci); (2) the type of stress (heating, freezing, drying, exposure to reduced pH and a_w, etc.); (3) some properties of the food in which the stressing took place; (4) media used to determine the organisms looked for; (5) temperature of incubation of media.

For fermentative Gram-negative rod-shaped bacteria, increased sensitivity to surface-active agents like bile salts, dyes, and similar components of selective media has been demonstrated. Recovery from injury and regained tolerance can, in many instances, be obtained in a time span of 1/2 to 2 hours at 25°C. In some instances, no beneficial effect or repair treatment can be observed, because during the preparation of the sample and particularly its dilutions, conditions and time of exposure were such that virtually complete recovery was achieved fortuitously (Mossel and Ratto, 1970). Another reason for not observing any benefit from resuscitation treatments might be that an increased resuscitation time is needed because of the greater severity of injury (van Schothorst and van Leusden, 1972), the extreme toxicity of one or more of the selective components present in the medium (van Schothorst and van Leusden, 1975), the antagonizing of molecular events by intrinsic food components, leading to complete repair (Mossel et al., 1965), or other factors not yet fully identified (Stiles et al., 1973). Enumeration procedures prolonging the period of repair require precautions against the proliferation of cells that either have not been stressed or have already recovered since this would lead to misleadingly high results.

Injury to *Staphylococcus aureus* often results in a loss of salt tolerance. Resistance to concentrations of about 7.5% sodium chloride can be restored by resuscitation treatments similar to those currently used for Gram-negative rods. *Staphylococcus* media that do not depend on salt for selectivity will recover injured cells. With such media, a resuscitation step is not necessary. Baird-Parker's tellurite glycine egg-yolk agar, for instance, allows consistently good recoveries of stressed cells by direct plating.

For all practical purposes well standardized resuscitation treatments, rather than fortuitous recovery, are recommended. In procedures intended to be used for the microbiological monitoring of foods in international trade this is imperative in order to obtain results of the required reproducibility.

The pre-enrichment step in *Salmonella* analyses (p. 164) is actually a resuscitation procedure.

ENUMERATION OF VIABLE SPORES

Bacterial spores are reluctant to germinate and grow out into active vegetative cells (Keynan et al., 1961). To enumerate spores by plating, it is necessary to break the dormancy, induce germination, and encourage cell growth. Sometimes spores will germinate but will not grow out and multiply (Hachisuka et al., 1954; Hyatt and Levinson, 1957; Hermier, 1962a, b), or they may fail to germinate, or they may germinate so slowly that colonies fail to form during a standard incubation period (Schmidt, 1957; Michalska, 1963; Vary and Halvorson, 1965; Keynan and Evenchik, 1969).

The simplest and most used procedure to encourage spores to germinate is heat activation, although exposure to low pH, reducing or oxidizing substances, ionizing irradiation, or dipicolinic acid may also be effective (Keynan et al., 1964; Finley and Fields, 1962; Schmidt, 1957). The necessary temperatures vary according to many factors, such as the age of the spores, the incubation temperature, the composition of the menstruum in which they were formed, the

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pH, the water activity, and the presence of metals or sugars in the medium during heating. The most important factor is, however, the species. What is an activation temperature for one species may be injurious to another. For example, 60°C is suitable for *Clostridium botulinum* type E, 65° to 80°C for mesophilic spore-forming bacteria, and 100°C for spore-forming thermophiles.

Certain nutrients in the plating medium frequently will encourage activated spores to grow out and reproduce – namely certain amino acids such as L-alanine, some sugars such as glucose, ribosides such as inosine or adenosine, various inorganic or organic ions, dipicolinic acid or its salts, surfactants, starch, and especially lysozyme.

Even damaged spores will often recover on transfer to nutritionally optimal media. For the recovery of clostridial spores, starch (Olsen and Scott, 1946) and bicarbonate have long been added (Wynne and Foster, 1948a, b, c). Busta and Ordal (1964) and J.L. Edwards et al. (1965) recommend adding calcium dipicolinate to the medium. Lysozyme in the medium improves outgrowth and multiplication of spores of *Clostridium perfringens* (Cassier and Sebald, 1969; Duncan et al., 1972a; Barach et al., 1974) and *C. botulinum* type E (Sebald and Ionesco, 1972).

PART II

Recommended methods for microbiological examination

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Introduction

Although they are widely used and generally accepted, the methods outlined below should be considered as interim procedures until comparative and collaborative studies dictate otherwise. The ICMSF is now conducting such studies as rapidly as limited resources permit, but welcomes help from other organizations.

These methods should provide the basis for legal judgments on the microbiological quality and safety of food, particularly of foods moving in international commerce. In making selections, therefore, the authors have emphasized sensitivity and reproducibility. For routine surveillance and factory control, and for 'screening' purposes, some methods require fewer media and manipulations, provide results more rapidly, and are cheaper than those proposed. These, however, may lack the precision desirable under arbitrational circumstances.

For several categories the Commission has recommended more than one method. In each case the alternative methods are judged to be equally suitable for the purposes intended, but may represent strong regional preferences often based on satisfactory usage by large numbers of technicians. No significance is attached to the order in which the methods are listed.

It is assumed that the methods will be used by, or the users directed by, a competent microbiologist. The quality of equipment and materials, and the preparation, organization, sterilization, aseptic technique, controlled testing, and proper cleaning and use of equipment and materials must be commensurate with good laboratory practice. The descriptions of the methods are as free from non-essential detail as is practical for a working manual.

For detailed specifications on apparatus and materials, see *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 1976), *Standard Methods for the Examination of Dairy Products* (Hausler, 1972), *Compendium of Methods for the Microbiological Examination of Foods* (Speck, 1976), or *The Bacteriological Examination of Water Supplies* (Ministry of Health and Ministry of Housing and Local Government, 1957). For recommendations on safety procedures in the laboratory, see Appendix IV.

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Preparation and dilution of the food homogenate

Methods of isolation and enumeration of the microorganisms present in all non-liquid foods usually require preliminary treatment of samples of the food to release into a fluid medium those microorganisms which may be embedded within the food or within dried or gelatinous surface films. The most common practice is to use an electrically driven mixing device with cutting blades revolving at high speed (blender). A homogeneous suspension of food and microorganisms is thus prepared which permits the preparation of dilutions appropriate for use in various determinative or enumerative procedures. Standardization of this preliminary procedure is important. Excessive speed of the cutting blades or unduly prolonged use of the mixer may cause injury to microbial cells, either mechanically or by the heat generated; thus excessive mixing may result in a reduced 'count.' Insufficient mixing, in contrast, may not release embedded bacteria or may provide a heterogeneous distribution within the suspension. Limitation of the speed of the cutting blades and of the duration of mixing time is necessary to provide optimal results. The times and speeds recommended for Methods 1 and 2 below were determined from many years of experience and experimental determinations (Barraud et al., 1967).

Care should be taken to minimize risk from aerosols when pathogens may be present in the test food. All mechanical blenders create aerosols.

Recently a new mixing procedure popularly known as 'stomaching' has been developed which is satisfactory for foods (Sharpe and Jackson, 1972; Baumgart, 1973; Kihlberg, 1974). In this technique the food sample and diluent are put into a sterile plastic bag which is then vigorously pounded on its outer surfaces by paddles inside a machine called a 'stomacher.' The resulting compression and shearing forces effectively break up solid pieces of food. After samples are removed for analysis, the bag and its remaining contents can be discarded and the machine is immediately ready for reuse. Labor involved in cleaning and sterilizing reusable homogenizer jars or flasks and cutting blades is thus eliminated. Additional advantages over the conventional blending technique include the low noise level, negligible temperature rise, and small storage space. The stomacher is not as efficient as the blender in breaking up fat globules, however, so that results on foods with fat levels above 20% are lower with the stomacher. Addition of 1% Tween 80 or other non-toxic surfactant will overcome the problem (Sharpe and Harshaman, 1976) (see Method 3 below).

Another factor highly important to accurate enumeration is the nature of the diluent used. Many commonly used diluents, such as tap or distilled water, saline solution, phosphate buffers, and Ringer solution, are toxic to certain microorganisms, particularly if the time of contact is unduly prolonged (Winslow and Brooke, 1927; Butterfield, 1932; de Mello et al., 1951; Gunter, 1954; Stokes and Osborne, 1956; King and Hurst, 1963). The presence of food material may protect many organisms from toxic effects, but one can by no means depend on this (Hartman and Huntsberger, 1961; Hauschild et al., 1967). Progressive dilution would also offset such a protective effect. A general purpose dilution medium in widespread use is 0.1% peptone (Straka and Stokes, 1957; King and Hurst, 1963; Hörter and Klein, 1969; Hall, 1970; P. Keller et al., 1974). When enumerating *Clostridium perfringens*, Hauschild et al. (1967) have shown a pronounced advantage in using peptone solution rather than standard phosphate buffer diluent (Hausler, 1972). Schmidt-Lorenz (1960) included fish products in the comparison of several dilution fluids. Physiological saline with 0.1% peptone added proved to be the most reliable. Peptone (0.1%) in saline (0.85% NaCl) is recommended by the International Standards Organization in its proposed procedure for aerobic counts at 30°C.

Where reproducibility rather than maximum sensitivity is the main objective in standard procedures (as for the plate count), various diluents may be adequate provided they are used in accordance with a defined composition and period of exposure, but for the isolation and enumeration of particular species or groups known to be sensitive to diluents containing inorganic salts or to distilled water, the least toxic diluent is to be preferred. Present knowledge suggests that 0.1% peptone in distilled water or in saline best meets this need. The validity of regional or personal preferences for particular diluents needs further investigation before a diluent can be defined for all enumerative purposes.

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Method 1[1]

I APPARATUS AND MATERIALS

1 Mechanical blender, two-speed model or single speed with rheostat control.
2 Glass or metal blending jars of 1-liter capacity, with covers, resistant to autoclave temperatures. One sterile jar (autoclaved at 121°C for 15 minutes) for each food specimen to be analyzed.
3 Balance with weights. Capacity at least 2500 g; sensitivity 0.1 g.
4 Instruments for preparing samples: knives, forks, forceps, scissors, spoons, spatulas, or tongue depressors, sterilized previous to use by autoclaving or by hot air.
5 A supply of 1-, 10-, and 11-ml pipettes (milk dilution specifications; Hausler, 1972).
6 Refrigerator, at 2–5°C.
7 Peptone Dilution Fluid (Medium 81), or Peptone Salt Dilution Fluid (Medium 83) sterilized in autoclave for each specimen: (i) 450 ml in flask or bottle, (ii) 90- or 99-ml blanks in milk dilution bottles (Hausler, 1972, specifications) or similar containers.

II PROCEDURE

1 Begin the examination as soon as possible after the sample is taken. Refrigerate the sample at 0–5°C whenever the examination cannot be started immediately after it reaches the laboratory. If the sample is frozen, thaw it in its original container (or in the container in which it was received at the laboratory) for a maximum of 18 hours in a refrigerator at 2–5°C. If the frozen sample can be easily comminuted (e.g., ice cream), proceed without thawing.
2 Tare the empty sterile blender jar, then weigh into it 50 ± 0.5 g representative of the food specimen (ICMSF, 1974). If the contents of the package are obviously not homogeneous (for example, a frozen dinner), take a 50-g sample from a mixture of the whole dinner, or analyze each different food portion separately, depending upon the purpose of the test.

1 From *Standard Methods for the Examination of Dairy Products* (Hausler, 1972); recommended by AOAC (1975), except that the latter calls for phosphate-buffered dilution water as the diluent.

3 Add to the blender jar 450 ml of Peptone Dilution Fluid or Peptone Salt Dilution Fluid. This provides a dilution of 10^{-1}.
4 Blend the food and dilute promptly. Start at low speed, then switch to high speed within a few seconds; or gradually within a few seconds increase to full voltage. Time the blending carefully to permit 2 minutes at high speed.[2] Wait 2 or 3 minutes for foam to disperse. However, once the diluent has been weighed into the sample, permit no unnecessary delays.
5 Measure 1 ml of the 10^{-1} dilution of the blended material, avoiding foam, into a 99-ml dilution blank, or 10 ml into a 90-ml blank. Shake this and all subsequent dilutions vigorously 25 times in a 30-cm arc. Repeat this process using the progressively increasing dilutions to prepare dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}, or those which experience indicates are desirable for the food under test.

Method 2[3]

I APPARATUS AND MATERIALS

1 Mechanical mincer, laboratory size, fitted with a plate with holes of diameter not exceeding 4 mm. Sterile.
2 Mechanical blender, operating at not less than 8000 rpm and not more than 45,000 rpm.
3 The requirements for Method 1 above, I, items 2, 3, 4, and 6.
4 A supply of 1-ml bacteriological pipettes.
5 Culture tubes for dilution fluid, 15–20 ml capacity.
6 Peptone Dilution Fluid (Medium 81) or Peptone Salt Dilution Fluid (Medium 83).

II PROCEDURE

1 Begin the examination as soon as possible after the sample is taken. Refrigerate the sample at 0–5°C whenever the examination cannot be started immediately after it reaches the laboratory. Preferably, start analysis of unfrozen samples within 1

2 For certain foaming foods, such as cream pies, a 1-minute blend is sufficient.

3 This method is used widely, particularly in European countries. As described, it follows closely the procedure being recommended by the International Standards Organization.

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hour after receipt. If the sample is frozen, thaw it in its original container (or in the container in which it was received in the laboratory) in a refrigerator at 2–5°C and examine as soon as possible after thawing is complete or at least sufficient to permit suitable subsamples to be taken (maximum thawing time, 18 hours).

2 If the sample is easy to cut and disperse, proceed to step 3; if it is difficult to blend (e.g., raw meat), grind and mix it twice in the mechanical mincer before proceeding to step 3.

3 Weigh into a tared blender jar at least 10 g of sample (to the nearest 0.1 g), representative of the food specimen (ICMSF, 1974).

4 Add 9 times as much dilution fluid as sample. This provides a dilution of 10^{-1}.

5 Operate the blender according to its speed for sufficient time to give a total of 15,000 to 20,000 revolutions. Thus, even with the slowest blender the duration of grinding will not exceed 2.5 minutes.

6 Mix the contents of the jar by shaking, and pipette duplicate portions of 1 ml each into separate tubes containing 9 ml of dilution fluid. Carry out steps 7 and 8 below on each of the diluted portions.

7 Mix the liquids carefully by aspirating 10 times with a sterile pipette.

8 Transfer with the same pipette 1.0 ml to another dilution tube containing 9 ml of dilution fluid, and mix with a fresh pipette.

9 Repeat steps 7 and 8 until the required number of dilutions are made. Each successive dilution will decrease the concentration 10-fold.

Method 3[4]

I APPARATUS AND MATERIALS

1 Colworth Stomacher, or similar machine, operating at about 230 rpm, with a capacity of 400 ml.

2 A supply of thin-walled (about 200 gauge) polyethylene base-welded bags, about 18 by 30 cm.

4 This method is not used specifically in any particular region of the world but is gaining in popularity generally because of its labor-saving advantages. The Colworth Stomacher 400 may be obtained from A.J. Seward and Co., Ltd., Blackfriars Road, London, England.

3 The requirements for Method 1 above, I, items 3, 4, and 6.

4 A supply of 1- and 10-ml bacteriological pipettes, sterile.

5 Peptone Dilution Fluid (Medium 81) or Peptone Salt Dilution Fluid (Medium 83), 9- or 90-ml blanks in culture tubes or dilution bottles, sterile.

II PROCEDURE

1 Begin the examination as soon as possible after the sample is taken. Weigh into a tared polyethylene bag at least 10 g of sample material (to the nearest 0.1 g), representative of the food specimen (ICMSF, 1974). Frozen specimens do not need to be thawed unless this is necessary for weighing the sample units; neither do foods like raw meats need to be minced. If thawing is necessary, thaw the sample in its original container (or in the container in which it was received in the laboratory) in a refrigerator at 2–5°C and proceed with the analysis after sufficient thawing has occurred to permit suitable sample units to be taken (maximum thawing time 18 hours).

2 Add 9 times as much dilution fluid as sample. This provides a dilution of 10^{-1}.

3 If pathogens are known or suspected to be in the food, place the bag inside another as a precaution against breakage.

4 For high-fat foods (over 20% fat) such as some meats, add 1% Tween 80 or some other non-toxic surfactant.

5 Place the bag into the stomacher and operate the machine for 60 seconds.

6 Shake the bag vigorously by hand and pipette 10 ml into a 90-ml dilution blank or 1 ml into a 9-ml dilution blank, to give a 10^{-2} dilution.

7 Shake the 100-ml suspension vigorously 25 times in a 30-cm arc, or aspirate the 10-ml suspension 10 times with a sterile pipette. Pipette 10 ml into a 90-ml blank or 1 ml into a 9-ml blank to give a 10^{-3} dilution. Repeat this process to prepare 10^{-4} and 10^{-5} dilutions, or those which experience indicates are desirable for the food under test.

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Enumeration of mesophilic aerobes: the agar plate methods

The number of aerobic mesophilic microorganisms ('plate count') has been one of the more commonly used microbiological indicators of the quality of foods, except where fermentation (as in cheese and certain types of sausage) or a natural 'ripening' process gives rise to very large numbers of bacteria. The aerobic mesophilic count has limited validity for assessing safety (cf. Part 1, p. 6). Nevertheless, it has value with many foods in a number of ways: e.g., in indicating the adequacy of sanitation and temperature control during processing, transport, and storage; in forming an opinion about incipient spoilage, probable shelf-life, uncontrolled thawing of frozen foods, or failure to maintain refrigerative temperatures for chilled foods; and in revealing sources of contamination during manufacture. The 'plate count' has special application to imported foods, where the importing country has no opportunity to control the standard of sanitation practiced in the manufacturing establishments.

The three methods in common use for enumerating aerobic mesophilic microorganisms are the Standard Plate Count, which is also called the Aerobic Plate Count or Pour Plate (reviewed by Hartman and Huntsberger, 1961; Angelotti, 1964); the 'surface plate' or 'spread drop' method (Reed and Reed, 1948; J.J.R. Campbell and Konowalchuk, 1948; Gaudy et al., 1963; D.S. Clark, 1967); and the Drop Plate method (Miles and Misra, 1938; Sharpe and Kilsby, 1971). The Plate Loop method is also now widely used for milk (D.I. Thompson et al., 1960). None of the procedures can be depended upon to enumerate all types of organisms present within the test specimens. Many cells may not grow because of specifically unfavorable conditions of nutrition, aeration, temperature, or duration of incubation.

The incubation temperature must be specified, for the agar plate methods apply to groups of microorganisms having different growth temperature ranges such as 0–7°C for psychrotrophs, 30–35°C for mesophiles, and 55°C for thermophiles. The temperature chosen would depend upon the purpose of the examination: for example,

the degree of spoilage and probable shelf-life of ice-packed chickens would best be estimated by using an incubation temperature of 0–5°C; similarly, the overall bacteriological quality of refrigerated farm bulk tank milk and/or the effectiveness and duration of its refrigeration would be best determined by a psychrotrophic count at 7°C for 10 days; a count at 25–30°C would provide the most information about general plant sanitation; one at 35–37°C would reflect the degree of contamination and/or growth by mesophiles; but 55°C would be the preferred incubation temperature for determining the thermophilic contamination from blanching equipment or from heated pumps in a food conduit system. Accurate control of the incubation temperature is essential in each procedure.

The thermoduric count measures the number of microorganisms that survive a pasteurization procedure of 63°C for 30 minutes. Such a test evaluates the build-up of bacteria in blanchers, or in other equipment held hot for long periods. Thermoduric organisms usually fail to grow at refrigeration or ambient temperatures.

Because microorganisms have very diverse temperature limits for growth, no one incubation temperature will absolutely exclude all organisms from another group. Many psychrotrophic organisms in foods have maximal growth temperatures between 30° and 32°C (Elliott, 1963; Ingraham and Stokes, 1959), and mosphiles can grow between 10°C and 37°C (Michener and Elliott, 1964). It follows, therefore, that within the mesophilic range the number of colonies usually decreases rapidly as the incubation temperature increases from 30° to 37°C. In most foods, the agar plate counts are highest when the incubator is set somewhere between 20° and 30°C. In milk, experience has shown the counts are highest when the incubator is set at about 32°C (Babel et al., 1955; Yale and Pederson, 1936). Temperature control is critical in the range from 30° to 37°C and, to obtain the needed precision, incubators are required with minimal fluctuation or variation of temperature throughout the incubation chamber. All incubators should be checked and calibrated frequently.

Many laboratories prefer to use the Surface Plate or Drop Plate procedures, particularly for routine screening tests, because they require less medium, fewer Petri plates, and less expensive pipettes. Apart from cost, the advantages and disadvantages of the Drop Plate and Surface Plate methods in comparison with the Standard Plate Count method are listed below.

Advantages: (1) It is not essential that the medium be translucent, as it is for the Standard Plate Count. (2) Because all colonies develop on the surface of the medium, colony appearance can gen-

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erally be studied more satisfactorily, and the proportion of the various colony types can be estimated more readily. (3) By dropping several dilutions onto a plate, the accuracy of the dilution system can be easily assessed. (4) Heat-labile organisms are not killed, as may occur when cells are exposed to melted agar (45°C) in the Standard Plate Count (van Soestbergen and Lee, 1969). (5) Prepoured plates can be stored and moved easily within a laboratory or to field laboratories for 'on-site' studies.

Disadvantages: (1) Because of the small volume of specimen used (0.02 or 0.1 ml), suspended food could interfere by causing a nutrient or inhibitory effect on microorganisms. (2) For the same reason, the Drop Plate and Surface Plate procedures are not entirely satisfactory for specimens with few organisms (i.e. fewer than 100 per gram). However, microbiological criteria for the mesophilic organisms in foods are likely to be well above this figure.

The Standard Plate Count procedure is used in many countries and almost exclusively in North America, where it has been specifically recommended for dairy products (Hausler, 1972) and other foods (AOAC, 1975). A modification of the method has been adopted by the International Dairy Federation (IDF Standard No. 3 [1958]; IDF Standard No. 49 [1970]) for determination of aerobic mesophiles in liquid and dried milk. In the dairy method, milk is added to Plate Count Agar (Standard Methods Agar; Medium 92), and the poured plates are incubated at 29–31°C for 3 days. To evaluate the overall bacteriological quality of milk, authorities in the United Kingdom use the reductase (methylene blue) test to replace the Standard Plate Count, particularly at the retail level. The Drop Plate method is also used by some laboratories in that country. The Surface Plate method is used in Europe and is officially recommended by the International Standards Organization for use with meat and meat products (Barraud et al., 1967). The Standard Plate Count procedure is so well established, and its accuracy and limitations so well understood, that the Commission recommends its retention, for purposes of arbitration. Nevertheless, one of the other methods may be chosen later, if warranted, after it has been compared with the Standard Plate Count method under sufficiently diverse circumstances.

Method 1[1] (The Standard Plate Count, Pour Plate, or Aerobic Plate Count)

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of the Food Homogenate (pp. 106–11), Method 1, 2, or 3.
2 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
3 1-, 5-, and 10-ml bacteriological pipettes (milk dilution specifications; Hausler, 1972).
4 Water bath or air incubator for tempering agar, 44–46°C.
5 Incubator, 29–31°C. (*Note:* The temperature constancy and uniformity of air incubators are notoriously bad. To check constancy, continuous or frequent interior temperature readings should be made over a period of several hours by suitable thermocouples or thermometers. To check uniformity, similar readings over a period of several hours should be made at various positions in the incubator while it is filled with agar plates [see also Hausler, 1972]. Stacks of Petri dishes inside the incubator should be separated from one another and from the walls and top of the incubator as well. Each stack should consist of no more than six, and preferably not more than four Petri dishes.)
6 Colony counter (Quebec dark field model or equivalent recommended).
7 Tally register.
8 Plate Count Agar (Standard Methods Agar; Medium 92).

II PROCEDURE

1 Prepare the food sample by one of the three procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 To duplicate sets of Petri dishes, pipette 1-ml aliquots from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions, and a 0.1-ml aliquot from the 10^{-5} dilution to give 10^{-1} to 10^{-6} g of food per Petri dish. This series is suggested when the approximate range of bacterial numbers in the specimen is not known. The level of dilution

1 The method follows that recommended for dairy products (Hausler, 1972) and for frozen, chilled, precooked, or prepared foods (AOAC, 1975) except that the latter specifies an incubation temperature of 35°C.

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prepared and plated can be varied to fit the expected bacterial count, but at least three levels should be plated.

3 Melt Plate Count Agar in flowing steam or boiling water, but do not expose it to such heat for a prolonged period. Temper the agar to 44–46°C and control its temperature carefully to avoid killing bacteria in the diluted sample. Promptly pour into the Petri dishes 10–15 ml of melted and tempered agar. Fewer than 20 minutes and preferably fewer than 10 minutes should elapse between making the dilution and pouring the agar.

4 Immediately mix aliquots with the agar medium by tilting and rotating the Petri dishes. A satisfactory sequence of steps is as follows: (a) tilt dish to and fro 5 times in one direction, (b) rotate it clockwise 5 times, (c) tilt it to and fro again 5 times in a direction at right angles to that used the first time, and (d) rotate it counterclockwise 5 times.

5 As a sterility check, pour one or several plates with uninoculated agar medium and with uninoculated diluent. After incubation, the number of colonies on these plates should not be enough to distort the count by more than one unit in the second significant figure (see step 8 below).

6 After the agar has solidified, invert the Petri dishes and incubate them at 29–31°C for 48 ± 3 hours.

7 Compute the Standard Plate Count or the Estimated Plate Count according to directions in steps 8 or 9 below. Observe instructions and comments on computing and recording, spreaders, inhibitors, reporting and interpreting, and repeatability and personal error in items 10–14 below.

8 *Computing Standard Plate Count* (SPC)

(a) Select two plates corresponding to one dilution and showing between 30 and 300 colonies per plate. Count all colonies on each plate, using the colony counter and tally register. Take the arithmetic average of the two counts and multiply by the dilution factor (i.e., the reciprocal of the dilution used). Report the resulting number as the SPC (see item 10 below).

(b) Both plates should be counted even if one of them should give a count of fewer than 30 or more than 300. Again, take the average of the two counts and multiply by the dilution factor and report the resulting number as the SPC.

(c) If plates from two consecutive decimal dilutions fall into the countable range of 30–300 colonies, compute the Standard Plate Count for each of the dilutions as directed above and report the average of the two values obtained, unless the higher computed count is more than twice the lower one, in which case report the lower computed count as the Standard Plate Count.

9 *Computing Estimated Standard Plate Count* (ESPC)

(a) If counts on individual plates do not fall within the range 30–300 colonies, report the calculated count as ESPC. Calculate the count according to the circumstances as directed in (b), (c), or (d) below.

(b) If plates of all dilutions show more than 300 colonies, divide each of the duplicate plates for the highest dilution into convenient radial sections (e.g., 2, 4, 8) and count all of the colonies in one or more sections. Multiply the total in each case by the appropriate factor to obtain an estimate of the total number of colonies for the entire plate. Average the estimates for the two plates, multiply by the dilution, and report the resulting count as the ESPC.

(c) If there are more than 200 colonies per one-eighth section of the plates made from the most dilute suspension, multiply 1600 (i.e., 200 × 8) by that dilution, and express ESPC as more than (>) the resulting number. In all such cases it is advisable to report the dilution used in brackets.

(d) In cases where there are no colonies on the plates made from the most concentrated suspension, report the ESPC as less than (<) 1 times the dilution.

10 *Computing and Recording* Only two significant digits should be used in reporting the SPC or the ESPC. They are the first and second digits (starting from the left) of the average of the counts. The other digits should be replaced by zeros. For example, if the actual count were 523,000, report the count as 520,000 (52×10^4). If the third digit from the left is 5 or greater add one unit to the second digit ('rounding off'). For example, if the actual count were 83,600, report the count as 84,000 (84×10^3).

11 *Spreaders*

(a) If spreading organisms are present on a plate, count the colonies lying outside the area of spread provided the total repressed growth area does not exceed one-half of the plate area. Correct the count of the plate to allow for the area not counted.

(b) Report the presence of spreaders (Spr) every time the area affected exceeds one-quarter of the plate area. When more than one plate out of twenty shows spreaders, take action to reduce the occurrence of spreaders (e.g., reduce air moisture inside incubator, or mix dilution fluid and tempered agar more thoroughly).

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4 Horse-Blood Agar (Medium 45) or Plate Count Agar (Medium 92).
5 Ringer Solution, 1/4 strength (Medium 97).

II PROCEDURE

1 Prepare dried plates of Horse Blood Agar or Plate Count Agar as described in Method 2, II, step 1 above.
2 Mark the plates on the bottom into three equal segments, and indicate for each segment the dilution to be used.
3 Prepare food samples by one of the procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11), using 1/4 strength Ringer Solution as the diluent.
4 Using the specially calibrated pipette, deliver 2 separate drops (0.02 ml each) of each dilution onto the surface of the relevant segment. Thus, with only two plates, six dilutions (10^{-1}, 10^{-2}, ..., 10^{-6}) can be plated in duplicate.
5 Allow the drops to dry for about 15 minutes at room temperature with the agar surface uppermost.
6 Incubate the plates inverted for 48 hours at 29–31°C.
7 Count all colonies for dilutions giving 20 or fewer colonies per drop.
8 Compute the Drop Plate Count per gram (ml) of specimen.

Method 4[6] (The Plate Loop; D.I. Thompson et al., 1960)

I APPARATUS AND MATERIALS

(Total equipment assembly, as shown in Figure 1, can be purchased as a unit from various suppliers, such as Etablissements Grosseron, 44000 Nantes, France)

1 Loop, 0.001 ml, preferably welded, calibrated to contain 0.001 ml, made of B & S gauge No. 26 platinum-rhodium (3.5%) wire,

and spherical with the cut end at right angles to the sides and having no protruding ridges or chips. When held vertically, these pipettes should deliver 50 drops per ml of distilled water (20°C) at the rate of one drop per second.

6 This method follows that recommended for dairy products (Hausler, 1972), with some modifications. It is intended for milk but can be used for other fluids as well.

true-circle loop, ID 1.45 mm ± 0.06 mm, attached to an 8-cm length of wire, and mounted in a suitable holder. Loops may be reshaped by being fitted over a No. 54 twist drill. Loops of proper size should not fit over a No. 53 twist drill.
2 Luer-Lok hypodermic needle, 13 gauge (sawed off 24–36 mm from the point where the barrel enters the hub). Insert the kinked end of the wire shank into the sawed-off needle to a point where the bend is about 12–14 mm from the end of the barrel.
3 Continuous-pipetting outfit: Becton, Dickinson & Co. No. 1251 (consisting of a metal pipetting holder, a Cornwall Luer-Lok syringe, and a filling outfit), 2-ml capacity. Adjust to deliver 1.0 ml. (The rubber tubing may be replaced with a longer tube for greater mobility, and a supply of tubing should be kept on hand, as the rubber deteriorates upon repeated autoclaving.) Attach the Luer-Lok hub to the Luer-Lok fitting. This apparatus and other parts may be sterilized by autoclaving at 121°C for 15 minutes, or sanitized by submerging the completely disassembled unit in boiling water for 10 minutes.
4 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
5 Water bath or air incubator for tempering agar, 44–46°C.
6 Incubator, 29–31°C. (See note concerning constancy and uniformity of incubators, p. 115.)
7 Colony Counter (Quebec dark field model or equivalent).
8 Phosphate-Buffered Dilution Water (Medium 90).
9 Plate Count Agar (Medium 92).

II PROCEDURE

1 Assemble the sterilized 0.001-ml measuring and transfer instrument ready for use (see Figure 1). Place the end of the rubber supply tube attached to the syringe in a bottle of sterile Phosphate-Buffered Dilution Water (Medium 90). Depress the syringe plunger rapidly several times to pump water into the glass syringe (which has previously been adjusted to deliver 1 ml with each depression of the plunger).
2 Before initial transfer is made in examining a series of samples, briefly flame the loop (preferably in a clean, high-temperature gas flame) and allow it to cool 15 seconds or more. Shake liquid samples carefully and always in the same standardized manner, and dip the loop into the sample (avoiding foam) as far as the bend in the shank (the bend serves as a graduation mark). To

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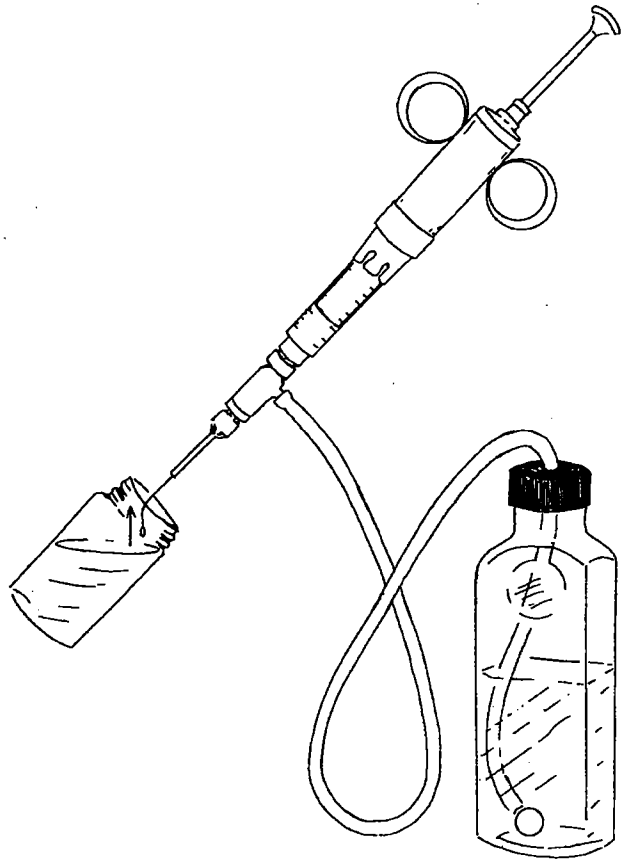


Figure 1 Plate loop outfit, showing vertical removal of loop (Hausler, 1972)

measure the sample, dip the loop vertically into the sample three times, moving the loop with a uniform up-and-down movement over a distance of about 2 1/2 cm. Remove the loop from the sample vertically at a uniform rate (55–60 dips per minute). This is necessary to ensure that the weight of milk on the loop is as constant as possible.

3 Raise the cover of a sterile Petri dish, insert loop and depress the plunger, causing sterile dilution water to flow across the charged loop and thus washing a measured 0.001 ml of sample into the dish. (*Caution*: Do not depress the plunger so rapidly that water fails to follow the shank and flow across the loop.)

4 Normally the residue remaining on the loop after discharging the sample is not significant. However, small imperfections in the welding of the loop or in smoothness of the metal surface might lead to incomplete rinsing. This possibility should be determined for each loop by running a series of control plates. If the loop is determined to be free-rinsing, no flaming between samples is necessary.

5 Pour plates as described under Method 1, II, 3 et seq., and incubate at 29–31°C for 48 hours.

6 Report results as Plate Loop Count per gram (ml).

MECHANIZATION AND AUTOMATION OF THE AGAR PLATE COUNT

Preparing dilutions, pipetting, pouring plates, and counting colonies can be tedious and expensive work. Some laboratories, analyzing samples in large volume, have successfully mechanized and automated these steps.

A mechanized system based on the Plate Loop method has been in use at the Milk Control Station in Zutphen, the Netherlands, since early 1973 (Posthumus, Klijn, and Giesen, 1974). The method permits the analysis of 12 samples simultaneously, to make a total of 5000 samples of milk each 2 weeks. The loop introduces 0.001 ml into melted agar in tubes, which are rolled as the agar solidifies (the 'roll tube' method; G.S. Wilson, 1922; Howard and Fischer, 1950; Clegg et al., 1951). Tests comparing this method with the Standard Plate Count have demonstrated its speed, reliability, accuracy, and economy.

Another mechanized method, developed by Jaartsveld and Swinkels (1974), also in the Netherlands, uses an automatic diluting device with pipettes instead of loops. It is also working satisfactorily on a practical scale.

In France the Eramic 25 instrument, which mechanizes all the operations of the Plate Loop method and uses Petri plates, has been in regular use since 1973 in several milk testing laboratories (Grappin, 1975).

In the United States the spiral system for plating and counting bacteria (Gilchrist et al., 1973) was developed in Food and Drug Administration Laboratories. It utilizes a single nutrient agar plate on which a known volume of the sample is deposited on the surface in ever increasing amounts in the form of an Archimedes spiral and in

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such a manner that the amount of solution contained on any portion of the plate is known. After the plate is incubated, it is viewed over a modified counting grid which relates areas of the plate to the contained volume of sample. By counting the number of colonies in an appropriate area of the plate, the number of bacteria in the sample thus can be estimated. Several significant improvements in the plating system have been made, and an electronic counter for use with the spiral plates has been developed. Although counts of bacteria in milk obtained with this system compare favorably to those obtained by the Standard Plate Count, it is too soon to make any recommendation about its general use.

Another sophisticated instrument – the Colworth 2000 (Sharpe et al., 1972a), now at the development stage – will automatically prepare decimal dilutions of the sample suspension in four different media, print on Petri plates the sample numbers and dilution numbers, and stack the plates for incubation. A novel feature of the machine is its ability to provide a fresh, sterile pipette for each dilution.

A simpler instrument is the Colworth Droplette Machine used to make serial dilutions in agar, which is then placed in Petri dishes as convex drops. The drop acts as a condenser lens to permit counting of the colonies using transmitted light and a ground glass gridded screen. The machine incorporates a pump for dilutions, a light source, and a screen for reading the results (Sharpe et al., 1972b; Sharpe and Kilsby, 1971).

At the present time, any laboratory contemplating mechanization or automation should make comparative studies against an accepted method to be certain that the new procedure gives results that are acceptable.

Coliform bacteria

Coliforms, fecal coliforms, and *Escherichia coli* are determined by three successive steps in determinative methodology, each more complex and costly. Three methods for the determination of coliforms by the most probable number procedure (MPN) are given here. Each has been used in many laboratories throughout the world. The first utilizes Lauryl Sulfate Tryptose Broth (LST) followed by confirmation of gas-positive tubes using Brilliant Green Lactose Bile Broth (BGLB), each being incubated at 35–37°C; the second method uses MacConkey Broth (MAC), alone; and the third method uses BGLB, followed by confirmation on Violet-Red Bile Agar or Endo Agar. Since publication of the first edition of this book, the Commission has supported collaborative and comparative research studies on these three procedures, the results of which are summarized at the end of this chapter.

LST was chosen in preference to Lactose Broth chiefly because of the results obtained from extensive analysis of many types of precooked frozen foods in which LST, in conjunction with BGLB, yielded 36% more positive results than Lactose Broth (Shelton et al., 1962). Many reports of similar advantages occur in the literature as reviewed by the Advisory Committee on Microbiology of Frozen Foods (AFDOUS, 1966) and by Hall (1964).

Two methods of determining fecal coliforms have been selected. The first uses E.C. Broth at 45.5° ± 0.2°C, and the inoculum is derived from gas-positive tubes of LST Broth. The second method, widely used in Europe (Buttiaux et al., 1956; Guinée and Mossel, 1963), employs BGLB incubated at 44° ± 0.1°C and the inoculum is derived from gas-positive tubes of MAC Broth. This is the method of E.F.W. Mackenzie et al. (1948).

The usual means for confirmation of *E. coli* is by use of IMViC tests as described below.

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ENUMERATION OF COLIFORMS: DETERMINATION OF MOST PROBABLE NUMBER (MPN)

Method 1[1] (North American)

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of Food Homogenate, Method 1, 2, or 3 (pp. 106–11).
2 Incubator, 35–37°C.
3 1-ml bacteriological pipettes.
4 Inoculating needle, with nichrome or platinum-iridium wire.
5 Brilliant Green Lactose Bile Broth 2% (Medium 17), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes (75 × 10 mm).
6 Lauryl Sulfate Tryptose Broth (LST Broth; Medium 55), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes or similar glass vials (75 × 10 mm).
7 Eosin Methylene-Blue Agar (Medium 36) or Endo Agar (Medium 34), for plates.

II PROCEDURE

1 Prepare food samples by one of the procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11). Dilution blanks remaining from the determination of plate count can be used. (*Caution*: Keep delays minimal to avoid growth or death of microorganisms suspended in the diluent.)
2 Pipette 1 ml of the decimal dilutions of food homogenate to each of three separate tubes of LST Broth.
3 Incubate tubes at 35–37°C for 24 and 48 hours.
4 After 24 hours, record tubes showing gas production.[2] Return tubes not displaying gas production to the incubator for an additional 24 hours.
5 After 48 hours, record tubes showing gas production.[2]
6 Select the highest dilution in which all three tubes are positive for gas production and the next two higher dilutions. For example, if the last three positive dilutions were 1:100, 1:1000, and 1:10,000 and the numbers of positive tubes in each dilution were 3, 1, and 0, respectively, the results are recorded as 1:100 = 3, 1:1000 = 1, and 1:10,000 = 0. Report MPN = 700.

If no dilution contains three positive tubes, select the three highest dilutions containing positive tubes. For example, if the last four positive dilutions were 1:10, 1:100, 1:1000, and 1:10,000, and the numbers of positive tubes in these dilutions were 2, 2, 1, and 1, respectively, the results are recorded as 1:100 = 2, 1:1000 = 1, and 1:10,000 = 1. Report MPN = 200.

If further dilutions beyond that showing three positive tubes were not made, select the last three dilutions made. For example, if the last three dilutions were 1:100, 1:1000, and 1:10,000, and the number of positives were 3, 3, and 3, respectively, the results are recorded as 1:100 = 3, 1:1000 = 3, and 1:10,000 = 3. Report MPN = >11,000.

7 Confirm that the tubes of LST Broth selected in step 6 above are positive for coliform organisms by transferring a loopful of each to separate tubes of Brilliant Green Bile Broth 2% or by streaking Eosin Methylene-Blue or Endo Agar plates. Incubate confirmatory tubes 24 and 48 hours at 35–37°C and note gas formation. The formation of gas confirms the presence of coliform organisms. Observe solid confirmatory media for typical coliform colonies after 24 and 48 hours at 35–37°C. The formation of black or black-centered colonies or the formation of mucoid pink-orange colonies on Eosin Methylene-Blue Agar confirms the presence of coliforms. Similarly, coliforms form red colonies surrounded by red halos on Endo Agar.
8 Record the number of tubes in each dilution that were confirmed as positive for coliform organisms.
9 To obtain the MPN, proceed as follows. Determine, from each of the three selected dilutions, the number of tubes that provided a confirmed coliform result. Refer to the MPN table (Table 6) and note the MPN based upon the levels of sample dilution and the number of confirmed positive tubes at each selected dilution. For example, if in the first illustration given in step 6 above all positive tubes in the three selected dilutions (1:100, 1:1000, and 1:10,000) yielded positive confirmatory results for coliform organisms, then the values for each dilution are 3, 1, and 0 respectively. To obtain the MPN of coliform organisms per gram of food, multiply the MPN value shown in the table by 10 (in this example, 40 × 10 = 400 per g).

1 The method follows that recommended for water (APHA et al., 1976) and foods (APHA, 1966; Lewis and Angelotti, 1964; AFDOUS, 1966; USFDA, 1972; AOAC, 1975).
2 If tests for fecal coliforms are to be made, select gas-positive tubes and follow the procedure described in Method 1 of the following section on Determination of Coliform Organisms of Fecal Origin.

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TABLE 6[a]
Most probable number (MPN) of bacteria; three tubes at each dilution[b]

Number of positive tubes at each dilution level				Confidence limits			
10^{-1} dilution	10^{-2} dilution	10^{-3} dilution	MPN per g	99%		95%	
0	1	0	3	<1	23	<1	17
1	0	0	4	<1	28	1	21
1	0	1	7	1	35	2	27
1	1	0	7	1	36	2	28
1	2	0	11	2	44	4	35
2	0	0	9	1	50	2	38
2	0	1	14	3	62	5	48
2	1	0	15	3	65	5	50
2	1	1	20	5	77	8	61
2	2	0	21	5	80	8	63
3	0	0	23	4	177	7	129
3	0	1	40	10	230	10	180
3	1	0	40	10	290	20	210
3	1	1	70	20	370	20	280
3	2	0	90	20	520	30	390
3	2	1	150	30	660	50	510
3	2	2	210	50	820	80	640
3	3	0	200	<100	1900	100	1400
3	3	1	500	100	3200	200	2400
3	3	2	1100	200	6400	300	4800

a Calculated from data of de Man (1975).
b At each dilution level, inoculate 1 ml into each of three tubes of media. To calculate MPN from dilutions greater than those shown, multiply the MPN by the appropriate factor of 10, 100, 1000, etc. For example, if tubes selected come from 10^{-2}, 10^{-3}, and 10^{-4} dilutions, multiply by 10; if from 10^{-3}, 10^{-4}, and 10^{-5} dilutions, multiply by 100.

Method 2[3] (British)

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of Food Homogenate, Method 1, 2, or 3 (pp. 106–11).
2 Incubator, 35–37°C.
3 1-ml bacteriological pipettes.
4 MacConkey Broth (Medium 61), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes (75 × 10 mm).

3 This method is used in many countries for the enumeration of coliforms in water (Ministry of Health and Ministry of Housing and Local Government, 1957).

II PROCEDURE

1 Prepare food sample by one of the procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11). Dilution blanks remaining from the determination of the plate count can be used. (*Caution*: Keep delays minimal to avoid growth or death of microorganisms suspended in the diluent.
2 Pipette 1 ml of each of the decimal dilutions of the food homogenate into each of three separate tubes of MacConkey Broth.
3 Incubate tubes at 35–37°C for 24 and 48 hours.
4 After 24 hours, record tubes showing gas production.[4] Return tubes not showing gas production to the incubator for an additional 24 hours.
5 After 48 hours record tubes showing gas production.[4] The formation of gas after 24 or 48 hours of incubation is considered sufficient evidence of the presence of coliforms.
6 To determine the MPN, record the highest dilution in which all three tubes are positive for gas production, as well as the next two higher dilutions. If this is not possible because none of the dilutions yielded three positive tubes, use the three lowest dilutions. Following the example used in Method 1 above, II, steps 6 and 9, and referring to Table 6, determine the MPN.

Method 3 (Brilliant Green Bile, Confirmed)

I APPARATUS AND MATERIALS

1 The requirements for Method 2 above, I, items 1–3.
2 Brilliant Green Lactose Bile Broth 2% (Medium 17), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes (75 × 10 mm).
3 Violet-Red Bile Agar (Medium 131) or Endo Agar (Medium 34), for plates.

II PROCEDURE

1 Prepare food samples by one of the three procedures recommended in the chapter on Preparation and Dilution of Food

4 To test for fecal coliforms, select gas-positive tubes and follow the procedure described in Method 2 of the following section on Determination of Coliform Organisms of Fecal Origin.

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Homogenate (pp. 106-11). Dilution blanks remaining from the determination of plate count can be used. (*Caution*: Keep delays minimal to avoid growth or death of microorganisms suspended in the diluent.)

2 Pipette 1 ml of each of the decimal dilutions of the food homogenate into three separate tubes of Brilliant Green Lactose Bile Broth 2%.

3 Incubate tubes at 35–37° C for 24 and 48 hours.

4 After 24 hours, record tubes showing gas production.[5] Return tubes not displaying gas to the incubator for an additional 24 hours.

5 After 48 hours, record tubes showing gas production.[5]

6 Select tubes for confirmation tests and MPN determination as described in Method 1 above, II, step 6.

7 Confirm tubes of Brilliant Green Bile Broth 2% selected in step 6 immediately above by streaking on the surfaces of Violet-Red Bile Agar or Endo Agar. Incubate plates at 35–37° C. Examine Violet-Red Bile Agar plates after 24 hours and Endo Agar plates after 48 hours. The formation of dark red colonies with diameters greater than 5 mm on Violet-Red Bile Agar or of red colonies surrounded by red halos on Endo Agar confirms the presence of coliform organisms.

8 Record the number of tubes in each dilution that were confirmed as positive for coliform organisms.

9 Determine the MPN of coliforms as described in Method 1 above, II, steps 6 and 9.

Method 4[6] (Violet-Red Bile Agar Plate)

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of Food Homogenate, Methods 1 and 2 (pp. 106-11).

2 Incubator, 35–37° C.

3 1-ml bacteriological pipettes.

5 To test for fecal coliforms, select gas-positive tubes and follow the procedure described in Method 2 of the following section on Determination of Coliform Organisms of Fecal Origin.

6 This direct plating technique is widely used as a rapid screening test. The method does not apply in the analysis of foods containing small numbers of coliforms, since the sensitivity is limited by the amount of food homogenate that can be introduced into a Petri dish.

4 Water bath for tempering agar, 44–46° C.

5 Violet-Red Bile Agar (Medium 131).

II PROCEDURE

1 Prepare food homogenate by one of the three procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106-11). Dilution blanks remaining from the determination of the plate count may be used. (*Caution*: Keep delays minimal to avoid growth or death of microorganisms suspended in the diluent.)

2 Transfer 1 ml of each dilution of food homogenate into a sterile Petri dish.

3 Add to each Petri dish 10–15 ml of Violet-Red Bile Agar tempered to 44–46° C.

4 Mix contents of plates thoroughly by tilting and rotating each dish. Allow the mixture to solidify (5–10 minutes) on a level surface; then distribute an additional 3–4 ml of plating medium as an overlay, completely covering the surface of the solidified medium and thus inhibiting surface colony formation.

5 Invert and incubate the plates for 24 hours at 35–37° C. Only the dark red colonies measuring 0.5 mm or more in diameter on uncrowded plates are considered to be coliform bacteria. If possible, choose for counting only those plates with not more than 150 of such colonies. Multiply the number of colonies by the dilution to obtain the number of coliform organisms per gram of sample.

DETERMINATION OF COLIFORM ORGANISMS OF FECAL ORIGIN

The following procedures should differentiate between coliforms of fecal origin (intestines of warm-blooded animals) and coliforms from other sources.

Method 1[7] (North American)

I APPARATUS AND MATERIALS

1 Inoculating loop, preferably of nichrome or platinum-iridium wire (3-mm diameter).

7 This method (modified Eijkman test; Eijkman, 1904) follows that recom-

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2 Agitated water bath with thermoregulation capable of maintaining a temperature of 44.5° ± 0.2°C.
3 E.C. Broth (Medium 32), 10-ml volumes in 150 × 15 ml tubes containing inverted Durham tubes (75 × 10 mm).

II PROCEDURE

1 Select tubes of Lauryl Sulfate Tryptose Broth (Medium 55) that are positive for gas production in Method 1 of the section on Enumeration of Coliforms above.
2 Inoculate a loopful of broth from each of the gas-positive cultures into a separate tube of E.C. Broth.
3 Incubate E.C. Broth tubes at 44.5° ± 0.2°C and read for gas production after 24 and 48 hours.
4 E.C. Broth tubes displaying gas production are presumed positive for fecal coliforms.

Method 2[8] (European)

I APPARATUS AND MATERIALS

1 Inoculating loop, 3 mm in diameter, preferably of nichrome or platinum-iridium wire.
2 Agitated water bath with thermoregulation capable of maintaining a temperature of 44° ± 0.1°C.
3 5-ml bacteriological pipettes.
4 Brilliant Green Lactose Bile Broth 2% (Medium 17), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes (75 × 10 mm).
5 Peptone Water (Medium 86), 10-ml volumes in 150 × 15 mm tubes.
6 Indole Reagent (Reagent 19).

mended for water (APHA et al., 1976; Lewis and Angelotti, 1964). Some laboratories prefer to adjust the incubating bath to 45.5° ± 0.2°C to isolate *E. coli* with fewer false positives. However, this higher temperature inhibits the growth of an occasional *E. coli* strain.

8 This method is used widely in several European countries in conjunction with Method 2 of the section on Enumeration of Coliforms above. As described, it follows the procedure outlined by E.F.W. Mackenzie et al. (1948).

II PROCEDURE

1 Select tubes of MacConkey Broth that are positive for gas production in Method 2 of the section on Enumeration of Coliforms above.
2 Inoculate a loopful of broth from each gas-positive culture into a tube of Brilliant Green Lactose Bile Broth 2% and a tube of Peptone Water.
3 Incubate tubes at 44° ± 0.1°C.
4 Read Brilliant Green Lactose Bile Broth 2% for gas production after 24 and 48 hours of incubation.
5 After 24 hours of incubation, pipette aseptically a 5-ml portion of each Peptone Water tube to a separate test tube and run the Indole Test (p. 135). Cultures showing gas production in Brilliant Green Lactose Bile Broth 2% and indole formation in Peptone Water are presumed positive for fecal coliform organisms.

IDENTIFICATION TESTS FOR COLIFORM ORGANISMS: THE IMViC PATTERN[9]

In routine analyses of foods, further confirmation of the presence of *E. coli* beyond that obtained from the tests described in Methods 1 and 2 of the preceding section is not usually feasible because of the time and labor involved. In special instances, however, where the extra effort is merited, the differentiation of the coliform group into species and varieties can be carried out on the basis of the results of four tests (indole, methyl red, Voges-Proskauer, and sodium citrate) referred to collectively as the 'IMViC tests.' A grouping of reaction combinations is presented in Table 7.

I APPARATUS AND MATERIALS

The media listed below include acceptable alternatives for the IMViC tests and for the purification of cultures. Decide on the procedures to follow before preparing the media.

9 The procedure for the IMViC tests follows closely those described in *Standard Methods for the Examination of Water and Wastewater* (APHA et al., 1976).

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TABLE 7[a]
Differentiation of coliforms (all of these organisms are capable of producing acid and gas from lactose in 48 hours at 35–37°C)

	Gas in lactose bile-salt medium at 44–45.5°C	Indole test	Methyl red test	Voges-Proskauer test	Growth in citrate
Escherichia coli					
Type I (typical)	+	+	+	–	–
Type II	–	–	+	–	–
Intermediates					
Type I	–	–	+	–[b]	+
Type II	–	+	+	–[b]	+
Enterobacter[c] *aerogenes*					
Type I	–	–	–	+	+
Type II	–	+	–	+	+
Enterobacter cloacae	–	–	–	+	+
Irregular					
Type I	–	+	+	–	–
Type II	+	–	+	–	–
Type VI	+	–	–	+	+
Irregular, other types		Reactions variable			

a This table is similar to the one given in Ministry of Health ... (1957).
b Weak positive reactions are occasionally found.
c The Judicial Commission of the International Committee on Nomenclature of Bacteria of IAMS has officially substituted the term *Enterobacter* as the generic designation for the genus previously known as *Aerobacter* (International Association of Microbiological Societies, 1963).

1 Incubator, 35–37°C.
2 Water bath for tempering agar, 44–46°C.
3 1-ml bacteriological pipettes with subdivisions of 0.1 ml or less.
4 Inoculating needle, preferably with nichrome or platinum-iridium wire.
5 Tryptone Broth (Medium 126), 5-ml volumes in tubes.
6 Peptone Water (Medium 86), 5-ml volumes in tubes.
7 Buffered Glucose Broth (Medium 21), 10-ml volumes (methyl red test) and 5-ml volumes (Voges-Proskauer test) in tubes.
8 Salt Peptone Glucose Broth (Medium 100), 5-ml volumes in tubes.
9 Koser Citrate Broth (Medium 49), 5-ml volumes in tubes.
10 Simmons Citrate Agar (Medium 106), slants with 2 1/2 cm butts in tubes.

11 Eosin Methylene-Blue Agar (Medium 36), for plates.
12 Endo Agar (Medium 34), for plates.
13 Nutrient Agar (Medium 73), slants in tubes and supply for plates.
14 Lactose Broth (Medium 51), 10-ml volumes in 150 × 15 mm tubes containing inverted Durham fermentation tubes (75 × 10 mm).
15 Indole Reagent (Reagent 19).
16 Methyl Red Solution (Reagent 24).
17 Voges-Proskauer Test Reagents (Reagent 38).
18 Materials for staining smears by Gram's method (Reagent 17).

II PROCEDURE FOR ISOLATION AND PURIFICATION OF CULTURES

1 Streak a loopful of each gas-positive broth tube from either Method 1 (E.C. Broth) or Method 2 (Brilliant Green Lactose Bile Broth 2%) of the section on Determination of Coliform Organisms of Fecal Origin (above) on a separate plate of Eosin Methylene-Blue Agar or of Endo Agar. Incubate plates inverted for 24 hours at 35–37°C.
2 Fish a representative colony (nucleated, with or without metallic sheen) from each plate and streak cells onto a Nutrient Agar plate. Incubate the plate inverted for 24 hours at 35–37°C.
3 Select individual colonies and transfer cells of each to a separate slant of Nutrient Agar and to a tube of Lactose Broth. Incubate cultures 24 hours at 35–37°C.
4 From those cultures producing gas in Lactose Broth, examine a smear stained by Gram's method to confirm the presence of Gram-negative non-spore-forming rods.
5 Use inoculating needle and 24-hour growth on slants of Nutrient Agar to inoculate IMViC media below.

III PROCEDURE FOR INDOLE TEST (Kovacs, 1928)

1 Inoculate tubes of Tryptone Broth or Peptone Water from pure cultures. Incubate tubes at 35–37°C for 24 hours.
2 Add 0.2–0.3 ml of Indole Reagent to each tube and shake.
3 Let tubes stand for 10 minutes and observe results. A dark red color in the amyl alcohol surface layer constitutes a positive test. An orange color indicates the probable presence of skatole and may be reported as a ± reaction.

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IV PROCEDURE FOR METHYL RED TEST (Ljutov, 1961)

1 Inoculate tubes of Buffered Glucose Broth from pure cultures. Incubate tubes at 35–37°C for 5 days.
2 Pipette 5 ml from each culture to a separate empty culture tube and add 5 drops of methyl red solution and shake.
3 Record a distinct red color as methyl red positive, a distinct yellow color as methyl red negative, and a mixed shade as questionable.

V PROCEDURE FOR VOGES-PROSKAUER TEST (Ljutov, 1963; Levine, 1916)

1 Inoculate tubes of either Buffered Glucose Broth or Salt Peptone Glucose Broth from pure cultures and incubate tubes at 35–37°C for 48 hours.
2 Pipette 1 ml of each culture to a separate empty culture tube and add 0.6 ml of naphthol solution and 0.2 ml of potassium hydroxide solution.
3 Shake the tubes, let them stand 2–4 hours, and observe results. Record the development of a pink to crimson color in the mixture as a positive test.

VI PROCEDURE FOR SODIUM CITRATE TEST (Koser, 1923; Simmons, 1926)

1 Inoculate tubes of Koser Citrate Broth or of Simmons Citrate Agar with cells from pure cultures. Use a straight needle and a light inoculum, since transfer of nutrients with the inoculum can invalidate the test. If Simmons Citrate Agar slants are used, stab the butts and streak the surfaces.
2 Incubate Koser Citrate Broth at 35–37°C for 72–96 hours. Incubate Simmons Citrate Agar at 35–37°C for 48 hours.
3 For either medium, record visible growth as a positive reaction and no visible growth as a negative reaction. Growth is usually indicated by a change in the color of the medium from light green to blue.

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ICMSF COLLABORATIVE TESTING OF COLIFORM METHODS

One of the principal aims of the International Commission on Microbiological Specifications for Foods is the evaluation of microbiological methods. The Commission has conducted comparative and collaborative testing programs on coliform methods, the objective being the selection of the 'best' method when more than one has been used for the enumeration of a particular organism or group.

In this chapter three MPN methods for the enumeration of coliforms and two methods for the determination of coliforms of fecal origin have been described. These were selected because each is used by many laboratories throughout the world. At the time of compiling the first edition of this book, the Commission was unable to select the 'best' method, but since then some progress has been made toward this goal.

In the first collaborative study the various methods were tested on eight artificially contaminated foods, using two levels of inoculation with coliforms and fecal coliforms. The samples were analyzed by two technicians in each of 14 or 15 laboratories in Europe, North America, and South America. The foods examined were frozen peas, frozen eggs, ice cream, frozen ground beef, meat meal, dried egg albumen, coconut, and non-fat dried milk. The range in coliform MPN values from the highest mean to the lowest (by laboratory) was from 0.86 log units for ice cream to 3.36 log units for non-fat dried milk. No significant differences were observed among the three presumptive methods for the determination of coliforms, nor were there significant differences between the two recommended methods for determination of fecal coliforms.

A second collaborative study was carried out with naturally contaminated peanut butter, dried buttermilk, and dried egg albumen. Samples were distributed to five laboratories. Two analysts in each laboratory analyzed triplicate aliquots of three subsamples from each master sample. The three recommended presumptive media were used, namely Lauryl Sulphate Tryptose Broth (LST), MacConkey Broth (MAC) and Brilliant Green Lactose Bile Broth 2% (BGLB). All gas-positive tubes were confirmed on Eosin Methylene-Blue Agar. There were no significant differences among the three presumptive media for peanut butter and buttermilk samples, but with egg albumen MAC values were significantly lower than LST or BGLB, indicating a greater sensitivity of MAC broth in this case. The results on confirmed tests indicated no significant differences, however. The 95%

confidence intervals for a single log value were as follows: peanut butter, ±0.88; buttermilk, ±1.03; egg albumen, ±0.87. Thus, despite exhaustive mixing of the samples, the confidence limit on each of the three samples was about two log cycles.

Partition of experimental error (analysis of variance) was done among replicate aliquots, subsamples, and analysts. For all three foods error due to replicate aliquots was greater than either the subsample or analyst error:

	Contribution to experimental error by		
Food	Replicate aliquots	Subsamples	Analysts
Peanut butter	96.0%	2.0%	2.0%
Buttermilk	62.0	11.0	27.0
Egg albumen	78.0	17.0	5.0

In view of the high contribution to experimental error traceable to replicate aliquots, a third collaborative study was carried out with naturally contaminated fluid milk, whole egg, and egg yolk. It was felt that in such samples the distributional error would be less than in dried products. Two technicians in each of four laboratories participated in the study. Each technician received four samples of each food, and each test on a given sample was conducted in duplicate. The test involved analysis using the MPN technique with LST and confirmation of gas-positive tubes in BGLB. Samples were also analyzed by direct plating on Violet-Red Bile Agar (VRB). The precision for a single aliquot on a single subsample for a single analyst within a laboratory on a given medium did not prove to be materially different than was observed on the dried foods:

	95% confidence limits ($\log_{10}$)	
Food	LST (confirmed in BGLB)	VRB
Milk	±1.01	±0.77
Whole egg	±0.77	±0.90
Egg yolk	±0.86	±0.77

Analysis of variance indicated the largest contribution to error could be traced to differences between subsamples within analysts and between aliquots within subsamples.

In summary, collaborative studies carried out by ICMSF have indicated no significant differences among the three methods recommended for detection of coliform organisms by the MPN procedure, or between the two procedures for detecting fecal coliforms. The greatest source of error appears to be variation among aliquots of the same sample. The magnitude of this effect is such that the 95% confidence limit for a given sample frequently exceeds two log cycles. This was found even with samples that were mixed with great care before submission for collaborative testing.

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Enterobacteriaceae

These methods involve enrichment of food samples in Buffered Peptone Water or Tryptone Soya Peptone Broth and detection of oxidase-positive organisms from subsequent growth on 1% Glucose Violet-Red Bile Agar. The methods provide a reasonable estimate of members of the family Enterobacteriaceae present in the food sample. For further discussion of the application of this method refer to Part I, Enteric Indicator Bacteria (p. 8).

PRESENCE OR ABSENCE TEST

I APPARATUS AND MATERIALS

1 Conical flasks, 100 ml.
2 Pipettes: 1-ml bacteriological pipettes with subdivisions of 0.1 ml or less; 10-ml bacteriological pipettes.
3 Water bath or air incubator for tempering agar, 44–46°C.
4 Incubator, 35–37°C.
5 Inoculating needle, preferably with nichrome or platinum-iridium wire.
6 Buffered Peptone Water (Medium 22).
7 Trypticase Soy Broth (Medium 123).
8 Enterobacteriaceae Enrichment Broth (Medium 35).
9 Same, double strength (Medium 35).
10 Violet-Red Bile Glucose Agar (Medium 132), to be pretested for productivity and selectivity.
11 Glucose Salt Medium (Medium 41).
12 Nutrient Agar (Medium 73).
13 Sterile Mineral Oil (Reagent 25).
14 Tetramethylparaphenylenediamine dihydrochloride, 1% aqueous solution.
15 Filter paper, Whatman No. 2, pieces 6 cm square.

II REFERENCE PROCEDURE

1 Prepare food sample by one of the three methods described in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 Pipette 1 ml or 1 g of food and 1 ml of a suitable series of food dilutions into tubes containing 10 ml of Buffered Peptone Water.
3 Mix tubes well and incubate at 35–37°C for 16–20 hours.
4 Mix tubes well and transfer 1 ml of each to tubes containing 10 ml each of Enterobacteriaceae Enrichment Broth. Mix tubes well and incubate at 35–37°C for 20–24 hours.
5 Mix well tubes showing turbidity due to bacterial proliferation, and streak onto plates of Violet-Red Bile Glucose Agar. Incubate at 35–37°C for 20–24 hours.
6 Colonies surrounded by a purple zone are considered tentatively to be Enterobacteriaceae.
7 Streak at least two typical colonies onto slants of Nutrient Agar, and stab them into tubes of Glucose Salt Medium; then cover the latter with Sterile Mineral Oil. Incubate at 35–37°C for 20–24 hours and carry out an Oxidase Test with the growth obtained on the slants.
8 *Oxidase Test* (Kovacs, 1956; J.E. Blair et al., 1970)
(a) Place a 6-cm-square piece of filter paper into an empty Petri dish and add 3 drops of tetramethylparaphenylenediamine dihydrochloride solution to its center.
(b) With an inoculating loop smear cells thoroughly onto the reagent-impregnated paper in a line 3–6 mm long. The Oxidase Test is positive if transferred cells turn dark purple in 5–10 seconds. If the outcome is negative while the Glucose Salt Agar has turned yellow, the presence of Enterobacteriaceae is proved.

III ROUTINE PROCEDURE

1 Prepare food sample by one of the three methods described in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 Pipette 1 ml or 1 g of food and 1 ml of a suitable series of food dilutions to 100-ml flasks containing 10 ml Trypticase Soy Broth. Incubate at 20–25°C for 2 hours, shaking the flasks every 15 minutes for 30 seconds. Add 10 ml double-strength Enterobacteriaceae Enrichment Broth. Mix flasks well and incubate at 35–37°C for 20–24 hours.

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3 Mix well flasks showing turbidity due to bacterial proliferation, and streak onto plates of Violet-Red Bile Glucose Agar. Incubate at 35–37° C for 20–24 hours.
4 Colonies surrounded by a purple zone of growth are considered tentatively to be Enterobacteriaceae.
5 Streak at least two typical colonies onto slants of Nutrient Agar, and stab them into tubes of Glucose Salt Medium; then cover the latter with Sterile Mineral Oil. Incubate at 35–37° C for 20–24 hours and carry out an Oxidase Test (above) with the growth obtained on the slants. If the outcome is negative while the Glucose Salt Agar has turned yellow, the presence of Enterobacteriaceae is proved.

ENUMERATION BY PLATE COUNT

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
3 1-, 5-, and 10-ml bacteriological pipettes, sterile.
4 Water Bath or air incubator for tempering agar, 44–46° C.
5 Incubator, 35–37° C.
6 Colony counter (Quebec dark field model or equivalent recommended).
7 Tally register.
8 Violet-Red Bile Glucose Agar (Medium 132) to be pretested for productivity and selectivity.
9 Glucose Salt Medium (Medium 41).
10 Nutrient Agar (Medium 73).
11 Tetramethylparaphenylenediamine dihydrochloride, 1% aqueous solution.
12 Filter paper, Whatman No. 2, pieces 6 cm square.

II PROCEDURE

1 Prepare food sample by one of the three procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 To duplicate sets of Petri dishes, pipette 1-ml aliquots from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. This series is suggested in

cases where the approximate range of bacterial numbers in the specimen is not known by experience. The level of dilution prepared and plated can be varied to fit the expected Enterobacteriaceae count.
3 Promptly pour into the Petri dishes 10–15 ml of Violet-Red Bile Glucose Agar, melted and tempered to 44–46° C.
4 Immediately mix aliquots with the agar medium by (a) tilting dish to and fro 5 times in one direction, (b) rotating it clockwise 5 times, (c) tilting it to and fro again 5 times in a direction at right angles to that used the first time, and (d) rotating it counterclockwise 5 times.
5 After solidification, pour a cover layer of approximately 10 ml of Violet-Red Bile Glucose Agar, tempered to 44–46° C, over all plate contents.
6 After the cover layer has solidified, invert the Petri dishes and incubate them at 35–37° C for 21 ± 3 hours.
7 Using the colony counter and tally register, count all purple colonies on plates containing 30–300 colonies.
8 Compute the number of presumptive colony-forming units of Enterobacteriaceae per gram of specimen.

III CONFIRMATION STEP

1 Streak at least two typical colonies onto plates of Violet-Red Bile Glucose Agar. Incubate at 35–37° C for 20–24 hours.
2 Streak at least two colonies surrounded by a purple precipitation zone onto slants of Nutrient Agar and also stab them into tubes of Glucose Salt Medium and cover the latter with Sterile Mineral Oil. Incubate at 35–37° C for 20–24 hours.
3 Carry out an Oxidase Test (p. 141) with the growth obtained on the slants. If the outcome is negative while the Glucose Salt Agar has turned to yellow, consider the examined colony as belonging to the Enterobacteriaceae.

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The enterococci

Among the streptococci, Lancefield's group D is composed of four species, namely, *Streptococcus faecalis*, *S. faecium*, *S. bovis*, and *S. equinus*. A fifth species, *S. avium*, possesses both the groups Q and D antigens (Nowlan and Deibel, 1967) and is physiologically similar to the group D streptococci.

Following its introduction by Thiercelin in 1899, the term *enterococcus* has received widespread usage among bacteriologists. Sherman (1937) classified the streptococci into four divisions according to their physiological and growth characteristics, as well as tolerance tests. One of the four divisions he established was the 'enterococcus' division, consisting of four species: *S. faecalis*, *S. liquefaciens*, *S. zymogenes*, and *S. durans*. The name used to designate this division, as employed by Sherman, was intended to denote a specific taxon without regard to habitat or source. In addition, Sherman recognized that all members of his enterococcus division belonged to Lancefield's serological group D.

Subsequent to Sherman's classical review of the streptococci, numerous investigators contributed toward a clearer understanding of the species that comprise the enterococci. Ultimately, the species were reordered into a more logical taxonomic scheme that comprised only two species and their respective varieties, namely, *S. faecalis* and *S. faecium* (see Deibel, 1964). This revised system of speciation in no way altered the taxonomic limits of the enterococci as designated by Sherman. The criteria for their identification as a specific taxonomic entity were retained.

Sherman (1938) noted that many streptococci classified as *S. bovis* and *S. equinus* cross-reacted with Lancefield's group D. Subsequent investigations (Shattock, 1949; D.G. Smith and Shattock, 1962) provided definitive evidence that these two species did, indeed, contain the group D antigen. As a result, these two species are now included among the group D streptococci, even though they differ in many respects from the other group D streptococci. They actually reside in Sherman's 'viridans' division, not in his enterococcus division.

The group D streptococci are found in the intestinal tract of warm-blooded animals. As a result of this common source or habitat, numerous investigators choose to include *S. bovis* and *S. equinus* among the enterococci. In other words, the term would become synonymous with the group D streptococci. As employed throughout this book, however, enterococcus refers only to the species *S. faecalis* and *S. faecium* and their varieties.

The term *fecal streptococcus* is used commonly in the literature but because it is so loosely defined it has largely lost its value for purposes of communication. When using this term some investigators refer only to the enterococci, while others would use the term to include any streptococcus that might be found in the intestine. More commonly, however, it is intended to be a vernacular term for all group D streptococci.

In some instances, the enterococci may be enumerated in food as an aid to estimating the sanitary conditions under which it was produced. However, a great deal of interpretation of the results is required for the various foods that may be examined. Although their habitat is considered to be the intestine, it is known that the enterococci can establish themselves in an epiphytic relationship among some plants (Mundt, 1970).

Many foods are expected to contain enterococci as part of their normal flora. Fermented foods such as cheeses and fermented sausages may contain very large numbers and yet be considered wholesome.

It is difficult to establish an acceptable level of enterococci in foods because they normally vary with the type of food, temperature of storage, time of holding, and other factors. In contrast to other streptococci, the enterococci are versatile with respect to temperature limits of growth, thermal tolerance, and tolerance to various chemicals and antibiotics. They survive times and temperatures employed to pasteurize milk, and some strains may grow at temperatures as low as 2°C. Their ability to survive in frozen foods or water is superior to that of the coliform bacteria.

The enterococci are occasionally associated with disease, although generally they are not considered to be pathogenic. More appropriately they could be considered as 'opportunists' and therefore are found associated with numerous low-grade infections. Their alleged ability to cause food-poisoning is discussed elsewhere in this book (pp. 41–2).

Numerous attempts have been made to devise selective or differential media for the quantitative determination of enterococci in

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foods. At least 30 such media have been used (Hartman et al., 1966) with varying degrees of success. Most such media capitalize on the relative tolerance of enterococci toward an adverse environment, and employ a chemical such as azide to inhibit other bacterial genera plus a second inhibitory substance or indicator to aid in distinguishing them from other streptococcus species. Most of the media and methods employed thus far lack selectivity or differential ability. Members of the genera *Lactobacillus*, *Pediococcus*, *Aerococcus*, and *Leuconostoc* frequently grow on such media. Quantitative recovery is difficult to achieve because of inherent differences among strains. Also, most foods to be examined may have been treated in a manner so as to injure the surviving cells (Emberger and Pavlova, 1971 a, b). Resuscitation is necessary if quantitative recovery is to be approached.

Two common plating media employed for enumerating the enterococci in foods are Packer's Crystal-Violet Azide Blood Agar (Packer, 1943) and KF Streptococcus Agar (Kenner et al., 1961). The KF Agar appears to be more selective and differential than Packer's medium, and is commercially available. On both media the enterococcus colonies are recognized by their characteristic size and coloration. Confirmation tests are performed on isolated cultures.

ENUMERATION OF PRESUMPTIVE ENTEROCOCCI

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
3 1-ml bacteriological pipettes.
4 Water bath or air incubator for tempering agar, 44–46°C.
5 Incubator, 35–37°C.
6 Colony counter (Quebec dark field or equivalent type recommended).
7 Tally register.
8 Packer's Crystal-Violet Azide Blood Agar (Medium 80), or KF Streptococcus Agar (Medium 48).

II PROCEDURE

1 Prepare food samples by one of the two methods described in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11), using the same techniques of dilution. Materials remaining from dilution blanks employed in the determination of the plate count can be used.
2 To duplicate sets of Petri dishes, pipette 1-ml aliquots of each of the decimal dilutions of the food homogenate.
3 Promptly add to each dish 15 ml of Packer's Crystal-Violet Azide Blood Agar or KF Streptococcus Agar, melted and tempered to 44–46°C.
4 Mix the samples and agar by rotating and tilting the dishes.
5 After the agar has solidified, incubate the plates inverted at 35–37°C for 72 hours (Packer's) or 48 hours (KF).
6 Using the colony counter and tally register, count all the small violet-colored colonies on the Packer's plates containing 30–300 colonies, or count all deep red (*S. faecalis*) and light pink (*S. faecium*) colonies on the KF plates containing 30–300 colonies.
7 Compute the number of presumptive enterococci per gram of food specimen.

CONFIRMATION OF ENTEROCOCCI

I APPARATUS AND MATERIALS

1 Incubator, 35–37°C.
2 Water bath, 44–46°C.
3 Inoculating needle, preferably with nichrome or platinum-iridium wire.
4 Brain Heart Infusion Broth (Medium 14).
5 Brain Heart Infusion Broth with added 6.0% (total 6.5%) sodium chloride (Medium 15).
6 Hydrogen peroxide, 3% aqueous solution.
7 Reagents for Gram stain (Reagent 17).

II PROCEDURE

1 Select 5 to 10 purple colonies from the Packer's plates or an equal number of red and/or light pink colonies from the KF plates. Pick individually into tubes of Brain Heart Infusion Broth and incubate at 35–37°C for 18–24 hours, or until turbidity appears.
2 Prepare Gram stains of each of the cultures and observe for typical Gram-positive, oval cocci, in pairs or short chains.

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3 Remove aseptically about 3 ml of each of the *Streptococcus* cultures and mix in another tube with about 0.5 ml of 3% hydrogen peroxide. Failure of bubbles to appear (catalase-negative) further confirms that the culture is a *Streptococcus.*

4 Inoculate one tube each of Brain Heart Infusion Broth with the confirmed *Streptococcus* isolates. Temper the tubes in a 44–46°C water bath and incubate at 44–46°C up to 48 hours, and look for growth.

5 Inoculate one tube each of Brain Heart Infusion Broth containing 6.5% NaCl with the confirmed *Streptococcus* isolates. Incubate at 35–37°C for 72 hours and look for growth.

6 A catalase-negative *Streptococcus* that grows at 44–46°C and in the presence of 6.5% NaCl confirms that the culture is an enterococcus.

IDENTIFICATION OF SPECIES

If necessary, the isolates can be identified as to species by conducting the tests outlined by Deibel and Seeley (1974). In addition, proof that the cultures belong to serological group D can be obtained by preparing extracts and testing with group D serum. The procedure to be followed for serological identification is described in the following chapter.

Hemolytic streptococci

Routine examination of foods for hemolytic streptococci is unwarranted, except perhaps for raw milk, which a few consumers still demand. However, special epidemiologic investigations of disease outbreaks may require extensive sampling and analysis of suspect foods.

Hemolytic streptococci, especially group A, are frequently found in the throats of apparently healthy individuals. Following infection, group A streptococci may persist in the throat, even in large numbers, for days or weeks. Their occurrence is so common that both laboratory examination of throat cultures and a study of clinical symptoms are necessary for accurate diagnosis of a streptococcal infection. Routine examination of food-processing plant personnel for the presence of hemolytic streptococci would be fruitless as a control measure, although their common occurrence in the throat provides a continuing source for possible food contamination.

Primarily, interest lies in the examination of suspect foods for group A hemolytic streptococci. Detection of this group in food in any quantity should be cause for serious concern. The other hemolytic streptococci are of considerably less significance to human health, but their presence may indicate an inadequate thermal process for the food, or subsequent contamination by humans. The enterococci may, however, survive milk pasteurization.

No satisfactory selective medium exists for the quantitative estimation of hemolytic streptococci in foods, particularly when they are present as a minority percentage of the overall flora. Blood agar (sheep or horse) serves as a differential medium, but it is not selective. Therefore, hemolytic streptococci in relatively small numbers may be completely undetectable because of overgrowth by other bacteria, particularly by those that grow rapidly and produce large colonies. Nevertheless, blood agar serves reasonably well for examining raw milk or similar dairy foods in which the predominant flora is most likely to be streptococci and other lactic acid bacteria that characteristically produce punctiform colonies. Hemolytic streptococci numbering as few as one percent of the flora can generally be

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detected and enumerated in milk. Blood agar also serves well to isolate causative organisms, including streptococci, from local abscesses and other suppurative material in raw meats.

Various attempts have been made to devise more selective media for the detection and enumeration of the pathogenic streptococci. Reasonable success has been achieved for the more hardy group D streptococci (see p. 144), but the other hemolytic streptococci generally are much more delicate and fastidious than the competing flora. Sodium azide in appropriate concentrations favors selective growth of the lactic acid bacteria and the pathogenic streptococci, but the direct addition of this chemical to blood agar destroys the differential value of blood agar.

Pike (1945) devised a selective enrichment broth for the qualitative detection of hemolytic streptococci. The medium, containing sodium azide and crystal violet as selective inhibitory agents, does favor hemolytic streptococci, although it appears to be more inhibitory toward the group A streptococci than the other serological groups. Since the primary interest, in most instances, is in the detection of group A streptococci, this medium has limited utility.

For the detection of group B streptococci in milk, Hauge and Ellingsen (1953) devised a blood agar medium which they termed T.K.T. (Thallium Sulfate - Crystal Violet-Toxin Blood Agar) and which depended upon the so-called CAMP reaction. The medium contains a titrated quantity of β-toxin from a *Staphylococcus aureus* culture. The toxin accentuates the hemolytic reaction of the group B streptococci and facilitates detection as well as presumptive identification. It is claimed that the T.K.T. medium, used as both an agar and broth, has proved to be of distinct value in detecting not only group B streptococci but other hemolytic streptococci as well.

The classic CAMP-esculin test (Slanetz et al., 1969) has been commonly used in the bovine mastitis control program but has received limited acceptance in clinical microbiology. Aside from the CAMP reaction the test relies upon the inability of the group B streptococci to hydrolyze esculin. This characteristic, along with the ability to hydrolyze sodium hippurate, is an exceptional combination among the streptococci.

Anaerobic incubation of the blood agar plates, especially in an atmosphere of 90% nitrogen and 10% carbon dioxide, increases the likelihood of detecting hemolytic streptococci, primarily by retarding the growth of competing aerobic or facultatively anaerobic microorganisms. Streptococci form punctiform colonies because they obtain energy for growth by fermentation, which is a relatively inefficient energy-yielding process. They grow equally well aerobically or anaerobically. Incubation under a high carbon dioxide tension enhances the growth rate, and therefore detection, of some streptococci, such as the 'minute streptococci' (group F; type 1, group G).

A few group A strains fail to produce streptolysin S; thus their surface colonies may fail to show the hemolytic reaction because of inactivation of the remaining oxygen-labile streptolysin O. Anaerobic incubation suppresses the greening reaction (alpha) of the viridans-type streptococci, although the green zones will appear shortly after the plates are exposed to the air. All in all, anaerobic incubation in a high carbon dioxide tension appears to have merit if the intent is to detect the greatest number of streptococci having hemolytic potential (Fry, 1933).

The nature of the Blood Agar Basal Medium is important. Historically, a beef infusion agar fortified with peptone or tryptone plus 0.5% sodium chloride, pH 7.2–7.4, has been employed. Commercially prepared infusion agar media, such as Trypticase Soy or Neopeptone, are now commonly used. No fermentable sugar should be added; as little as 0.05% will tend to destroy the differential value of the blood agar. This fact places a limitation upon the minimal dilutions of foods containing sugar that can be examined. For group A streptococci neopeptone-containing agar is generally preferred because it contains an inhibitor to a protease produced by some group A strains which destroys the type-specific M antigen.

In his original description and designation of the blood agar reactions by different streptococci, Brown (1919) employed horse blood, and used the pour plate technique in order to obtain isolated embedded colonies. Horse blood is still commonly used, especially in England, although sheep blood is gaining more widespread acceptance among clinical microbiologists. Sheep blood is inhibitory to *Haemophilus haemolyticus*, an organism commonly present in the human throat and easily confused with beta-hemolytic streptococci. Rabbit and human blood can also be used although some believe that the possible presence of antistreptolysin O in human blood might interfere with the detection of those streptococci that elaborate this lysin (groups A, C, G). Updyke (1957) reported that 88% of the hemolytic group D strains that she tested gave an alpha reaction in sheep blood agar, but were beta-hemolytic in rabbit, horse, and human blood agar.

The pour plate technique as originally employed by Brown aids in the recognition of beta-hemolytic colonies. The perpendicular radius of the hemolytic zone from a surface colony may not be sufficiently deep to penetrate the entire thickness of the blood agar, thus

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partially masking the hemolysis when viewed with transmitted light. Nevertheless, the 'drop' plate technique gives reasonably satisfactory results. For convenience the clinical microbiologist generally relies upon streaking specimens, e.g. throat swabs, onto the surface of pre-poured blood agar plates. The streaking should produce isolated colonies; also, stabbing the agar several times over the inoculated surface facilitates detection of beta-hemolysis. The characteristics of surface colonies aid in the identification of the strain. A low-power microscope or hand lens is most helpful in examining the individual colonies and hemolytic zones. The virulent group A *Streptococcus* produces rather characteristic surface colonies – either mucoid or 'matt' (dehydrated mucoid) – as a result of its synthesis of hyaluronic acid and the type-specific M antigen.

If isolation and identification of the hemolytic colonies are required, the colonies should be picked into a suitable broth medium. Trypticase soy, brain-heart infusion, or other commercially prepared broths are satisfactory for this purpose. For preparing extracts for serological group identification, any medium that supports excellent growth will suffice. If M typing of group A cultures is desired, cultures for preparing extracts should be grown in a neopeptone-containing medium or other suitable medium (e.g. Todd-Hewitt Broth) that will retard autolytic destruction of the M antigen.

Extracts for precipitin tests to determine the serological group, or M type within group A, are generally prepared by the acid-heat method (Lancefield, 1933; Swift et al., 1943). Alternatively, extracts for group identification may be prepared by Fuller's formamide method (Fuller, 1938), by lysis with phage (Maxted, 1957), by digestion with an enzyme from *Streptomyces albus* (except for groups D and K) (Maxted, 1948), or by simple autoclaving (Rantz and Randall, 1955). The fluorescent antibody technique for the identification of group A streptococci (Moody et al., 1963) is gaining increasing acceptance in the diagnostic laboratory.

Testing for bacitracin sensitivity identifies group A streptococci presumptively with approximately 90% accuracy (Maxted, 1953). A broad zone of growth inhibition indicates that the *Streptococcus* is group A. Control cultures confirm the validity of the test. Over 90% of the group A streptococci show growth inhibition, while only a small percentage of non-group A streptococci are sensitive.

In some instances, cultural and physiological characteristics help identify pure cultures of hemolytic streptococci. Refer to Deibel and Seeley (1974) for such procedures. Other helpful references are

R.E.O. Williams (1958), Bodily et al. (1970), and Wannamaker and Matsen (1972).

Some other organisms hemolyze blood, and may confuse the inexperienced. *Staphylococcus aureus*, alpha-type streptococci, Gram-negative cocci, and *Haemophilus haemolyticus* frequently do so, and numerous other species of *Escherichia*, *Clostridium*, or *Bacillus* may at times yield hemolytic colonies. Colony appearance, Gram stain, and a few simple tests on the isolated cultures generally will suffice to eliminate these microorganisms.

ENUMERATION OF PRESUMPTIVE HEMOLYTIC STREPTOCOCCI

I APPARATUS AND MATERIALS

1 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
2 1-ml bacteriological pipettes.
3 Water bath or air incubator for tempering agar, 44–46°C.
4 Aerobic and anaerobic (90% N_2, 10% CO_2) incubators or jars, 35–37°C.
5 Colony counter (Quebec or similar), and low-power microscope or hand lens.
6 Blood Agar Basal Medium (Medium 45, Formula 2), for plates.
7 Sterile defibrinated sheep blood (preferably) or horse blood.

II PROCEDURE

1 Prepare food samples by one of the three methods described in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11). Materials remaining from dilution blanks employed in the determination of plate count can be used.
2 To duplicate sets of Petri dishes, pipette 1-ml aliquots of each of the decimal dilutions of the food.
3 Add 5 ml of defibrinated sheep blood (or horse blood) to each 100 ml of melted blood agar base tempered to 44–46°C. Agitate without incorporation of air bubbles.
4 Add 15 ml (not over 20 ml) of the blood agar to each of the Petri dishes. Mix the dilutions and agar promptly by rotating and tilting the dishes.

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5 After the agar has solidified, invert the plates and incubate one set aerobically at 35–37°C for 24 hours, and the other in an atmosphere of 90% N_2 and 10% CO_2 at 35–37°C for 24 hours.
6 Count punctiform colonies having beta-hemolytic zones. Examine colonies under a hand lens or low-power microscope to confirm beta-hemolysis. Reincubate for another 24 hours under the respective atmospheres and count again.

CONFIRMATION OF HEMOLYTIC STREPTOCOCCI

I APPARATUS AND MATERIALS

1 Incubators, 35–37°C: one aerobic, one anaerobic (90% N_2, 10% CO_2).
2 Inoculating needle, preferably nichrome or platinum-iridium wire.
3 Brain Heart Infusion Broth, tubes (Medium 14).
4 Brain Heart Infusion Agar, slants (Medium 12).
5 Prepoured Sheep Blood Agar, plates (Medium 105).
6 Filter paper disks each containing 0.02 unit bacitracin.[1]
7 Pure culture of known group A *Streptococcus*, as well as another serological group, e.g. group B.
8 Hydrogen peroxide solution, 3%.
9 Reagents for Gram stain (Reagent 17).

II PROCEDURE

1 Pick suspicious and representative colonies (up to 10 per sample depending on numbers present on plates) from Blood Agar plates (aerobic and anaerobic), after either or both incubation periods, into tubes of Brain Heart Infusion Broth. Incubate tubes at 35–37°C for 24 hours, or until distinct turbidity appears.
2 Prepare Gram stains from each of the cultures and eliminate those that are not Gram-positive streptococci.
3 From each of those cultures appearing to be streptococci, streak duplicate prepoured Sheep Blood Agar plates in a manner to obtain isolated colonies. Drop a bacitracin disk onto each plate in an area where moderate growth is expected. Incubate the duplicate plates, one aerobically, the other anaerobically, for 24 hours. Observe plates for apparent purity of culture. If not pure, reinoculate colonies into Brain Heart Infusion Broth and restreak. Colonies with a broad zone of growth inhibition surrounding the bacitracin disk may be group A *Streptococcus*.
4 From each of those cultures appearing to be streptococci, inoculate Brain Heart Infusion slants and incubate 24 hours at 35–37°C. Observe nature of growth and confirm growth as typical of *Streptococcus*. Add 1 to 2 ml of 3% hydrogen peroxide to the tube. If no bubbles appear, the culture is confirmed as a *Streptococcus* (negative catalase test).

1 Available from Difco Laboratories, Detroit, Michigan, or other commercial sources. Store the sterile disks below 8°C.

IDENTIFICATION OF SPECIES

I APPARATUS AND MATERIALS

1 Vaccine capillary tubes, both ends open and lightly fire polished; OD, 1.2–1.5 mm; length, about 140 mm.
2 Centrifuge.
3 Boiling water bath with racks.
4 Brain Heart Infusion Broth (Medium 14), 30-ml in 40-ml centrifuge tubes.
5 Metacresol Purple Indicator Solution (Reagent 23).
6 *N*/5 HCl in 0.85% NaCl.
7 *N*/5 NaOH in distilled water.
8 Group-specific antisera (commercially available).
9 1:500 merthiolate solution.

II PROCEDURE FOR EXTRACT PREPARATION

1 Inoculate centrifuge tubes containing Brain Heart Infusion Broth from each of the pure cultures confirmed to be hemolytic streptococci. Incubate 24 hours at 35–37°C, or until excellent growth appears.
2 Centrifuge the tubes at about 2000 rpm for 30 minutes, or until cells are well sedimented. Decant and discard supernatant liquid. *Use care! This material may be infectious.*
3 Add 1 drop each of the Metacresol Purple Indicator and 0.3 ml *N*/5 HCl in 0.85% NaCl to the cells. Mix and transfer to a 15-ml conical centrifuge tube. Make certain that the suspension has a distinct pink color. If not, add another drop of HCl.

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4 Place in a boiling water bath for 10 minutes with intermittent agitation.
5 Cool, then centrifuge for another 30 minutes or until the supernatant liquid is reasonably clear.
6 Decant supernatant liquid to another small centrifuge tube. Neutralize to pH 7.4–7.8 (slightly purple indicator) by adding *N*/5 NaOH dropwise. Do not add an excess.
7 Centrifuge another 30 minutes or until supernatant liquid is crystal clear. Decant carefully into a capped vial and store refrigerated. Preserve the extract with one drop of 1:500 merthiolate solution if necessary.

III PROCEDURE FOR SEROLOGICAL TEST

1 Dip one capillary tube into each grouping serum until about a 1-cm column is drawn into the capillary. Wipe outside clean with facial tissue, but do not permit air to enter the end containing the serum.
2 Dip each of the capillaries into the extract to be tested and allow about a 1-cm column to be drawn in under the serum. If an air bubble separates the two fluids, discard and repeat.
3 Carefully wipe outside of capillary and plunge the end containing the fluids into plasticine or similar putty material to plug the end. Invert and insert other end into a plasticine-containing rack to hold the capillary in an inverted position.
4 Observe for up to 30 minutes for precipitate formation at the interface of the two fluids. Strongly positive reactions may appear almost immediately. A positive precipitin test indicates that the *Streptococcus* from which the extract was prepared is a member of that serological group. Test known control cultures to determine relative potencies of the sera and to avoid false readings. To identify the serotype, repeat, using properly absorbed typing sera. If typing sera are not available, send the culture or extract to a central laboratory that is equipped to conduct the test. Ordinarily, serotyping is not necessary except for epidemiological investigations or other research projects.

The enumeration of yeasts and molds

All molds and yeasts grow well at pH values of 5.0 and lower and thus generally outgrow bacteria in acid foods. In addition, many molds and some yeasts tolerate low a_w values (below approximately 0.95) much better than the majority of bacteria; indeed at values below 0.75 some yeasts and molds are the only organisms that can grow. Thus the spoilage agents for a substantial number of foods are yeasts and/or molds.

Waksman (1922) suggested the use of acidified media for the enumeration of molds in soil, and for approximately the next 30 years food microbiologists adopted this technique for counting yeasts and molds in foods. However, it eventually became clear that the use of low-pH media for this purpose has two distinct shortcomings: (1) many bacteria will grow at low pH value, even below 4 (Mossel et al., 1962; Koburger, 1970); and (2) some molds and yeasts grow poorly at pH values of many of the media that have been suggested for use (Holwerda, 1952; Mossel et al., 1962; Ingram, 1959; Koburger, 1970, 1971, 1972, 1973; B. Jarvis, 1973), especially when the cells have been recently stressed (Nelson, 1972). Hence low-pH media are not entirely satisfactory for enumerating yeasts and molds.

Over the past 20 years antibiotics have been used effectively, in place of low pH, to suppress the growth of bacteria in media developed for enumerating yeasts and molds. Various antibiotics have been found useful, for example, penicillin + streptomycin, chloramphenicol, chlortetracycline, oxytetracycline, and gentamicin (W.B. Cooke, 1954; Beech and Carr, 1955; Flannigan, 1973). For general purposes, the broad spectrum antibiotic oxytetracycline has been useful (Mossel et al., 1962; Put, 1964; Saincliver and Roblot, 1966), and is preferred over chlortetracycline because of its greater stability. Gentamicin suppresses Gram-negative bacteria, and is therefore effective in such foods as chilled meats (Hup and Stadhouders, 1972; Mossel et al., 1970, 1975). Rose bengal will retard the growth of molds which normally form an abundance of aerial mycelia, such as *Neurospora* and *Rhizopus* (B. Jarvis, 1973). However, it also inhibits some

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yeasts (Mossel et al., 1975). The best incubation temperature is about 22°C (Mossel et al., 1962; Koburger, 1970, 1972; Flannigan, 1973), and the most suitable incubation period is about 5 days.

Mold colonies appear either from strands of hyphae or from spores. Therefore a heavily spored mold thallus will give a very much higher plate count than one of similar size without spores. For a method to measure the actual amount of vegetative hyphal growth, use the Howard Mold Count (AOAC, 1975) instead of the Pour Plate method that follows.

THE POUR PLATE YEAST AND MOLD COUNT METHOD

I APPARATUS AND MATERIALS

1 Requirements for Preparation and Dilution of the Food Homogenate (pp. 106–11).
2 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm).
3 1-, 5-, and 10-ml bacteriological pipettes.
4 Water bath or air incubator for tempering agar, 44–46°C.
5 Incubator, 20–24°C.
6 Colony counter (Quebec dark field model or equivalent recommended).
7 Tally register.
8 Oxytetracycline Gentamicin Yeast Extract Glucose (OGY) Agar (Medium 79).

II PROCEDURE

1 Prepare food sample by one of the three procedures recommended in the chapter on Preparation and Dilution of the Food Homogenate (pp. 106–11). (*Caution*: Keep delays minimal to avoid growth or death of microorganisms suspended in the diluent.)
2 To duplicate sets of Petri dishes, pipette 1-ml aliquots from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions. This series is suggested in cases where the approximate range of colony-forming units in the sample is not known by experience. The dilutions prepared and plated can be varied to fit the expected colony count.
3 Promptly pour into the Petri dishes 10–15 ml of OGY Agar, melted and tempered to 44–46°C.

4 Immediately mix aliquots with the agar medium by (a) tilting dish to and fro 5 times in one direction, (b) rotating it clockwise 5 times, (c) tilting it to and fro again 5 times in a direction at right angles to that used the first time, and (d) rotating it counterclockwise 5 times.
5 After the agar has solidified, invert the Petri dishes and incubate them at 20–24°C for 3–5 days.
6 Using the colony counter and tally register, count all colonies on plates containing 30–300 colonies, making microscopic examination where required to identify yeast colonies.
7 Compute the number of yeasts and molds per gram or ml of food.

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Salmonellae

All salmonellae should be considered as potential pathogens of man. The oral route is virtually the exclusive means of entry of these organisms into the body; therefore, the examination of foods for their presence is of great importance. The procedures recommended here are based upon the experience of the members of the Commission as well as other competent workers. However, no method has yet been developed which can be guaranteed to recover all *Salmonella* serotypes under the diverse conditions presented by food products.

Methods for the isolation and identification of salmonellae in foods may consist of a sequence of six stages:

1 Non-selective enrichment.
2 Selective enrichment.
3 Plating on selective and differential agar media.
4 Screening of suspect colonies on media revealing key biochemical characteristics of the organisms.
5 Antigenic analysis in two steps: (a) the use of polyvalent O and polyvalent H antisera, and (b) the use of group-specific O and H antisera.
6 Typing by means of bacteriophage.

Stages 1–3 are for isolation, and 4–6 for identification. Step 1 is for the purpose of rejuvenating salmonellae injured by processing or storage conditions. The second step is selective enrichment, which favors the growth of salmonellae in an environment which may contain large numbers of bacteria other than *Salmonella*. Step 3 permits the 'sorting' of suspect salmonellae, based upon biochemical characteristics. The fourth step permits further screening, based upon biochemical attributes. Step 5, through serological tests, leads to the ultimate identification of the suspect isolates as a member of the genus *Salmonella*. Step 6 encompasses even more refined identification of the isolate, such identification being of particular value for epidemiological purposes, because serological types of a common

bacteriophage pattern are likely to have a common origin. Conversely, isolates showing the same antigenic structure but dissimilar bacteriophage patterns are probably not of common origin.

In the past, non-selective enrichment has been used only when it was believed that a process, such as heating, drying, irradiation, or freezing, or a condition such as low pH, had caused 'injury' to salmonellae in food products. Recently Edel and Kampelmacher (1973) and Gabis and Silliker (1974a) have reported that even with food samples such as raw meats and liquid eggs pre-enrichment of the sample in a non-selective medium results in more *Salmonella* isolations than direct enrichment in selective media. At this time, based upon these findings, the Commission recommends that all samples be pre-enriched in a non-selective medium prior to selective enrichment.

Non-selective enrichment has traditionally involved culturing samples in Lactose Broth. This practice was initiated by North (1961) for the analysis of dried egg albumen. Subsequently various workers have explored modifications in the composition of the pre-enrichment medium, and have found that the medium is not critical. For example, European workers commonly employed Buffered Peptone Water in place of Lactose Broth (Edel and Kampelmacher, 1968). In some foods, such as dried yeast, the composition of the sample required only its reconstitution in distilled water. With non-fat dry milk, the sample is reconstituted in distilled water containing Brilliant Green dye. Candy and candy-coating are reconstituted in non-fat dry milk containing Brilliant Green dye. With high-fat foods, such as rendered animal by-products, the inclusion of Tergitol 7 facilitates dispersion of the fat in the non-selective medium. In general, Lactose Broth and Buffered Peptone Water are interchangeable. Table 8 is a partial summary of the pre-enrichment media employed.

A selective enrichment broth encourages the multiplication of salmonellae and reduces or inhibits growth of competitive organisms, such as coliforms, *Proteus*, and *Pseudomonas*, which might otherwise outgrow salmonellae, particularly if the ratio of competitors to salmonellae is high. The use of more than one *Salmonella* selective broth will increase recovery, and further, the temperature of incubation may profoundly affect the rate of *Salmonella* recovery. Edel and Kampelmacher (1968) reported that with incubation of selective broths at 43°C more *Salmonella*-containing meat samples were found than with incubation at 37°C, particularly in samples containing a high level of competing organisms. Silliker and Gabis (1974) confirmed these results. Since it is necessary to restrict the number of selective variables, the Commission recommends the use of Selenite

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TABLE 8
Pre-enrichment procedures for salmonellae

Product	Pre-enrichment broth
Dried whole eggs, dried egg yolks, dried egg whites, pasteurized liquid and frozen eggs, prepared powdered mixes, biscuit, infant formula, raw eggs, raw meats, egg-containing pasta, dyes and colouring substances	Lactose Broth or Buffered Peptone Water (medium 51 or 22, respectively)
Dried yeast (inactive)	Distilled water
Non-fat dry milk and dry whole milk	Distilled water containing 2 ml Brilliant Green Solution 1% (Reagent 5) per liter[a]
Candy and candy coating	Reconstituted dry milk with Brilliant Green (Medium 95)
Rendered animal by-products, coconut	Lactose Broth with 1% Tergitol (Medium 52) or Buffered Peptone Water with 0.22% Tergitol (Medium 23)

a Aseptically weigh 100 g of sample into sterile 2-liter flask (or wide-mouth screw-cap 2-qt jar). Add 1 liter of sterile distilled water and mix well. Determine pH with test paper (if pH is < 6.6, adjust to 6.8 ± 0.2 with 1*N* NaOH). Add Brilliant Green Solution, and mix well. Incubate as instructed in the text.

Cystine Broth and Tetrathionate Brilliant Green Broth, both incubated at 43°C.

Selective agars usually contain inhibitors that render the media selective and an indicator system that imparts colors to the colonies or the surrounding medium, which make such media differential, i.e., capable of segregating biochemical characteristics of colonies growing on the medium. Laboratories have preferences for specific differential media; collaborative tests have shown that laboratories have greatest success with those media with which they are most familiar. Accordingly, the Commission recommends that each laboratory use one differential medium of its choice but that in addition it use Bismuth Sulfite Agar and Brilliant Green Agar. Among other media which might be selected are Brilliant Green Sulfadiazine Agar, Brilliant Green MacConkey Agar, Desoxycholate Citrate Agar, *Salmonella-Shigella* Agar, or other differential and selective media of the laboratory's choice.

As with any analytical procedure, the significance of the result will depend upon the adequacy of the sample in representing the lot or quantity of food sampled. The National Academy of Sciences–National Research Council of the United States (1969) recommended

sampling plans for detection of *Salmonella* in foods based upon the degree of risk presented by a food. These plans, modified somewhat, have been recommended by ICMSF (1974).

Research sponsored by the ICMSF and others has shown that it is unnecessary to analyze each of several samples or analytical units drawn from a particular food lot (Silliker and Gabis, 1973). The multiple analytical units may be combined or 'lumped' to provide larger units for testing purposes. For example, the sixty 25-g units selected from a particular product lot may be combined or 'lumped' to produce three 500-g samples for analysis. If all three 500-g samples are found to be negative by test, then the inference is that the level of *Salmonella* is no greater than one in 500 g. Similarly, foods presenting a lesser degree of consumer risk, such as those requiring the analysis of thirty 25-g samples, may be 'lumped' into a smaller number of units for analysis. For 25-g samples, two 'lumped' samples each weighing 375 g would be analyzed. If the two were negative, then the probability would be 95% that the level of contamination in the lot analyzed was no greater than one *Salmonella* in 250 g, which is the same probability that would exist if each of the thirty 25-g samples had been analyzed and found negative. The practical importance of the 'lumping' approach is obvious. It permits statistical quality control over the *Salmonella* defect with analytical costs that are economically feasible.

ISOLATION OF SALMONELLAE

This part includes three main stages: (i) non-selective enrichment, (ii) selective enrichment, and (iii) plating on selective agar media.

1 APPARATUS AND MATERIALS

1 Mechanical blender, operating at not less than 8000 rpm and not more than 45,000 rpm.
2 Blending jars of 1-liter capacity, with covers, resistant to pasteurization temperatures: one jar for each food specimen to be analyzed.
3 Balance with weights, capacity at least 2500 g, sensitivity 0.1 g.
4 Instruments for preparing samples: knives, forceps, forks, scissors, spoons, spatulas, sterilized or pasteurized prior to use.
5 Incubator, 35–37°C.
6 Circulating water bath, 43° ± 0.05°C.

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7 Covered containers, resistant to sterilizing or pasteurizing temperatures, with capacity capable of holding largest volume of pre-enrichment medium to be used, including the sample.
8 1-ml bacteriological pipettes.
9 Inoculating needle and inoculating loop with nichrome or platinum-iridium wire.
10 Petri dishes, glass (100 × 15 mm) or plastic (90 × 15 mm) or equivalent.
11 Lactose Broth (Medium 51), bulk.
12 Buffered Peptone Water (Medium 22).
13 Reconstituted Non-Fat Dry Milk with Brilliant Green (Medium 95).
14 Lactose Broth containing 1% Tergitol 7 (Medium 52).
15 Selenite Cystine Broth (Medium 103), bulk and in 10-ml volumes in tubes as required.
16 Tetrathionate Brilliant Green Broth, Kauffmann modification (Medium 113), bulk and in 10-ml tubes as required.
17 Brilliant Green Agar (Medium 16), for plates.
18 Bismuth Sulfite Agar (Medium 8), for plates.
19 Laboratory's 'medium of choice' for plates.
20 Buffered Peptone Water with 0.22% Tergitol (Medium 23).
21 Triple Sugar Iron (TSI) Agar slants (Medium 118).
22 Lysine Iron Agar (LIA) Agar slants (Medium 59).

II PRE-ENRICHMENT OF SALMONELLAE

1 Mix the sample with 9 volumes of enrichment medium (w/v), chosen from Table 8. If the sample is not soluble in the enrichment medium, or if it will not readily disperse in it, blend the sample with an appropriate volume of medium and add the blended sample to the total volume of enrichment broth.[1]

1 Sterilize the pre-enrichment medium in the autoclave; however, if this is difficult because the volume of liquid is too large, pasteurize it with its container instead. It is necessary merely to destroy all non-sporing bacteria; this occurs at or above 80°C. Many cheap plastic containers can withstand this much heat. Use a pasteurized medium on the same day it is prepared. Cool or warm the pre-enrichment medium to 35–37°C before inoculation. If the sample contains a high concentration of sugar or salt, or other inhibitory substance, adjust appropriately the volume of the pre-enrichment medium to promote the growth of salmonellae. Similarly, if the pH of the sample is so high or low that it may impair the growth of salmonellae in the pre-enrichment medium, adjust the pH of the medium by adding 1% sulfuric acid or 1% potassium hydroxide solution. The pH of the inoculated medium should be 6.6 to 7.0 prior to incubation.

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2 Incubate the pre-enrichment medium for 18–24 hours at 35–37°C.

III SELECTIVE ENRICHMENT OF SALMONELLAE

1 Pipette 1 ml of the pre-enrichment culture to 10 ml of Selenite Cystine Broth. Incubate in a water bath at 43° ± 0.05°C for 24 hours.
2 Transfer 1 ml of the pre-enrichment culture to 10 ml of Tetrathionate Brilliant Green Broth. Incubate at 43° ± 0.05°C for 24 hours.

IV PLATING ON SELECTIVE AGAR MEDIA FOR SALMONELLAE

1 Transfer a 5-mm loopful of each of the two selective enrichment media to the surface of one plate of each of the three selective agar media listed below, and spread in a manner to obtain isolated colonies.
2 Incubate Brilliant Green Agar plates for 24 hours at 35–37°C. Typical colonies of *Salmonella* are colorless, pink to fuchsia, or translucent to opaque with the surrounding medium pink to red.
3 Incubate Bismuth Sulfite Agar plates at 35–37°C for 48 hours. Typical *Salmonella* colonies on Bismuth Sulfite Agar appear brown or gray to black, sometimes with a metallic sheen. The medium surrounding the colony is usually brown at first, then turns black as the incubation time increases. Some strains produce green colonies with little or no darkening of the surrounding medium.
4 Incubate and interpret the 'laboratory's choice' plates as directed by the supplier.

IDENTIFICATION OF SALMONELLAE

This section includes three stages in the identification of suspected *Salmonella* cultures: (1) screening of suspect colonies by use of determinative biochemical tests; (2) serological recognition of suspect isolates by use of polyvalent O, polyvalent H, group-specific O, and Spicer-Edwards H antisera; and (3) typing by means of bacteriophage.

Identification of an isolate as a member of the genus *Salmonella* is within the capabilities of any laboratory that possesses commercially available laboratory media and antisera. Laboratories concerned

with the routine detection of salmonellae in foods should use biochemical tests to screen suspect isolates, then subject such isolates to serological testing in order to determine whether they are actually salmonellae. Ultimate identification of salmonellae as to serotype requires the expertise of a typing center.

Biochemical screening for salmonellae

I APPARATUS AND MATERIALS

1 Inoculating needle, preferably made of platinum-iridium or nichrome wire.
2 Thermoregulated water bath, 35–37°C.
3 Colonies of suspect *Salmonella* on plates of Brilliant Green, Bismuth Sulfite, or 'laboratory choice' agars.
4 Lysine Iron Agar (Medium 59), slants with 25–30 mm butts in tubes.
5 Triple Sugar Iron Agar (Medium 118), slants with 25–30 mm butts in tubes.

II PROCEDURE

1 Pick, with a sterile inoculating needle, two or more of each suspect colony-type from Brilliant Green, Bismuth Sulfite, and the 'laboratory choice' medium to slants of Triple Sugar Iron Agar (TSI) and Lysine Iron Agar (LIA). Inoculate the media by streaking back and forth on the slant and then by stabbing the butt. After innoculating one medium, without flaming the needle, inoculate the second medium in the same fashion.
2 Incubate the TSI and LIA slants 24 hours at 35–37°C. *Salmonella*-suspect cultures on TSI show alkaline (red) slants and acid (yellow) butts, with or without H_2S production (blackening of the agar). *Salmonella*-suspect cultures on LIA show an alkaline (purple) reaction throughout the medium, with or without H_2S production (blackening). The 'suspect' reaction on TSI indicates that the organism ferments glucose (yellow butt) but fails to ferment lactose or sucrose (red slant). These reactions are typical of the salmonellae. However, a few salmonellae can ferment sucrose or lactose, to produce an acid (yellow) slant or butt.

The 'suspect' reaction on LIA, in contrast, results from the decarboxylation of lysine, producing an alkaline (purple) reaction in the butt of the tube. Organisms failing to decarboxylate lysine,

but which ferment glucose, will produce an acid (yellow) reaction in the butt of the tube. Organisms which ferment neither glucose nor decarboxylate lysine will produce an alkaline (purple) slant and butt. Organisms showing this reaction are generally strict aerobes of the genus *Pseudomonas*. These isolates will fail to show growth in the butt of the tube, because of their aerobic metabolism. They may be further 'screened' from consideration by means of the Oxidase Test (see p. 141). Lactose and sucrose-fermenting salmonellae will give typical 'suspect' reactions on LIA, since this medium contains neither lactose nor sucrose. *Arizona* organisms may give *Salmonella*-suspect reactions on TSI, due to late fermentation of lactose. These organisms will give *Salmonella*-suspect reactions on LIA, since they decarboxylate lysine and ferment glucose. More important, both *Arizona* and salmonellae which ferment lactose and/or sucrose will give typical *Salmonella* reactions on LIA, even though the same isolates may show non-*Salmonella*-type TSI slants. Similarly, rare strains of *Salmonella* fail to decarboxylate lysine and thus show an acid butt and an alkaline slant on LIA. These organisms show typical *Salmonella* reactions on TSI.

Serological tests for salmonellae

(P.R. Edwards and Ewing, 1972; AOAC, 1967)

I APPARATUS AND MATERIALS

1 Glass slides (5 × 7.5 cm) or Petri dishes (100 × 15 mm).
2 Inoculating needles and loops, preferably made of platinum-iridium or nichrome wire.
3 Pipettes: 0.2-ml capacity with 0.01-ml graduations; 1.0-ml capacity with 0.01-ml graduations; and 5-ml and 10-ml sizes with 0.1-ml graduations.
4 Serological test tubes, 75 × 10 mm or 100 × 13 mm.
5 Thermoregulated water bath, 50° ± 1°C.
6 Cultures of suspect salmonellae or *Arizona* on TSI or LIA slants.
7 H Broth (Medium 43), 5-ml volumes in 13 × 100 mm tubes.
8 Sodium chloride (0.85% aqueous solution).
9 0.6% Formalinized Saline Solution (0.85% saline solution containing 0.6 ml of formalin per 100 ml).
10 *Salmonella* antisera
(a) *Salmonella* polyvalent O (somatic) antiserum which contains agglutinins for at least the O antigens: 1–16, 19, 22, 23, 24, 25, and Vi.

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(b) *Salmonella* individual O (somatic) antisera for at least each of the groups A, B, C_1, C_2, D, E (E_1, E_2, E_3, E_4), F, G, H, I, and Vi.

(c) *Salmonella* polyvalent H (flagellar) antiserum containing agglutinins for at least the following H antigens: a, b, c, d, eh, en, enx, fg, fgt, gm, gmq, gms, gp, gpu, gq, gst, gt, i, k, lv, lw, lz_{13}, lz_{28}, mt, r, y, z, z_4z_{23}, z_4z_{24}, z_4z_{32}, z_6, z_{10}, z_{29}, 1, 2; 1, 5; 1, 6; and 1, 7.

(d) *Salmonella* Spicer-Edwards H (flagellar) antisera consisting of seven pooled antisera which react as follows:

(i) *Salmonella* H antiserum Spicer-Edwards 1 which reacts with antigens a, b, c, d, eh, fg, fgt, gm, gms, gmt, gp, gpu, gq, gst, ms, mt, and i.

(ii) *Salmonella* H antiserum Spicer-Edwards 2 which reacts with antigens a, b, c, k, r, y, and z_{29}.

(iii) *Salmonella* H antiserum Spicer-Edwards 3 which reacts with antigens a, d, eh, k, z, z_4z_{23}, z_4z_{24}, z_4z_{32}, and z_{29}.

(iv) *Salmonella* H antiserum Spicer-Edwards 4 which reacts with antigens b, d, fg, fgt, gm, gms, gmt, gp, gpu, gq, gst, ms, mt, k, r, z, and z_{10}.

(v) *Salmonella* H antiserum e, n complex which reacts with antigens enx and enz_{15}.

(vi) *Salmonella* H antiserum L complex which reacts with antigens lv, lw, lz_{13}, lz_{28}.

(vii) *Salmonella* H antiserum 1 complex which reacts with antigens 1, 2; 1, 5; 1, 6; 1, 7; and z_6.

II PROCEDURE FOR POLYVALENT O (SOMATIC) TEST (SLIDE OR PLATE TEST)

1 Dilute and adequately pretest antisera with known test cultures, to ensure reliability of test results with unknown cultures.

2 Using a wax pencil mark off two sections about 1 × 2 cm on the inside of a glass Petri dish or on a 5 × 7.5 cm glass slide.

3 Place a small amount (loopful) of culture from a Nutrient Agar or Triple Sugar Iron Agar slant (24 or 48 hours) directly onto the dish or slide in the upper part of each marked section.

4 Add 1 drop of 0.85% sodium chloride solution to the lower part of each marked section. With a clean, sterile transfer loop or needle emulsify the culture in the saline solution for one section and repeat for the other section.

5 Add a drop of *Salmonella* polyvalent O antiserum to one section of emulsified culture and mix with a sterile loop or needle.

6 Tilt the mixture in both sections back and forth for 1 minute and observe against a dark background. A positive reaction is indicated by a rapid, strong agglutination.

7 Classify the polyvalent O (somatic test) as:

(a) Positive when there is agglutination in the culture-saline-serum mixture and no agglutination in the culture-saline mixture.

(b) Negative when there is no agglutination in the culture-saline-serum mixture.[2] These cultures should be tested also with polyvalent H (flagellar) antiserum (III, immediately below).

(c) Non-specific when both mixtures agglutinate. This result requires additional testing as described in *Identification of Enterobacteriaceae* by P.R. Edwards and Ewing (1972).

III PROCEDURE FOR POLYVALENT H (FLAGELLAR) TEST

1 Dilute and adequately pretest antisera with known cultures to ensure reliability of results with unknown cultures.

2 To 5 ml of a 24-hour H Broth culture of the organism under test, add 5 ml of 0.6% Formalinized Saline Solution. Let stand 1 hour before use. This formalinized broth culture can be stored at 5–8° C for several days if necessary.

3 Place 0.02 ml of an appropriately diluted *Salmonella* polyvalent flagellar H antiserum into a small serological test tube (10 × 75 mm or 13 × 100 mm) and add 1 ml of the formalinized broth culture (antigen) from step 2 above.

4 Prepare a control, substituting Formalinized Saline Solution in place of antiserum: place 0.02 ml of Formalinized Saline Solution into the same size serological test tube as used in the previous test (step 3 above) and add 1 ml of the formalinized broth culture from step 2 above.

5 Incubate the antigen-serum mixture (step 3 above) and the corresponding antigen-saline mixture (step 4 above) at 50° C for 1 hour in a water bath. Observe preliminary results at 15-minute intervals and read final results at the end of 1 hour of incubation.

2 The polyvalent O antisera do not contain agglutinins for antigens of some salmonellae. Therefore, expect negative tests from certain strains, such as: *S. cerro*, group K (18); *S. minnesota*, group L (21); *S. alachua*, group O (35).

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6 Classify the polyvalent H test as:
(a) Positive when there is agglutination in the culture-formalinized-saline-serum mixture (step 3 above) and no agglutination in the culture-formalinized-saline mixture (step 4, above).
(b) Negative when there is no agglutination in the culture-formalinized-saline-serum mixture.[3]
(c) Non-specific when both mixtures agglutinate. This result requires additional testing as described in *Identification of Enterobacteriaceae* by P.R. Edwards and Ewing (1972).

7 Cultures that are non-motile or cultures that are *Salmonella* polyvalent H (flagellar) negative when retested are classified according to the results of other tests as described in the manual *Identification of Enterobacteriaceae* by Edwards and Ewing (1972).

IV PROCEDURE FOR INDIVIDUAL O GROUP TEST

This test is performed to determine the O (somatic) group to which the culture belongs.

1 Dilute and adequately pretest antisera with known test cultures to ensure reliability of results with unknown cultures.
2 Perform O group test on culture as in II, steps 2–6, using individual group O antisera (including Vi) in place of the *Salmonella* polyvalent O antiserum. Repeat the test using each O group antiserum.
3 Suspend cultures that are positive with Vi serum in 1 ml of physiological saline to make a heavy suspension, then heat in boiling water for 20–30 minutes and allow to cool. Retest heated culture suspension using O group D, C_1, and Vi sera. Vi-positive cultures which react with somatic group D serum are probably *Salmonella typhi* and Vi-positive cultures which react with somatic group C_1 serum are probably *Salmonella paratyphi* C. Heated Vi-positive cultures which do not react with any individual somatic serum but continue to react with Vi serum probably belong to the *Citrobacter* group and are not salmonellae.
4 Record as positive for that group cultures that give a positive somatic test when tested with one of the individual O groups.

3 The polyvalent H antiserum does not contain agglutinins for antigens of some salmonellae. Therefore, expect negative tests from certain strains, such as: *S. simsbury*, z_{27}; *S. wichita*, z_{37}; *S. chittagong*, z_{35}.

record as negative cultures that do not react with any individual O antisera.

V PROCEDURE FOR SPICER-EDWARDS H (FLAGELLAR) TESTS

These tests may be performed in place of the polyvalent H test (III above) to determine the presence or absence of H antigens.

1 Dilute and adequately pretest antisera with known cultures to ensure reliability of results with unknown cultures.
2 Test each culture using each of the seven Spicer-Edwards H antisera. Perform this test as for the polyvalent H test (III above) using one of the seven Spicer-Edwards H antisera for each test instead of the *Salmonella* polyvalent H antiserum.
3 Positive agglutination[4] indicates the presence of an H antigen. Identify the antigen by comparing the pattern of agglutination reactions obtained with the agglutinins known to be present in each of the seven Spicer-Edwards antisera (Table 9). The manufacturer also supplies the results of these reactions in a table that lists the Spicer-Edwards antisera and the H antigens with which each reacts.

Phage typing of salmonellae

Phage typing is a desirable epidemiological aid in the identification of salmonellae organisms for which acceptable phages are available. Production of *Salmonella* phages is under the aegis of the World Health Organization, but international distribution even of those developed to date is not yet feasible.

4 If the culture produces a positive agglutination when tested with each of the four Spicer-Edwards antisera Nos. 1, 2, 3, and 4 (a four plus pattern), then the results indicate the presence of a non-specific antigen other than a *Salmonella* antigen, or the presence of more than a single *Salmonella* H antigen, which cannot be identified with these antisera until the antigens are separated. This may require submission of the culture to an appropriate reference laboratory.

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TABLE 9
Positive reactions of Spicer-Edwards *Salmonella* H antisera with H antigens

H antigen	Positive reaction in Spicer-Edwards *Salmonella* H antisera
a	1, 2, 3
b	1, 2, 4
c	1, 2
d	1, 3, 4
eh	1, 3
G complex	1,4
i	1
k	2, 3, 4
r	2, 4
y	2
z	3, 4
z_4 complex	3
z_{10}	4
z_{29}	2, 3
enx, enz_{15}	en complex
lv, lw, lz_{13}, lz_{28}	L complex
1, 2; 1, 5; 1, 6; 1, 7; z_6	L complex

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Shigellae

Very little work has been done to develop and evaluate enrichment and plating media specifically for the isolation of shigellae from foods. Procedures used for the recovery of shigellae from human fecal samples have been applied, employing such media as Desoxycholate Citrate-, *Salmonella-Shigella*-, and XL-agars (Lewis and Angelotti, 1964; Hartsell, 1951; Nakamura and Dawson, 1962). These are considered superior to MacConkey, Brilliant Green, or Bismuth Sulfite agars; selenite broths are preferable to tetrathionate broths (G.S. Wilson and Miles, 1964). Often methods routinely used for the isolation of salmonellae are applied for the detection of shigellae, but these methods are not fully satisfactory (W.I. Taylor, 1965). Obviously research is needed to develop enrichment and plating media that are satisfactory for the isolation of shigellae from various food products.

The genus *Shigella* consists of Gram-negative, aerobic and facultatively anaerobic, non-sporulating, non-motile, rod-shaped bacteria in the family Enterobacteriaceae. They do not decarboxylate lysine, and gas production is restricted to one serotype (*S. flexneri* 6). Lactose is usually not utilized and, when it is, fermentation is usually delayed for several days. The shigellae consist of four species: *Shigella dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D). Organisms formerly described as *S. alkalescens* and *S. dispar* now constitute the alkalescens-dispar group which are non-motile, anaerogenic variants of *Escherichia coli*. For a complete description of this genus see P.R. Edwards and Ewing (1972).

Shigellae are among the more difficult enteric pathogens to isolate. Their ability to survive in specimens is considerably less than that of salmonellae. In clinical specimens, they may survive only a few hours so that long shipment cannot be tolerated without jeopardizing the result of the investigation. Buffered Glycerol Saline has given the best results as a transport medium (Morris et al., 1970).

Food

BAM: Food Sampling/Preparation of Sample Homogenate

April 2003

Bacteriological Analytical Manual
Chapter 1
Food Sampling and Preparation of Sample Homogenate

Authors

The adequacy and condition of the sample or specimen received for examination are of primary importance. If samples are improperly collected and mishandled or are not representative of the sampled lot, the laboratory results will be meaningless. Because interpretations about a large consignment of food are based on a relatively small sample of the lot, established sampling procedures must be applied uniformly. A representative sample is essential when pathogens or toxins are sparsely distributed within the food or when disposal of a food shipment depends on the demonstrated bacterial content in relation to a legal standard.

The number of units that comprise a representative sample from a designated lot of a food product must be statistically significant. The composition and nature of each lot affects the homogeneity and uniformity of the total sample mass. The proper statistical sampling procedure, according to whether the food is solid, semisolid, viscous, or liquid, must be determined by the collector at the time of sampling by using the *Investigations Operation Manual* (5). Sampling and sample plans are discussed in detail in ref. 6.

Whenever possible, submit samples to the laboratory in the original unopened containers. If products are in bulk or in containers too large for submission to the laboratory, transfer representative portions to sterile containers under aseptic conditions. There can be no compromise in the use of sterile sampling equipment and/or the use of aseptic technique. Sterilize one-piece stainless steel spoons, forceps, spatulas, and scissors in an autoclave or dry-heat oven. Use of a propane torch or dipping the instrument in alcohol and igniting is dangerous and may be inadequate for sterilizing equipment.

Use containers that are clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product. Containers such as plastic jars or metal cans that are leak-proof may be hermetically sealed. Whenever possible, avoid glass containers, which may break and contaminate the food product. For dry materials, use sterile metal boxes, cans, bags, or packets with suitable closures. Sterile plastic bags (for dry, unfrozen materials only) or plastic bottles are useful containers for line samples. Take care not to overfill bags or permit puncture by wire closure. Identify each sample unit (defined later) with a properly marked strip of masking tape. Do not use a felt pen on

plastic because the ink might penetrate the container. Whenever possible, obtain at least 100 g for each sample unit. Submit open and closed controls of sterile containers with the sample.

Deliver samples to the laboratory promptly with the original storage conditions maintained as nearly as possible. When collecting liquid samples, take an additional sample as a temperature control. Check the temperature of the control sample at the time of collection and on receipt at the laboratory. Make a record for all samples of the times and dates of collection and of arrival at the laboratory. Dry or canned foods that are not perishable and are collected at ambient temperatures need not be refrigerated. Transport frozen or refrigerated products in approved insulated containers of rigid construction so that they will arrive at the laboratory unchanged. Collect frozen samples in pre-chilled containers.

Place containers in a freezer long enough to chill them thoroughly. Keep frozen samples solidly frozen at all times. Cool refrigerated samples, except shellfish and shell stock, in ice at 0-4°C and transport them in a sample chest with suitable refrigerant capable of maintaining the sample at 0-4°C until arrival at the laboratory. Do not freeze refrigerated products. Unless otherwise specified, refrigerated samples should not be analyzed more than 36 h after collection. Special conditions apply to the collection and storage of shucked, unfrozen shellfish and shell stock (1). Pack samples of shucked shellfish immediately in crushed ice (no temperature specified) until analyzed; keep shell stock above freezing but below 10C. Examine refrigerated shellfish and shell stock within 6 h of collection but in no case more than 24 h after collection. Further details on sample handling and shipment may be found in the *Investigations Operation Manual* (5) and the *Laboratory Procedures Manual* (3). The *Investigations Operation Manual* (5) contains sampling plans for various microorganisms. Some of those commonly used are presented here.

A. **Sampling plans**

1. *Salmonella* species

a. Sample collection

Because of the continuing occurrence of *Salmonella* in foods, sampling plans for these organisms have received the attention of committees of national and international organizations (6,7). Each of these committees has recommended varying the number of samples from a particular lot of food according to the sampling category to which a food is assigned. Generally, the assignment to a sampling or food category depends on 1) the sensitivity of the consumer group (e.g., the aged, the infirm, and infants); 2) the possibility that the food may have undergone a step lethal to *Salmonella* during the manufacturing process or in the home; and 3) the history of the food. The selection of a sampling plan depends mainly on the first 2

criteria cited. The history of the food would be important in deciding whether to sample, i.e., whether there was a past history of contamination. For the *Salmonella* sampling plan discussed here, 3 categories of foods are identified.

Food Category I. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants.

Food Category II. - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

Food Category III. - Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.

In certain instances, it may not be possible to fully conform to the sampling plan. Nonetheless it is still important to ascertain whether or not Salmonella is present in the suspect food. Therefore, the analyst should still try to analyze as many analytical units as is required for the food of interest, i.e., 60 analytical units for Category I foods, 30 analytical units for Category II foods, and 15 analytical units for Category III foods. Individual 25 g analytical units may be combined into 375 g composites as described above unless otherwise indicated in Chapter 5 or the OMA. Below are examples of situations that might confront the analyst.

1) The number and weights of the sample units is correct.

Each sample should be mixed to ensure homogeneity before withdrawing a 25 g analytical unit. The analytical units can be composited (fifteen 25 g units into a 375 g composite), unless otherwise indicated in Chapter 5 or in the OMA. Samples should be preenriched at a 1:9 sample-to-broth ratio.

2) The number of sample units is correct, but several of the sample units have been damaged and are unusable.

For example, fifteen 1 lb bags of pasta have arrived for testing, but 5 of the bags are torn and unusable. In this case, the analyst should only sample from the 10 intact bags. The contents of each intact bag should be mixed to ensure homogeneity before the analytical units are withdrawn. Since the analyst needs one 375 g composite, ten 37.5 g analytical units, from the remaining 10 intact bags, should be used to form the composite. The composite should be combined with its preenrichment medium at a 1:9 sample-to-broth ratio (375

g sample/3375 ml preenrichment) as directed in Chapter 5 of the OMA.

3) The number of sample units is incorrect, but the total weight of the sample unit(s) is greater than what would be necessary to perform the sample analysis.

For example, a single 10 lb wheel of cheese has arrived for testing. Since cheese is a Category II food, thirty 25 g analytical units must be analyzed. These analytical units should be taken randomly from a wide variety of locations around the wheel. If *Salmonella* is present in a food, then the odds of detecting it will be enhanced if two 375 g composites are analyzed rather than a single 25 g analytical unit, as would be the case if the analyst were to treat the entire wheel as a single sample.

4) There is less sample available than is necessary to form the required number of composites.

For example, an 8 oz (226.8 g) bag of almonds has arrived for testing. Almonds are a Category II food. Category II foods require thirty 25 g analytical units (750g), so it is impossible to analyze the amount of almonds required by the sampling plan. In this case, the analyst should analyze all of the almonds at a 1:9 sample-to-broth ratio (226.8g sample/2041 ml preenrichment medium).

If, in the above example, the total weight of the almonds had been less than 2 composites (750 g), but more than 1 composite, then the analyst should analyze both a whole and a partial composite. The analytical units comprising these composites should be taken randomly from a wide variety of locations in the lot of almonds. Both composites, should be preenriched at a 1:9 sample-to-broth ratio.

This sampling plan applies to the collection of finished products under surveillance and/or for determination of compliance for regulatory consideration. It also applies to the collection of factory samples of raw materials in identifiable lots of processed units and/or finished products where regulatory action is possible. It does not apply to the collection of in-line process sample units at various stages of manufacture since those samples do not necessarily represent the entire lot of food under production. The actual techniques involved in sampling are covered in the *Investigations Operation Manual* (5).

A sample unit consists of a minimum of 100 g and is usually a consumer-size container of product. Take sample units at random to

ensure that a sample is representative of the lot. When using sample containers, submit a control consisting of one empty sample container that has been exposed to the same conditions as those under which the sample was collected. Collect more than one sample unit from large institutional or bulk containers when the number of sample units required exceeds the number of containers in the lot. A sample unit will consist of more than one container when containers are smaller than 100 g (e.g., four 25 g containers could constitute a sample unit).

The numbers of sample units to be collected in each food category are as follows: Food Category I, 60 sample units; Food Category II, 30 sample units; Food Category III, 15 sample units. Submit all samples collected to the laboratory for analysis. Advise the laboratory in advance of perishable sample shipments.

b. Sample analysis

The laboratory will analyze each sample for the presence of *Salmonella* according to methods described in this manual, or in *Official Methods of Analysis* (2). Take a 25 g analytical unit at random from each 100 g sample unit. When a sample unit consists of more than one container, aseptically mix the contents of each container before taking the 25 g analytical unit. To reduce the analytical workload, the analytical units may be composited. The maximum size of a composite unit is 375 g or 15 analytical units. The minimum number of composite units to be tested for each food category is as follows: Food Category I, 4 composite units; Food Category II, 2 composite units; Food Category III, one composite unit. For each 375 g composite, the entire amount of 375 g is analyzed for *Salmonella*.

Keep the remainder of the sample unit in a sterile container for compliance requirements as per section 702(b) of the Federal Food, Drug, and Cosmetic Act as amended through February, 1993. Refrigerate perishable samples and samples supporting microbial growth. An analytical control is required for each sample tested. The sampled lot is acceptable only if analyses of all composite units are negative for *Salmonella*. If one or more composite units are positive for *Salmonella*, the lot is rejected, provided that the analytical control is negative for *Salmonella*. A lot will not be resampled unless the environmental control for *Salmonella* is positive. For all samples positive for *Salmonella*, determine the somatic group. See Chapter 5 for information on further handling of these cultures. Recommendations for regulatory action may be based on the identification of the *Salmonella* somatic group and will not require definitive serotyping before initiation of regulatory action.

c. Imports.

These sampling plans apply to imported food products intended for human consumption.

d. Classification of food products for sampling purposes

Foods that have been classified into the 3 categories described above for regulatory sampling are listed in the categories according to the Industry Product Code sequence and nomenclature (4). Listing does not necessarily mean that these products are probable sources of *Salmonella*. **Food Category I.** - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the aged, the infirm, and infants. **Food Category II.** - Foods that would not normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption. Examples are as follows:

Industry Product Code

2 Milled grain products not cooked before consumption (bran and wheat germ)

3 Bread, rolls, buns, sugared breads, crackers, custard- and cream-filled sweet goods, and icings

5 Breakfast cereals and other ready-to-eat breakfast foods

7 Pretzels, chips, and other snack foods

9 Butter and butter products, pasteurized milk and raw fluid milk and fluid milk products for direct consumption, pasteurized and unpasteurized concentrated liquid milk products for direct consumption, dried milk and dried milk products for direct consumption, casein, sodium caseinate, and whey

12 Cheese and cheese products

13 Ice cream from pasteurized milk and related products that have been pasteurized, raw ice cream mix and related unpasteurized products for direct consumption

14 Pasteurized and unpasteurized imitation dairy products for direct consumption

15 Pasteurized eggs and egg products from pasteurized eggs, unpasteurized eggs and egg products from unpasteurized eggs for consumption without further cooking

16 Canned and cured fish, vertebrates, and other fish products; fresh and frozen raw shellfish and crustacean products for direct consumption; smoked fish, shellfish, and crustaceans for direct consumption

17 Meat and meat products, poultry and poultry products, and gelatin (flavored and unflavored bulk)

20-22 Fresh, frozen, and canned fruits and juices, concentrates, and nectars; dried fruits for direct consumption; jams, jellies, preserves, and butters

23 Nuts, nut products, edible seeds, and edible seed products for direct consumption

24 Vegetable juices, vegetable sprouts, and vegetables normally eaten raw

26 Oils consumed directly without further processing; oleomargarine

27 Dressings and condiments (including mayonnaise), salad dressing, and vinegar

28 Spices, flavors, and extracts

29 Soft drinks and water

30 Beverage bases

31 Coffee and tea

33 Candy (with and without chocolate; with and without nuts) and chewing gum

34 Chocolate and cocoa products

35 Pudding mixes not cooked before consumption, and gelatin products

36 Syrups, sugars, and honey

37 Ready-to-eat sandwiches, stews, gravies, and sauces

38 Soups

39 Prepared salads

54 Nutrient supplements, such as vitamins, minerals, proteins, and dried inactive yeast

Food Category III: Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption. Examples are as follows:

Industry Product Code

2 Whole grain, milled grain products that are cooked before consumption (corn meal and all types of flour), and starch products for human use

3 Prepared dry mixes for cakes, cookies, breads, and rolls

4 Macaroni and noodle products

16 Fresh and frozen fish; vertebrates (except those eaten raw); fresh and frozen shellfish and crustaceans (except raw shellfish and crustaceans for direct consumption); other aquatic animals (including frog legs, marine snails, and squid)

18 Vegetable protein products (simulated meats) normally cooked before consumption

24 Fresh vegetables, frozen vegetables, dried vegetables, cured and processed vegetable products normally cooked before consumption

26 Vegetable oils, oil stock, and vegetable shortening

35 Dry dessert mixes, pudding mixes, and rennet products that are cooked before consumption

2. Aerobic plate counts, total coliforms, fecal coliforms, *Escherichiacoli* (including enteropathogenic strains), *Staphylococcus* spp., *Vibrio* spp;, *Shigella* spp., *Campylobacter* spp., *Yersinia* spp., *Bacilluscereus*, and *Clostridium perfringens*

   a. Sample collection

      From any lot of food, collect ten 8-oz subsamples (or retail packages) at random. Do not break or cut larger retail packages to obtain an 8-oz subsample. Collect the intact retail unit as the subsample even if it is larger than 8 oz.

   b. Sample analysis.

      Analyze samples as indicated in current compliance programs.

B. **Equipment and materials**

1. Mechanical blender. Several types are available. Use blender that has several operating speeds or rheostat. The term "high-speed blender" designates mixer with 4 canted, sharp-edge, stainless steel blades rotating at bottom of 4 lobe jar at 10,000-12,000 rpm or with equivalent shearing action. Suspended solids are reduced to fine pulp by action of blades and by lobular container, which swirls suspended solids into blades. Waring blender, or equivalent, meets these requirements.
2. Sterile glass or metal high-speed blender jar, 1000 ml, with cover, resistant to autoclaving for 60 min at 121°C
3. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
4. Sterile beakers, 250 ml, low-form, covered with aluminum foil
5. Sterile graduated pipets, 1.0 and 10.0 ml
6. Butterfield's phosphate-buffered dilution water (RII), sterilized in bottles to yield final volume of 90 ± 1 ml
7. Sterile knives, forks, spatulas, forceps, scissors, tablespoons, and tongue depressors (for sample handling)

C. **Receipt of samples**

1. **The official food sample is collected by the FDA inspector or**

investigator. As soon as the sample arrives at the laboratory, the analyst should note its general physical condition. If the sample cannot be analyzed immediately, it should be stored as described later. Whether the sample is to be analyzed for regulatory purposes, for investigation of a foodborne illness outbreak, or for a bacteriological survey, strict adherence to the recommendations described here is essential.

2. **Condition of sampling container.** Check sampling containers for gross physical defects. Carefully inspect plastic bags and bottles for tears, pinholes, and puncture marks. If sample units were collected in plastic bottles, check bottles for fractures and loose lids. If plastic bags were used for sampling, be certain that twist wires did not puncture surrounding bags. Any cross-contamination resulting from one or more of above defects would invalidate the sample, and the collecting district should be notified (**see** C-5, below)
3. **Labeling and records.** Be certain that each sample is accompanied by a completed copy of the Collection Report (Form FD-464) and officially sealed with tape (FD-415a) bearing the sample number, collecting official's name, and date. Assign each sample unit an individual unit number and analyze as a discrete unit unless the sample is composited as described previously in this chapter.
4. **Adherence to sampling plan.** Most foods are collected under a specifically designed sampling plan in one of several ongoing compliances programs. Foods to be examined for *Salmonella*, however, are sampled according to a statistically based sampling plan designed exclusively for use with this pathogen. Depending on the food and the type of analysis to be performed, determine whether the food has been sampled according to the most appropriate sampling plan.
5. **Storage.** If possible, examine samples immediately upon receipt. If analysis must be postponed, however, store frozen samples at -20°C until examination. Refrigerate unfrozen perishable samples at 0-4°C not longer than 36 h. Store nonperishable, canned, or low-moisture foods at room temperature until analysis.
6. **Notification of collecting district.** If a sample fails to meet the above criteria and is therefore not analyzed, notify the collecting district so that a valid sample can be obtained and the possibility of a recurrence reduced.

D. Thawing

Use aseptic technique when handling product. Before handling or analysis of sample, clean immediate and surrounding work areas. In addition, swab immediate work area with commercial germicidal agent. Preferably, do not thaw frozen samples before analysis. If necessary to temper a frozen sample to obtain an analytical portion, thaw it in the original container or in the container in which it was received in the laboratory. Whenever possible, avoid transferring the sample to a second container for thawing. Normally, a sample can be thawed at 2-5°C within 18 h. If rapid thawing is desired, thaw the sample at less than 45°C for not more than 15 min. When thawing a sample at elevated temperatures, agitate the sample continuously in thermostatically controlled water bath.

E. **Mixing**

Various degrees of non-uniform distribution of microorganisms are to be expected in any food sample. To ensure more even distribution, shake liquid samples thoroughly and, if practical, mix dried samples with sterile spoons or other utensils before withdrawing the analytical unit from a sample of 100 g or greater. Use a 50 g analytical unit of liquid or dry food to determine aerobic plate count value and most probable number of coliforms. Other analytical unit sizes (e.g., 25 g for *Salmonella*) may be recommended, depending on specific analysis to be performed. Use analytical unit size and diluent volume recommended for appropriate *Bacteriological Analytical Manual* method being used. If contents of package are obviously not homogeneous (e.g., a frozen dinner), macerate entire contents of package and withdraw the analytical unit, or, preferably, analyze each different food portion separately, depending on purpose of test.

F. **Weighing**

Tare high-speed blender jar; then aseptically and accurately (± 0.1 g) weigh unthawed food (if frozen) into jar. If entire sample weighs less than the required amount, weigh portion equivalent to one-half of sample and adjust amount of diluent or broth accordingly. Total volume in blender must completely cover blades.

G. **Blending and diluting of samples requiring enumeration of microorganisms**

1. **All foods other than nut meat halves and larger pieces, and nut meal**. Add 450 ml Butterfield's phosphate-buffered dilution water to blender jar containing 50 g analytical unit and blend 2 min. This results in a dilution of 10^{-1}. Make dilutions of original homogenate promptly, using pipets that deliver required volume accurately. Do not deliver less than 10% of total volume of pipet. For example, do not use pipet with capacity greater than 10 ml to deliver 1 ml volumes; for delivering 0.1 ml volumes, do not use pipet with capacity greater than 1.0 ml. Prepare all decimal dilutions with 90 ml of sterile diluent plus 10 ml of previous dilution, unless otherwise specified. Shake all dilutions vigorously 25 times in 30 cm (1 ft) arc in 7 s. Not more than 15 min should elapse from the time sample is blended until all dilutions are in appropriate media.
2. **Nut meat halves and larger pieces**. Aseptically weigh 50 g analytical unit into sterile screw-cap jar. Add 50 ml diluent (G-l, above) and shake vigorously 50 times through 30 cm arc to obtain 10^0 dilution. Let stand 3-5 min and shake 5 times through 30 cm arc to resuspend just before making serial dilutions and inoculations.
3. **Nut meal**. Aseptically weigh 10 g analytical unit into sterile screw-cap jar. Add 90 ml of diluent (G-l, above) and shake vigorously 50 times through

30 cm arc to obtain 10^{-1} dilution. Let stand 3-5 min and shake 5 times through 30 cm arc to resuspend just before making serial dilutions and inoculations.

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Exhibit U

statistical aspects of the microbiological analysis of foods

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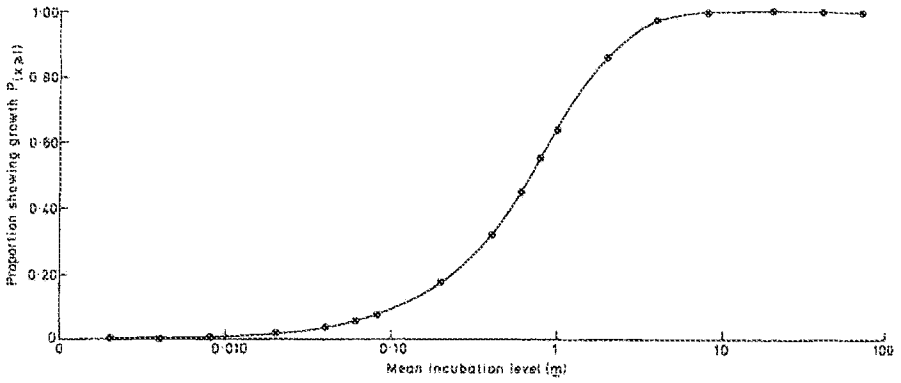
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TABLE 8.1

EXPECTED PROPORTIONAL RESPONSE IN A MULTIPLE-TUBE DILUTION SERIES. INITIAL INOCULUM CONTAINS 2×10^5 PROPAGULES/ml; DILUTION FACTOR = 10

Dilution tested	Mean inoculum level/tube* (m)	Expected proportion tubes positive ($P_{x>1}$)	Expected proportion tubes negative ($P_{x=0}$)	Expected no. tubes positive for 1 tubes inoculated	5	10
10^{-3}	200	0.999	0.001	1	5	10
10^{-4}	20	0.999	0.001	1	5	10
10^{-5}	2	0.865	0.135	1	4	9
10^{-6}	0.2	0.181	0.819	0	1	2
10^{-7}	0.02	0.020	0.980	0	0	0
10^{-8}	0.002	0.002	0.998	0	0	0

* Assuming 1 ml of dilution added to medium in each tube.



8.1 Expected proportion of cultures giving positive response for various mean inoculum levels (m)

8.2 Single tube dilution tests

If only one tube is inoculated at any given dilution level, only two responses are possible, i.e. negative ($P_{x=0}$) or positive ($P_{x>1}$). Where presence or absence tests (P/A) are used at various dilution levels, it is not uncommon for "skips" to occur, e.g.

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Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Response	+	+	+	-	+

The probability for a negative response (i.e. less than one "viable propagule/tube") is dependent upon the level of organisms in the original sample. For instance, if the above series were derived from a sample containing (say) 5×10^3 viable propagules/ml, the probability for the observed results at the 10^{-4} and 10^{-5} dilutions (assuming 1 ml inoculum) would be 0.62 and 0.05, respectively. Hence, the result obtained on the 10^{-4} dilution might be expected to occur in 61 tests out of 100 (i.e. 6 in 10) and that on the 10^{-5} dilution in 5 tests out of 100 (i.e. 1 in 20). Neither of these results would be improbable. However, if the original inoculum contained only 1×10^3 viable propagules, the relative frequencies for the observed results in the 10^{-4} and 10^{-5} dilutions would be 0.90 and < 0.01, respectively. Hence, the observed result at 10^{-4} dilution could be expected to occur in 9 tests out of 10, but that at the 10^{-5} dilution might reasonably be expected only in one out of more than 100 tests. Such a result would be improbable, but not impossible. The probabilities for such an observation are listed for various values of m in Table 8.2, from which it can be seen that a "skip" could occur by chance at least once in every 20 tests for mean inoculum levels between 0.5 and 3 viable propagules/culture at the 10^{-z} dilution. When such skips occur, the analyst is not able to decide whether the observed result could have been expected to occur merely by chance (i.e. $P > 0.05$) or whether the probability of its occurrence is so remote that the result should be ignored.

Calculation of the initial contamination level using data derived from a single-tube assay, at best, can give only an approximate indication, as illustrated in Ex. 8.1. However, it should be noted that the occurrence of a "skip" can reduce the range of the 95% confidence limits (Ex. 8.1.B) compared with the sharp demarcation shown in Ex. 8.1.A. Such results should therefore always be recorded, though in general it is dubious whether it is justifiable to attempt to derive probable contamination levels for single-tube dilution (P/A) tests.

It should be noted that the calculation of these confidence limits assumes Poisson distribution (which may not be correct) and ignores any effects of dilution or other technical errors. In the single-tube, as in multiple-tube series (§ 8.3.2) the confidence limits for the results can be improved by reducing the dilution interval (i.e. 1 in 2 dilution is better than 1 in 4, which is better than 1 in 10), since the response curve obtained would be smoothed out to some extent (e.g. the 95% confidence limits for an initial dilution of 10^{-3} followed by sequential 2-fold dilutions, positive at 2^1 and 2^2 but negative at 2^3 and 2^4 would be 200 to 12,000 propagules/unit of original sample, c.f. Ex. 8.1.A.

TABLE 8.2

PROBABILITIES OF OCCURRENCE OF "SKIPS" IN A SINGLE-TUBE DILUTION SERIES FOR VARIOUS INOCULA LEVELS

Mean inoculum level in dilution 10^{-z} † ($\bar{m}$)	Probability* of occurrence of	
	negative result ($P_{x=0}$) at dilution 10^{-z} †	positive result ($P_{x\geq 1}$) at dilution $10^{-(z+1)}$ †
0.01	0.99	<< 0.01
0.05	0.95	0.01
0.1	0.90	0.01
0.2	0.82	0.02
0.3	0.74	0.03
0.4	0.67	0.04
0.5	0.61 (ca. 1 in 2)	0.05 (1 in 20)
0.6	0.55	0.06
0.7	0.50	0.07
0.8	0.45	0.08
0.9	0.41	0.09
1.0	0.37	0.10
2.0	0.14	0.18
3.0	0.05 (1 in 20)	0.26 (1 in 4)
4.0	0.02	0.33
5.0	< 0.01	0.40
30.0	<< 0.01	0.95

* To 2 significant places.
** 95% confidence limits for both events to occur.
† 10^{-z} = dilution level e.g. 10^{-1} ; $10^{-(z+1)}$ = next dilution level, e.g. 10^{-2}.

Example 8.1. Calculation of initial contamination level from a single-tube dilution series

Assume 10-fold serial dilutions, with 1 ml inoculum from each dilution and the following results obtained:

Dilution		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Response in series:	(A)	+	+	-	-	-
	(B)	+	+	-	+	-

(A) series: the presumed initial contamination level lies between 1×10^3 and 1×10^4 propagules/ml of original sample. But from Table 8.2 the probability that a positive result $P_{(x\geq 1)} = 1 - P_{(x=0)}$ will occur in at least 1 in 20 tests at dilution 10^{-z} (i.e. 10^{-3} in this example) is associated with a mean inoculum level ranging upwards from 0.05 propagules/unit volume of dilution (10^{-z}). The 1 in 20 chance of a negative result $P_{(x=0)}$ at the $10^{-(z+1)}$ dilution ranges downwards from a mean inoculum level of 3 propagules/unit volume of dilution $10^{-(z+1)}$, i.e. < 30 propagules/unit volume of dilution 10^{-z}.

Hence, although the apparent contamination level is 1000- 10,000 propagules per unit of original sample, the 95% confidence limits range from 0.05×10^3 (i.e. 50) to 30×10^3 (i.e. 30,000). Therefore result (A) indicates, with 95% confidence, that the contamination level lies between 50 and 30,000 viable propagules/unit of original sample!

(B) series: the apparent contamination level is either $\geq 10^3$ and $< 10^4$ or $\geq 10^5$ and $< 10^6$ propagules, (i.e. between 10^3 and 10^6 propagules/unit of original sample.) Applying the same approach as that applied for the (A) series, the 95% limits for the count of 10^3 - 10^4 are 50 - 30,000 propagules, whilst the limits for the count being 10^5 - 10^6 are 5,000 - 3,000,000 propagules. The likelihood of the combinations occurring in at least 1 in 20 tests is limited so that the most probable contamination level (95% confidence limit) lies between 5,000 and 30,000 propagules/unit of original sample. These limits are shown (**) in Table 8.2.

8.3 Multiple-tube dilution series

8.3.1 Multiple tubes at a single dilution level

If more than one tube is inoculated at a given dilution level, either all tubes show a positive or negative reaction (when numbers of organisms inoculated are very high or very low, respectively), or a graded response is achieved with some tubes remaining sterile whilst others exhibit growth (see last 2 columns, Table 8.1). For a single dilution level, it may be shown that the probability for no organisms (i.e. <1 fertile propagule) occurring in a sample of volume v when the true density = m organisms/unit volume is given by $p = e^{-vm}$. If n samples each of volume v are tested and, of these, s samples are sterile, then the proportion of sterile samples (s/n) provides an estimate of p. Hence:

$$\hat{p} = s/n = e^{-vm} \quad \text{and}$$

$$m = -(1/v)\ \ln\ (s/n) = -(2.303/v)\ \log\ (s/n)$$

where ln and log are logarithms to bases e and 10, respectively. The value m is the most probable number ($\hat{m}$) of organisms per ml.

If the probability that a sample is "sterile" is p_0, then the probability that s/n samples are sterile is given by the binomial expansion as

$$[n!/s!(n - s)!]\ p_0^s(1 - p_0)^{n-s}$$

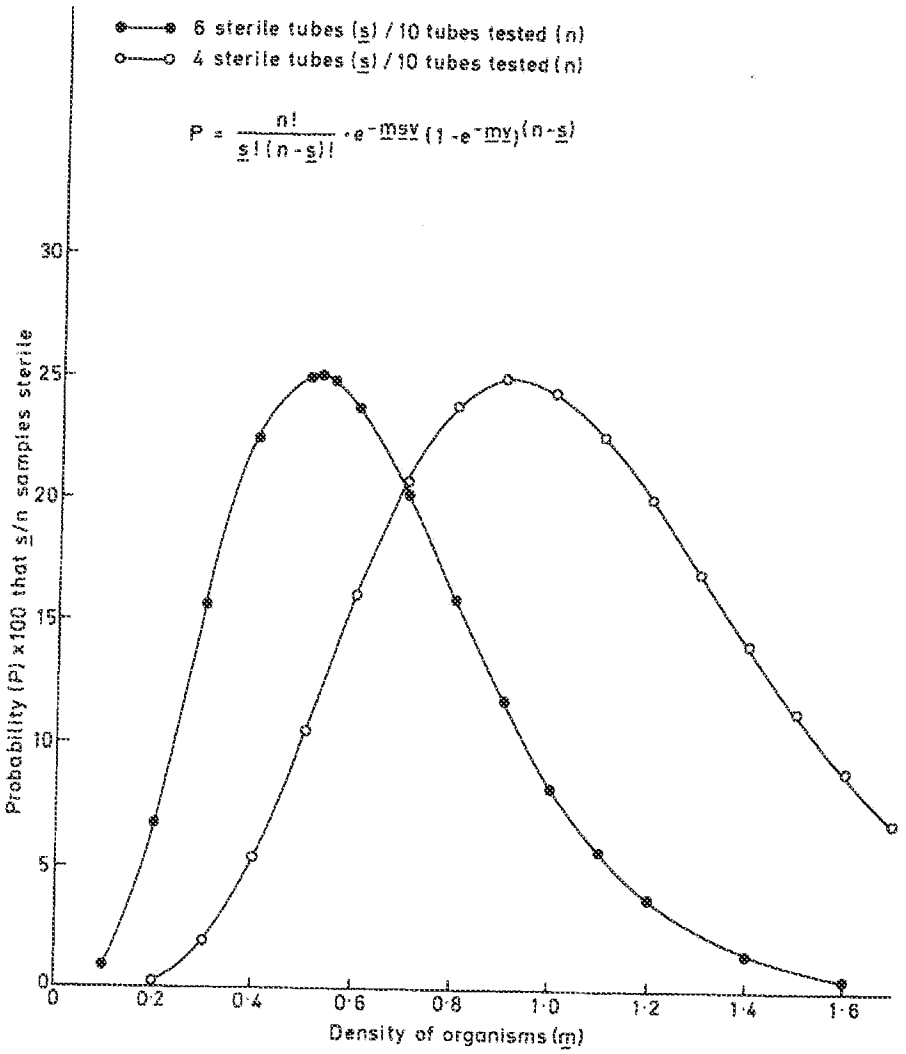
but since $p_0 = e^{-vm}$, this expression may be rewritten as:

$$[n!/s!(n - s)!]\ e^{-vms}\ (1 - e^{-vm})^{(n-s)}$$

The derived terms from this expansion permit the construction of a curve showing probability (p) against density of organisms (m). This is illustrated (Fig. 8.2) for two values of s/n and assuming v = 1 ml; when s/n = 0.6, the

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most probable number (MPN) of organisms inoculated ($\hat{m}$) = 0.51 propagules/ml; when s/n = 0.4, MPN ($\hat{m}$) = 0.92 propagules/ml. These values are the same as those derived using $\hat{m} = -(1/v)\ln(s/n)$. From the shapes of the curves it is clear that many other densities of organisms could also give the observed ratios (s/n) of 0.4 or 0.6, though with lower probability.



8.2 Probability distribution for multiple-tube dilution test at single dilution level

8.3.2 Multiple-tubes at several dilution levels

In practice, more than one dilution level is necessary since the precision of the MPN is very poor when the number of organisms inoculated is likely to give either all positive or all negative results. When all replicate tests are positive, maximum probability occurs when m is infinite, so that the estimated density ($\hat{m}$) is also infinite. When all results are negative, the estimated density is zero at maximum probability. Thus, a single dilution is of value only if the numbers of organisms inoculated (mv) is chosen so that some tests give a positive and others a negative result.

When more than one dilution level is used, an estimate of population density ($\hat{m}$) can be derived for each dilution level. In a series with inoculum volume v_i and s_i tubes sterile out of n_i tubes tested, the estimated density of organisms for the ith dilution is given by:

$$\hat{m}_i = -(2.303/v_i) \log (s_i/n_i)$$

However, since the best estimate for each dilution will vary in precision, it is not possible to take the arithmetic mean of the estimates for each dilution level in order to derive an overall estimate. Two primary methods exist for deriving the best estimate of numbers.

8.3.2.1 Stevens' method for multiple dilution levels. Fischer and Yates (1954) published Tables based on the mean fertile level (x) and the mean sterile level (y) where x = number of positive results at all dilution levels divided by the number of cultures (n) at each level, and y = number of levels minus x. Using the same notation as before (i.e. s_i is the number of sterile cultures (negative results) out of n_i tubes at the ith dilution level), then

$$x = \Sigma [(n_1 - s_1) + (n_2 - s_2) + \ldots + (n_i - s_i)]/n$$

and

$$y = i - x$$

One then enters the appropriate Table (Table 8.3) for values of x or y according to the number of dilution levels and the dilution ratio in order to determine a factor K. The estimated number of organisms is then given by:

$$\log \lambda = x \log a - K$$

where λ = estimate of number of organisms (= $\hat{m}$) and a = dilution ratio. Hence for 10-fold dilutions:

$$\log \lambda = x - K$$

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TABLE 8.3

K VALUES FOR DETERMINING THE DENSITY OF ORGANISMS BY THE DILUTION METHOD (from Fisher & Yates, 1954)

Value of x	Value of K for				
	4-fold dilutions with			10-fold dilutions*	
	4 levels	5	> 6	(> 3 levels)	
				$x \leqslant 1$	$x > 1$
0.0	-	-	-		0.763
0.1	-	-	-		0.768
0.2	-	-	-		0.768
0.3	-	-	-		0.760
0.4	0.704	0.706	0.707	0.761	0.747
0.5	-	-	-	0.740	0.736
0.6	0.615	0.617	0.618	0.733	0.733
0.7	-	-	-	0.736	0.736
0.8	0.573	0.576	0.577	0.744	0.749
0.9	-	-	-	0.753	0.753
1.0	0.555	0.558	0.559	0.763	0.763
1.5	0.545	0.551	0.553		
2.0	0.537	0.548	0.551		
2.5	-	0.545	0.552		

* When $x > 1$ enter Table with decimal part of x only.

The variance of the estimate of log λ is given by:

$$1/n \ (\log 2) \ (\log \alpha)$$

Hence for 10-fold dilutions:

$$s^2_{\log \lambda} = 1/n \ (\log 2) = 0.301/n$$

This method is applicable to 2-, 4- and 10-fold dilution ratios with not less than 4, 4 or 3 dilution levels, respectively. Unfortunately, the method does not discriminate in any way between valid and invalid series of results (see § 8.3.2.2 below).

8.3.2.2 Most probable number method for multiple dilution levels. The most probable number method is an extension of the concept used above (§ 8.3.1) for multiple tubes at a single level. Assuming that for i dilution levels we have the following proportions of sterile cultures:

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s_1/n_1, s_2/n_2, s_3/n_3 ... s_i/n_i

then the probability that these events should all happen at once can be derived from the product of the probabilities that each individual event would occur. As before, a graph of the probability against m shows a single maximum, and the value of m at that maximum probability is taken as the Most Probable Number ($\hat{m}$).

As pointed out by Cochran (1950), the value of $\hat{m}$ cannot be written down explicitly, but can be derived from the equation:

$$s_1v_1 + s_2v_2 + s_3v_3 + \ldots + s_iv_i = [(n_1 - s_1)\, v_1\, e^{-v_1m}/(1 - e^{-v_1m})] +$$

$$+ [(n_2 - s_2)\, v_2\, e^{-v_2m}/(1 - e^{-v_2m})] + [(n_3 - s_3)\, v_3\, e^{-v_3m}/(1 - e^{-v_3m})] + \ldots +$$

$$+ [(n_i - s_i)\, v_i\, e^{-v_im}/(1 - e^{-v_im})]$$

Methods for solving this equation by iteration have been given by Halvorson and Ziegler (1933) and Finney (1947). More recently De Man (1975, 1983) used computer techniques to derive the composite probability distribution curves for various combinations of results, dilution levels and culture numbers.

For routine purposes, standard Tables can be used to derive the most probable number of organisms in a sample. Such Tables, cited in the publications of Woodward (1957), DHSS (1969), AOAC (1975), APHA (1976), ICMSF (1978), do not always provide data on the confidence limits which can be applied to the MPN values and in some instances include many unacceptable and improbable results (i.e. nonsense codons; De Man, 1975).

Both Woodward (1957) and De Man (1975) have pointed out that the occurrence of many improbable results indicates some malfunction in the performance of the MPN tests. As an extreme example De Man (1975) cites the case of a result 0, 0, 10 positive results from a 10-tube 3-dilution series (10^0 - 10^{-2}). The MPN for such a result can be calculated as 0.9 propagules/ml with 95% confidence limits of 0.5 - 1.7. However, the probability of obtaining such a result is only 0.17×10^{-24} when the mean inoculum level is 0.9 organisms/ml. Furthermore, it should be noted also that the method of Stevens (§ 8.3.2.1) would give the same estimate of the number of organisms (λ = 1.73; 95% CL = 0.78 - 3.85) for this codon (0-0-10) as for the codons 0-10-0 and 10-0-0; however, only the latter would be an acceptable result and the MPN according to De Man (1975) would be 2.3 with 95% CL of 1.2 - 5.8.

In order to assess whether an improbable result has been obtained, it is possible to test the goodness of fit of observed results with expected results using the χ^2 test. However, Moran (1954 a,b; 1958) has proposed a more severe

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test which is applicable, however, only when the extreme dilutions give approximately 0% and 100% positive results. Moran's test is performed by calculating a value $T_{obs.}$ which is given by:

$$T_{obs} = \Sigma\ fm\ (n - fm)$$

where fm = the number of positive results at each dilution and n is the total number of tubes at each dilution. The expected value of T (T_{exp}) and the standard error of T (SE_T) are then obtained from Table (8.4) and a value M is derived for the significance of the difference between T_{obs} and T_{exp}, viz:

$$M = (T_{obs} - T_{exp})/SE_T$$

Values of $M \geqslant 1.645$ or $\geqslant 2.326$ occur by chance with probabilities of $\leqslant 0.05$ and $\leqslant 0.01$ respectively. The use of this method is illustrated in Ex. 8.2.

TABLE 8.4

EXPECTED VALUES OF T (T_{exp}) AND STANDARD ERROR OF T (SE_T) FOR MORAN'S TEST

n	T_{exp}			SE_T		
	2*	4*	10*	2*	4*	10*
5	20	10	6.02	5.69	4.02	3.12
6	30	15	9.03	7.63	5.40	4.19
7	42	21	12.64	9.75	6.89	5.35
8	56	28	16.86	12.02	8.50	6.60
9	72	36	21.67	14.46	10.22	7.93
10	90	45	27.09	17.03	12.04	9.34
11	110	55	33.11	19.74	13.96	10.83
12	132	66	39.74	22.58	15.97	12.39
13	156	78	46.96	25.55	18.07	14.02
14	182	91	54.79	28.63	20.24	15.71
15	210	105	63.22	31.83	22.51	17.74
16	240	120	72.25	35.14	24.85	19.28
17	272	136	81.88	38.56	27.27	21.16
18	306	153	92.12	42.07	29.75	23.08
19	342	171	102.95	45.70	32.31	25.08
20	380	190	114.39	49.41	34.94	27.11
30	870	435	261.90	91.53	64.72	50.22
40	1560	780	469.61	141.49	100.05	77.63

n = number of tubes inoculated from each dilution.
* Dilution factor.

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Example 8.2. Use of Moran's test for validity of multiple-tube dilution counts

Assume the following results obtained in a multiple-tube dilution assay, using 10 tubes at each dilution level:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
No. tubes positive (A):	10	10	8	4	0
(B):	10	9	7	5	0

For series (A):

$$T_{obs} = 10\ (10 - 10) + 10\ (10 - 10) + 8\ (10 - 8) + 4\ (10 - 4) + 0\ (10 - 0)$$

$$= 0 + 0 + 16 + 24 + 0$$

$$= 40$$

From Table 8.4:

$$T_{exp} = 27.09 \quad \text{and} \quad SE_T = 9.34$$

then:

$$M_A = (40 - 27.09)/9.34 = 1.38$$

Since $M_A < 1.45$, the observed results for series (A) do not differ significantly ($P > 0.05$) from those expected and the series is valid for a MPN calculation.

For series (B):

$$T_{obs} = 10\ (10 - 10) + 9\ (10 - 9) + 7\ (10 - 7) + 5\ (10 - 5) + 0\ (10 - 0)$$

$$= 0 + 9 + 21 + 25 + 0$$

$$= 54$$

From Table 8.4:

$$T_{exp} = 27.09 \quad \text{and} \quad SE_T = 9.34$$

then

$$M_B = (54 - 27.09)/9.34 = 2.88$$

Since $M_B > 2.326$, the observed number of positive tubes differs significantly ($p < 0.01$) from the expected number and therefore series (B) provides an improbable result from which to calculate a MPN.

Cochran (1950) pointed out that in planning an MPN test the following decisions are required: (1) the range of volumes to be tested; (2) the dilution factor to be used; and (3) how many tests to be inoculated at each dilution level. In planning such a test, the aim is to obtain equal precision across a number of possible levels of cell density, i.e. the ratio between the standard error and the true density should be unity. When the density of organisms is expected to be reasonably constant (e.g. in analysis of a water supply of known

quality) it is possible to select inoculum levels such that the lowest dilution volume (i.e. largest volume of inoculum) should contain at least one propagule and that the highest dilution (i.e. smallest volume) should contain not more than two propagules. However, when the upper density of organisms nears the limit of the test for the dilutions chosen, the probability of obtaining all positive cultures is greater for a small number of replicates at each dilution level than for a large number of replicates. Hence, it is safer to use the rule that the maximum number should not exceed 1 propagule per volume of the highest dilution. This can be done by "guesstimating" the limits ($\hat{m}_L$ and $\hat{m}_H$) between which the true density would be expected to lie; then $V_H > 1/\hat{m}_H$ and $V_L < 1/\hat{m}_L$; where V_H = largest volume (or volume at lowest dilution level) and V_L = smallest volume (or volume at highest dilution level).

For example, if the probable true density (m) lies between 10 and 800 propagules/ml, the largest volume to be inoculated per test should not exceed 1/10 ml (0.1 ml of original sample or 1 ml of the 10^{-1} dilution) and the smallest volume should not exceed 1/800 (0.00125 ml original sample or 1.25 ml of the 10^{-3} dilution). In practice, this range could be covered either by using 1 ml per test of each of three 10-fold dilutions (i.e. 1/10, 1/100 and 1/1000) or by using 1 ml per test of (say) one 10-fold and four 4-fold dilutions (1/10, 1/40, 1/160, 1/640, 1/2560).

The choice of dilution ratio affects precision significantly over the range of dilution ratios between two and ten only if the total number of tests is not kept constant. However, use of the lower dilution ratio results in the precision being kept more constant throughout the range of densities tested (i.e. $1/V_H$ - $1/V_L$). This is illustrated in Fig. 8.3 for dilution ratios of two and ten.

The standard error of the MPN can be derived (Cochran, 1950) from the following equation (assuming a cell density between $1/V_H$ and $1/V_L$, a limited number of tests at each dilution and a dilution ratio of five or less):

$$SE_{\log \hat{m}} = 0.55 \sqrt{(\log a)/n}$$

where a = dilution ratio and n = number tests at each dilution. For 10-fold dilutions, where the standard error may peak at the value for $\hat{m}$,

$$SE_{\log \hat{m}} = 0.58 \sqrt{\log a/n} = 0.58 \sqrt{1/n}$$

Values for various levels of a and n, together with the factors for deriving standard errors and 95% confidence limits are given in Table 8.5.

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TABLE 8.5

STANDARD ERROR OF LOG $\hat{m}$ AND FACTOR FOR CONFIDENCE LIMITS (COCHRAN, 1950)

No. of samples per dilution	$SE_{(\log_{10}\hat{m})}$ for dilution ratio (a)				Factor* for 95% confidence limits with dilution ratio (a)			
n	2	4	5	10	2	4	5	10
1	0.301	0.427	0.460	0.580	4.00	7.14	8.32	14.45
2	0.213	0.302	0.325	0.410	2.67	4.00	4.47	6.61
3	0.174	0.246	0.265	0.335	2.23	3.10	3.39	4.68
4	0.150	0.214	0.230	0.290	2.00	2.68	2.88	3.80
5	0.135	0.191	0.206	0.259	1.86	2.41	2.58	3.30
6	0.123	0.174	0.188	0.237	1.76	2.23	2.38	2.98
7	0.114	0.161	0.174	0.219	1.69	2.10	2.23	2.74
8	0.107	0.151	0.163	0.205	1.64	2.00	2.12	2.57
9	0.100	0.142	0.153	0.193	1.58	1.92	2.02	2.43
10	0.095	0.135	0.145	0.183	1.55	1.86	1.95	2.32

* In deriving the 95% confidence limits (CL) the MPN should be multiplied or divided by the appropriate factor. For example if the MPN for a 5-tube dilution series with a 10-fold dilution ratio is 22 propagules/ml, the 95% CL are given by (22 x 3.30) and (22/3.30) i.e. 72.5 - 6.7.

The standard error increases (precision decreases) on either side where the likelihood of all positive or all negative results increases. The precision is greatest when approximately equal numbers of positive and negative results are most likely to occur. The optimum mean inoculum (about 1.6 propagules/culture) is more likely to be missed by chance in a ten-fold than in a two-fold dilution series, hence the graph for ten-fold dilutions fluctuates more than does that for two-fold dilutions (Fig. 8.3).

These CL assume that the logarithm of the MPN is normally distributed and differ from those cited by De Man (1975), who derived CL from the computer-printed histograms of the distribution functions for different combinations of tube numbers and positive results. However, De Man's Tables (Tables 8.6 and 8.7) cover only 10-fold dilution ratios; hence the data in Table 8.4 at least allow some confidence limits to be assigned to MPNs derived from other dilution series.

TABLE 8.6

MPN TABLE FOR 3 x 1, 3 x 0.1, AND 3 x 0.01 g (ml) (DE MAN, 1983)

Number of positive results			MPN	Category when No. of tests					Confidence limits			
				1	2	3	5	10	⩾ 95%	⩾ 95%	⩾ 99%	⩾ 99%
0	0	0	< 0.30						0.00	0.94	0.00	1.40
0	0	1	0.30	3	2	2	2	1	0.01	0.95	0.00	1.40
0	1	0	0.30	2	1	1	1	1	0.01	1.00	0.00	1.60
0	1	1	0.61	0	3	3	3	3	0.12	1.70	0.05	2.50
0	2	0	0.62	3	2	2	2	1	0.12	1.70	0.05	2.50
0	3	0	0.94	0	0	0	0	3	0.35	3.50	0.18	4.60
1	0	0	0.36	1	1	1	1	1	0.02	1.70	0.01	2.50
1	0	1	0.72	2	2	1	1	1	0.12	1.70	0.05	2.50
1	0	2	1.1	0	0	0	3	3	0.4	3.5	0.2	4.6
1	1	0	0.74	1	1	1	1	1	0.13	2.00	0.06	2.70
1	1	1	1.1	3	3	2	2	2	0.4	3.5	0.2	4.6
1	2	0	1.1	2	2	1	1	1	0.4	3.5	0.2	4.6
1	2	1	1.5	3	3	3	3	2	0.5	3.8	0.2	5.2
1	3	0	1.6	3	3	3	3	2	0.5	3.8	0.2	5.2
2	0	0	0.92	1	1	1	1	1	0.15	3.50	0.07	4.60
2	0	1	1.4	2	1	1	1	1	0.4	3.5	0.2	4.6
2	0	2	2.0	0	3	3	3	3	0.5	3.8	0.2	5.2
2	1	0	1.5	1	1	1	1	1	0.4	3.8	0.2	5.2
2	1	1	2.0	2	2	1	1	1	0.5	3.8	0.2	5.2
2	1	2	2.7	0	3	3	3	3	0.9	9.4	0.5	14.2
2	2	0	2.1	1	1	1	1	1	0.5	4.0	0.2	5.6
2	2	1	2.8	3	2	2	2	1	0.9	9.4	0.5	14.2
2	2	2	3.5	0	0	0	0	3	0.9	9.4	0.5	14.2
2	3	0	2.9	3	2	2	2	1	0.9	9.4	0.5	14.2
2	3	1	3.6	0	3	3	3	3	0.9	9.4	0.5	14.2
3	0	0	2.3	1	1	1	1	1	0.5	9.4	0.3	14.2
3	0	1	3.8	1	1	1	1	1	0.9	10.4	0.5	15.7
3	0	2	6.4	3	3	2	2	2	1.6	18.1	1.0	25.0
3	1	0	4.3	1	1	1	1	1	0.9	18.1	0.5	25.0
3	1	1	7.5	1	1	1	1	1	1.7	19.9	1.1	27.0
3	1	2	12	3	2	2	2	1	3	36	2	44
3	1	3	16	0	0	0	3	3	3	38	2	52
3	2	0	9.3	1	1	1	1	1	1.8	36.0	1.2	43.0
3	2	1	15	1	1	1	1	1	3	38	2	52
3	2	2	21	2	1	1	1	1	3	40	2	56
3	2	3	29	3	3	3	2	2	9	99	5	152
3	3	0	24	1	1	1	1	1	4	99	3	152
3	3	1	46	1	1	1	1	1	9	198	5	283
3	3	2	110	1	1	1	1	1	20	400	10	570
3	3	3	> 110									

TABLE 8.7

MPN TABLE FOR 5 x 1, 5 x 0.1, AND 5 x 0.01 g (ml) (DE MAN, 1983)

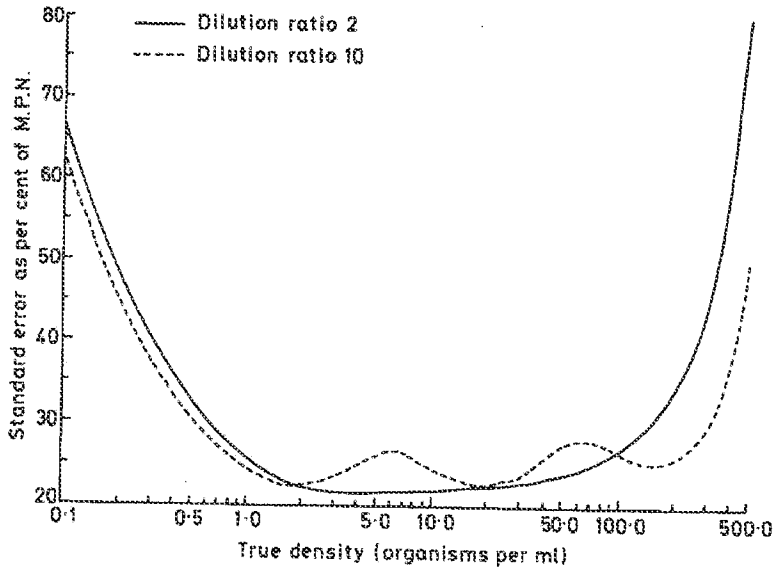
Number of positive results			MPN	Category* when No. of tests					Confidence limits			
				1	2	3	5	10	≥95%	≥95%	≥99%	≥99%
0	0	0	< 0.18						0.00	0.65	0.00	0.93
0	0	1	0.18	2	2	2	1	1	0.00	0.65	0.00	0.93
0	1	0	0.18	1	1	1	1	1	0.01	0.65	0.00	0.93
0	1	1	0.36	3	3	3	2	2	0.07	0.99	0.02	1.40
0	2	0	0.37	3	2	2	2	1	0.07	0.99	0.02	1.40
0	2	1	0.55	0	0	0	3	3	0.17	1.40	0.09	2.10
0	3	0	0.56	0	3	3	3	3	0.17	1.40	0.09	2.10
1	0	0	0.20	1	1	1	1	1	0.02	0.99	0.01	1.40
1	0	1	0.40	2	1	1	1	1	0.07	1.00	0.02	1.40
1	0	2	0.60	0	0	3	3	3	0.17	1.40	0.09	2.10
1	1	0	0.40	1	1	1	1	1	0.07	1.10	0.03	1.40
1	1	1	0.61	3	2	2	2	1	0.17	1.40	0.09	2.10
1	1	2	0.81	0	0	0	0	3	0.33	2.20	0.20	2.80
1	2	0	0.61	2	1	1	1	1	0.18	1.40	0.09	2.10
1	2	1	0.82	3	3	3	3	2	0.35	2.20	0.20	2.80
1	3	0	0.83	3	3	3	3	2	0.33	2.20	0.20	2.80
1	3	1	1.0	0	0	0	0	3	0.3	2.2	0.2	2.8
1	4	0	1.1	0	0	0	0	3	0.3	2.2	0.2	2.8
2	0	0	0.45	1	1	1	1	1	0.08	1.40	0.04	2.10
2	0	1	0.68	2	1	1	1	1	0.18	1.50	0.09	2.10
2	0	2	0.91	0	3	3	3	3	0.33	2.20	0.20	2.80
2	1	0	0.68	1	1	1	1	1	0.19	1.70	0.10	2.30
2	1	1	0.92	2	2	1	1	1	0.33	2.20	0.20	2.80
2	1	2	1.2	0	0	3	3	3	0.4	2.5	0.2	3.4
2	2	0	0.93	1	1	1	1	1	0.34	2.20	0.20	2.80
2	2	1	1.2	3	3	2	2	2	0.4	2.5	0.2	3.4
2	2	2	1.4	0	0	0	0	3	0.6	3.4	0.4	4.4
2	3	0	1.2	3	2	2	2	1	0.4	2.5	0.2	3.4
2	3	1	1.4	0	3	3	3	3	0.6	3.4	0.4	4.4
2	4	0	1.5	0	3	3	3	3	0.6	3.4	0.4	4.4
3	0	0	0.78	1	1	1	1	1	0.21	2.20	0.12	2.80
3	0	1	1.1	1	1	1	1	1	0.4	2.2	0.2	2.9
3	0	2	1.3	3	3	3	2	2.	0.6	3.4	0.4	4.4
3	1	0	1.1	1	1	1	1	1	0.4	2.5	0.2	3.4
3	1	1	1.4	2	1	1	1	1	0.6	3.4	0.4	4.4
3	1	2	1.7	3	3	3	3	2	0.6	3.4	0.4	4.4
3	2	0	1.4	1	1	1	1	1	0.6	3.4	0.4	4.4
3	2	1	1.7	2	2	2	1	1	0.7	3.9	0.5	5.1
3	2	2	2.0	0	3	3	3	3	0.7	3.9	0.5	5.2
3	3	0	1.7	2	2	1	1	1	0.7	3.9	0.5	5.2
3	3	1	2.1	3	3	3	2	2	0.7	3.9	0.5	5.2
3	3	2	2.4	0	0	0	3	3	1.0	6.6	0.7	9.4
3	4	0	2.1	3	3	2	2	2	0.7	4.0	0.5	5.2
3	4	1	2.4	0	3	3	3	3	1.0	6.6	0.7	9.4
3	5	0	2.5	0	0	0	3	3	1.0	6.6	0.7	9.4
4	0	0	1.3	1	1	1	1	1	0.4	3.4	0.3	4.4

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TABLE 8.7 (continued)

Number of positive results			MPN	Category* when No. of tests					Confidence limits			
				1	2	3	5	10	⩾95%	⩽95%	⩾99%	⩽99%
4	0	1	1.7	1	1	1	1	1	0.6	3.4	0.4	4.4
4	0	2	2.1	3	2	2	2	2	0.7	3.9	0.5	5.2
4	0	3	2.5	0	0	0	0	3	1.0	6.6	0.7	9.4
4	1	0	1.7	1	1	1	1	1	0.6	3.9	0.4	5.1
4	1	1	2.1	1	1	1	1	1	0.7	4.1	0.5	5.3
4	1	2	2.6	3	3	2	2	2	1.0	6.6	0.7	9.4
4	1	3	3.1	0	0	0	0	3	1.0	6.6	0.7	9.4
4	2	0	2.2	1	1	1	1	1	0.7	4.8	0.5	6.1
4	2	1	2.6	2	1	1	1	1	1.0	6.6	0.7	9.4
4	2	2	3.2	3	3	3	2	2	1.0	6.6	0.7	9.4
4	2	3	3.8	0	0	0	0	3	1.3	10.0	0.9	14.7
4	3	0	2.7	1	1	1	1	1	1.0	6.6	0.7	9.4
4	3	1	3.3	2	2	1	1	1	1.0	6.6	0.7	9.4
4	3	2	3.9	3	3	3	3	2	1.3	10.0	0.9	14.7
4	4	0	3.4	2	2	1	1	1	1.3	10.0	0.9	14.7
4	4	1	4.0	3	3	2	2	2	1.3	10.0	0.9	14.7
4	4	2	4.7	0	0	0	3	3	1.4	11.3	0.9	14.7
4	5	0	4.1	3	3	3	3	2	1.3	10.0	0.9	14.7
4	5	1	4.8	0	0	3	3	3	1.4	11.3	0.9	14.7
5	0	0	2.3	1	1	1	1	1	0.7	6.6	0.5	9.4
5	0	1	3.1	1	1	1	1	1	1.0	6.6	0.7	9.4
5	0	2	4.3	3	2	2	2	1	1.3	10.0	0.9	14.7
5	0	3	5.8	0	0	0	3	3	2.1	14.9	1.4	20.0
5	1	0	3.3	1	1	1	1	1	1.0	10.0	0.7	14.7
5	1	1	4.6	1	1	1	1	1	1.4	11.3	0.9	14.7
5	1	2	6.3	2	2	1	1	1	2.1	14.9	1.4	20.0
5	1	3	8.4	3	3	3	3	2	3.4	22.0	2.1	27.0
5	2	0	4.9	1	1	1	1	1	1.5	14.9	0.9	20.0
5	2	1	7.0	1	1	1	1	1	2.2	16.8	1.4	23.0
5	2	2	9.4	2	2	1	1	1	3.4	22.0	2.1	28.0
5	2	3	12	3	3	2	2	2	3	24	2	32
5	2	4	15	0	0	0	0	3	6	35	4	45
5	3	0	7.9	1	1	1	1	1	2.3	22.0	1.5	27.0
5	3	1	11	1	1	1	1	1	3	24	2	32
5	3	2	14	1	1	1	1	1	5	35	3	45
5	3	3	17	3	2	2	2	1	7	39	4	51
5	3	4	21	3	3	3	3	2	7	39	4	51
5	4	0	13	1	1	1	1	1	3	35	3	45
5	4	1	17	1	1	1	1	1	6	39	4	51
5	4	2	22	1	1	1	1	1	7	44	4	57
5	4	3	28	2	1	1	1	1	10	70	6	92
5	4	4	35	2	2	2	1	1	10	70	6	92
5	4	5	43	0	0	3	3	3	15	106	9	150
5	5	0	24	1	1	1	1	1	7	70	4	92
5	5	1	35	1	1	1	1	1	10	106	6	150
5	5	2	54	1	1	1	1	1	15	165	10	223
5	5	3	92	1	1	1	1	1	23	253	15	338
5	5	4	160	1	1	1	1	1	40	460	20	620
5	5	5	> 160									

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8.3 Composition of dilution ratios 2 and 10. (From Cochran, 1950)

1: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have the greatest chance of being obtained. There is only at most 5% chance of obtaining a result that is less likely than the least likely one in this category.

2: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 1, but there is only at most 1% chance of obtaining a result that is less likely than the least likely one in this category.

3: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 2, but there is only at most 0.1% chance of obtaining a result that is less likely than the least likely one in this category.

0: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 3. There is only a chance of 0.1% to obtain a result in this category, without anything being wrong.

Before starting testing it should be decided which category will be acceptable, i.e., only 1, 1 and 2 or even 1, 2 and 3. When the decision to be taken on the basis of the result is of great importance, only category 1 or at most 1 and 2 results should be accepted. Category 0 results should be considered with great suspicion.

Caution: The confidence limits given in the tables are meant only to provide some idea about the influence of statistical variations on results. There will always also be other sources of variation, which may sometimes even be more important.

8.3.3 Differences between MPN values

From time to time it may be necessary to establish the significance of the difference between two MPN estimations. This can be determined using the combined standard errors for the two MPNs by reference to standard statistical tables of Students "t", viz:

$$t = (\log \hat{m}_1 - \log \hat{m}_2)/0.55 \sqrt{(\log a_1/n_1) + (\log a_2/n_2)}$$

or:

$$t = (\log \hat{m}_1 - \log \hat{m}_2)/0.58 \sqrt{(\log a_1/n_1) + (\log a_2/n_2)}$$

when dilution ratio (a) is 10-fold. The use of the method is illustrated in Ex. 8.3.

Example 8.3. Calculation of Most Probable Numbers for two dilution series and the significance of the differences between the MPN estimates

Assume: series (A), 10-fold dilutions with 5 tubes per dilution (each inoculated with 1 ml of dilution) giving the following results:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Result	5 + 0-	2 + 3-	1 + 4-	0 + 5-

Then from Table 8.7, MPN = 7 x 10 = 70 propagules/ml, with 95% confidence limits of 22 - 168/ml.

Assume: series (B), 10-fold dilutions, with 3 tubes per dilution (each inoculated with 1 ml of each dilution) giving the following results:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Result	3 + 0-	3 + 0-	0 + 3-	0 + 3-

The MPN from Table 8.7 is 170 propagules/ml using codon 3-3-0 with 95% CL of 70 - 390/ml.

These two test series provide MPNs of 70 and 170 propagules/ml but, although different, the 95% CLs of the MPNs overlap. The significance of the difference is tested by:

$$t = (\log \hat{m}_1 - \log \hat{m}_2)/0.58\sqrt{(\log a_1)/n_1 + (\log a_2)/n_2}$$

where:

$\hat{m}_1$ and $\hat{m}_2$ = MPNs for series (A) and (B), respectively;

$a_1 = a_2$ = dilution ratio = 10;

n_1 and n_2 = number of tubes for series (A) 5, and (B) 3, respectively.

Then:

$$t = (\log 170 - \log 70)/0.58\sqrt{(\log 10)/5 + (\log 10)/3}$$

$$= (2.2304 - 1.8451)/0.58 \sqrt{(0.2 + 0.33)} = 0.3853/0.4236 = 0.9097$$

From Pearson and Hartley's (1966) Table 12, for $\nu = (5 + 3 - 2) = 6$ degrees of freedom, the observed value of t would be expected to occur on about 4 occasions in 10 ($p \approx 0.40$). Hence, the difference between these observed MPN values could occur by chance and is not significant at the 5% level.

8.3.4 Special applications of multiple-tube dilution tests

For control purposes it may be required to make a decision to accept or reject a consignment of food on the basis of a MPN test (e.g. for *Enterobacteriaceae*). For such purposes it is possible to construct an operating characteristics (OC) curve (§ 5.2.3) for acceptance or rejection at specific confidence levels of tube dilution counts. The best control system would be based on the use of a limit permitting "acceptance of the 'lot' if less than c tubes out of n at dilution i show evidence of growth" (i.e. if the number of sterile tubes at dilution i is $> c_i$).

The shape of the OC curve will be dependent upon the values c and n for the dilution level giving the critical density of propagules. On the assumption that log n is normally distributed when n is large, the steepest part of the OC curve will occur at the 50% acceptance point (approximate value of $c = 0.2\ n$). Lower percentage acceptance would be associated with $c < 0.2\ n$ and vice versa. The appropriate choice of c will therefore lie in the range $0 - 0.5\ n$, assuming that the cumulative binomial distribution is a valid model.

8.4 Presence or absence tests based on single or multiple samples

In the analysis of foods for low numbers of pathogenic organisms (e.g. salmonellae), it is normal to inoculate one or more samples into a suitable selective or enrichment medium prior to streaking on to a diagnostic medium to determine whether or not the specific organism sought is present. Apart from purely technical errors, presence/absence tests are subject to a number of statistical considerations concerning the distribution of organisms.

A quality control procedure based on this concept has been proposed by Aspinall and Kilsby (1979). In their scheme a critical value (c) is chosen such that when: (1) the cell density exceeds c the probability of rejection is at least 0.95; and (2) when the cell density is less than $0.2\ c$, the probability of acceptance is 0.95.

For a critical value (c) of 10^{x+1} g the material is diluted $10^{(x-1)}$-fold and 0.5 ml of this suspension is inoculated into each of two tubes of culture medium. A further 10-fold dilution of the suspension (i.e. a 10^{-x}-fold dilution) is then prepared and 0.5 ml is added to each of seven tubes of media. Following incubation, the MPN and confidence limits can be derived from the data in Table 8.8;

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note that the CL cited are twice those quoted in the original paper of Aspinall and Kilsby (1979) (Kilsby and Aspinall, personal communication, 1983). If all tubes give a positive response, the material is rejected; if a result is obtained which is not shown in Table 8.8 the test must be repeated. The scheme is illustrated in Ex. 8.4.

TABLE 8.8

ESTIMATES OF MPN/g (MODIFIED FROM ASPINALL AND KILSBY: 1979)

Numbers of positive tubes at dilution		MPN/g	95% Confidence limits	
$10^{(a-1)}$	10^{a}	$\times 10^{-(a-1)}$	lower $\times 10^{-(a-1)}$	upper $\times 10^{-(a-1)}$
0	0	< 0.76 (0)	-	2.8
0	1	0.76	0.014	2.8
1	0	0.92	0.038	4.8
1	1	1.9	0.32	6.4
2	0	2.7	0.48	10.8
1	2	3.0	1.34	6.8
2	1	4.6	0.90	16.2
2	2	7.4	1.92	22.0
2	3	11.0	2.8	30.0
2	4	17.0	4.0	40.0
2	5	25.0	6.8	58.0
2	6	39.0	11.2	98.0
2	7	> 39 (∞)	19.6	∞

Example 8.4. Use of Multiple Tube MPN for Quality Acceptance/Rejection Purposes

Suppose that for a food product the quality specification requires rejection of product containing more than 1000 organisms/g. Then $c = 10^3 = 10^{(a+1)}$, hence $a = 2$.

Prepare product dilutions of $10^{-(a-1)}$ (i.e. 10^{-1}) and 10^{-a} (i.e. 10^{-2}) and inoculate 0.5 ml of the 10^{-1} dilution into each of 2 tubes and 0.5 ml of the 10^{-2} dilution into each of 7 tubes of broth. Following incubation, examine for evidence of growth in the tubes of media.

Suppose the following results are obtained on three lots of product:

Product	Result at dilution of		MPN/g*	Decision
	10^{-1}	10^{-2}		
A	2+	7+	$> 39 \times 10^1 = > 390$	Reject lot
B	1+	5+	Not calculable	Repeat test
C	0+	1+	$0.76 \times 10^1 = 7.6$	Accept lot

* From Table 8.8.

A potential problem in the application of the methods as described is that if all nine tubes give positive results (i.e. 2+, 7+) the MPN cannot be calculated, yet requires that the product be rejected. However, if only eight positive tubes occur (i.e. 2+, 6+) the product would be accepted with an MPN of 390/g (Table 8.8). If, as in Ex. 8.4, the critical level (c) is an MPN of 1000/g, the cut-off of 390/g would appear to be somewhat low. However, the (2,7) scheme of Aspinall and Kilsby (1979) is much less likely to reject satisfactory material than is a 3 level, 3 tube (3,3,3) scheme such as is frequently used for control purposes (ICMSF, 1978).

8.4.1 Quantification based on relative occurrence of defectives

In Chap. 5, the use of the binomial distribution to describe the operating characteristics (OC) curve for sample plans was described. The binomial relationship describing the probability of occurrence of defective samples can be used also to quantify the probable level of occurrence of organisms in the lot of food under test.

On the assumption that the organisms in a "lot" of food are distributed randomly and that a number (n) of samples is tested, then the probability (P) of no defective test samples (i.e. no positive tests) is given by:

$$P = 1 - [1 - (d/100)]^n$$

where d = actual % defectives in the lot. This will be true provided that n is a small fraction (< 25%) of the total lot. The equation may be rearranged to give the % defectives for a given probability and number of samples:

$$d = 100\ (1 - \sqrt[n]{(1 - P)}$$

Knowing the incidence of defective samples (d) and the size of each sample (W) it is then possible to derive the maximum contamination level (C) for the lot:

$$C = (d/100)\ (1/W) \text{ organisms/g at probability level } P$$

$$= [1 - \sqrt[n]{(1 - P)}]/W$$

This calculation is illustrated below in Ex. 8.5. Derived values for the maximum incidence of contamination at $P = 0.95$ and $P = 0.99$, assuming a sample size of 25 g, are presented in Table 8.9 for various sample sizes (n).

From the data in Table 8.9, it can be seen that in testing for salmonellae, a negative test result on a single sample (i.e. $n = 1$) of 25 g will merely indicate, on average, that on 19 occasions out of 20 the lot will contain < 38 salmonellae/kg. Increasing the number of samples tested to 10 or 20 increases

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the confidence for freedom from salmonellae since, on average, on 19 occasions out of 20 the lot would be expected to contain < 11 or ≯ 6 salmonellae/kg, respectively, if all test results were negative. The data also indicate on average that on 99 occasions out of 100, the maximum contamination levels would correspond to 40, 15 or 9 salmonellae/kg, respectively, for 1, 10 or 20 samples tested and found to be "negative".

TABLE 8.9

PERCENTAGE DEFECTIVES AND MAXIMUM CONTAMINATION LEVELS ASSUMING RANDOM DISTRIBUTION OF ORGANISMS

No. samples tested	Maximum % defectives* at		Maximum no. organisms/kg*† at	
n	$P = 0.95$	$P = 0.99$	$P = 0.95$	$P = 0.99$
1	95	99.0	38	40
2	77.6	90.0	31	36
3	63.2	78.5	26	32
4	52.7	68.4	21	28
5	45.1	60.2	18	24
10	25.9	36.9	11	15
20	13.9	20.6	6	9
30	9.5	14.2	4	6
50	5.8	8.8	3	4
100	3.0	4.5	2	2

* Assuming no positives detected.
† Assuming sample size = 25 g; rounded up to the nearest whole number.

Quantification based on positive results can be derived as the most probable number (MPN) (§ 8.3.1 above) for multiple tests at a single dilution. Hence, using $\bar{m} = -(1/v)\ \ln\ (s/n)$, a single positive from 10 tests each of 25 g provides a MPN of 4.2 organisms (e.g. salmonellae)/kg. This value is well below the maximum number which would be expected at $P = 0.95$ if all tests had been negative but the 95% confidence limits of the MPN range from < 1 to > 60 salmonellae/kg

8.4.2 Effect of subculture volumes and incubation times

When samples are tested for presence/absence of specific microbes, the analytical procedure may involve the use of pre-enrichment and selective enrichment stages.

Different methodological details are recommended by different authorities. Using the recommendations of ICMSF (1978) for salmonellas testing, the initial sample is dispersed in 9 volumes of pre-enrichment medium, and after a suitable incubation period 1 ml is transferred to 10 ml of each of two selective enrichment media. Thereafter, one 5 mm loopful (about 0.02 ml) for each selective enrichment is streaked on to one plate of each of three selective diagnostic media and, after incubation, selected colonies are picked for typing.

Hence, three dilution stages occur. The first stage results in a 10-fold dilution of the sample. Organisms inoculated with the sample will grow during incubation, but the number growing will depend upon (a) the initial contamination level and the dispersion both of the organism being sought and of competitive organisms; (b) the condition of the organisms (i.e. whether cells have been "stressed" by the environmental conditions in the food); (c) the population doubling time for the organism sought in the conditions of the test (i.e. culture medium composition, incubation temperature and time).

The second and third stages (selective enrichment) will be affected similarly, but in the third stage the individual propagules will be required to form discrete colonies on the diagnostic medium. The fourth stage (picking of colonies) is considered below (§ 8.4.3).

Clearly, the possibilities for isolation of specific organisms will depend not only on the initial incidence of contamination but also on the manner in which the test is done. A too short period of pre-enrichment may result in no recovery of stressed organisms, thereby reducing the statistical probability of transferring resuscitated organisms to the enrichment broth. Too long a period in a non-selective pre-enrichment may provide opportunity for overgrowth by competitive organisms, leading to suppression of the organism sought. A hypothetical example of the effects of incubation time is shown in Ex. 8.6 and 8.7 and in Fig. 8.4.

Example 8.5. Calculation of maximum probable contamination level for presence/absence tests

Assume 10 x 25 g samples are tested for salmonellae and all tests are negative. What are the maximum contamination levels at probabilities of 95% and 99%. Using the formula:

$d = 100\ (1 - \sqrt[n]{(1 - P)})$, for (A): $P = 0.95$ and (B): $P = 0.99$:

(A) $d = 100\ (1 - \sqrt[10]{(1 - 0.95)}) = 100\ (1 - \sqrt[10]{0.05})$

$= 100\ (1 - 0.741)$

$= 25.89\%$

Hence, at 95% probability, maximum contamination of the lot by salmonellae = $C_A = 25.89/(100 \times 25) = 10.4$ salmonellae/kg.

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(8) $d = 100\ (1 - \sqrt[10]{0.01}) = 36.90\%$

Hence at 99% probability, the maximum contamination of the lot by salmonellae = 14.8 salmonellae/kg = $c_B = 36.9 \times 1000/(100 \times 25)$.

Example 8.6. Effect of incubation time in pre-enrichment medium on numbers of salmonellae for subculture.

Assume: (1) 25 g dry food sample inoculated into 225 ml nutrient broth for pre-enrichment; (2) initial contamination level = x salmonellae/g; (3) length of lag phase (L) for resuscitation = 6 h; (4) population doubling time (T_d) = 30 min = 0.5 h), at the appropriate incubation temperature; (5) synchronous growth occurs.

Using:

$$N = N_0\ 2^{(T-L)/T_d}$$

where:

N = no. salmonellae/ml after time T (h)

N_0 = no. salmonellae/ml initially

L = length of lag phase (h)

and

T_d = doubling time (h)

Then since:

$N_0 = 25\ x/250 = 0.1\ x$

$L = 6$ h

and

$T_d = 0.5$ h

After:

6 h, $N = 0.1\ x\ 2^{(6-6)/0.5} = 0.1\ x\ 2^0 = 0.1\ x$

9 h, $N = 0.1\ x\ 2^{(9-6)/0.5} = 0.1\ x\ 2^6 = 6.4\ x$

12 h, $N = 0.1\ x\ 2^{(12-6)/0.5} = 0.1\ x\ 2^{12} = 410\ x$

24 h, $N = 0.1\ x\ 2^{(24-6)/0.5} = 0.1\ x\ 2^{36} = 6.9\ x \times 10^9$

Hence, the expected number of organisms likely to be transferred in an inoculum of pre-enrichment medium and the probability of transferring at least 1 viable organism, assuming random distribution when $x = 0.1$ (i.e. 10 organisms/100 g) is

Incubation time (h)*	No. organisms** in 1 ml pre-enrichment medium	Probability of ≥ 1 organism** in	
		1 ml	10 ml
6	0.01	< 0.01	0.095
9	0.64	0.47	> 0.99
12	41.0	> 0.99	> 0.99
18	1.7×10^5	> 0.99	> 0.99
24	6.9×10^8	> 0.99	> 0.99

* In pre-enrichment, assuming conditions as above.
** Assuming α = 0.1 salmonellae/g food sample

Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be ≥ 0.95, assuming Poisson distribution, with $\hat{m}$ =3: $P_{(\alpha=0)} = 0.05$, therefore $P_{(\alpha\geq1)} = 0.95$. However, at lower numbers, the chances of transferring at least 1 viable organism will be affected not only by the initial contamination level (α) but also by the volume of medium inoculated into the enrichment culture. In methods which permit only resuscitation, not growth, during pre-enrichment, it is essential to use the entire pre-enrichment culture to inoculate double-strength enrichment medium (Hall, 1975), although selectivity may then be affected by the food constituents.

Obviously the level of salmonellae in the pre-enrichment culture after any given incubation period will depend upon the cultural conditions used, the initial contamination level, the extent of sublethal cell damage and the relative numbers and types of competitive organisms in the sample.

8.4.2.1 Compositing of samples. When a number of samples is to be tested it is sometimes recommended that the samples be composited in order to reduce the work load on the laboratory. Several methods of compositing can be undertaken:

(1) Combine all samples at pre-enrichment stage; i.e. for 10 x 25 g samples inoculate all 250 g directly into 2250 ml of pre-enrichment medium. After incubation, carry out tests on the same basis as for a single sample (i.e. inoculate 10 ml into 100 ml enrichment medium).

(2) Prepare 10 pre-enrichment series (i.e. one for each 25 g sample) and after incubation composite equal volumes of each pre-enrichment medium (e.g. 10 x 1 ml) to inoculate the 100 ml enrichment broth.

(3) A combination of (1) and (2) above where, say, 5 samples are composited at pre-enrichment stage and aliquots of the duplicate pre-enrichment media are composited at the stage of inoculating the enrichment medium.

(4) Composite all 10 samples and then test one (or rarely two) 25 g aliquots from the bulk composited samples.

Methods (1), (2) and (3) provide the same degree of statistical confidence as would be attained by testing the 10 samples separately, in so far as a negative result indicates no sample contamination and therefore < 11 organisms/kg (at P = 0.95). However, if a positive result is obtained, it is not possible to estimate (even approximately) the degree of contamination. Method (2), at least in theory, permits the tests to be repeated on stored pre-enrichment cultures

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so that some indication of the proportion of contaminated samples can be obtained. Method (3) is a compromise between Methods (1) and (2).

Method (4) is based on a fallacious concept, since by compositing the original sample and testing only one aliquot of the composite sample, the results will provide no more information about the lot than would testing of a single sample of, say, 25 g. It is totally incorrect to interpret a negative result as indicating (at $P = 0.95$) a maximum contamination level of 11 organisms/kg, which would have been given by 10 negative results.

8.4.3 Selection of colonies for identification

After development of isolated colonies on the surface of the selective-diagnostic medium, it is necessary to select for further identification colonies showing typical morphology. The chances of isolation of specific organisms from an almost pure culture will of course be high. However, when mixed cultures are present and some of the organisms of different types have apparently similar colonial morphologies, then several colonies may need to be picked. In some laboratory manuals (Hall, 1975) no reference is made to the number of colonies to pick; others (ISO, 1979) recommend selection of the square root of the number of "typical" colonies, whilst ICMSF (1978) recommend picking of at least two typical colonies and ISO (1978) recommends picking five typical colonies.

It can be shown that the chance of picking any one colony of the type sought is dependent upon the number (n) of the specific type relative to the total number (N). Since N is not large the relationship is governed by the hypergeometric distribution. Table 8.10 shows the number of colonies of the type sought (e.g. *E. coli*) which must be present in order to select, with a given statistical probability, at least one colony of the type sought from among a population of otherwise typical colonies. For instance, if the true incidence of *E. coli* is 50% of the colonies having "typical" coliform morphology, then if one picks 5 colonies out of a population of 30 colonies, the chance of picking at least one *E. coli* is slightly greater than 95% ($p > 0.95$). If, however, the true incidence is only 10% (i.e. 3 *E. coli*/30 colonies) then the chance that at least one *E. coli* colony is included in the sample of 5 colonies is somewhat less than 50% ($P < 0.50$).

Example 8.7. Statistical Probability of Transferring Salmonellae during Enrichment and Isolation.

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Exhibit V

COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

EDITED BY
FRANCES POUCH DOWNES
KEITH ITO



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Chapter 10

Rapid Methods for Detection, Identification, and Enumeration

Phyllis Entis, Daniel Y.C. Fung, Mansel W. Griffiths, Lynn McIntyre, Scott Russell, Anthony N. Sharpe, and Mary Lou Tortorello

10.1 INTRODUCTION

Microbiological testing is both time- and labor-intensive, and there is a need to develop easier, faster methods for detecting, identifying and enumerating microorganisms in foods. The reasons for the difficult nature of microbiological testing are well known. First, the microorganism of interest (MOI) is often present at low levels relative to the mixed community of species in which it exists; i.e., finding and recognizing it are difficult tasks. Second, food is a complex matrix, which causes interference with analytical methods; i.e., some degree of separation or purification of the MOI is required. Most standard microbiological methods employ measures that address both problems. Selective enrichment allows the smaller MOI population to increase in number while keeping in check the growth of other species. The enrichment culture is then plated onto a differential medium to obtain isolated colonies, and in doing so, the MOI is effectively purified from the food matrix. Detection of typical colonies is followed by subculture and a variety of tests for species identification. Enumeration of the MOI is an additional task that is often needed, and in most cases it is even more difficult than detection and identification.

Rapid methods for detection, identification and enumeration of microorganisms offer savings in both time and labor. While many rapid detection and identification methods have been developed and brought to market, the choices available in rapid methods of enumeration are much more limited. The world of rapid methods is expanding and changing, however, and innovations undoubtedly will continue to be made.

This chapter covers selected rapid procedures for detection, identification and enumeration of microorganisms in foods. The various sections of the chapter cover methods based on antibody and nucleic acid recognition; biochemical and enzymatic methods; phage probes; membrane filtration; and impedance. Many of these rapid methodologies have been commercialized and/or have undergone collaborative testing. Details for performing rapid assays for specific microorganisms or groups of microorganisms appear in many other chapters in this book.

Because of its dynamic nature, it is difficult to portray the field of rapid methods with the currency to last until the next edition of the *Compendium*. Updates may be obtained from more frequently published information sources, such as the trade journals, the publications of professional organizations, newsletters and catalogs of commercial suppliers, and the Internet.

10.2 ANTIBODY-BASED METHODS

10.21 Introduction

Antibodies are protein molecules which bind to specific cellular structures or products, generally called antigens. The identification of an antigen that is unique to a particular microbial group (genus, species, strain, etc.) is the first step in the design of an antibody-based method. Antibody-based methods are available for a wide variety of microbial groups, and in fact, have been used for microbial identification for decades. Improved assay design and the use of monoclonal antibodies have significantly reduced the specificity problems which plagued many of the first-generation antibody-based methods. Now these methods are among the most commonly used techniques in food microbiology for rapid detection and identification. Antibodies are also used in affinity separation applications, in which microbial cells or their products are purified from the food matrix for use in subsequent assays. The applications of immunoassay technology to food analysis are broad; however, their most significant impact in food microbiology, both commercially and from a food safety perspective, has been in the detection of foodborne pathogens or their toxins.[7,30,42] A list of antibody-based rapid methods for foodborne pathogen detection has been published.[18] Because so many antibody-based methods have been commercialized, their individual descriptions will not be attempted in this chapter. Many of the subsequent chapters in this book provide details of the methods as they pertain to particular microorganisms.

10.22 Immunofluorescence

Immunofluorescence methods utilize fluorescent antibodies, i.e., antibodies coupled to fluorescent dyes, for specific identification of microorganisms. Fluorescent antibody techniques were among the first rapid methods available in food microbiology.[2] In the traditional immunofluorescence assay, microbial cells from an enrichment culture are fixed to a microscope slide, and the fixed cells are treated with fluorescent antibody. After excess reagent is removed, the slide is observed using a fluorescence microscope. The presence of the specific microorganisms is visualized by fluorescent staining of the cells. Sensitive quantitation of specific bacteria may be achieved by including membrane filtration to concentrate the sample, prior to performing the fluorescent antibody reaction on the surface of the filter.[40] Immunofluorescence is the

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basis for flow cytometry, a technology used for many years in clinical applications, e.g. recognition of lymphocyte subpopulations based on antigen expression. Flow cytometers have been modified so that detection of cell sizes on the order of microorganisms is possible. The instrumentation involves detection of fluorescent cells moving in a fluid stream past an optical sensor. Cell sorting capability also provides the possibility of isolating the fluorescent cells from a mixture. Applications to the microbiological analysis of foods, including pathogen detection, have been limited but promising.[9,14,26,28,43]

10.23 Latex Agglutination

Antibodies coupled to latex microspheres are the reagents used in latex agglutination assays for microbial identification. When in the presence of reactive microbial cells, the microspheres agglutinate due to formation of molecular cross-bridges, resulting in a visible "clumping" reaction. The microbial cells are generally obtained from an isolated colony, although a centifuged sample of an enrichment culture may also be used. The sensitivity of the latex agglutination test is generally in the range of 10^7–10^8 cells per mL of sample. Latex agglutination assays have been described for several foodborne pathogens, including *Salmonella*,[13] *Shigella*,[29] *Campylobacter*,[23] and *E. coli*.[27] The assays are often used to confirm the identity of colonies from selective agar. It is important to perform appropriate controls each time to ensure that the microspheres have retained reactivity and to detect false-positives caused by spontaneous agglutination of the microspheres.[6]

In reverse passive latex agglutination (RPLA), antibodies attached to latex microspheres react with soluble antigens (such as toxins) in test tubes or wells of microtiter plates. If an antigen-antibody reaction occurs, a diffuse pattern is observed at the bottom of the tube or well. In the absence of a reaction, a ring or "button" is observed.[20] RPLA assays have been commercially developed for detection of enterotoxins, including those produced by *Staphylococcus*,[31] *Bacillus cereus*,[3] *Clostridium perfringens*,[21] *E. coli*,[4] and *Vibrio cholerae*.[1]

10.24 Immunoimmobilization

Antibodies directed against cellular flagella may be used in immobilization assays to detect specific motile microorganisms. Motile microbial cells traverse a semisolid medium until they meet the antiflagellar antibodies diffusing from the opposite direction. A visible arc, which forms at the antibody/microbial cell interface, is the indication that target microorganisms are present.[12,15,36] Non-motile variants of the microorganisms, or those expressing different flagellar antigens, are not detected by the assay.

10.25 Enzyme Immunoassay

Enzyme immunoassay (EIA), also known as enzyme-linked immunosorbent assay (ELISA), is a common technique for antibody-based detection of microorganisms or their products. Since its origin in the 1970's, the EIA has become a standard tool in many food microbiology laboratories. Many different EIA formats have been developed.[8,37,39] However, the common element in all of the formats is the involvement of antibody recognition of a target antigen, with some type of linkage to an enzyme-substrate reporter system. The enzyme's activity upon its substrate produces a measurable product, which indicates whether the antigen-antibody reaction took place and, therefore, whether the target antigen was present in the sample. In food microbiology, the "sandwich" assay is a familiar format and is illustrated in Figure 1. In the sandwich assay, antibody that is immobilized onto a solid phase, such as the well of a microtiter plate, captures the specific antigen, e.g. the target microbial cells present in an enrichment culture. After rinsing to remove unbound substances, a second antibody that is conjugated to an enzyme, such as horseradish peroxidase or alkaline phosphatase, is added. Another wash is then performed. After addition of an appropriate substrate for the enzyme, a visible product of the enzyme-substrate reaction is formed, which indicates the presence of the target microbial cells in the original sample. Most often, the reaction product causes a visible color change. The intensity of the colored product formed is directly proportional to the concentration of the target antigen, within a certain range, and may be quantified by optical instrumentation. Variations on the EIA theme employ fluorescent or chemiluminescent reaction signals instead of color development.[19,25,45,47]

An essential element in EIA interpretation is the numerical value, obtained from the optical instrumentation, that is used to discriminate between a positive test and a negative test, i.e., the "cut-off" value. The terms "positive" and "negative" are used to indicate the presence or absence of target antigen, respectively. The cut-off value is often set to be 2–3 times the mean of several negative control results.

Perhaps the most widely used support for the EIA is the microtiter plate, but test tubes, blotting membranes, and even micropipet tips have been used.[38,46] EIAs have become so popular that fully automated systems have been developed for their performance.[11,24]

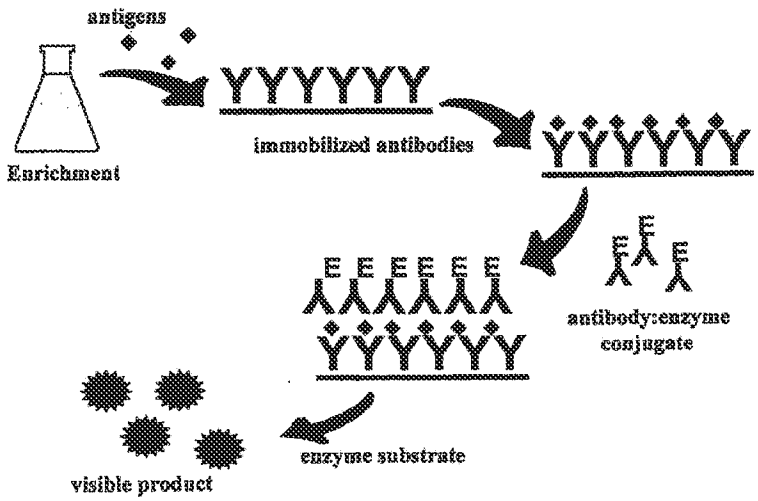


Figure 1. The "sandwich" enzyme immunoassay (EIA) format

10.26 Immunoprecipitate Detection

Recent entries to the world of rapid assays are the immunoprecipitate detection devices (Figure 2), in which an aliquot of enrichment culture is added to the sample port on a "paddle," and the sample is wicked through a chromatographic matrix to a reaction area containing antibodies conjugated to some type of precipitable material, such as colored latex particles or colloidal gold. If antigen is present,

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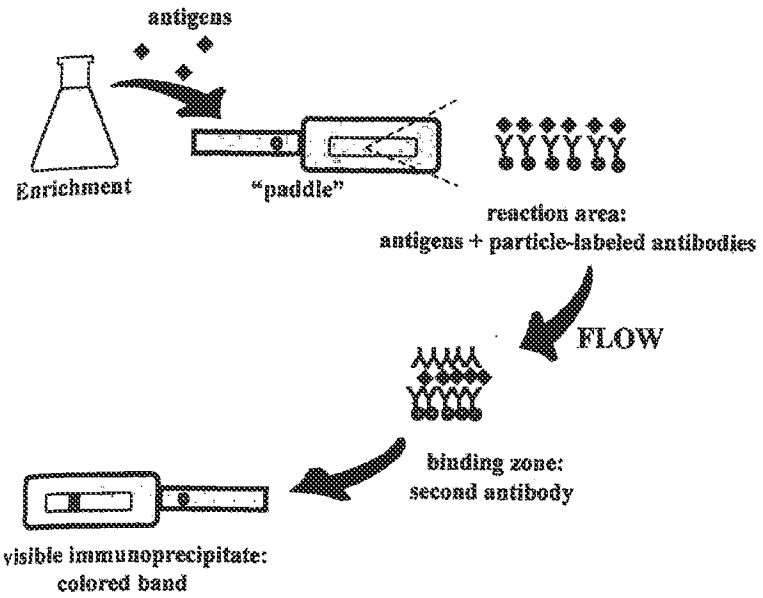


Figure 2. Generalized design of an immunoprecipitate detection device.

the antigen-antibody complex flows through the matrix to a binding zone, where it binds to an immobilized second antibody. The localized concentration of the antibody-antigen-antibody complex results in a visible immunoprecipitate, indicated by a colored band on the matrix. A control reaction area also exists on the paddle to provide visible assurance that the detection device is working correctly. A band in both reaction areas is a positive test for the presence of the target antigen. A band only in the control area is a negative test. Immunoprecipitate detection devices have been described for several food pathogens.[5,16,17] The reaction is extremely rapid, with an assay time as short as 5 minutes.

10.27 Immunoaffinity Chromatography and Immunomagnetic Separation

In addition to detection and identification of target antigens, antibodies have been used to achieve separation of antigens from complex matrices by their specific binding characteristics. Immunoaffinity chromatography is a quick way to separate and purify soluble antigens, and immunomagnetic separation allows isolation of particulate antigens, i.e., microbial cells, from a mixture in one step.

Antibodies coupled to a solid inert matrix are used in immunoaffinity chromatography. The sample containing the target antigen is allowed to flow through the immunoaffinity matrix, which is often packed in a column for ease of use. The target antigen is retained in the immunoaffinity matrix while other materials are removed by extensive washing of the column with buffer. The target antigen is then eluted by changing the composition of the buffer appropriately. Immunoaffinity columns have been used for detection of toxins in foods.[34]

Immunomagnetic separation (IMS) is performed by reaction of microbial cells, usually from an enrichment culture, with antibody-coated magnetic microspheres, or beads (Figure 3). The antibody molecules "capture" the microbial cells through their specific binding capability. After a magnet is applied to the side of the test tube, the magnetic beads are drawn to the wall of the tube, effectively concentrating and purifying the captured cells from the mixture. Non-target cells and food debris are washed away, leaving a purified suspension of target cells, which may then be used in subsequent assays. They may be plated onto selective agar to achieve isolation of the microorganism,[35,44] or used as a purification step prior to use in other assays, e.g., immunoassays,[10] nucleic acid assays,[23] or optical methods such as microscopy, flow cytometry and electrochemiluminescence detection.[32,43,48] IMS improves the efficiency of colony isolation and confirmation so that shorter enrichment times may be possible.[33] Applications for the use of these handy reagents will no doubt continue to increase in number in the future.

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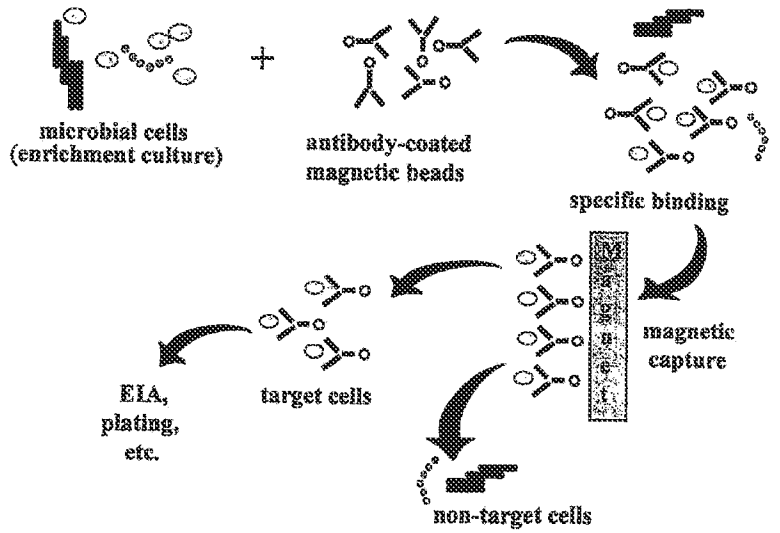


Figure 3. *Immunomagnetic separation of target microbial cells from food matrix and other microorganisms.*

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10.3 NUCLEIC ACID-BASED METHODS

10.31 Introduction

The sequence of nucleotide bases in the DNA of a microorganism is ultimately its most defining feature. The entire DNA molecule, however, contains too much information to process; therefore, shorter nucleotide sequences that are unique but more readily analyzed are the targets of nucleic acid-based detection methods. Hybridization and the polymerase chain reaction (PCR) are the nucleic acid-based methods that are most commonly used in food microbiology,[33] and they will be described in this section. Both methods depend on the rules of complementary nucleotide base pairing, i.e: adenine binds to thymine, and cytosine binds to guanine. In hybridization, the target sequence is recognized by a complementary nucleotide probe that is labeled in some way for direct detection of the sequence. In PCR, two nucleotide primers bind to complementary regions that flank the target sequence, which is then enzymatically amplified for detection. In either method, the DNA molecule must first be denatured to separate the double-stranded helix into single strands so that the probe or primers can bind to the appropriate sequence.

Whereas hybridization requires approximately 10^5–10^6 copies of the target DNA for detection, PCR is much more sensitive, producing millions of copies of a single gene target in a few hours.[21] Hybridization can be quantitative, if performed as a colony hybridization using the same format as agar plate counting. Quantitation is possible but difficult with PCR, which is most commonly used as a qualitative or presence/absence assay. Compared to antibody-based methods, there are relatively few commercialized versions of nucleic acid-based methods, which will be described in this section.

10.32 Hybridization

Hybridization is the process by which a DNA probe binds to a complementary region of single-stranded DNA or RNA. A DNA probe is usually a 20–2000 nucleotide base sequence of DNA that is unique to a particular microbial group. Probes can identify specific genes, e.g. toxin genes; regions of ribosomal RNA (rRNA); or DNA sequences that have unknown function but are common to particular microbial groups.

DNA probes may be prepared synthetically or biologically. Short oligonucleotide probes (20–30 bases in length) are easily prepared with the aid of a DNA synthesizer, and the proliferation of commercial suppliers offering such services has made oligonucleotide probes both widely available and affordable. Biological preparation of probes involves cloning of a nucleotide sequence into a plasmid that replicates many copies of the sequence. The entire plasmid may be used as a probe, but better specificity is likely if the nucleotide sequence is isolated by restriction endonuclease digestion and subsequent gel electrophoresis. In either case the probe sequence must be labeled so that it provides a signal that can be detected in the hybridization assay.

The radioactive isotope ^{32}P was the first probe label to be used. Although highly sensitive, ^{32}P as a probe label has all the disadvantages of handling and disposal of radioactive material, as well as a short shelf-life. There has been a trend toward development of non-isotopic methods of probe detection, such as fluorescent labels. Non-isotopic methods often involve enzymes, e.g., horseradish peroxidase or alkaline phosphatase, which can act upon substrates, producing color or chemiluminescence. Most commonly the enzyme is conjugated to a protein that binds to a specific ligand on the DNA probe to form an enzyme-binding protein-ligand-DNA complex. Many variations on the theme have been applied. A commonly used ligand-binding protein pair is biotin-streptavidin. Antibodies have also been used as binding proteins for ligands on the DNA probe.

There are two basic hybridization formats: solid-phase and liquid-phase. The phase refers to the location of the target sequence during hybridization. In solid-phase hybridization, the target sequence is bound to a solid support, e.g. a membrane filter. In liquid-phase hybridization, the target sequence is suspended in liquid, and after the hybridization reaction has occurred, it is removed from solution for detection.

Solid-phase hybridization may be performed as colony blots or dot-blots. Colony blotting involves transfer of individual colonies that have grown up on agar plates to a membrane filter. In dot-blotting, microbial growth from liquid or solid culture is transferred, e.g. with a loop or other device, as a "dot" onto a localized area of a membrane. In either case, the membrane is then treated with reagents that lyse the cells and denature the DNA. The released single strands of target DNA stick in place to the membrane, and then the probe is added. After the probe hybridizes with the matching DNA sequence on the membrane, unhybridized DNA is washed away. The composition of the hybridization buffer, temperature, and the conditions of washing are all critical to the specificity of the reaction and may be varied to affect the accuracy of the probe binding. After hybridization, an appropriate method is used for detection of the probe-target hybrid. If the probe is labeled with ^{32}P, a piece of X-ray film is placed on the membrane. The radioactive label exposes the film and develops spots where the probe hybridized to the DNA. If a non-isotopic probe is used, the reagents are added to develop visible spots directly on the membrane.

Colony blots may be used to enumerate target microbes in a food, if the food is plated directly. After development of the probe signal on the membrane, the reactive spots are counted and may be related directly to colony forming units of the target microbe. Dot blots are not quantitative. A generalized illustration of a colony hybridization is shown in Figure 4. Detailed procedures have been published for performing colony hybridizations for major foodborne pathogens, including *E. coli*, *Shigella*, *Listeria*, *Staphylococcus*, *Vibrio*, and *Yersinia*.[20] Hydrophobic grid membrane filtration (section 10.5) has been combined with probe hybridization to provide improved identification of *Listeria* and *E. coli* colonies.[35,39]

In liquid-phase hybridization, both probe and target DNA are suspended in solution, where the hybridization proceeds. Following hybridization, the target DNA with its hybridized probe is separated from the solution through capture of the hybrids on a solid support. Capture may be effected on hydoxylapatite, because of the relatively high affinity to hydroxylapatite that double-stranded DNA shows compared to single-stranded DNA. A streptavidin-coated matrix may be used to capture DNA that has been labeled with biotin. A solid support coated with polydeoxythymidylic acid (poly-dT) may be used in conjunction with a "capture probe," which contains a polydeoxyadenylic acid (poly-dA) tail at the end of a sequence complementary to the target nucleic acid. The probe can bind to the target nucleic acid and to the poly-dT by complementary base pairing, resulting in removal of the probe with its hybridized target nucleic acid from solution.

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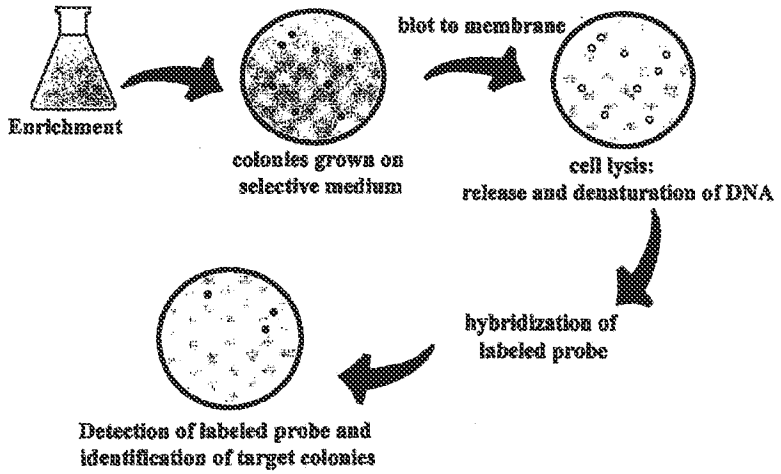


Figure 4. Generalized diagram of colony hybridization.

Liquid-phase hybridization has been commercialized in the GENE-TRAK® Assays, which are dual-probe systems based on 16S ribosomal RNA detection in enrichment culture samples.[13,14] The target ribosomal RNA hybridizes on one end to a poly-dA-tailed probe and on the other end to a fluorescein-labeled detector probe. The hybrids are captured on a poly-dT-coated "dipstick." After binding of an enzyme-conjugated anti-fluorescein antibody, detection is accomplished colorimetrically. GENE-TRAK® Assays have been developed for various food pathogens, including *Salmonella, Listeria, E. coli, Staphylococcus, Campylobacter* and *Yersinia*.[16] Another commercial assay based on liquid-phase hybridization is the AccuPROBE® test, which uses a chemiluminescence detection system. Aimed primarily at the clinical market, the AccuPROBE® test is designed for colony identification, and is not useful for screening of enrichment cultures.[34] *Campylobacter, Staphylococcus* and *Listeria* are the food pathogen species represented in the AccuPROBE® line.[18]

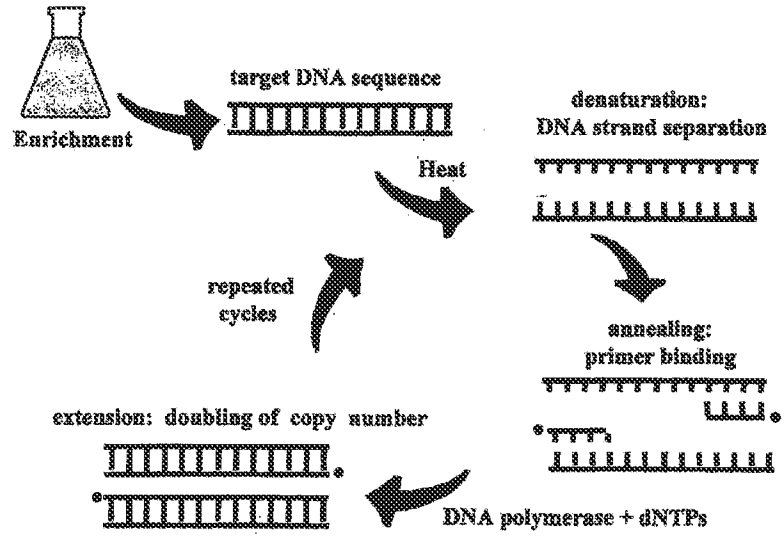


Figure 5. Steps of the polymerase chain reaction

10.33 PCR

Since its development in 1983,[30] PCR has become an essential tool in molecular biology, and its use is increasing in food microbiology. PCR uses enzymatic amplification to produce, within a few hours, millions of copies of a target nucleic acid sequence. The enzyme DNA polymerase copies the target sequence, which is refered to as the template. Oligonucleotide primers are needed to provide starting points for the DNA polymerase, which adds nucleotide bases to the primers in the correct order according to the sequence of the template. The primers are designed to flank the target sequence; i.e., they define the ends or boundaries of the target sequence, and they are oriented in such a way that the DNA polymerase copies the template in the correct direction.

The PCR procedure consists of repeating cycles of alternating high and low temperatures that bring about the amplification. Thermostable DNA polymerase enzymes are used because of their ability to withstand the temperature fluctuations. Each cycle consists of three steps: denaturation, annealing and extension (Figure 5). Denaturation at high temperature causes the double stranded target DNA to separate into single strands. In the annealing step, the temperature is lowered, and the primers bind to their complementary sequences on opposite strands of the target DNA. The temperature is raised again, and DNA polymerase adds nucleotide bases onto the ends of both primers (extension), building two new polynucleotide chains according to the sequences within the target region. Thus, the original target is copied. The newly made copies then serve as templates for additional rounds. Because it is doubled in each successive cycle, the amount of target DNA is increased exponentially. The cycle is repeated until an amount of product sufficient for detection is made. Detailed protocols for PCR may be found in Chapter 11 of this book, where its use in several microbial typing methods is described.

Gel electrophoresis is typically used to detect the amplified product. Because the primers bind to specific sites surrounding the target DNA, the size of the amplified product can be anticipated and detected as a band of known size on the gel. Proof of the band's identity may be obtained by blotting of the gel and DNA probe hybridization to the band.

The appeal of PCR technology lies in the amplification of small quantities of DNA to detectable levels in a short amount of time. Theoretically, a single gene target can be amplified a million-fold for detection in a few hours. Thus, direct detection, i.e., without the need for a lengthy enrichment step, is possible. In practice, however, direct detection of a single gene target is difficult to achieve. The small sample volumes used in PCR (microliter-scale) limit the sensitivity of the procedure for examining foods directly. In addition, inhibitory substances that interfere with the PCR are present in many foods. Thus, a preliminary enrichment culture step to increase target levels is common in PCR detection protocols. Enrichment culture also provides some assurance that the DNA detected is derived from viable cells. The superior sensitivity of the

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PCR compared to conventional culture methods may allow shorter enrichment times, however.[19,40]

Sample preparation procedures to remove inhibitory substances are critical to the success of the PCR, whether it is to be used as a direct assay or coupled with an enrichment culture step. PCR inhibitors may be removed to some extent by optimizing DNA extraction protocols or including purification steps such as immunoprecipitation or membrane filtration.[6,15,23,27,32] DNA polymerases from various sources show different degrees of susceptibility to interference from inhibitors.[1]

The intricacies of preparing materials for performing the PCR have been eased by the availability of commercialized systems that supply many of the reagents in ready-to-use form. The BAX™ systems supply tableted PCR reagents in prepackaged tubes, buffers and other materials for detection of the amplified product by gel electrophoresis using pre-cast gels. BAX™ systems have been used for screening food samples for *Salmonella*,[3] *E. coli*,[24] and *Listeria*.[38] Other commercialized systems have incorporated alternatives to gel electrophoresis detection. Probelia™ utilizes colorimetric detection of amplified product in microtiter plate wells. After PCR, the amplified product is hybridized in solution to a capture probe linked to the microplate well surface and to an enzyme-labeled detector probe. Detection is then achieved by adding the enzyme substrate and colorimetric development. Probelia™ has been used for *Salmonella* detection,[12] and a system for *Listeria* is also available. TaqMan™ is based on fluorescence detection of the PCR product, with the amplification and detection performed in a single tube. The unique TaqMan™ probe is labeled with two different fluorescent dyes, which function as reporter and quencher dyes. If no product is present, the fluorescent signal of the quencher dye is detected. As amplification proceeds, the probe hybridizes to the product, and the quenching effect is relieved; thus, the fluorescence of the reporter dye is detected. The ratio of reporter to quencher dye is measured in an automated system and indicates whether the PCR product was present. TaqMan™ detection of *Salmonella*,[25] *Listeria*[5] and *E. coli*[31] has been tested.

PCR procedures have been described for detecting many of the bacterial foodborne pathogens in food matrices, including *Salmonella*,[7,11,28] *E. coli*,[29] *Clostridium perfringens*,[17,43] *C. botulinum*,[2,16] *Listeria monocytogenes*,[4] *Staphylococcus aureus*,[44] *Shigella*,[26] and *Campylobacter*.[42,45] Multiplex PCR protocols, which are designed to test for more than one gene target simultaneously, can be used to detect the presence of more than one microoganism or to identify a microorganism based on multiple gene markers.[8,9,23,37]

The detection of foodborne viruses is an area in which PCR may have its most significant impact. Because of the extreme difficulty or inability to culture many viruses in the laboratory, PCR provides an opportunity for sensitive detection of viral nucleic acid in food. Sample preparation, as discussed above, is crucial, because an enrichment step for increasing the amount of target nucleic acid is not feasible. Procedures have addressed the need for both purification and concentration of virus particles from food matrices.[22] The viruses of importance in foodborne illness have RNA, rather than DNA, as genomic material.[10] For their detection, RT-PCR has been applied, a procedure which uses the enzyme reverse transcriptase (RT) to make a DNA copy of the RNA genome before proceeding with the standard PCR protocol. Combining purification/concentration procedures with RT-PCR has provided improved detection of viral disease agents in foods.[36,41]

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10.4 BIOCHEMICAL AND ENZYMATIC METHODS

10.41 Introduction

Biochemical and enzymatic methods are among the oldest methods in microbiological analysis. They have been used since the dawn of bacteriology to study the metabolic activities of microorganisms for identification purposes. These methods eventually became standard tests in bacteriology laboratories. As time passed, improvements were made to obtain results more rapidly or easily. Fung[5] made a historical account of the development of rapid bacteriological procedures, including miniaturized and automated systems. A comprehensive treatment of rapid testing is provided in the *Handbook for Rapid Methods and Automation in Microbiology*.[6]

10.42 Miniaturized Microbiological Methods

Hartman[15] compiled over 1,200 citations of rapid miniaturized techniques for microbiological testing in his classical book *Miniaturization of Microbiological Methods*. A significant advance in miniaturization came with the development of the microtiter plate format, which was introduced in the 1960s. Ninety-six reactions could be performed in the wells of the microtiter plate, each holding approximately 0.2 mL of media or reagents. The reduced volume compared to conventional test tubes, which held 5–10 mL, and the multiple tests which could be performed in a single plate provided a great savings in time, materials, space, and labor.

Miniaturized methods have been used to study large numbers of isolates from foods,[7,17,18] including yeasts and molds.[3,8,13,14,19,20] Useful microbiological media were developed using the miniaturized format,[11,23] which is also ideal for doing challenge testing of chemicals against many types of microorganisms.

10.43 Commercial Miniaturized Diagnostic Kits ("Minikits")

A host of commercial diagnostic kits were marketed beginning in the early 1970s, as a result of the development of miniaturized

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methods. Some of the systems survived the test of time. Many fell by the wayside due to various reasons, e.g. poor performance or design. The following is a synopsis of currently available commercial minikits. A recent review on this subject was made by Russell et al. [21]

10.431 Agar Media Minikits

10.4311 Enterotube (Roche Diagnostics, Nutley, NJ). The only agar-based diagnostic kit system still widely used is the Enterotube II, a self-contained plastic tube containing twelve different conventional media in separate compartments. The tube is inoculated with a wire threaded through each compartment along the length of the tube. The unit permits assay of 15 standard biochemical tests from a single bacterial colony. Reagents are added into the Indole test and Voges-Proskauer test compartments before recording of the color reactions and gas formation. Table 1 describes the color reactions of the tests, which are recorded on data sheets (Figure 6). A five-digit number is generated from the test results and matched to the manufacturer's code book, which provides the identification of the isolate.

Similar color reaction charts, data recording sheets and code book formats are available for the other minikits described in this section. Some systems utilize a seven- or ten-digit code number. The code book of one system cannot be used to interpret data from another minikit. Sometimes an isolate cannot be identified from the code generated. An impure or insufficient inoculum may be the cause, or perhaps an incorrectly prepared diluent. After exhaustive testing, if the isolate is still not identifiable, a culture may be sent to the manufacturer for further testing, and the results may be added to the data base. Nevertheless, it is very unusual to "discover" a new organism in this way.

The Enterotube II was developed to identify species of the Enterobacteriaceae only. A similar unit called Oxi/Ferm Tube was designed for gram-negative nonfermenters.

The accuracy of the Enterotube II was 94% compared to the conventional method.[20] A 90–95% accuracy of a minikit against the standard conventional methods is considered good to excellent. When accuracy drops to 85%, the system is considered marginally acceptable, and any value below that is not acceptable.

Advantages of the Enterotube II include speed and ease of inoculation. There is no need to prepare an inoculum suspension, and a single colony can be used for identification. Disadvantages include difficulty in stacking of the units in the incubator, short shelf-life and a limited data base for food isolates.

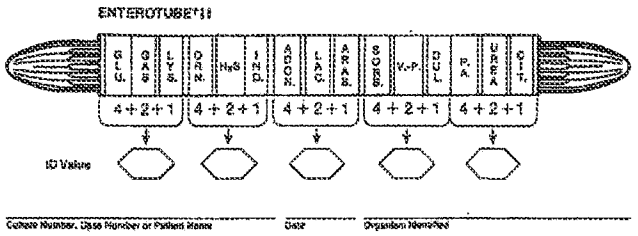


Figure 6. Enterotube II data sheet

10.432 Dehydrated Media Minikits

A variety of minikits utilize dehydrated media, which become hydrated upon inoculation. Longer shelf-life, minimal contamination, and lighter weight of the units are major advantages of dehydrated versus agar media kits.

10.4321 API (bioMerieux Vitek, Inc., Hazelwood, MO). The API is probably the most widely used diagnostic system in the world for the *Enterobacteriaceae*. Originally, the data base was very strong for clinical isolates, but in recent years, it has been expanded to cover food, environmental and industrial isolates as well. A flow sheet on the operation of the API system is shown in Figure 7.

The API 20E has 20 small elongated wells housed in a plastic panel, each containing a dehydrated medium, and is designed to perform 23 miniaturized biochemical tests from a pure agar plate culture. Colony growth is mixed with buffer to make a suspension of cells, which is used to inoculate the wells of the API strip using a small pipet. The unit is then placed into a plastic chamber, into which water is added to avoid moisture loss during incubation. After incubation for 4 hr or 18 hr at 35–37°C, the unit is removed from the incubator, and reagents are added into certain wells. After the color reactions are recorded, a 7-digit code is generated. The unit can also be read and interpreted by a computerized system. The API has been tested extensively, with reported accuracies ranging from 90.2%[12] to 93%.[18]

Advantages of the API include an excellent data base, a proven record of testing over the past 25 years, and long shelf-life. Disadvantages include a difficult and time-consuming inoculation into the units, especially if many are to be prepared simultaneously. Handling and stacking of the flexible trays and lids are inconvenient. Reading and interpretation of reactions require experience and training.

The following systems are available in formats similar to the API 20E:

API NFT, for gram negative non-*Enterobacteriaceae*

API CAMP, for *Campylobacter* spp.

API Staph-IDENT, for staphylococci and micrococci

API 20A, for anaerobes

Table 1. Enterotube II Color Reactions

Abbreviation	Test	Positive Reaction	Negative Reaction
GLU	Glucose utilization	Yellow	Red
Gas	Gas production	Wax lifted	Wax not lifted
LYS	Lysine decarboxylase	Purple	Yellow
ORN	Ornithine decarboxylase	Purple	Yellow
H_2S	H_2S production	Black	Beige
IND	Indole formation	Pink-Red	Colorless
ADON	Adonitol fermentation	Yellow	Red
LAC	Lactose fermentation	Yellow	Red
ARAB	Arabinose fermentation	Yellow	Red
SORB	Sorbitol fermentation	Yellow	Red
VP	Voges-Proskauer	Red in 20 minutes	No red color
DUL	Dulcitol fermentation	Yellow	Green
PA	Phenylalanine deaminase	Black to smoky gray	Green
UREA	Urease	Red-Purple	Yellow
CIT	Citrate utilization	Deep blue	Green

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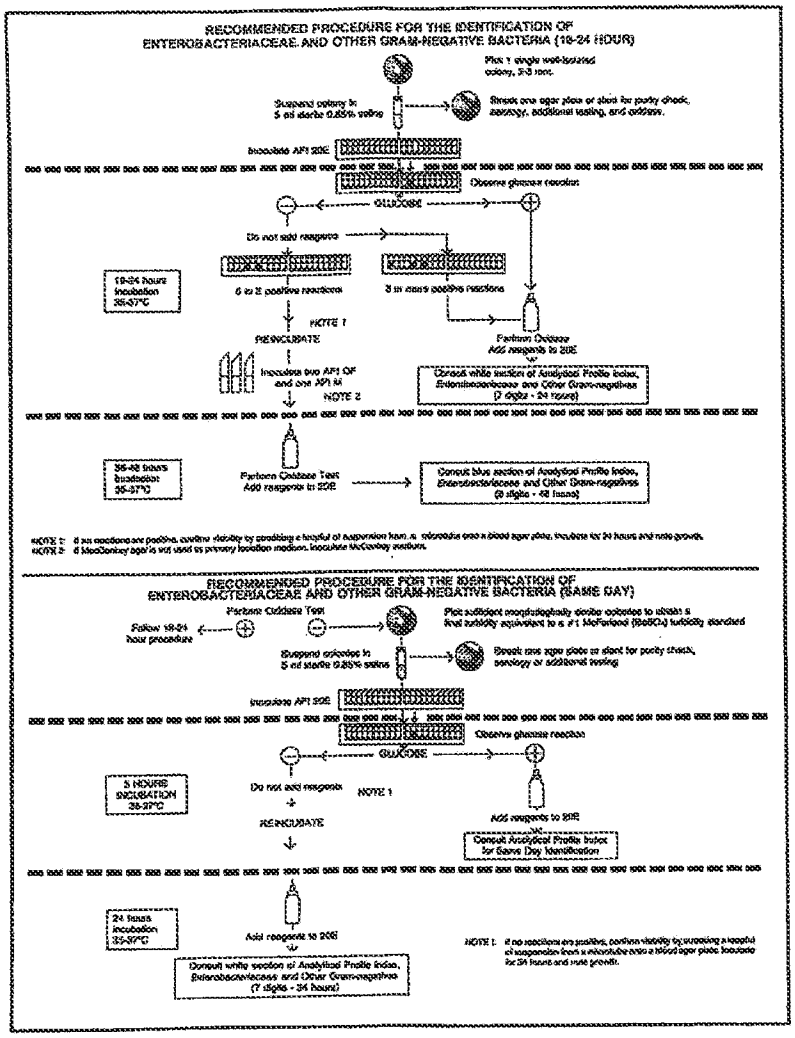


Figure 7. *Procedure for isolate identification using API 20E system.*

API 50 CH, for *Lactobacillus*
API 20C, for yeasts
Rapid STREP, for streptococci
Rapid CORYN, for *Corynebacterium*

10.4322 ATB (bioMerieux Vitek, Inc., Hazelwood, MO). The ATB is a system for testing assimilation of 32 different substrates by a microbial isolate. Cells are suspended in liquid and introduced into the unit. After incubation for 4–24 hr, depending on the culture, the tests are read manually or automatically. Test strips are available for anaerobes, staphylococci, micrococci, yeast, *Enterobacteriaceae*, streptococci, and gram negative bacilli.

10.4323 Minitek (Becton Dickinson Microbiology, Franklin Lakes, NJ). The Minitek system consists of a plastic unit of ten wells into which are placed paper discs impregnated with different substrates. A suspension of bacterial cells is injected into the wells by a calibrated "gun." Mineral oil is overlayed in some anaerobe tests, and the units are incubated at 35–37°C for 18 hr before reactions are read. A code is generated and the identification is made using the Minitek code book.

Thirty-six different substrates are available for use with the system. A special dispensing chamber may be used to allow 10 types of discs to be dispensed into the 10 test wells of the unit. Two units may be used for the 20 common tests for identification of the *Enterobacteriaceae*.

Fung et al.[10] rated the accuracy of the Minitek at 97%. The system has the flexibility to allow the analyst to use different substrate discs for characterization of genera and species other than the *Enterobacteriaceae*. The many steps involved in setting up the units and the need for several instruments for inoculation of the wells are disadvantages of the system.

10.4324 BBL Crystal (Becton Dickinson Microbiology, Franklin Lakes, NJ). The two-part unit design of the BBL Crystal system provides convenience in use and reading of results. The bottom chamber of the unit is for holding one mL of a liquid suspension of the test culture. The top unit contains 30 small rods with dehydrated substrate housed at the tip of each rod. The rods are clipped into the bottom unit and in one motion the test culture comes into contact with 30 different substrates. The unit is then incubated overnight at 37°C, and the results are read either manually or by using the Crystal light box. The 10-digit code generated by the results can be interpreted using the Crystal code book or by computer.

The Enteric/Nonfermentor ID Kit with 30 tests is designed for the identification of both *Enterobacteriaceae* and nonfermentative gram negative organisms, and the gram Positive ID kit allows identification of 121 gram positive cocci and bacilli. Advantages include sturdiness of the units, ease of operation and computer-assisted identification. There are no significant disadvantages.

10.4325 MicroID (Organon Teknika, Durham, NC). The first minikit capable of gram negative isolate identification in four hours was the MicroID (Figure 8). The system consists of a molded, styrene tray containing 15 reaction chambers and a hinged cover. The first five chambers contain a substrate disc and a detection disc in the lower and upper portions of the chamber, respectively, and the other ten chambers contain combination substrate/detection discs. Except for the Voges-Proskauer test, the discs contain all the substrates and detection reagents required to perform the biochemical tests. Bacterial cells are suspended into liquid, then 0.2 mL is inoculated into the top of each chamber. The inoculum flows to the bottom of the chamber, where it hydrates the disc. After incubation for 4 hr at 35°C, two drops of 20% KOH are added to the Voges-Proskauer chamber. The unit then is rotated 90 degrees, so that the substrate and detection reagents of the first five chambers are mixed. The reactions of the 15 chambers are read, the first five from the upper portion of the chamber. The MicroID code book is used to interpret the code number generated from the results. The accuracy of the MicroID has been reported as 93.5%[12] and 97%.[20] A similar format with different substrates is available to identify *Listeria spp.*

Advantages of the MicroID are its accuracy, speed of reaction, convenience in operation, addition of only one reagent, and long shelf-life. Disadvantages include having to individually inoculate each of the 15 chambers and difficulty in reading the color of marginal reactions.

10.4326 RapID One System (Remel, Lenexa, KS). The RapID One System consists of a chamber with an inoculum trough con-

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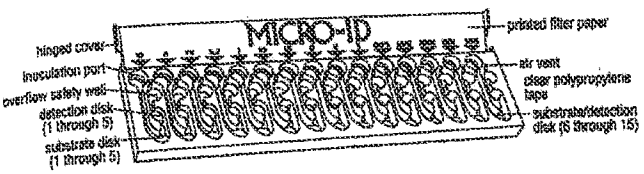


Figure 8. Micro ID system.

nected to 20 separate wells, each housing a different dehydrated substrate. A bacterial cell suspension is introduced into the trough, then the unit is tilted so that the inoculum flows into the 20 test wells in one motion. The reactions can be read after a 4-hr incubation. Remel markets test kits for *Enterobacteriaceae, Leuconostoc, Pediococcus, Listeria, Neisseria, Haemophilus,* nonfermentors, yeast, anaerobes, streptococci, and urinary tract pathogens. Identification accuracies of various anaerobes to the genus level using the anaerobic RapID system range from 83–97%, and to the species level, 76%–97%.[18]

Advantages of the RapID One System include the reaction speed and one-step inoculation. A disadvantage is the skill required in reading the color changes.

10.44 Chromogenic Media

Microorganisms growing in the presence of certain dyes and organic compounds may fluoresce under UV light or produce distinctive colors. Such differential color reactions have been exploited in the development of growth media for rapid screening and identification of microorganisms.

Although not a new concept,[19] recently there has been interest in developing novel differential media that utilize a new generation of chromogenic substrates. The new chromogens and media ingredients allow differential coloring of different bacterial groups on the same agar plate. BBL CHROMagar (Becton Dickinson, Franklin Lakes, NJ), BCM (Biosynth, Naperville, IL), Chromocult and Fluorocult Agars (Merck, Darmstadt, Germany) and Rainbow Agar (Biolog, Hayward, CA) are representative. *Salmonella, Listeria, E. coli* O157:H7 are a few of the microorganisms for which chromogenic media have been developed.

10.45 Quantitative Enzymatic Methods

The previous sections described the use of biochemical and enzymatic activities for the identification of microorganisms to the genus and species levels in rapid assays. These metabolic activities may also be used as rapid indicators of biomass, providing an estimation of the size of the microbial population in foods.

Metabolic enzyme activity can be measured directly or indirectly. For example, a direct enzymatic test is the widely-used MUG test for *E. coli*, which detects action of the enzyme β-glucuronidase on the compound 4-methylumbelliferyl-β-D-glucuronide (MUG), resulting in production of a fluorescent compound visible under UV light. Theoretically, the more MUG-positive *E. coli* cells present in the liquid, the more fluorescence will be generated in the presence of the MUG reagent. An example of an indirect test is pH change, caused by acid formation by the microbial population in a food. The amount of acid formed, and therefore the magnitude of the pH drop, would be indicative of the microbial load in a food.

Enzyme activity tests are valuable because of the speed with which a biomass estimate can be obtained. There are drawbacks, however. (1) Food components may interfere with the measurement of enzyme activity. (2) The food may not provide physiologically optimal conditions; therefore, enzyme activity may be suboptimal and may not directly reflect the size of the microbial population. The following sections describe several enzyme activity tests used for rapid evaluation of biomass in foods and the food production environment.

10.451 ATP Bioluminescence

Bioluminescence is the emission of light from viable cells and has been used as an estimation of microbial load, as well as an index of general cleanliness of the food production environment. Bioluminescence has been coupled to an ATP-dependent enzymatic reaction. Because ATP is a marker for viable cells, its measurement in a bioluminescence reaction indicates the presence of biomass. In the presence of luciferin-luciferase, oxygen and magnesium, ATP promotes the light-generating reaction. The amount of light generated is proportional to the amount of ATP, and therefore biomass, in the sample.

Many attempts have been made to correlate ATP levels with the total microbial count, with mixed success. The amount of light generated does not always reflect microbial numbers for various reasons: biological food components (e.g. meat or vegetable tissue) may have an intrinsic ATP content; the microbial population may be metabolically inactive or slow; ATP levels may vary among different groups of a mixed microbial population. Siragusa[22] reviewed applications of ATP bioluminescence technology for microbial monitoring in a wide variety of materials. Recently, ATP research and application work has been focused on sanitation monitoring in the food plant environment. ATP indicates the presence of biomass, and after cleaning, ATP levels drop dramatically.

A number of companies currently manufacture portable handheld instruments for measuring ATP levels from environmental swabs. After the swab is reacted with the enzyme reagents in the self-contained instrument, results are obtained in a matter of minutes. The technology allows sanitation monitoring in "real-time." Some of the instruments can detect as little as 100 fg ATP.

The following companies market bioluminescence-based instruments for ATP measurement:

Biotrace-Ecolab Inc. (St. Paul, MN)
Celsis Inc. (Evanston, IL)
Charm Sciences Inc. (Malden, MA)
Cogent Technologies (Cincinnati, OH)
EM Science (Gibbstown, NJ)
GEM Biomedical Inc. (Hamden, CT)
IDEXX Laboratories Inc. (Westbrook, ME)
Lumac (Silver Spring, MD)
Millipore Corp. (Bedford, MA)
New Horizons Diagnostics (Columbia, MD)

10.452 Limulus Amebocyte Lysate Assay

All gram negative organisms produce endotoxin as a structural part of the cell wall. In the presence of endotoxin, amebocytes from the horseshoe crab *Limulus* form a gel. The gel formation is the basis of the endotoxin measurement system known as the Limulus Amebocyte Lysate (LAL) Assay. Jay[16] used this simple and sensitive assay to estimate Gram negative cell number in ground beef, but it has also been used to monitor the presence of endotoxin in the hospital environment, pharmaceuticals, air, water and other materials.

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10.453 Catalase Test

Catalase is an enzyme that breaks down hydrogen peroxide (H_2O_2) into water and oxygen gas (O_2). The O_2 liberated in the reaction can be detected as bubbles in the solution of H_2O_2, and the test can be performed in a simple capillary tube.[4] Not all microorganisms produce the catalase enzyme, but many of the food spoilage species do, e.g. *Pseudomonas spp., Micrococcus, Staphylococcus*, as well as a number of enterics. The test may be used as a rapid method for estimating levels of these microbial populations in certain applications. Because catalase is also present in other biological materials, e.g. meat, the test may be useful in sanitation monitoring in the same way that ATP bioluminescence is used (see section 10.451). The catalase test has also been used to monitor the end-point heating temperature of food. Ang et al.[1] showed that heating poultry meat to 71°C destroys both bacterial and animal catalase. The test is 99% accurate and is simple and easy to perform.

10.454 CO_2 Measurement

Many organisms produce CO_2 as a metabolic by-product, and its measurement may be used to estimate microbial population levels. The Bactec system (Becton Dickinson, Franklin Lakes, NJ) uses radiolabeled substrates in selective media to monitor radiolabeled CO_2 production, and therefore measure population sizes of the microbial groups capable of growth in the media.

10.46 References

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10.5 MEMBRANE FILTRATION

10.51 Hydrophobic Grid Membrane Filter

The hydrophobic grid membrane filter (HGMF) was first introduced to extend the counting range of conventional membrane filters, thus reducing the number of dilutions required to analyze a sample.[37,38] At first, its applicability was limited to water samples and other suspensions that did not contain a significant amount of debris.[22] Later, prefilters were introduced to eliminate particles from the food homogenates prior to filtration,[21,32] and a variety of diluents and enzyme digestions techniques were introduced to enable more efficient filtration of the prefiltered homogenates.[21,31,40]

The HGMF has numerous advantages over other food microbiology methods. In most cases, its counting range eliminates the need for multiple dilutions[4,38]; the process of filtration separates the microorganisms from the food matrix, thus eliminating interference with growth as well as with interpretation of differential reactions[18]; confining colonies inside the individual growth squares on the HGMF usually results in taller, denser colonies which are easier to detect[4,37] and restrains fast-growing organisms from spreading and overgrowing other colonies; the non-staining nature of the membrane filter allows introduction of non-toxic dyes into the culture medium, enhancing visibility of the colonies[17,19,27]; the ordered arrangement of colonies on the filter reduces the need for subjective interpretation during counting, improving between-analyst reproducibility[37,41]; and the filter can be transported from medium to medium (or staining reagent) without disturbing the colonies, allowing for preliminary incubation of an injured population on a repair medium prior to selective incubation[2,16] or for sequential reading of two or more differential reactions on the same filter.[6,11,16,24]

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The disadvantages of the HGMF are the need to clean and resterilize the filtration apparatus, the difficulty encountered in filtering certain types of foods, and the eye fatigue involved in counting up to 1600 individual grid squares. The labor required to clean and resterilize filtration units is largely offset, in most cases, by the elimination of multiple dilutions. Handling protocols to facilitate rapid filtration must be established for each food product prior to incorporating that product into the test routine, but, once established, they remain consistent unless the formulation of the food product changes. Finally, both manual (ISO-GRID Line Counter, see Figure 9) and automated[42] counting aids have been developed to assist in interpreting growth patterns on HGMF.

Soon after the introduction of food filtration techniques, several procedures were developed for the detection or enumeration of microorganisms of interest to the food microbiologist, including coliforms and *Escherichia coli*,[3,38] aerobic plate count,[4] yeast and mold count[4], *Staphylococcus aureus*,[3] enterococci[3] and *Salmonella*.[22] Later, HGMF-based tests for *Vibrio parahaemolyticus*,[6,16] fluorescent pseudomonads,[26] *Zygosaccharomyces bailii*,[23] *Aeromonas hydrophila*,[45] lactic acid bacteria,[5,24,28] *Listeria monocytogenes*,[35] and *E. coli* O157:H7[7,20] were also described. Of these, the coliform, *E. coli*, aerobic plate count, yeast and mold, *Salmonella* and *E. coli* O157:H7 procedures have been accepted as Official Methods by AOAC International.[8-15]

The hydrophobic grid membrane filter, with its ordered growth patterns, has been used by several researchers as the basis for enzyme-labeled antibody (ELA) enumeration and presence/absence tests for organisms of interest in food microbiology. Early efforts in this area were directed towards *S. aureus*[33] and *E. coli* O157.[7,43] ELA-HGMF methods have also been described for verotoxigenic *E. coli*,[29] *Clostridium botulinum*[30] and *Salmonella*.[44] The ELA procedure is carried out either directly on the HGMF or on a "blot" or replica of HGMF colonies on another membrane filter. When a replica is used, the colonies on the original filter remain viable and can be subcultured for further detailed investigation, if desired.

Colony hybridization methods have also been developed for use with HGMF. A chromogen-labeled DNA probe was developed and used to detect colonies of *Listeria monocytogenes* directly on the filter.[34,35] Also, a colony hybridization technique has been developed to enumerate and differentiate *Vibrio parahaemolyticus* and *V. vulnificus*.[25] In this last procedure, the HGMF was used for primary isolation of suspect colonies from samples and a blot replica of the filter was probed with a chromogen-labeled DNA probe.

10.511 Equipment Needed

ISO-GRID Hydrophobic Grid Membrane Filters (QA Life Sciences, Inc.)

ISO-GRID Filtration Units with built-in 5µm pore size prefilter (QA Life Sciences, Inc.)

ISO-GRID stainless steel clamp (QA Life Sciences, Inc.)

Membrane Filter forceps (blunt-tipped, no gripping ridges)

Vacuum manifold or 1 liter vacuum flask

Vacuum pump (1/6 hp. minimum for use with vacuum flask; 1/3 hp. minimum for use with manifold

Vacuum tubing with Y-connector and tubing clamp

Counting instrument (ISO-GRID Line Counter; QA Life Sciences, Inc.). Quebec Colony Counter or other illuminated magnifiers are acceptable alternatives

10.512 Sample Preparation for Quantitative Analyses

Prepare a 1:10 sample homogenate. Ideally, a minimum 10 g sample should be used. The choice of diluent will depend on the nature of the sample. For example, most meat and dairy products filter best when prepared in a diluent containing 1% Tween 80, whereas most other products are best prepared in a surfactant-free diluent such as 0.1% peptone or Butterfield's Phosphate Buffer. Homogenates can be prepared by using a conventional lab blender, by shaking, or by using a "stomacher" or equivalent instrument. Some foods produce viscous homogenates which are difficult to filter. These can often be handled simply by diluting the homogenate to 1:100 and filtering 10 mL of the diluted homogenate. Dilution reduces the viscosity of the sample homogenate, allowing it to pass through both the prefilter and membrane filter more easily, while filtration reconcentrates the sample, allowing the analyst to obtain a sensitivity level of 10 MPN/g. A number of foods require digestion with one or a combination of enzymes to enable 1 mL of a 1:10 dilution of the homogenate to filter. Detailed recommendations on choice of diluent, enzyme digestion and sample dilution for optimum filtration, together with instructions on enzyme preparation and use, can be found elsewhere.[1,36]

10.513 General Procedure for Quantitative Analysis

The process of setting up samples for analysis by HGMF is very consistent, regardless of the analysis. It is comprised of the following steps:

1. Prepare the sample homogenate for filtration.
2. Place a sterile filtration unit on the vacuum flask or manifold and rotate the hinged funnel to the open (horizontal) position.
3. Use sterile forceps to aseptically position a filter on the base of the filtration unit. Rotate the hinged funnel to the shut (vertical) position.
4. Slide the stainless steel clamp over the flanges protruding from both sides of the filtration funnel and base and rotate the clamp arm to the shut (horizontal) position (see Figure 10).

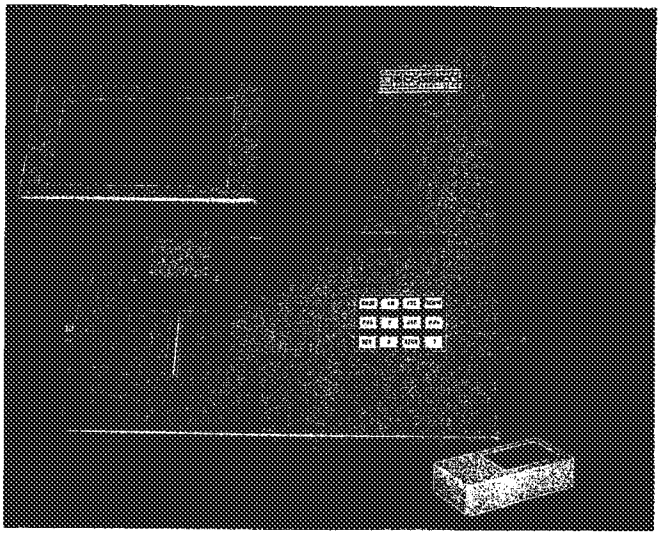


Figure 9. *ISO-GRID Line Counter*

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5. Dispense approximately 10–15 mL of sterile water or diluent into the top of the funnel. This will begin to drip through the prefilter onto the membrane filter, preventing the sample from sticking to either filter.
6. Without waiting for the sterile liquid to drip through the prefilter, measure the desired volume of sample homogenate or diluted homogenate (typically, 1 mL of 10^{-1} homogenate to obtain 10 MPN/g sensitivity) into the funnel. Dispense an additional 10-15 mL of sterile water or diluent into the funnel to ensure uniform dispersal of the sample.
7. Using a Y-branched vacuum tubing, gently apply vacuum to the filtration funnel just below the prefilter to draw liquid quickly into the lower chamber of the funnel. Vacuum should also be applied simultaneously to the filtration unit base below the HGMF to prevent the membrane filter from being dislodged during the prefiltration step.
8. Apply vacuum to the base of the filtration unit to draw the sample liquid through the membrane filter.
9. Remove the membrane filter from the filtration unit, place it on the desired culture medium and incubate as appropriate for the test.
10. Following incubation, examine the membrane filter for squares containing target colonies, count the number of positive squares, convert to the corresponding most probable number (MPN) index and multiply by the appropriate dilution factor to obtain the MPN per gram or milliliter. MPN conversions can be obtained by using the formula MPN = $1600 \times \log_e (1600/[1600 - x])$, where x represents the number of positive squares.

If multiple quantitative analyses are to be run on a sample, a single filtration unit can usually be used for all those tests. After the first filtration is complete and the membrane filter has been placed on the appropriate medium, a second membrane filter can be inserted into the filtration unit and a second filtration carried out. The number of filtrations that can be run for a single sample on a single filtration unit without washing is limited only by the ease with which the sample homogenate passes through the 5 µm stainless steel prefilter. Table 2 summarizes culture media, incubation conditions (duration and temperature) and target colony colors for several common quantitative analyses.

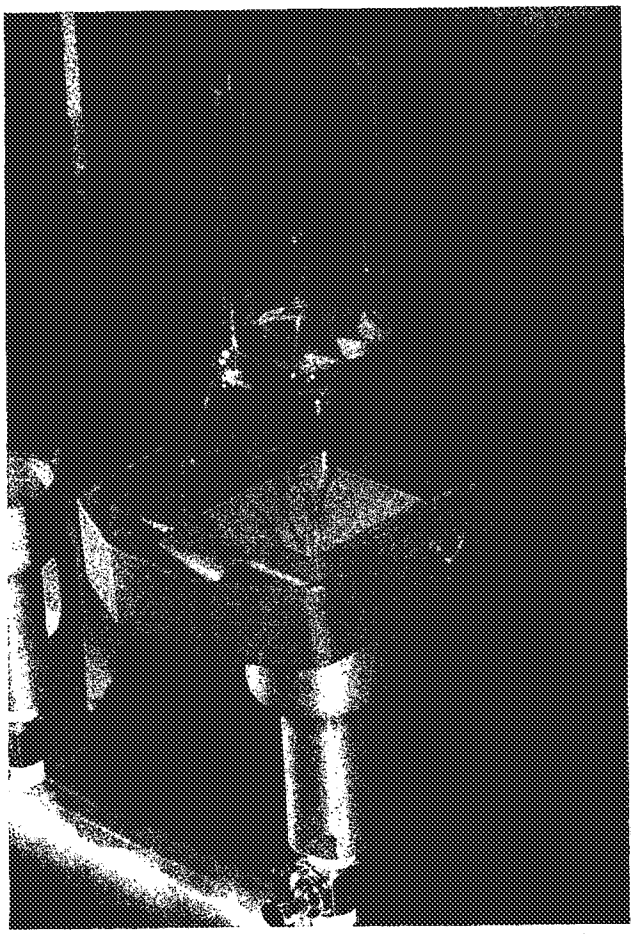


Figure 10. *ISO-GRID Filtration Unit clamped shut with filter in place.*

10.514 Counting and Dilution Strategies for Quantitative Analyses

The HGMF counting range spans nearly 4 $\log_{10}$ cycles, although counts obtained at the upper end of the range should be treated with caution. When 95% or more of the squares are positive, the filter produces an underestimate of the true population. Therefore, any result exceeding 1520 positive squares should be reported as an estimated count. In the vast majority of cases, filtering a single dilution will suffice to cover the desired counting range (as compared to running three consecutive $\log_{10}$ dilutions as is done with most conventional procedures). There are two approaches to deciding what sample dilution should be filtered for any analysis. The simplest is to choose the lowest dilution that would be run if a conventional method was being used. For example, if a coliform test is usually run on 1:10, 1:100 and 1:1,000 sample dilutions, then 1 mL of the 1:10 dilution should be filtered for the HGMF analysis. Similarly, if an Aerobic Plate Count on a particular product is usually carried out on the 1:1,000, 1:10,000 and 1:100,000 dilutions, then the filtration should be run on the 1:1,000 dilution.

A second method of determining dilutions is based on the quantitative product specification for the analysis in question. For example, if an Aerobic Plate Count result is considered unsatisfactory at levels above 10^5 CFU/g, then a dilution should be chosen to enable accurate and convenient counting in the vicinity of that count level. Filtering 1 mL of a 1:1,000 dilution from a sample with an Aerobic Plate Count of 10^5 CFU/g would produce approximately 100 positive squares. This level of growth is relatively easy to count, is well within the accurate counting range of the HGMF, and allows for easy and accurate counting both above and below the critical target level, giving the analyst a clear picture of how "clean" or, conversely, how far out of specification the sample is. Alternatively, if only an occasional sample is expected to be out of specification and the analyst wishes more information at the lower end of the count range, 1 mL of a 1:100 dilution can be filtered instead.

Occasionally, almost all of the squares will contain target colonies. When nearly all squares are positive, one should count the number of negative squares and subtract from 1600 to determine the number of positive squares. On other occasions, a filter may contain several hundred positive squares. If the growth is randomly distributed over the entire 1600 squares, and if at least 25% of the squares (400 squares) appear to be positive, the number of positive squares over the entire filter can be estimated by counting the number of positive squares contained in eight contiguous rows and multiplying by five to determine the estimated number of positive squares on the entire filter.[26]

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Table 2. Hydrophobic Grid Membrane Filter Applications for Quantitative Analysis

Analysis	Ref.	AOAC Official Method	Culture Media	Incubation Conditions	Interpretation
Aerobic plate count	10	986.32	TSAF Agar	48 ± 3 hours; 35–37°C	Count all squares containing growth (usually appear as green colonies)
Total coliform count	8, 17	990.11	LMG Agar	24 ± 2 hours; 35–37°C	Count all squares containing blue colonies
E. coli count	8, 17	990.11	BMA Agar	2–5 hours[a]; 35–37°C	Count all squares containing fluorescent colonies under long wave UV light
Fecal coliform count	31	983.25	m-FC Agar	24 ± 2 hours; 44.5 ± 0.5°C	Count all squares containing blue colonies
Yeast & mold count	34	995.21	YM-11 Agar	50 ± 2 hours; 25 ± 1°C	Count all squares containing growth (usually appear as blue colonies)
E. coli O157:H7	36	997.11	SD-39 Agar	24 ± 2 hours; 44.0–44.5°C	Count all squares containing pink colonies; subculture and confirm serologically
Enterococci	20		m-Enterococcus Agar	48 ± 3 hours; 35–37°C	Count all squares containing pink or red colonies; confirm using catalase test
S. aureus count	20		Baird-Parker Agar	48 ± 3 hours; 35–37°C	Count all squares containing black or dark gray colonies; confirm using coagulase test
Lactic acid bacteria	15		MRSD Agar	48 hours (anaerobic); 25°C	Count all squares containing growth
Zygosaccharomyces bailii	23		ZBM Agar	2–3 days; 30°C	Count all squares containing smooth, moist, round-convex colonies (usually appear blue)
Fluorescent pseudomonads	22		TSA Agar, then S-1Agar	4 + 48 hours[b]; 25°C	Count all squares containing colonies that fluoresce under long wave UV light

[a] After counting coliform colonies on LMG Agar, transfer filter to BMA and incubate 2–5 hours.

[b] First incubate on TSA for 4 hours, then transfer to S-1 Agar and incubate 48 hours.

Regardless of the dilution filtered, whether positive or negative squares are counted, and how many rows are examined, calculations must always proceed in the following sequence:

a) Convert any negative square count or estimated count to obtain a total number of positive squares on the filter ("Score");
b) Convert the Score to the corresponding MPN either by application of the formula given above or by using a look-up table[36]; and
c) Multiply the resultant MPN by the dilution factor to obtain the MPN/g.

While the reproducibility of HGMF results is usually sufficient to avoid the need for duplicate filtrations,[9-11,14,15] greater precision might occasionally be desired. When calculating results obtained from replicate filters, determine the Score and MPN separately for each filter, take the average of the MPN values and multiply the resultant average MPN by the dilution factor to obtain the MPN/g.

10.515 *Procedure for* Salmonella *Presence/Absence Testing*

The details of this procedure are given elsewhere.[1] The *Salmonella* method can be summarized in the following steps:

1. Suspend a minimum 25 g sample in 9 volumes of nonselective enrichment broth. Mix as appropriate, adjust the pH, if necessary, to 7.0 ± 0.2, and incubate for 18–24 hours at 35–37°C.
2. Transfer 0.1 mL (100 µL) of overnight nonselective enrichment into a 10 mL volume of Mueller-Kaufmann Tetrathionate Broth. Incubate 6–8 hours at 35–37°C in a water bath.
3. Using the filtration technique described in the quantitative analysis procedure (steps 2–9), filter 0.1 mL (100 µL) of Tetrathionate Broth culture. Place the membrane filter onto EF-18 Agar and incubate 18–24 hours at 42 ± 0.5°C.
4. Examine the membrane filter for the presence of green colonies. These are presumptive *Salmonella* colonies. If no growth is evident on the membrane filter, or if only yellow colonies are present, the sample may be reported as "*Salmonella* Not Detected."
5. If presumptive positive colonies are present, subculture a representative number of these (three is usually sufficient) for biochemical and, if appropriate, serological confirmation.

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10.53 Commercial References

Commercial Supplier for ISO-GRID Hydrophobic Grid Membrane Filters and Related Products
QA Life Sciences, Inc., 6645 Nancy Ridge Drive, San Diego, CA 92121

10.6 IMPEDANCE, CONDUCTANCE, AND CAPACITANCE

10.61 Theory

The use of electrical methods to determine bacterial growth dates back to the late 19th century.[60] In the early 1900s, conductivity was found to be useful for measuring ammonia produced by clostridia in various enviroruxents,[61] and was used for measuring bacterial proteolysis.[1] Although electrical methods for enumerating bacteria were evaluated throughout the 1900s, it was not until the early 1980s that these methods became automated and were used to estimate bacterial populations on a variety of foods.[17,18,19,21,23,26,34,64]

Impedance is defined as the resistance to the flow of a sinusoidal alternating current through a conducting material. Determination of impedance is complex in that it consists of a resistive component (R) and a reactive component (X), which, for the purposes of monitoring bacterial growth, is measured as capacitive resistance (X_c). Impedance (Z) is calculated using the equation $Z = \sqrt{(R^2 + X_c^2)}$ where $X_c = 1 / 2\pi fC$ (f = frequency; C = capacitance).[9] The conductance (G) of a bacteriological growth medium is a function of the resistance (R) of the medium, where $G = 1/R$ and is dependant upon the ionic strength of the solution.[19,28] Capacitance changes in a growth medium occur very close to the electrode. The electrode serves as one side of the capacitor and a layer of ions serves as the opposite side. Smaller ions produce a thinner layer and this is reflected as a change in capacitance.[13] Capacitance has been proven to be useful in high conductivity media used for estimation of yeasts and molds.[28]

As bacteria multiply in a growth medium, they convert large molecules into smaller, more mobile ionic metabolites, which change the impedance of the medium.[18] These metabolites increase the conductance and decrease the impedance of the medium. Once microbial populations reach a level of 10^6 to 10^7 cells/mL, the impedance, conductance, or capacitance (depending on which parameter is being measured) of the medium shifts to the extent that the instrument can detect the change.[18] At this point, a detection time (DT) in hours is automatically reported and recorded by the instrument. Hence, DT is a function of the temperature at which the assay is conducted, the medium, the generation time of the bacterium or group of bacteria able to grow in the medium, the extent of repair the organisms need to begin multiplying, and the inhibitors in the sample or medium.

A common misconception about impedance microbiology is that the method is incapable of enumerating low numbers of bacteria from samples. This confusion is due to the misunderstanding about the sensitivity of the instrument versus the sensitivity of the assay. The sensitivity of the instrument is 10^6 to 10^7 cells, because there must be sufficient metabolite accumulation to enable a large impedance shift. The sensitivity of the assay is one viable cell because, as one cell multiplies to 10^6 to 10^7, its presence may be detected.

The time required for the monitoring instrument to detect the impedance shift depends upon the number of microorganisms in the sample initially. For example, if a sample contains bacterial populations at a level of 10^5 cells/mL, the detection time would be reached rapidly because only four to eight cell divisions would be required to exceed the detection threshold level.[28] However, for samples that contain only 1 microorganism/mL, more time would be required for microbial populations to multiply to the detection threshold. Hence, detection times are inversely proportional to initial bacterial numbers.

Most commercial systems (section 10.62) can be programmed such that samples that detect rapidly and are "highly contaminated" may appear red on the report screen of the computer. This allows for immediate recognition by the technician that a sample is highly contaminated and the product should be held. Likewise, detection time ranges that would be considered "caution zones" can be programmed to appear yellow on the screen and ranges that would be considered "acceptable" would be programmed to appear green. Using this technique, a technician can determine the disposition of many products being tested by glancing at the computer screen.

10.62 Commercial Systems

10.621 The Bactometer

The Bactometer Microbial Monitoring System is a computer controlled impedance, conductance, and capacitance monitoring device. The Bactometer can be used to: 1) perform total microbial, coliform, yeast and mold, lactic acid bacteria, *Escherichia coli*, and *Pseudomonas fluorescens* counts, 2) detect the presence of *Salmonella* and *Listeria*, 3) predict shelf-life, 4) conduct challenge and functional sterility testing, and 5) monitor environmental samples.

The Bactometer includes the following components: a computer, printer, and Bactometer Processing Unit (BPU). The BPU is comprised of two incubators that can be set independently of one another with a temperature range from 10°C below ambient temperature to 55°C. Each BPU can simultaneously monitor from 1 to 128 samples. Three additional BPU's (each containing two independent incubators) can be added to bring the total capacity of the system to 512 samples.

Test samples (total of 2.0 mL) are loaded into the Bactometer disposable module wells (16 wells per module) which are inserted into the BPU. The system is capable of monitoring conductance, capacitance, or total impedance for each sample. Readings on each sample are taken every six minutes.

The Bactometer software utilizes an algorithm that determines the onset of acceleration in the sample's impedance curve. The DT's are displayed as soon as they are available. Samples exceeding the microbial specification level appear in red, borderline or marginal samples appear in yellow, and samples that are acceptable and below the specification level appear in green. The software allows the user to print reports, view and plot curves, and perform statistical analysis required for the creation of calibration curves.

10.622 The RABIT

RABIT for Windows™ is a compact and versatile system for rapid enumeration of bacteria. The basic system is composed of a PC with Windows™ software and a 32-channel incubation block. The test cells appear to be similar to test tubes; however, the bottom of the cell contains an electrode assembly that may be removed and disassembled for cleaning, autoclaving, and reuse. Thus, the

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cost per test is very low because the cells are reusable rather than disposable. A working volume of 2–10 mL of medium may be added to each test cell. The design of the test cell allows the user to conduct direct or indirect impedance assays (section 10.648).

The 32-channel incubation block is composed of two sides which may be individually controlled with regard to temperature. The system can be expanded to provide a total of 512 test channels by increasing the number of incubation modules. The user has full access to information about each individual cell throughout incubation, allowing each test to be set up or terminated on an individual basis.

The Windows™ based software facilitates sample entry and analysis of results. Impedance data may be exported to spreadsheets and database programs for manipulation. Statistical Process Control (SPC) software is available for companies that utilize SPC programs. Final impedance readings may be viewed on screen or printed.

10.623 The Malthus

The Malthus-V can be used to detect a wide range of microorganisms. Up to 256 samples can be tested simultaneously for the presence of aerobic, microaerophilic, and anaerobic microorganisms.[28] The computer-based instrument is designed for continuous automated analysis. Liquid, colloidal, and solid samples require no special preparation. The samples are analyzed using reusable electrodes over a temperature range of 4°C to 47°C. Color-coded pass/fail/suspect results are automatically displayed on an integral HP-Vectra microcomputer, and programmed checks ensure precise analysis. The Malthus Analyzer has the capability for on- and off-line communications with other computers.[28]

10.624 The BacTrac

The BacTrac may be used to detect the proliferation of various types of bacteria, yeast, and mold. Included with the system are a 386 or 486 computer, an incubation block that can contain 20 or 40 measuring cells with working volumes of 10 or 100 mL. The test cells are similar to the RABIT in that they contain an electrode assembly that may be removed and disassembled for cleaning, autoclaving, and reuse. The design of the test cell allows the user to conduct direct or indirect impedance assays (section 10.648). Up to six incubators may be linked to allow for a maximum system capacity of 240 samples.

As with other impedance methods, the impedance of the medium (M-value) may be monitored. However, this system is also able to simultaneously monitor the impedance associated with the area around the electrode (E-value). While the Bactometer is able to monitor impedance, conductance, and capacitance (signal around the electrode), it is unable to separate these signals and interpret them individually for each individual sample. Monitoring these two signals independently is called the Impedance-Splitting method. This method is especially useful for monitoring bacteria that are grown in high salt media, such as *Staphylococcus aureus*, because, at high salt concentrations, the M-value does not significantly decrease as the bacteria multiply; however, the E-value decreases drastically.[44]

10.63 General Considerations and Precautions

The total aerobic plate count (APC) method is different than impedance, conductance, and capacitance with regard to how estimations of bacterial populations are performed. With the APC method, each individual bacterium on the surface of a plate containing bacteriological growth medium with agar must multiply to the extent that a visible biomass (colony) is reached. The colonies are then counted and an estimate of the original number of bacteria in the sample is determined. Impedance, conductance, and capacitance, are based on the electrical changes that occur in medium as bacteria break down large molecules into smaller, more mobile metabolites. Thus, preparation procedures, media, and growth conditions for conducting electrical assays are different than those used for the APC method. Detailed reviews of approaches to the development electrical methodologies are presented elsewhere.[19,53,54]

10.631 Media

The growth medium used to conduct electrical assays greatly influences the electrical shift that must be interpreted by the instrument. If medium components are not designed to optimize the electrical shift during bacterial growth, impedance curves will be inferior. The following circumstances may lead to curves that are not able to be interpreted by the instrument: 1) the baseline may drift, 2) the percent change in impedance may reverse direction during the growth phase of the organism, 3) the acceleration of the impedance change may be weak, 4) a lower maximum impedance change value may be obtained, 5) the curve may be bi-phasic which cannot be interpreted, 6) erratic curves may develop if bacteria are able to produce gas while growing in the medium.[19] Thus, media must be developed to optimize the signal by controlling the use of ingredients that contribute to erratic curves such as certain types of salts and carbohydrates.

10.632 Incubation Temperature

Incubation temperature is one of the most important considerations when conducting an impedance assay. Temperature is used as a selective component of the assay. For example, if a sample containing 1 coryneform and 10^5 pseudomonads were placed into an impedance instrument and allowed to incubate at 30°C, the coryneform would be the organism that is detected and enumerated.[19] Although the pseudomonads in the initial sample exceeded the coryneform by a factor of 100,000 in the initial sample, they did not contribute significantly to the impedance change of the medium and, thus, were not enumerated. This example illustrates how temperature may be used to select for the growth of a particular species of bacteria to be enumerated in samples containing mixed populations. Detailed methods for developing and optimizing media have been reported.[47,53]

10.633 Sample Preparation and Handling

Because time to impedance shift or "detection time" is the parameter being measured, sample preparation and handling must be extremely strict when conducting impedance assays. Samples should always be placed into growth medium that is a consistent temperature. The amount of time to inoculate the medium, place the sample into the module well, place the module well into the incubation chamber, and initiate the impedance readings must be held constant from sample to sample. For example, if a sample is placed into cold growth medium, and held in a refrigerator for 1 hr before being placed into the module and then incubated, this sample will have a much different DT than an identical sample that is placed into a warm medium and incubated immediately. Thus, every effort must be made to standardize sample preparation and handling procedures.

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10.634 Generation Times

When using impedance to estimate bacterial numbers in samples containing mixed populations, it is essential that the media and temperature be selected so that differences in generation times between the different species of bacteria in the sample are minimized.[18] Firstenberg-Eden and Tricarico[21] revealed that 18°C is the appropriate temperature for monitoring samples that contain mixed flora (mesophilic and psychrotrophic) when enumerating total populations of bacteria using impedance. This is due to the fact that 18°C is the temperature at which generation times for the mesophiles and psychrotrophs are most similar at 1.5 and 1.2 hr, respectively.[21] Minimization of differences in generation times allows most of the genera in the sample to multiply at a similar rate and hence, gives a more accurate indication of the total populations present.

10.635 Inhibitors

Some foods contain natural compounds that are inhibitory to the growth of bacteria. Other foods such as processed products, may contain preservatives. Natural bacterial inhibitors or preservatives may prolong the lag time of the bacteria being enumerated and delay detection, giving a false estimation of the number of organisms in the original sample. Interference by inhibitors or preservatives may be detected by conducting assays on serial dilutions of the sample. If the diluted samples have similar detection times or shorter detection times than concentrated samples, then the presence of an inhibitor may be suspected.[19]

Ions in foods may interfere with the impedance signal and cause excessive drift by increasing or decreasing the impedance readings, or by reacting with components in the medium to produce compounds which mimic bacterial metabolites. Specifically, chloride (Cl^-) ions cause excessive drift and destroy the oxide layer coating on electrodes in the Bactometer module.[19] Bisulfite (HSO_3^-) ions can form $SO_3^=$ in neutral medium, which changes the conductivity component of the medium.

Inhibitory substances should be neutralized using dilution or separation procedures. Dilutions should only be conducted on those samples that contain a sufficient number of bacteria initially.

10.636 Selective Components

Selective components in commercial media may act as inhibitors when conducting impedance assays. A general knowledge of the components of the medium to be used is necessary for determining those species of bacteria that will be inhibited if selective components are present during impedance monitoring.

10.637 Damaged Cells

Sublethal injury to microorganisms may be caused by heating, cooling, freezing, or exposure to inhibitory or toxic substances. Sublethally injured cells would not be detected using a standard APC that is incubated for 48 hr, because in that time interval the bacteria would not have sufficient time to recover, reproduce, and produce a visible biomass. In an electrical assay, the accumulation of metabolites could be detected for cells which have been injured and must repair themselves before initiating replication. For processed foods that contain injured cells, it is useful to develop separate calibration curves.

10.638 Particulate Matter

Some foods contain significant quantities of particulate matter such as spices, that interfere with the electrical impulse as it passes between electrodes. For these foods, the sample should be diluted (if there are enough organisms to allow dilution) or filtered to remove the particulate matter. Another technique involves covering the electrodes with hot sterile plate count agar and then layering the inoculated medium over the plate count agar. As bacteria multiply and produce metabolic by-products, the metabolites diffuse into the plate count agar and change the impedance between the electrodes.

10.64 Typical Applications

In general, impedance assays require much less time and effort to conduct than traditional plate counts. With traditional plate counts, the sample must be diluted using serial dilutions, plated using duplicate plates, incubated, and then counted. For impedance assays, samples can be analyzed without using serial dilutions, provided they contain at least one viable bacterial cell and no more than 10^7 cells. Samples that contain greater than 10^7 cells must be diluted because the impedance threshold will be reached within 1 hr and the instrument will not be able to interpret such rapid impedance changes. In most cases, with impedance, the sample is diluted 1:10 into a microbiological growth medium and 1 mL of the growth medium is then placed into the Bactometer module well (Bactometer) or tube (RABIT, Malthus, and BacTrac). The module or tube is then incubated and impedance is read automatically by the instrument.

10.641 Estimation of Aerobic Plate Counts

To estimate APC using impedance, differences in the generation times of the species in the sample must be minimized by choosing the proper medium and temperature of incubation.[19] By minimizing differences in generation times of species in a sample, the microorganisms, although different, will multiply at a similar rate and will reach the detection threshold of 10^6–10^7 at similar times. Using this method, correlations between impedance and plate counts will be highest. By correlating DT to APC, future APC can be estimated based on impedance readings.

A commonly used procedure to estimate APC from foods of animal origin was described by Firstenburg-Eden.[19] A 10 g sample is placed into 90 mL of brain heart infusion broth (BHI) and stomached. One milliliter of the strained homogenate is then placed into a module well (Bactometer) or tube (RABIT, Malthus, and BacTrac). The module or tube is then incubated in the instrument at 30°C for 48 hr. Depending upon the initial microbial load in the sample, results may usually be obtained in 1 to 12 hours.

10.642 Estimation of Selected Groups of Microorganisms

Impedance has been used as a means of enumerating coliforms,[20,22,31,53,61,62] fecal coliforms,[33,46,58] mesophiles,[49] and *Enterobacteriaceae*[11] from samples of wastewater, dairy products, poultry, and meat. The growth of lactic acid bacterial starter cultures have been monitored using impedance.[5] Other researchers used impedance as a means of monitoring the metabolic activity of lactic acid bacteria in milk.[30] Direct and indirect impedance techniques have been used to estimate yeast populations in frozen fruit juices.[13,14] Using specific media and temperature, the growth of these groups of bacteria or fungi may be enhanced over competitors in mixed samples. By enhancing their growth, these organisms are able to multiply, produce enough metabolites to change the impedance of the medium, and be responsible for the detection time.

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10.643 Estimation of Specific Bacteria

Using media and temperature that are exclusively selective for a particular microorganism, impedance may be used as a means of detecting and enumerating the organism from samples containing mixed populations. Media have been developed specifically for detecting *Salmonella* in foods using impedance.[2,8,12,15,24,35,36,38,41,42,59] Because bacterial reduction of trimethylamine oxide to trimethylamine is generally repressed under aerobic conditions, it was originally thought that detection of *Salmonella* using impedance would be optimal using anaerobic medium.[57] However, another researcher found that for *Salmonella* detection, impedance changes increased and time to detection decreased when impedance was conducted in aerobic conditions.[37]

Immunomagnetic separation procedures were combined with impedance as a means of capturing and concentrating *Salmonella* from preenriched broth medium.[40] The medium used was selenite cysteine/trimethylamine oxide/mannitol medium (SC/T/M). The authors demonstrated that *Salmonella* detection was enhanced by eliminating competition from other bacteria in dried milk samples using selective medium.[40]

Impedance methods for detecting *Salmonella* have been compared to conventional methods and have been found to be suitable for screening, since negative results can be obtained in less than 38 hr.[44] Others found that impedance outperformed rapid microbiological techniques, such as the Gene-Trak System and *Salmonella*-Tek.[45] Impedance as a means of detecting *Salmonella* has been given AOAC first action approval following a 17-laboratory collaborative trial.[25]

Listeria spp. have been detected by conducting impedance assays using a modified growth medium containing acriflavine, ceftazidime, nalidixic acid and esculin.[43] Other species of bacteria were able to grow in the medium; however, *Listeria* spp. could be differentiated from the others by the shape of the impedance curve. Glucose-enriched nutrient broth supplemented with proflavine and moxalactam has been used to detect *Listeria* spp. in spiked (10^3 cfu/gm) cheese.[27] Only Bolton and Gibson[7] have described a method for detecting *Listeria* spp. from unspiked foods. For further information regarding the use of impedance as a means of detecting pathogenic bacteria, please refer to an excellent review by Silley and Forsythe.[57]

A procedure to enumerate *Escherichia coli* (*E. coli*) from foods of animal origin in as little as 1 to 7 hours has been developed.[16] A significant linear correlation ($r^2 = 0.92$) was observed between $\log_{10}$ Petrifilm™ *E. coli* Count Plates (AOAC International, approved methodology number 991.14) and *E. coli* detection times (DT) using CM Medium. *E. coli* were enumerated from broiler chicken carcasses in 1 to 7.6 hours, which would allow processors to analyze products prior to shipment.

A capacitance method for enumerating *Pseudomonas fluorescens* from broiler carcass rinses was described by Russell.[47] Brain heart infusion broth (BHI) containing the following additives: nitrofurantoin (4 µg), carbenicillin (120 µg), and Irgasan (25 µg) per mL (BHI-PSA-patent accepted and pending) was able to effectively select for the growth of *Pseudomonas fluorescens* in samples containing mixed microflora, such as a broiler chicken carcass rinse. The time required to enumerate *P. fluorescens* was <22.4 hr. This method may be useful for enumerating *P. fluorescens* on chicken as a means of predicting the potential shelf-life of fresh products.

10.644 Estimation of Psychrotrophic Plate Counts

Psychrotrophic plate counts at 7°C for milk samples containing high numbers of psychrotrophs were highly correlated ($r = -0.95$) to impedance detection times, when samples were incubated at 18°C.[21] The authors found that by reducing the incubation temperature to 18°C, while conducting impedance measurements, the generation time of the psychrotrophic bacteria was similar to the mesophilic bacteria in the sample. By reducing the generation time, the psychrotrophs were able to multiply and change the impedance of the medium, which resulted in a detection time that was not influenced by the presence of mesophilic bacteria.[21]

A selective medium was developed for rapidly enumerating *Pseudomonas fluorescens* from broiler chicken carcasses using capacitance.[47] $\log_{10}$ detection times were highly correlated ($r^2 = 0.96$) to psychrotrophic plate counts conducted at 7°C. The author concluded that capacitance, in combination with selective media, can be used to enumerate psychrotrophs from fresh chicken in less than 18 hours, as opposed to 10 days, as is required by the standard method.[48]

10.645 Estimation of Shelf-life

Estimation of shelf-life of fresh foods is based on enumerating the organism or group of organisms responsible for producing spoilage defects (odor, slime, discoloration) on the food. Mead and Adams[32] described the challenge of rapidly enumerating small numbers of psychrotrophs from samples containing much higher numbers of mesophiles by stating "the rapid isolation of psychrophilic spoilage bacteria is of interest in relation to various foods and numerous attempts have been made previously to develop selective media which would permit more rapid growth of the organisms at higher incubation temperatures whilst inhibiting the growth of mesophiles." The traditional method for enumerating psychrotrophs to predict shelf-life is described in section (Chapter 13) and requires 10 d at 7°C. After 10 d, most foods would have already been purchased and consumed before results could be obtained.

A rapid impedance based method for determining the potential shelf-life of milk has been reported.[5] In this study, milk samples were preincubated in plate count broth at 18 or 21°C for 18 hr. After preincubation, the samples were placed in module wells containing modified plate count agar (MPCA) and were incubated at 18° or 21°C. In another study, high correlation coefficients ($r^2 = 0.87$ and 0.88) were observed between the shelf-life of milk and impedance readings taken at 18 and 21°C, respectively.[4]

To rapidly predict the shelf-life of fresh fish, samples have been analyzed using conductance at 25°C in trimethylamine oxide nitrogen medium (TMAO).[29] Using this method, H_2S producing bacteria enumerated using conductance were highly correlated ($r^2 = 0.96$) to the shelf-life of fresh fish.

The potential shelf-life of fresh poultry can be predicted in less than 18 hr.[48] In this study, $\log_{10}$ capacitance detection times were highly correlated to day of storage ($r^2 = 0.86$).

10.646 Determination of Temperature Abuse

A series of studies were conducted to develop a method to rapidly determine the occurrence of temperature abuse of fresh poultry.[49–54] By incubating samples at 42°C, increases in populations of mesophilic bacteria as a result of temperature abuse were detected. To make the procedure more sensitive to small fluctuations in temperature abuse, coliforms were monitored at 43°C, such that significant increases of coliforms on groups of chicken carcasses were considered to be due to temperature abuse because, under refrigeration, populations of coliforms remain constant or decrease in number.[53]

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10.647 Sterility Testing of Ultra-High Temperature (UHT) Products

Many shelf-stable foods are sterilized using ultra-high temperature processing (UHT). The impedance procedure is often used as a screen to insure that all viable cells or spores have been destroyed during the sterilization process. These assays are conducted to determine if any microbial growth can be detected, not for enumeration purposes; thus, equilibration of generation times is not an issue. Food samples may be pre-incubated with or without growth medium to allow any viable organisms or spore-forming bacteria to proliferate. Samples are then placed in one or more growth media and incubated at one or more temperatures (often 35° and 55°C are used to allow mesophiles and thermophiles to grow). Procedures for screening dairy products,[55] fruit juices,[56] and low acid canned foods[10] have been reported.

10.648 Indirect Methods

A method for indirectly monitoring yeast growth using conductance has been described.[63] As yeast proliferate, they evolve CO_2. The evolution of CO_2 causes the conductance of an alkaline solution, which is contained in an insert containing electrodes, to change. The indirect method is advantageous for measuring the growth of yeasts because the electrical signal is greatly magnified.[13] Using indirect methods, culture media containing high concentrations of salt or other inhibitory agents, that negatively influence the electrical signal when conducting direct impedance measurements, can be used for the detection of microorganisms without interference.[6] An indirect impedance procedure has also been reported for detecting the presence of *Escherichia coli* in potable water.[63] The authors stated that when compared with the Colilert® method, indirect impedance eliminated the need for confirmation and was found to be more rapid than Colilert®. It is also possible for the method to be used with samples containing high solids/low volume where membrane filtration would be inappropriate.

10.65 Interpretation of Results

10.651 Calibration Curve Method

Before using electrical methods for estimation of bacterial numbers in foods samples, a calibration curve should be developed for each "group" of products that will be assayed in the future. The calibration curve is used to define the relationship between the electrical method and a standard method (usually APC) or the electrical method and some other parameter, such as shelf-life.

To develop a calibration curve, 80 to 100 samples of a food product with varying levels (3–5 $\log_{10}$ cycles) of the organism(s) to be assayed are analyzed using the electrical and conventional methodologies. For example, to enumerate *E. coli* from milk, 100 samples of milk should be obtained that contain *E. coli* at levels of 10^1 to 10^5 and should be tested using electrical and conventional techniques.[19] These data are then analyzed using linear or quadratic regressions. Because there is error associated with using electrical methods and conventional methods, and the two methods are not measuring identical things (metabolites versus visible colonies), the data points will generally be scattered about the regression line. The correlation coefficient (r) is used to describe the correlation between the two methods. The closer r approaches –1.0, the higher the level of agreement between the two methods. The ideal r is negative as opposed to positive 1.0, because DTs are inversely proportional to APC or other bacterial counts. The correlation between electrical and conventional methods will vary based on the number of samples tested and the range of the concentration of microorganisms on the samples. In general, on samples where the generation time differences of the target organisms can be minimized, a correlation coefficient of r = 0.85 or higher is achievable if 80 to 100 samples containing levels of 10^1 to 10^5 are tested.[28]

When developing a calibration curve, several factors must be considered. Samples that contain few (< 10) microorganisms are subject to sampling error. Curves generated from these samples contain more scatter and lower correlation coefficients than samples containing higher numbers of microorganisms. In addition, when a sample contains few microorganisms, extended lag times may result in long and variable DTs.[28] For samples that contain more than 10^7 microorganisms/mL, the detection threshold is reached so rapidly, that the number of microorganisms cannot be distinguished. It is often necessary to remove extreme data points (low or high) from the calibration curve equation, so as to reduce the amount of error associated with the curve. Any data point that lies far outside the standard error should be evaluated to determine if 1) an error was made in calculating or recording the colony count, 2) an error was made in entering the data into the computer, 3) an inaccuracy of DT resulted from an abnormal curve, and 4) the data point was derived by assay of an unusual sample source.[28]

Most food products do not contain a naturally broad range of microorganisms to be used for calibration curve purposes. To increase counts artificially, food samples are temperature abused. If samples must be temperature abused to develop a calibration curve, every effort should be made to mimic actual abuse situations (temperature, moisture, and oxygen conditions) that would occur naturally, so as not to accidentally select for the growth of a microorganism or group of microorganisms that will interfere with the desired counts. Any alteration of the natural flora may result in inaccurate DTs.[28]

Calibration curves may also be influenced by the type of food being analyzed. A separate calibration curve must be developed for foods that contain ingredients that increase or decrease the growth of bacteria, i.e., sugars or preservatives. Foods should be grouped according to these factors and a calibration curve should be developed for each group.

10.652 Sterility Method

Foods that contain low levels of microorganisms may be tested using an "industry sterility" protocol. This protocol was developed such that DTs are achieved only if the product is contaminated with more microorganisms than the specified level. An appropriate dilution scheme is used to dilute out the normal background flora and allow the detection of higher numbers of organisms. Because dilutions are made, any detection means that the sample is contaminated with the organism at a level that exceeds the specified level. For example if samples normally contain 1 to 50 organisms and the specified level is 1000 cells/mL, a 1:100 dilution would be used. This method may be used for various foods and different levels of contamination, depending on the dilution scheme chosen. It is effective for foods in which a calibration curve cannot be developed because of low- or high-count samples, inability to calibrate, presence of inhibitors, etc. For the sterility technique to be effective, however, a large difference in counts must exist between acceptable and unacceptable samples.[28]

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10.67 Commercial References

Bactometer Microbial Monitoring System M128 (BioMérieux Vitek, Inc., Hazelwood, MO)
Rapid Automated Bacterial Impedance Technique (RABIT-Don Whitley Scientific Limited, UK)
Malthus V (Malthus Diagnostics, Inc., North Ridgeville, OH)
BacTrac 4100 (Sy-Lab, Vertriebsges.m.b.H., Purkersdorf, Austria)
Gene-Trak Systems (Hopkinton, MS)
Salmonella Tek™ (Organon Teknika Corporation, Durham, NC)
Petrifilm E. coli Count Plates (3M Microbiology Products, St. Paul, MN)
CM Medium (bioMérieux Vitek, Inc., Hazelwood, MO)
Colilert® (IDEXX Laboratories, Inc., Westbrook, ME)

10.7 PHAGE PROBES

10.71 Introduction to Bacterial Viruses

10.711 History of Discovery

The existence of bacterial viruses known as bacteriophages—*eaters of bacteria*—was first reported by the British scientist F.W. Twort in 1915, and somewhat controversially in 1917 by the Canadian bacteriologist F. d'Herelle. Twort's publication in the Lancet documented the phenomenon of "glassy transformation" observed in colonies of micrococci grown on agar in the presence of small pox vaccine. He postulated that the "transparent dissolving material" might be caused by: i) an enzyme; ii) could be part of the life cycle of the bacterium; or iii) was caused by a *virus*, a term first used vaguely by Jenner in 1796 and used more specifically in 1898 by the Dutch botanist Martinus Beijerinck in his study of Tobacco Mosaic Virus.[114]

d'Herelle, however, was much more certain of the viral nature of this bacteriolytic agent, subsequently published a book in 1921 entitled *Le bactériophage: son rôle dans immunité*. However, he mistakenly assumed that a single bacteriophage existed,[40] and that it could act against a number of different species of bacteria due to adaptive variation.[88] This is, in hindsight, not a surprising assumption when one considers that virus taxonomy and classification was still many years away from the generally accepted system in use today.[39] Australian scientist Burnett, between 1924 and 1934, disproved d'Herelle's theory of a single bacteriophage, and went on to identify a myriad of viruses, which while exhibiting different physical and biological properties, were able to maintain these characteristics despite repeated subculturing.[88]

Significant scientific advances were to be made in the study of bacteriophages during the late 1930s to 50s. In particular, the formation of the Phage Group—a group of scientists to include Max Delbruck, Alfred D. Hershey, Salvador E. Luria, and James D. Watson—played an enormous role in both the beginnings of molecular biology and the elucidation of the structure of DNA. During this period, researchers showed that viruses were composed of either DNA or RNA with a protein component, and in 1946, Hershey demonstrated that bacterial viruses could undergo genetic recombination. In 1952, Zinder and Lederberg first demonstrated host transduction (the transfer of genetic information via a virus particle) by bacteriophage P22, while Brenner, Jacob and Meselson discovered messenger RNA in 1961.[40] The presence within the Phage Group of the electron microscopist Anderson enabled the identification of viral architecture, a feature now used by the International Committee on Taxonomy of Viruses (ICTV) to aid in identification and classification of viruses along with nucleic acid information.[39] The attachment of bacteriophages to bacterial cells was also demonstrated by electron microscopy. Ultimately this led to the elucidation of replication of these parasites within the host cell.

10.712 Phage Replication

Phages, in common with other viruses, do not possess the biochemical machinery required to replicate themselves, and thus rely on the assistance of an actively metabolizing bacterial cell to do the job for them. As phages have no means of actively seeking out replicative hosts, coming into contact with one is a completely random event, relying on their interaction during movement through a medium.[112] Replication will only follow if the cell

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contains specific receptor sites for the phage in question, such as cell wall lipopolysaccharides, proteins, teichoic acids, pili or flagella. Variations within receptor groups further increase the phage's specificity for a particular host, with the result that bacteriophages are typically specific in their host requirements to at least the genus level, and usually beyond.[35] The replicative cycle can differ depending on the phage, but there are sufficient commonalities to use T4[100] and Lambda[22] phages as general examples of the lytic and lysogenic replicative cycles respectively.

10.7121 Attachment and infection. Attachment, as described above, occurs when a phage encounters a bacterial cell possessing a particular receptor specific for that particular phage. In the case of the extensively studied DNA bacteriophage T4, fibers associated with its contractile tail mediate attachment, and as more fibers make contact with the receptor, the tail plate of the phage settles down onto the surface of the cell. Thus attachment is generally a two-step event involving first a reversible step, followed by irreversible binding. For each phage-host combination, various salt concentrations (in particular calcium and magnesium ions) govern the rate of attachment, influencing the formation of electrostatic bonds between tail fibers and cell receptors. Either reduction or removal of these ions, or receptor mutations will effectively prevent adsorption and subsequent phage multiplication. After attachment the next step of phage replication follows: infection of the host.

First, conformational changes occur to the adsorbed tail base plate and sheath, and the tail sheath contracts. The central tube of the phage tail then enters through the bacterial wall and DNA is pushed out of the phage head, similar to the action of a syringe, and into the host cell. This constitutes infection of the cell, although it can also occur by other means, such as attachment to pili and transfer of DNA either through or along the pilus. Shortly after introduction of phage DNA into the bacterium, synthesis of host DNA and protein is suppressed. Two events govern the subsequent synthesis of phage DNA: first, the degradation of host DNA into its nucleotide components occurs, providing building blocks for viral DNA synthesis and preventing further host gene expression. Second, modification of bacterial RNA polymerase occurs, with the result that it now recognises viral promoters, thus promoting the transcription of viral genes rather than host genes. Viral DNA synthesis commences within 5 minutes of infection, followed by synthesis of proteins responsible for phage structure, assembly, and cell lysis and phage release. It takes approximately 15 minutes for the first complete T4 particles to appear following infection, and another 7 minutes for cell lysis.[112]

10.7122 The lytic versus lysogenic cycle Two modes of replication have been observed for bacteriophages: i) the lytic cycle, where replicated DNA is packaged into phage heads and subsequently released from the bacterial host by cell lysis; such phages (e.g. T4) are described as virulent, and ii) the lysogenic cycle, where phage DNA is incorporated into and replicated within the bacterial genome; in this case phages are termed temperate, the best characterized being the double-stranded DNA phage lambda. Virulent phages will typically lyse and destroy susceptible hosts (although some filamentous phages are secreted without cell lysis), while temperate phages can adopt either mode. The choice between the lytic and lysogenic cycle depends on the relative expression rates of phage repressor encoded by the *cII* gene (promoting lysogeny) and the *cro* protein, capable of turning off repressor gene expression and starting the lytic pathway.[22] Following infection of susceptible cells with lambda phage, a very small proportion will be lysed but the majority will survive to become lysogens. These infected cells continue to replicate as normal, creating a clone of cells containing phage DNA. The latent form of the phage genome remaining in the host is termed the prophage, and its presence prevents superinfection of the host with the same virus. Temperate phages can be induced, in particular by agents causing DNA damage, such as UV light or chemical mutagens, and once induced will enter the lytic cycle, resulting in production of phage at the expense of the bacterial host. Two classes of genes are involved in lysogeny and induction; those establishing the lysogenic state, and those maintaining the prophage in a repressed state within the bacterial genome. Mutations in either group of genes will result in loss of lysogenic function, and depending on the class involved mutants can either resort to lytic activity, lose the prophage completely, or be unable to release viable phage particles.[45]

10.7123 Host Implications. The lysogenic relationship between host and phage can be mutually beneficial. The incorporation of phage DNA into the bacterial genome is considered a survival tactic of the virus, and typically occurs under adverse conditions such as host nutrient deprivation or a high multiplicity of infection where the host cells are in danger of being completely destroyed, exposing phage to environmental hazards. One of the most significant properties conferred to host bacteria following lysogenization by a temperate phage is that of toxin production. Toxins including Shiga-like toxins in *Escherichia coli*, botulinum toxins in *Clostridium botulinum*, enterotoxins in *Staphylococcus aureus*, diphtheria toxin in *Corynebacterium diphtheriae*, and pyrogenic toxins in *Streptococcus pyogenes* have all been identified as proteins produced as a result of "phage conversion" of the bacterial host.[63] Another benefit of lysogenic infection has been reported in the literature as a type of "suicide bombing" where a small proportion of *Bacillus subtilis* cells infected with a phage release virus, thus killing off any susceptible strains present and giving the remaining bacteria (those which can safely harbor the phage) a competitive edge.[3]

Although it is mentioned above that phage infection can be an advantage to the host, a proportion of the bacterial population is going to be lysed as the virus takes over the replicative machinery of the cell and puts it to the task of manufacturing more viral particles. This has been problematic in fermentation processes, where starter culture failures can occur as a result of bacteriophage infection, and control is difficult particularly when a large number of variable phages are continually introduced into the processing environment via raw materials such as raw milk,[38] and even from the starter cultures themselves.[124]

10.713 Microbiological Applications of Bacteriophages

10.7131 Bacterial typing. The use of characterized bacteriophages to type bacteria beyond the species and serotype level is well known, and perhaps the best recognized system is the International Typing System for *Listeria monocytogenes* defined in 1985 by Rocourt *et al.*[119] developed following the successful application of phage typing to other bacteria such as *Staphylococcus aureus*.[122] Identification of new bacteriophages has resulted in an optimized typing system,[90] as well as the development of alternate bacteriophage typing sets[78] and a reversed phage typing procedure.[38,79] Bacteriophage typing has also proven useful as an epidemiological tool in the study of food poisoning outbreaks associated with *L. monocytogenes*, *Bacillus cereus*,[1] *Salmonella enteritidis*,[17,153] and *E. coli* O157:H7.[8,49,123] This allows the epidemiologist to confirm the relationship between outbreak strains isolated

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from infected patients and those cultured from suspected food samples. In addition, environmental sources of contamination can be identified, their movement within food processing plants monitored, and persistent problems distinguished from sporadic contaminants.[80]

10.7132 Biological indicators. The most frequently used phages as biological indicators are those of the coliphage group. Their use as indicators of water quality was proposed based on their occurrence in waters along with their bacterial hosts, with numbers of phage theoretically reflecting the numbers of host bacteria present. Several studies have utilized these phages instead of the host strains as an indication of fecal pollution,[18,103] and evidence suggests that they are as good as, or indeed better indicators than bacterial systems currently in use. A coliphage-based water assay is described in the Standard Methods for the Examination of Water and Wastewater,[2] and using *E. coli* strain C as the host bacterium, five or more phages/100 mL of water can be detected within 4–6 hours. Problems have been encountered with naturally occurring coliphages in environmental waters, which affect the correlation between phages and fecal pollution. However, viruses tend to survive better than coliforms in water, and have been shown to exhibit a higher resistance to chlorine. Their application as indicators for coliforms in foods has seen limited study.[68,69] Overall this method may be useful as an alternative to *E. coli* or coliform determinations, or as an indicator of enterovirus survival.[67]

10.7133 Cloning vectors. Extensive genetic characterization of certain bacteriophages has seen their extensive application as cloning vectors for amplification of DNA in *E. coli*. Indeed, phage Lambda was the prototype vector long before recombinant molecules could be made *in vitro*. Lambda phage offers a number of attractive features for cloning: recombinant genomes can be efficiently recovered in packaged phage heads, plaques provide a large amount of DNA for hybridization studies, and cloned genes can be easily analyzed. Phage M13 has been particularly useful in cases where single-stranded DNA is required, such as in site-directed mutagenesis, while phage P1 offers a large cloning capacity of up to 100 kilobases. P1 has also been demonstrated to have a wide host range, which could facilitate the transfer of DNA to bacteria other than *E. coli*.[101]

10.7134 Phage therapy. A prophetic aspect of the discovery of bacteriophages was the suggestion by d'Herelle that bacteriophage could be used as a form of therapy for the treatments of 'intestinal disturbances', bacillary dysentery, staphylococcal infections, and bubonic plague.[53] While this idea was viewed, certainly by d'Herelle himself, as perhaps the universal cure for all bacterial infections, it was never explored to any great extent, and indeed with the advent of antibiotics was all but discarded. However, with the increasing incidence of bacterial resistance to certain antibiotics, bacteriophage therapy has been revisited in the 1990s.[75,98,115,132]

While scientists such as Lederberg[73] have been reluctant to completely rule out the usefulness of phage therapy, a number of pertinent questions remain unanswered in the wake of work by Merril et al.[98] to isolate and utilize long-circulating mutants of *Escherichia coli* phage and *Salmonella typhimurium* phage P22. An identified alteration to the phage head protein was found to be responsible for the mutants' success in avoiding elimination by the reticuloendothelial system of test mice, one of the more important problems to be addressed in phage therapy. Despite this work, scepticism remains for many reasons such as the potential for bacteria to develop resistance to phages (a phenomenon which may be slowed by the use of phage combinations) and a lack of hard data despite claims that phage therapy has been successfully used in parts of Europe for many years.[115]

10.7135 Bacterial detection. As a result of the usefulness of phages in the typing of bacteria, and as cloning vectors, their ability to identify microorganisms to at least the genus level has seen their increased application in techniques for the detection of important food-associated bacteria. Ultimately, their specificity dictates the success of any phage-based system, and thus identification of useful phages for species- or strain-specific detection of bacteria is of the utmost importance. The next section of this chapter will focus on the use of phages in a variety of molecular, biochemical and physical methods applicable to the microbiological analysis of foods, some of which are in use, and their imminent application in more recently developed technologies.

10.72 Bacteriophage-Based Detection Methods

10.721 Phage-Based Reporter Genes

10.7211 *lux*-based bioluminescence. The use of bacterial bioluminescence, in particular the application of *lux* genes, and aspects of its molecular organization and regulation have been extensively reviewed,[7,59,60,96,97,127,136-139] and readers are referred to these publications for a comprehensive introduction. Briefly, bacterial bioluminescence is a light-emitting reaction involving the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aliphatic aldehyde, such as tetradecanal (considered the natural substrate of the reaction) or dodecanal, by molecular oxygen. The reaction is catalysed by intracellular luciferase according to the following equation:

$$FMNH_2 + RCHO + O_2 \; FMN + RCOOH + H_2O + LIGHT$$

where light emission is in the form of a glow with an estimated quantum yield (photons of light produced per molecule of substrate) of 0.1.[19] Given that the production of $FMNH_2$ from flavin mononucleotide is essential to the reaction, only living cells capable of electron transport will be able to produce this blue-green light (490nm emission), and bioluminescence is thus an excellent means of establishing the metabolic status of an organism. Light can be measured by instrumentation including luminometers, scintillation counters and sensitive photon counting imagers such as the BIQ Bioview Image Quantifier (Cambridge Imaging, Cambridge, U.K.) which is capable of detecting single bioluminescing cells.[64] Naturally bioluminescent bacteria have been isolated from marine, freshwater, and terrestrial environments, and the majority belong to one of four genera: *Vibrio*, *Alteromonas*, *Photobacterium*, or *Xenorhabdus*.[7] In symbiotic relationships, bacterial bioluminescence is thought to serve as a means of communication for the host, but its purpose is less clear for free-living bacteria.[97]

A heterodimeric enzyme, bacterial luciferase is composed of two subunits, (40kDa) and (36kDa), respectively encoded by the *luxA* and *luxB* genes. These genes are flanked by *lux* genes *C*, *D*, and *E*, respectively coding for reductase, transferase, and synthetase proteins responsible for the production of aldehydes from fatty acids. These five genes compose the *lux* operon, transcribed in the order *luxCDABE*, and these genes have been cloned and sequenced for some luminescent *Vibrio*, *Photobacterium*, and *Xenorhabdus* spp.[97] Other *lux* genes have since been identified in *V. fischeri*, including a second operon consisting of regulatory genes *luxI* and *luxR* located immediately upstream from the *lux* operon. These genes code for an autoinducer and receptor required for activation and expression of luminescence, in conjunc-

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tion with another gene, *luxG*, encoding a reductase involved in the production of $FMNH_2$.[97]

Transfer of *lux* genes has been approached from a number of angles, depending on both the organisms to be transformed, and the application of the bioluminescent phenotype. Plasmids, containing either the complete *luxCDABE* operon, *luxAB* luciferase genes, or fused *luxAB* genes, have been constructed and employed to transfer a bioluminescent phenotype to a desired bacterial recipient.[60] The complete operon encodes substrate in addition to luciferase, thus providing a continuously luminescent organism. Innate substrate production provides a non-destructive means of real-time monitoring of luminescent bacteria in mixed culture and within eukaryotic cells, but is only useful for gram-negative organisms capable of recognizing transcription and translation signals originating from bioluminescent bacteria. Additionally, continuous expression of substrate places energy constraints on the cell, and is therefore not suitable for physiological studies.

Alternatively, only the genes for luciferase are required if substrate is added exogenously, and this does not significantly influence cell metabolism. Bacteria expressing *luxAB* are therefore non-luminescent before substrate is added, and this less complicated procedure is favored for use with gram-positive bacteria where a promoter also has to be engineered for expression. Another option when working with gram-positive bacteria is the use of *luxAB* fusions. A monomeric fusion product was reported to increase bioluminescence levels in *Bacillus subtilis* about 100 times more than the use of unfused *luxAB* genes. Furthermore, this increased light output was comparable to that obtained from a control *E. coli* strain demonstrating optimal bioluminescence levels.[60] Transposon vectors have also been engineered to contain *lux* genes without promoters, such that the genes are only expressed when the transposon inserts into the bacterial chromosome beside a promoter region.[60] In addition, the source of luciferase genes influences the temperature stability of the resulting enzyme, and should be carefully considered based on the growth temperature of the microorganism of interest and the application of the bioluminescent phenotype. It is worth noting that fusion genes produce products with temperature optima 7–10°C lower than that of the wild type.[60]

Such a variety of alternatives has resulted in the transformation of a number of both gram-negative and gram-positive bacteria, and these now bioluminescent microorganisms have most commonly been used as reporters of, for example, bacterial injury and repair, biocide efficacy,[158] regulation of gene expression[36,107,127] and survival of pathogens such as *Salmonella enteritidis*[27] and *Escherichia coli* O157:H7 in food processing.[65,146]

Bioluminescence-based detection methods for bacteria have also been developed by the use of a third gene vector: bacteriophage engineered to carry *lux* genes within their genome. Depending on the host-specificity range of these phage vectors, generic or specific detection of microorganisms can be achieved by bacterial expression of bioluminescence within 30–50 minutes of infection and transduction by *lux*$^+$ phage unable to express the bioluminescent phenotype themselves.[71] This approach to bacterial detection, first described by Ulitzer and Kuhn,[149] has led to a number of microbiological applications, in particular the detection of various foodborne and clinically important pathogens.

A near on-line method for detecting enteric bacteria using recombinant *lux* bacteriophage was subsequently proposed by Kodikara et al.[71] Using a slaughterhouse processing pig carcasses as a test environment, the authors reported the ability of recombinant *lux*$^+$ Enterobacteriaceae-specific phages to convert enteric bacteria sampled from carcasses, meat-contact factory surfaces, and various abattoir areas into bioluminescent bacteria within one hour of sampling. The limit of detection of this assay was around 10^4 target cells per g or cm^2, but was substantially improved by the incorporation of a 4 hour enrichment in Luria broth at 37°C, reducing the detection limit to less than 10 enterics per g or cm^2 with a required total assay time of 5 hours. Despite these promising results and the applicability of such a rapid detection system in a Hazard Analysis Critical Control Points (HACCP) system, industrial application of such on-line analysis has not been adopted.[136] Regardless, this research prompted significant research activities in the area of bacterial detection, and alternative applications have been forthcoming.

In 1993, Turpin et al.[147] reported the development of a phage-based luminescent most-probable-number (MPN) method for the detection of *Salmonella typhimurium*, employing a commercially available *lux*$^+$ P22 *Salmonella* phage (Amersham International). A 15-tube MPN test protocol was established, with each of 0.1, 1, and 10 mL volumes of samples (including soils, sewage sludge and water) artificially inoculated with *S. typhimurium* pipetted into five tubes of sterile buffered peptone water. After overnight enrichment, samples were tested by both the MPN method (inoculated into Rappaport-Vassiliadis broth) and the *lux*-based protocol where samples were inoculated into Luria broth containing 6.9×10^{10} plaque forming units (PFU) of *lux*-P22 phage. MPN samples were incubated at 43°C overnight, streaked onto xylose lysine desoxycholate and brilliant green agars after 24 and 48 hour incubations, and presumptive colonies were biochemically confirmed using the API system of identification. *lux* samples were incubated for 90 minutes at 30°C and aldehyde substrate was then added. Subsequent luminescence was measured using a luminometer, with positive tubes producing relative light outputs 100 to 1000 times greater than negative samples. When compared with the MPN method, the *lux*-based method had an efficiency of 100% with no false positive or negative results. A *S. typhimurium* detection limit of 10 colony forming units (CFU) per gram of soil or per 100 mL of lake water was demonstrated in the presence of large numbers of competing bacteria, thereby reducing the requirements for selective enrichment and decreasing the duration of the protocol to less than 24 hours.

Three recombinant *lux* phages, containing either *luxAB* or the entire *lux* operon, were later constructed by Chen and Griffiths[26] to expand the detection range to *Salmonella* isolates belonging to different serogroups. One hundred percent of the *Salmonella* strains belonging to groups B and D, and selected group C isolates tested were identified using a recombinant phage plaque assay, while all of the 22 non-*Salmonella* isolates tested negative. The optimum adsorption time and temperature for subsequent phage infection and luminescence was determined to be 30 minutes at 42°C, producing detectable luminescence in less than one hour for *Salmonella* concentrations of 10^8 CFU/mL. Employing a 6 hour pre-incubation step, as few as 10 CFU/mL of *Salmonella* could be reliably detected, which was reported to agree well with previous estimates using a similar detection system. The method was then evaluated in a food system by directly inoculating *S. enteritidis* into the white of eggs followed by recombinant phage addition. After a 16 hour incubation period, luminescence was detected in eggs inoculated at an initial level of about 10^3 CFU, but lower concentrations of 100 and 10 CFU of *S. enteritidis* per egg went undetected. However, increasing the incubation period to 24 hours resulted in luminescence detection at all levels, with the opportunity to observe the internal location of luminescent

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organisms by CCD imaging of the whole egg. Additional protocols using both i) *E. coli* Petrifilm and X-ray autoradiography, and ii) liquid media and luminometry were evaluated and demonstrated to be useful alternatives to the original methodology, offering a less expensive means of light detection and a more portable assay system.

Similarly, Loessner et al.[83] reported the construction and use of a *lux*$^+$ reporter bacteriophage, based on the specificity of *Listeria* phage A511 for about 95% of *Listeria monocytogenes* isolates belonging to serogroups 1/2 and 4. A *V. harveyii luxAB* gene fusion was inserted just downstream of the strong promoter for the major capsid protein gene *cps*. Using the recombinant phage A511::*luxAB*, as few as 500 CFU/mL of tested isolates could be detected after phage infection and a two hour incubation at 20°C. To increase the sensitivity even further, *Listeria* selective enrichment broth was utilized to enrich artificially contaminated lettuce samples over a 16 hour period at 30°C before phage-based testing. The sensitivity of detection was subsequently increased to less than one cell of *L. monocytogenes* Scott A per gram of salad, with a total time investment of 22–24 hours. While the bacteriophage used was reported to be highly specific for certain *L. monocytogenes* serogroups, it has a very broad host range with demonstrable activity against *L. ivanovii* and *L. seeligeri* isolates. Therefore, this particular phage will only gain application as a generic detection method for *Listeria* spp., assuming that it is also capable of infecting other species within the genus such as *L. innocua*, which has been proposed as a useful indicator for the presence of *L. monocytogenes*.

The detection of *L. monocytogenes* Scott A in various artificially contaminated foods by the above method was further examined by Loessner's research group,[85] and a MPN-based method was also evaluated. The method was shown to reliably detect *L. monocytogenes* at a level of 100 CFU/g or less in all tested foods following a 20 hour selective incubation, with even lower detection limits for liverwurst, shrimp, pasteurized milk, cottage cheese, ricotta cheese, chocolate pudding, and cabbage. Detection of lower levels of target cells was improved for minced meat, liverwurst, shredded Swiss cheese, and cabbage samples following an increase in enrichment time to 44 hours, although detection sensitivity never improved beyond 10 CFU/g for minced meat and Camembert, likely due to interference by a large microbial background flora. Naturally bioluminescent bacteria were also responsible for non-specific light emission by a shrimp sample, but this was easily identified due to light emission by a negative sample not containing *lux* phage. Naturally contaminated food samples were also tested, with 57 determined to be *Listeria*-positive by selective plating, and 55 positive by the *lux* phage method. Differences were observed in the number of positive results each method identified, with the phage-based method identifying more positive dairy products and dairy plant environmental isolates, while the plating method identified 20% more positive poultry samples. Failure to isolate *Listeria* cells by plating was proposed to be related to sensitivity of some strains to selective agents used in Oxford agar, while failure to detect poultry isolates by phage-mediated luminescence was due to the fact that phage A511::*luxAB* was determined not to infect those isolates.

A modified *lux*-MPN method was developed and evaluated, using *Listeria* selective broth as the growth medium in a 3 × 3 tube method, with artificially contaminated samples of minced meat, shrimp, ricotta cheese, and cabbage.[85] Samples were removed from positive tubes after 20 and 44 hours of enrichment at 30°C, and tested using the *lux*$^+$ phage method. Numbers calculated from the luminescent MPN method agreed well with the levels of *L. monocytogenes* Scott A initially inoculated into food samples following both 20 and 44 hour enrichment, with only minced meat samples showing slightly higher counts after 44 hours, demonstrating the rapidity and reliability of such an assay. Furthermore, it was demonstrated to be easily modifiable to a microtitre plate format, enabling a large number of samples to be processed simultaneously.

10.7212 *luc*-based bioluminescence. The natural bioluminescence of the North American firefly *Photinus pyralis* has been similarly exploited in bacterial systems, following the successful cDNA-cloning and expression of the *luc* gene, encoding a single polypeptide, 62 kDa luciferase, in *E. coli*.[82] The production of light by this means relies on the interaction of ATP, present in all living cells as an energy transfer molecule, with substrate, heterocyclic carboxylic acid luciferin, in the presence of molecular oxygen and magnesium, and is catalysed by the firefly luciferase enzyme according to the following equations:

$$\text{Luciferase} + \text{luciferin} + \text{ATP} \rightarrow \text{luciferase–luciferyl–AMP} + \text{PPi} \quad \text{(Eq. 1)}$$

$$\text{Luciferase–luciferyl–AMP} + O_2 \rightarrow \text{luciferase} + \text{oxyluciferin} + \text{AMP} + CO_2 + \text{light} \quad \text{(Eq. 2)}$$

This reaction results in the highly efficient production of light, in the form of a flash, with an estimated quantum yield of 0.88, substantially more efficient than that generated by bacterial luciferase.[19] The luciferase of *P. pyralis* emits yellow-green light with peak emission at 560 nm, but other firefly and insect species (including click beetles) have been shown to emit different colors of light, ranging from green (547 nm) to red (604 nm).[102] This reporter system is particularly attractive for use with bacteria because they are not capable of natural *luc*-encoded luminescence, and therefore will not interfere with detection of *luc*-transformed bacteria. Any natural bioluminescence will be of *lux* origin, and can be differentiated from *luc* by their differing wavelengths of light emission. Given that only one gene is involved in encoding insect luciferases, they also offer the advantage over *lux* genes of being somewhat simpler to work with. *luc*-encoded bioluminescence can be measured by the same instrumentation used for *lux*$^+$ detection, or a customized apparatus such as the system reported by Gailey et al.,[43] who developed an imaging system based on a single-photon counting photomultiplier tube. This system is capable of real-time monitoring of luciferase gene expression in cancer cells but at a substantially reduced cost.

There have, however, been problems associated with the use of firefly luciferase relating to its pH and temperature stabilities, sensitivity to proteolysis, and the rapid decay of light output during the bioluminescence reaction. Given that different species of insects can produce different light emissions while sharing a common substrate, differences have been attributed to enzyme structure.[102] This, along with the recent publication of the structure of firefly luciferase, has been the impetus for genetic manipulation studies of the structure-function relationship in an attempt to create enzyme derivatives with improved light emission, and more applicable pH and thermal stabilities. For example, substitution of the amino acid serine at position 286 in the firefly luciferase sequence by either leucine, valine, glutamine, tyrosine, lysine or phenylalanine results in a shift in the luciferase emission spectrum producing red-orange light as opposed to the wild-type yellow-green.[5,87] Additionally, amino acid substitutions can decrease or improve thermal stability, as demonstrated by White et al.[159] who generated a series of mutant enzymes by replacing glutamate at position 354 with all other possible amino acid residues. With the exception of glycine, proline and aspartate, any

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amino acid replacing the glutamate residue produced an enzyme with enhanced thermostability as compared to the wild-type firefly luciferase. The flash kinetics of the reaction, caused by the interaction of luciferase and oxyluciferin, have been addressed to some degree by the use of chemicals such as coenzyme A, reported to prolong the half-life of light to ten minutes. Another system of reagents, known as LucLite™, has been developed and it has been estimated to improve the half-life of light output to over 5 hours, with the production of a glow-type light signal.[121] In addition, Thompson et al.[144] reported that a single serine to threonine substitution at position 198 resulted in an enzyme with a 150-fold increase in half-time for light emission decay, as well as a higher pH optimum.

The permutations are numerous, and offer an approach which will be able to produce enzymes with more desirable properties for a particular application, along with the simultaneous differentiation of bacteria based on the emission spectra of the *luc* genes used. The use of dual bio- and chemi-luminescence reporter genes for luciferase and β-galactosidase has also been reported,[91] and current advances in characterisation of luciferases from other species of insects[152,163] will no doubt advance the application of *luc*-based bioluminescence even further.

As with the *lux* genes, the firefly *luc* gene has been engineered into both plasmid and bacteriophage delivery vectors, facilitating gene cloning of many eukaryotic and prokaryotic cells, including *E. coli* O157:H7[61,106] and *Staphylococcus aureus*.[134]

10.7213 Ice nucleation activity. Ice nucleation, i.e., formation of ice nuclei in pure water supercooled below its freezing point, by bacteria was first reported in 1973 by Schnell and Vali, and this frost damage phenomenon was subsequently demonstrated to be caused by the plant pathogen *Pseudomonas syringae*. Since then, a number of strains of *P. syringae* as well as strains of *Xanthomonas campestris*, *Erwinia ananas*, *E. herbicola*, *P. fluorescens* and *P. viridiflava* have been identified as ice nucleation active (INA) bacteria.[89] Bacterial ice nucleation has been shown to occur at threshold temperatures ranging from –2 to –10°C, higher than those associated with the ice nucleation activity of lichens, freeze-tolerant amphibians and insects[156] and these threshold values have been used to further classify bacterial ice nuclei into three groups.[128]

Although it is not known what advantage this phenotype conveys to INA gram-negative bacteria, a single chromosomally-encoded gene has been determined to be responsible for ice nucleation activity, and a variety have already been sequenced, including *inaA* (*E. ananas*), *iceE* (*E. herbicola*), *inaLI* (*E. uredovora*), *inaW* (*P. fluorescens*), *inaX* (*X. campestris* pathovar *translucens*) and *inaZ* (*P. syringae*), ranging in size from 3.4kb to 7.5kb.[77] Recently, a new ice nucleation gene, *inaV*, of about 3.5kb in size was isolated from *P. syringae*.[128] This gene encodes a 1200 amino acid residue protein, InaV, and shares 92.4% homology with *inaZ*. Based on 77% sequence conservation of their protein products,[128] it is thought that these individual *ina* genes from different bacteria evolved from a common gene. Also, their action seems to be host independent in nature, since antibodies raised against the inaW protein of *P. fluorescens* will recognize and react with the inaZ protein of *P. syringae* expressed in *E. coli*.[89] Ina proteins are located in the outer membrane of bacterial cells, and have a functional requirement for lipids.[50] It has been proposed that ina proteins act as templates for ice formation, and that differing aggregation of these proteins dictates variations in threshold temperatures.[156]

Ice nucleation genes cloned into *Escherichia coli* have been shown to convey ice nucleating properties to their host,[105] and this has led to the application of bacterial ice nucleation in a variety of ways, ranging from freezing processes in the food industry,[156] artificial snow production,[50] a reporter for gene expression in moderately halophilic bacteria[6] and promoter analysis in *Zymomonas mobilis*,[34] to a reporter for the detection of bacteria such as *Salmonella* spp. in food.[160] It has been estimated that the use of *ina* gene fusions for the detection of gene expression is 100- to 1000-fold more sensitive than the *lux* detection system.[50] With a low number of proteins (300 or less) required for ice nucleation to occur, such activity could be a useful proposition for the detection of injured cells still capable of minimal cellular activity.

By cloning an ice nucleation gene into a delivery vector such as a bacteriophage, which cannot express the INA phenotype on its own, this phage can be used to transduce susceptible cells. An INA phenotype can thus be passed on to infected cells, with ice nuclei synthesized within 15 minutes of *ina* gene transcription. This principle was employed to develop a sensitive and rapid method for the detection of *Salmonella* spp., known as the BIND™ (Bacterial Ice Nucleation Diagnostic) assay, marketed by IDEXX Laboratories, Inc., located in Westbrook, Maine, USA. This test employs a proprietary phage mixture, engineered to contain *ina* genes, to detect ice nucleation in susceptible *Salmonella* cells. Phage and samples being tested for the presence of *Salmonella* are mixed in the presence of a freezing-indicator dye, carboxyfluorescein, and incubated at 37°C to allow phage attachment and transduction of susceptible bacteria. Samples are then incubated at 23°C to optimize expression of the ice nucleation protein and formation of ice nuclei. Ice nucleation activity is subsequently detected by chilling treated samples and controls to a temperature of –9.5°C. Freezing of cells is indicated by an orange, non-fluorescent appearance, while negative samples appear fluorescent green. The level of bacterial contamination can be calculated, based on the concentration of ice nuclei determined by the droplet freezing assay.[160]

The performance of the BIND™ prototype assay in foods such as milk, meat, gelatin and eggs was most encouraging, with little or no interference from the food samples themselves or from background ice nuclei. In an optimized prototype assay using an *ina*-transduced phage constructed from P22 (with a similar host specificity to the parent phage), a sensitivity of 10 cells per mL or less was obtained in test samples containing either *S. enteritidis*, *S. typhimurium*, *S. dublin*, *S. paratyphi* B, or *S. gallinarium*. A similar detection limit was achieved for *S. Dublin* in the presence of high numbers of non-*Salmonella* bacteria. The test is also capable of detecting sublethally injured cells which are still able to produce ice nuclei. Any enrichment step can thus be relatively short, enhancing the rapidity of the method, which, at around two hours for a positive result is a considerable time saver as compared to other detection methods for *Salmonella* spp.[160]

Information regarding the BIND™ assay and the agreement of assay-positive isolates with identification by the API 20E biochemical system was presented in 1995 by Wolber at the 95th general meeting of the ASM.[161] Ten food matrices were spiked at low (20 to 50 cells/25g) and high (10^2 to 10^3 cells/g) levels with 20 of the commonest *Salmonella* serotypes isolated in the U.S., and after a non-selective pre-enrichment using buffered peptone water, samples were tested for ice nucleation ability. Positive isolates were then identified by the API 20E system, and agreed 100% with the results obtained by the BIND™ assay.

More recent information from IDEXX regarding the BIND™ assay claims an assay sensitivity of one viable *Salmonella* cell per 25g of sample, following a single overnight enrichment step. Handling time for the assay is estimated at less than one minute

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per sample, and including enrichment, results are obtained within 22 hours. The test can now detect 500 different *Salmonella* spp. without producing false-positive results.[4]

10.7214 Green Fluorescent Protein. Another reporter system of tracking gene expression and protein localization in a variety of organisms and cells is the green fluorescent protein (GFP), naturally occurring in the bioluminescent jellyfish *Aequorea victoria*. GFP is activated in *A. victoria* by the absorption of blue chemiluminescence generated from the calcium binding activation of photoprotein aequorin, and produces a stable, strong green fluorescence.[25] It has been proposed that GFP evolved as a means of amplifying the relatively low levels of chemiluminescence produced by photoproteins into higher intensity fluorescence with no extra energy expenditure on the part of the cell.[31] The 27-kDa GFP, encoded by the *gfp* gene, is 238 amino acid residues in size and absorbs light at 395nm (with a smaller peak at 475nm). The formation of GFP is favored by lower temperatures (~22°C) and takes about four hours in *E. coli*.[58] Once GFP is formed, it is stable at temperatures up to 65°C, at alkaline pH, and is resistant to proteases. Its crystal structure has been determined to consist of 11 beta-sheets forming a barrel-like arrangement around a central alpha-helix containing the fluorescence center.[99] Its activity requires the presence of oxygen, but does not need additional genes, co-factors or substrates for fluorescence to occur. This allows for the detection of sub-lethally injured cells, making it an attractive alternative to the use of substrate-dependent marker systems such as *lux* and *luc*.

Research interest in GFP soared following the demonstration by Chalfie et al.[25] that the *gfp* gene engineered into a plasmid vector could produce fluorescence in both *E. coli* and eukaryotic cells when the transformed cells were excited by near-U.V. or blue light. Additionally, the *gfp* gene can be fused to genes of interest in living cells without interfering with cell function and growth, although it has been suggested that the functionality of some fusion proteins may be affected by the size of the GFP tag.[99] This makes it a potentially useful marker system in the study of, for example, bacterial biofilm formation. Such breakthroughs, together with the availability of fluorescence detection methods such as flow cytometry, epifluorescent microscopy, spectrofluorimetry, and confocal laser microscopy, have led to the application of GFP technology in a number of research areas.

In a special issue of the journal *Gene*, dedicated to GFP, some bacterial applications were reported. For example, host-pathogen interactions following infection of live mammalian cells with either *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, or *Mycobacterium marinum*, were monitored by bacterial expression of the GFP phenotype.[150] Bacterial movement through environmental materials such as quartz sand could be tracked by fluorescence[21] and plasmid transfer was studied using GFP as a marker of genetic transfer between bacterial cells.[28] Recent publications have focused on GFP as a successful marker system for evaluating movement[145] and survival of genetically engineered microorganisms in the environment,[74] the survival of *E.coli* O157:H7 in orange juice and apple cider,[41] and detection of individual *P. aeruginosa* cells in a mixed biofilm population.[15]

Despite the obvious attractiveness of this marker system, it has yet to be reported as a bacteriophage-based detection method. Promising results have been published reporting the use of virus-based *gfp* vectors such as potato virus X,[16] tobacco mosaic virus,[24] and retroviruses,[70] and it is anticipated that bacteriophage-mediated delivery of GFP will be available in the near future. Inevitably, problems associated with this new technology have arisen, which have in part already been addressed by researchers. One of the most crucial aspects is the fluorescence intensity of the protein, and efforts have been made to improve fluorescence by single point mutations targeting primarily the serine65-tyrosine66-glycine67 chromophore. Replacement of Ser65 with alanine, valine, leucine, cysteine, and threonine residues resulted in loss of the 395nm excitation emission peak, and moved the more photostable 475nm peak slightly towards a longer wavelength, producing spectra similar to that of the sea pansy *Renilla* GFP. When Ser65 was replaced by threonine, a six-fold increase in brightness of GFP was observed as compared to wild-type GFP, with decreased photobleaching.[58] Cormack et al.[30] reported the generation of GFP variants, by random amino acid substitution, which when expressed in *E. coli*, showed a 100-fold increase in fluorescence intensity, attributed to shifts in excitation maxima and more efficient protein folding than that of wild-type GFP.

The color emission of GFP has also been studied, and it was demonstrated that replacement of Tyr66 and Tyr145 with histidine and phenylalanine, respectively, produced isoforms emitting blue instead of green light.[54,99] A red-shifted variant, RSGFP4, has also been created by mutagenesis,[162] and similarly, photoactivation of GFP in low oxygen environments produced red instead of green fluorescence.[37] This provides exciting advantages to the use of GFP and its variants in simultaneous detection of cells expressing different fluorescence emissions as demonstrated by Yang et al.,[162] who used dual colour microscopy to visualize cells expressing red and green fluorescence. While the use of GFP has been praised due to the absence of interfering background fluorescence, care should be taken in the application of its variants, as reported by Lewis and Errington.[76] These researchers found that a blue *gfp* variant was not useful in the study of sporulation of *Bacillus subtilis*, due to the innate fluorescence of the spore-former in the blue portion of the spectrum.

Little information is currently available regarding the detection limits of GFP. While it has been estimated as being 10^5 bacterial cells using a derivative Tn5GFP1 transposon vector[21] and as low as 1 CFU/mL using a commercial cDNA vector,[61] the use of a strong promoter and modified *gfp* genes[140] should improve sensitivity greatly.

10.722 Phage-Based ATP Bioluminescence

The use of ATP (adenosine-5′-triphosphate) bioluminescence as a rapid method for the detection of bacterial biomass and its applications in food microbiology, hygiene monitoring and HACCP programs have been extensively reviewed.[46,47,52,148] The ATP bioluminescence reaction has already been discussed with regard to *luc*-encoded bioluminescence and readers are directed to this section for details of the reaction. Protocols for the analysis of food samples typically include primary chemical treatments, filtration and washing steps to remove background and/or non-microbial ATP. Bacterial cells are concentrated on the filter and are then chemically lysed to release microbial ATP which can be quantified by the addition of the luciferin-luciferase complex. This assay can be performed either in liquid form using a luminometer, or by direct application of ATP extractants and luminescent reagents onto filters, followed by image processing.[142] Light output, quantified in terms of relative light units, can thus be used to estimate biomass[48] based on the generally accepted fact that the ATP content of each bacterial cell is reasonably constant. Alternatively, in hygiene monitoring, both microbial ATP and ATP present in food debris are of relevance and thus a total ATP estimate is desirable to evaluate hygiene levels relating to microbial contamination and overall cleanliness.[129]

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While ATP bioluminescence has been widely accepted as a rapid and convenient method for the analysis of overall bacterial load, the lack of specificity of the procedure is a major drawback in its application to many areas of food microbiology. Chemical extraction of microbial ATP is inherently non-specific, and while measurement of total ATP levels has been shown to be sufficient for the purposes of quality assessment of food products and factory hygiene,[9] the detection of specific groups of microorganisms in mixed cultures, for example, foodborne pathogens, starter cultures, and spoilage bacteria, has been impossible to achieve. Two approaches have been studied in an attempt to improve the specificity of the ATP bioluminescence reaction, both of which exploit the specificity of lytic bacteriophages. These breakthroughs could make ATP bioluminescence a more complete tool for the microbiological analysis of foods.

10.7221 Lytic phages. The first approach has focused on the replacement of chemical extractants with bacteriophages capable of specifically lysing groups of microorganisms. Very simply, the lysis of bacterial cells by phage will result in release of intracellular ATP which can be detected by bioluminescence. A sample containing added bacteriophage and bacterial cells susceptible to phage infection will show an increased amount of ATP in the system, as compared to a control sample containing no phage. This approach was first published in 1995 by Sanders,[125] who reported the use of a bacteriophage for the specific detection of *Listeria monocytogenes* even in the presence of the closely related species *Listeria innocua*. In a broth system, the presence of *L. monocytogenes* ATCC 23074 was confirmed within 80 minutes of addition of bacteriophage ATCC 23074-B1, when the light output was at its maximum. This method was also capable of detecting 10^5 CFU/g of *L. monocytogenes* in artificially contaminated lettuce within 15 hours, incorporating a selective enrichment step.

Despite the specificity of the assay, the lack of sensitivity has become an overriding concern. The bioluminescence assay itself has a detection limit of around 10^3–10^4 bacterial cells, and is unreliable for cell numbers below this level. For the phage assay, even in pure culture, at least 10^5–10^6 cells are required to produce a detectable increase in ATP as compared with a control in which no phage lysis has occurred. For optimum results an even higher cell count is required. As an alternative to pre-enrichment techniques, suggestions to improve sensitivity have focused on i) the use of adenylate kinase (AK) and excess amounts of ADP as a means of driving the equilibrium of AMP, ADP and ATP towards the anabolism of ATP,[135] and ii) recycling of ATP by the use of specific substrate-enzyme mixtures to effectively increase the net production of ATP.[53] The AK assay, employing lytic phage to release intracellular AK, currently has a detection limit of less than 10^4 cells/mL for both *E. coli* and *S. newport*. Results were achievable in less than one hour for *E. coli* while the *Salmonella* assay took up to two hours.[13] The ATP recycling method was demonstrated to improve the sensitivity of the standard ATP assay from 550nM to 250pM ATP.[53] However, this was achieved using pure ATP standards and not by ATP extraction from microorganisms, as the publication title suggests.

10.7222 Bacteriolysins. A second approach to improving the ATP bioluminescence assay has also exploited the activity of lytic bacteriophages, more specifically the endolysins they produced as late gene products during phage replication. Endolysins have been demonstrated to facilitate the release of progeny phage from the bacterial cell; this is known as 'lysis from within'.[143] These lysins fall into one of at least four categories of enzymes: lysozymes (as is the case with phage T4), transglycosylases, endopeptidases, and amidases. The enzymes gain access to the peptidoglycan layer by the formation of non-specific membrane lesions or holes, produced by a group of membrane proteins known as holins.[165]

The genes responsible for endolysin production have been isolated and cloned for a number of phages,[164] and subsequent information gained from characterization of these lytic enzymes has led the way towards more applied uses for phage endolysins. More recently, Loessner et al.[81] reported the use of a recombinant phage L-alanoyl-D-glutamate peptidase endolysin ply118 of *L. monocytogenes*, overexpressed in *E. coli* to very high levels, as an efficient means of gently releasing DNA, RNA and proteins from *Listeria* cells. This procedure was subsequently modified to incorporate a 12 amino acid purification tag without adversely affecting the lytic activity of the protein, and enabled the production of lysins purified to more than 90% homogeneity.[84] This endolysin was shown to be specific for *Listeria* cells, but did not lyse other gram-positive and gram-negative bacteria. It thus provides an efficient and rapid means of lysing specific cells. Analysis of gene sequences upstream of endolysin genes *ply*118 and *ply*500 revealed highly conserved holin (*hol*) genes, which the endolysins require for transportation across the cell membrane.[82]

The continued characterization of phage lytic enzymes[58,86,130] will greatly enhance our knowledge of the molecular action of such enzymes, but as well, will increase their applicability in other areas of microbiology. For example, such recombinant lysins are currently being evaluated as ATP extractants for use in bioluminescence assays,[84] in which intact target cells could be selectively lysed and rapidly detected by firefly luciferase. In addition, they offer the potential of being used as antimicrobial agents to eliminate pathogens in the food industry and in medical applications.

10.723 Phage-Based Fluorescence

10.7231 Labeling of intracellular phage nucleic acid. Fluorescent stains have been reported as a useful accessory to the use of phage probes, as a means of labeling phage nucleic acid within infected cells. Sanders et al.[126] reported the use of two fluorescent dyes, chromomycin A3 (CA3) and Hoechst 33258 (H33258), to bind and label respectively G-C- and A-T-rich regions of phage T4 DNA present inside infected *E. coli* cells. Following infection of *E. coli* ATCC 11303 with phage T4, samples were placed on ice to interrupt viral replication, washed and ethanol-fixed in preparation for fluorescence staining. Cells were then analyzed by dual-laser flow cytometry, and within 35 minutes of phage infection, T4-infected cells could be differentiated from uninfected cells based on fluorescent detection of phage DNA. While this technique was developed to study viral DNA within the infected bacterial host, it has also provided an excellent means of fluorescent labeling of bacterial cells infected by phage. Thus it could be used in conjunction with phages of known host specificity as a detection method for particular groups of microorganisms.

10.7232 Labeling of virus-packaged nucleic acid. Fluorescent labeling of nucleic acid packaged within the virus head has also been used to directly count viruses in samples from freshwater and marine environments using epifluorescence microscopy.[56] This labeling technique was employed to create differential phage probes using fluorescent stains with different excitation wavelengths for the identification and enumeration of specific groups of marine bacteria.[57] More recently, labeling of phage DNA has been adapted to enable fluorescent detection of *E. coli* O157:H7 in ground beef, in conjunction with immunomagnetic separation and flow cytometry.[44] Prior to sampling, phage DNA was fluor-

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escently labeled with the benzoxazolium-4-quinolinium fluorescent dye YOYO. Ground beef and milk samples were artificially inoculated with low levels of *E. coli* O157:H7 and pre-enriched in non-selective media for 6 hours. Target bacteria were subsequently removed from the food matrices by immunomagnetic separation with *E. coli* O157:H7 Dynabeads, and the fluorescent phage preparation was then used to label the recovered cells. Using both confocal laser scanning and epifluorescent microscopy techniques, in addition to a more automated flow cytometric method, target *E. coli* O157:H7 cells were counted based on their fluorescence. The detection limits achieved were approximately 2.2 CFU/g in ground beef and 10^5 CFU/mL in milk. The potential application of this method for detection of other food-borne pathogens is enormous, given the large number of fluorescent stains commercially available for phage labeling and the adaptability of magnetic separation techniques for the detection of a wide variety of bacteria.[104]

10.724 Metabolic Inhibition Assays

A number of methodologies, including electrical impedance, turbidimetry and colorimetry, that rely on metabolic activity and microbial growth as a means of investigating microbial populations are available. Similar to cultural methods, total counts of microorganisms can be determined by using non-selective media, while specific detection of microorganisms has involved the development and use of suitable differential or selective culture media. By incorporation of pH indicators, fermentable carbohydrates, organic acids and alcohols, or other inhibitors such as antibiotics, preferential growth conditions for the microorganism of interest are provided at the expense of the background microbial flora. Such technologies have found many applications in food microbiology, particularly with advancement in the automation of such systems.[151] They are particularly promising for the study and application of bacteriophages, where metabolically active bacterial cells are required for viral replication.[159]

10.7241 Electrical impedance. Of the metabolic methods to be considered in this section, electrical impedance, though more complicated in theory, has been the most widely studied and developed in terms of its microbiological applications. Detection by impedance is based on changes to the growth medium associated with the increase in biomass of metabolically active microorganisms. The components of growth media, such as proteins, carbohydrates and lipids, are uncharged or weakly charged substrates, but as microorganisms metabolize these compounds, they are converted into more highly charged molecules such as amino acids, lactate, and acetate. These metabolic products increase the conductivity of the medium. However, this increase is small, therefore requiring a microbial level of at least 10^6 cells/mL to be reached before significant changes can be detected. The period between initiation of the test and detection is known as the detection time (D.T.), the value of which is inversely proportional to the number of cells initially present in the test sample.

A large number of reports regarding the electrical detection of specific microorganisms have been published, particularly since the introduction of automated systems such as the Vitek Bactometer® Microbial Monitoring System and the Malthus Microbial Growth Analyzer. There are a number of differences between the two systems, but the most pertinent is their electrical signal measurement. The Malthus Analyzer measures only conductance, while the Vitek system offers more flexibility by measuring either conductance, capacitance, or total impedance. Applications utilizing these systems have focused primarily on microbial enumeration, detection of contaminants[66,72] and pathogens, particularly Enterobacteriaceae[11,29,42,108,111] and *Listeria* spp.,[51,110,120] shelf-life studies,[12,108] sterility testing of UHT products,[141] and detection of bacteriophages implicated in cheese starter culture failures.[23,153]

Despite its many uses, the direct impedance technique has disadvantages, the most crucial being that it does not operate under high salt concentrations found in some growth media, and this has prompted the development of alternative methods. An indirect approach, where metabolic production of CO_2 is considered as an indicator of microbial growth rather than biomass, prompted the development of the RABIT (Section 10.6) automated bacterial impedance technique.

10.7242 Turbidimetry. Turbidimetry is a relatively simple means of estimating bacterial generation times. The use of turbidity measurements takes advantage of the increase in optical density of sterile microbiological growth media associated with growth of inoculated bacteria. Although this method is used to estimate the rate of growth of microorganisms, generated optical density data can also be used to estimate cell populations when compared to total plate counts. The automation of optical density readers has been particularly useful in increasing the number of samples capable of being analyzed at one time, and computer interfacing has provided an easier means of graphing and analyzing generated growth curves. One particular automated system, the Bioscreen C microbiology plate reader (Labsystems, Helsinki, Finland), has provided food microbiologists with a useful tool for the evaluation of turbidity as a means of estimating bacterial levels in food samples. Capable of incubating up to 200 samples at various temperatures, the Bioscreen C can be programmed to read the optical density of each sample well a particular number of times over a defined incubation period. In the case of food samples, dilutions are often required to reduce the background turbidity to a minimum, and the resulting growth curves can be analyzed using a number of parameters appropriate to the application. These include the time taken for optical density to increase by a defined amount, maximum optical density, slope of the logarithmic growth phase, or turning point time (the middle of the logarithmic phase).

The Bioscreen C has been used by researchers to, for example, predict colony forming units in raw milk[93] and other foods,[92] and assess the effects of antimicrobials such as bacteriocins[131] on bacterial growth. In addition it has proved very useful in the development of data for predictive modeling,[94] and most recently has been used to investigate recovery of single heat-injured *Salmonella* cells by measuring the lag phase of cells pre-enriched in non-selective media.[135] To date bacteriophage-based turbidimetric methods for the detection of foodborne pathogens have not been reported.

10.7243 Colorimetry. The suitability of color changes to indicate the status of biochemical reactions is evident by their extensive application in numerous methodologies used in microbiology, such as differential and selective culture media, biochemical tests for identification of isolates, and enzyme-linked immunosorbent assays (ELISA). This approach has also been used in the development of media for both electrical impedance, where the incorporation of a color change is typically used in conjunction with detection times and curve characteristics to confirm a particular microorganism, and colorimetric assays, where the color of growth media containing pH indicators is monitored over time for changes corresponding to, for example, fermentation of carbohydrates. This has been applied particularly in dairy microbi-

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ology where colorimetric tests have been successfully developed to evaluate the presence of antibiotics, coliforms, pathogens or psychrotrophs, enzyme activity, stale flavor development, lactic culture activity, and shelf life of dairy products. Instrumentation includes the the Omnispec™ 4000 bioactivity monitor[117] and the versatile LabSMART™ color scanner and software package.[116] Both instruments operate on the basis of reflectance color readings, which eliminates any problems associated with background turbidity of food samples.[118] Phage-based colorimetric assays have not been reported to date.

10.7244 Phage-based applications. What these methods have in common (regardless of their mode of detection), and what has primarily limited their application in pathogen detection in foods, is their requirement for differential or selective media. The use of such media can increase testing time considerably, and does not always allow for the detection of stressed and injured bacterial cells. Generally, they do not differentiate to the species level, and can also be affected by the growth of contaminating microorganisms associated with the background microflora of foods, resulting in false-positive detections. One approach to these problems is the use of bacteriophages, specifically their effect on the metabolic activity of their hosts, which can offer an alternative means of detecting specific microorganisms in the presence of the background flora of foods. Based on the target population's inability to replicate (in the presence of specific phage) to similar cell concentrations achieved by a negative control not exposed to phage, positive detection of pathogens can be confirmed. If target cells are present, they will be infected and lysed by the phage, resulting in delayed detection in samples with added phage. Control samples without phage will have normal metabolism and growth, resulting in a shorter detection time.

This approach was evaluated by Pugh and Arnott[113] in their development of impedimetric media for the detection of *Salmonella* spp. in confectionery products. A combination of two impedimetric media, selenite-cystine-trimethylamine oxide-dulcitol medium (SC/T/D) and modified lysine decarboxylase broth (MLD), was used in an attempt to take into account those *Salmonella* strains not capable of fermenting dulcitol, but able to decarboxylate lysine. In addition, phage Felix O-1, estimated to be lytic for 96.1% of tested *Salmonella* strains, and phage G47, included to compensate for the tendency of Felix O-1 not to infect O groups E1 to E4, were employed to confirm the positive genus identification made by impedance microbiology. Of 81 *Salmonella* strains tested in the Bactometer system by both media and the phage test, all strains except one strain of *S.* Houten, were positively identified by at least two of the three protocols, while false-positive reactions by *Citrobacter freundii, E. coli, Proteus vulgaris,* and *Pseudomonas aeruginosa* were obtained in either SC/T/D or MLD, but never both broths. The non-*Salmonella* strains were all negative according to the phage test. A pre-enrichment step using brilliant green-milk broth was incorporated into the assay when artificially inoculated samples were analysed. In all cases but one, where *Salmonella* isolates were identified by conventional selective enrichment and plating, positive identifications were made by at least two of the three test protocols. The one exception was an isolate of *S. typhimurium* which was positively identified by the phage test but neither by the conventional nor the Bactometer methods. Despite the apparent usefulness of this phage-based impedimetric method, confirmation of presumptive positives made the method more time consuming, and no further application of bacteriophages to impedance has been reported.

Recently, these metabolic methods were reevaluated by McIntyre and Griffiths[95] in an effort to i) develop specific pathogen detection methods using bacteriophages specific for *Listeria* spp., *E. coli,* and *Salmonella* spp., ii) to demonstrate the ability of particular phages to differentiate particular isolates, and iii) to investigate whether decreased selectivity of media could be counter-balanced by the incorporation of specific bacteriophages for the bacteria of interest. This attempted to reduce the amount of time required for testing, and take into account the deleterious effect of selective media on stressed or injured cells, while the phage test would indicate if target cells were present.

Impedimetric and colorimetric methods were successfully developed for the detection of bovine isolate *E. coli* G2-2 in raw milk and ground beef using Vitek Coliform Medium (CM), a lactose-containing medium designed to change color from purple to yellow in the presence of lactose-fermenting coliforms, and phage AT20, demonstrated to be lytic for isolate G2-2. Less than 10 CFU of *E.coli* per mL of raw milk could be detected by both phage-based methods within 10 hours, while a detection limit of less than 1000 CFU/g of ground beef was achieved using a test-tube based phage colorimetric assay in 10.25 hours. Using phage AT20, specific for non-shiga toxin (stx) *E. coli* but not stx-positive isolates, the presence of non-stx-producing *E. coli* G2-2 in raw milk and ground beef was subsequently confirmed by an increase in the time required to reach the detection threshold in the presence of phage as compared to identical samples without added phage. Equivalent impedimetric detection limits were achieved for skim milk powders artificially contaminated with various *Salmonella* spp. using phage Felix 0-1 and CM (able to support the growth of *Salmonella* but with no lactose fermentation), but the specificity of the assay was greatly enhanced by the use of a second bacteriophage, SJ2, with a host range narrow enough to positively detect only *S. typhimurium* and *S. enteritidis.*

The effect of using less selective media for the impedimetric detection of *L. monocytogenes* ATCC 23074 in combination with specific phage ATCC 23074-B1 gave rather interesting results. In pure culture using both Listeria selective impedimetric broth and less selective Demi-Fraser broth, large differences in detection times for *L. monocytogenes* were observed in the presence and absence of phage, with, as expected, slower detection occurring in the presence of phage. In addition, detection was substantially faster in the less selective medium. However, when raw milk was artificially contaminated with *L. monocytogenes* and tested in a similar fashion, the reverse result was obtained in Demi-Fraser broth where slightly faster detection times were achieved in the presence of phage. It is likely, in this case, that the lower selectivity of the medium plus the lowering of the numbers of *Listeria* present due to the lytic action of the phage actually allowed faster growing members of the background microflora to dominate. These microorganisms were thus able to reach a detectable level faster than was the case in the absence of phage, where original *Listeria* levels were competing with the raw milk microflora. Interpretation of results by this method should thus be considered carefully, depending on the selective nature of the medium, the food material being tested, and the growth rates of the microorganisms in question. Phage-based turbidimetric detection of pathogens has also been demonstrated using similar media and phage combinations, but the need to dilute food samples at least 100–1000 times detracts from the sensitivity and overall usefulness of this type of assay. However, the Bioscreen C has been used effectively in pure culture work to assess the susceptibility of

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bacterial strains to lytic phages, and has been useful in establishing optimum phage concentrations for use in other assays.

10.725 Miscellaneous Phage Probe Applications

10.7251 High-performance liquid chromatography. A high-performance liquid chromatography (HPLC) method for the detection of *Salmonella* spp. utilising the Felix O-1 phage was reported in 1983.[61] Using an E-1000 m-Bondagel gel permeation column, it was demonstrated that HPLC could be used to distinguish and detect *Salmonella* spp. based on the detectable increase in bacteriophage titres associated with phage replication and cell lysis. Susceptible host cells and Felix O-1 phage were mixed and allowed to interact for two hours during which replication of phages would occur. The aqueous phase was removed, treated with chloroform, and centrifuged to remove cell debris. This bacteriophage supernatant was then injected into the HPLC column and the resulting chromatograph was analysed for a specific peak corresponding to the retention of bacteriophage previously determined to occur 3.2 minutes after injection. It was determined from the results obtained that a minimum bacteriophage concentration of approximately 10^9 PFU/mL was required to give a reliable detection (response of at least 10mm over background), corresponding to around $3x10^6$ bacteria per mL required in the reaction mixture. This peak occurred only when *Salmonella* spp. were present in the reaction mixture, and a positive detection was demonstrated even when non-*Salmonella* spp. were also present. The high levels of bacteria required for detection was a definite shortcoming of this method, and was subsequently addressed by the use of filtration and enrichment in follow-up research outlined below.

These modifications were incorporated in order to detect *Salmonella* spp. in milk using this HPLC method.[62] Large pore electropositive filters were employed to remove bacteria from artificially-inoculated milk samples, and subsequently treated with alkaline broth media to elute the trapped microorganisms from the filter. This method was estimated to recover about 40% of the bacteria originally added to the milk, and subsequent overnight enrichment in the presence of brilliant green dye was adequate to ensure that 75-100% of the total bacterial population consisted of salmonellae. Following enrichment, the cells were centrifuged and washed, resuspended in $CaCl_2$-supplemented nutrient broth, and bacteriophage was added to defined cell densities. Following preparation of bacteriophage supernatants (as previously described), 25 mL volumes were injected into the HPLC column, and the presence of a 3.2 minute bacteriophage peak was indicative of the presence of *Salmonella* in the original sample. Less than five salmonellae per mL of milk were detectable by this method, resulting in a positive detection result within 24 hours of sampling. However, as previously reported,[61] problems arose when certain *Salmonella* serotypes were not infected by this phage. The false-negative results were explained by the potential presence of either prophages, known to limit or inhibit replication of Felix O-1, or bacteriophage-restricting plasmids. Despite the improved detection limit achieved in this research, the use of this assay has not been more recently reported.

10.7252 Immobilized-phage capture assay. More recently, the 'Sapphire' lytic phage (Amersham International) has been evaluated in a novel immobilized capture method for the separation and concentration of *Salmonella* strains by Bennett et al.[10] Using both a microtitre plate and dipstick method, phage particles were allowed to adhere to the solid phase, washed several times to remove unbound virus, and treated with bovine serum albumin to minimize non-specific binding of cells. The phage-coated solid phase was then exposed to several *Salmonella* serotypes including *S. typhimurium* and *S. enteritidis*, and non-salmonellae including *E. coli*, *Proteus mirabilis*, and *Hafnia alvei* in liquid suspensions. Using the polymerase chain reaction (PCR), the method was evaluated for i) its ability to remove cells from the suspensions and ii) specifically distinguish and remove *Salmonella* cells from mixed cultures of Enterobacteriaceae. A commercially available *Salmonella*-specific BAX PCR system was chosen, but used in a procedure slightly different from that suggested by the manufacturer, previously demonstrated to significantly increase the sensitivity of the system. Data obtained showed that capture of *Salmonella* spp. was possible using both solid phases mentioned above, but, based on the detection limits of the PCR assay, high numbers of cells (around 10^7 CFU/mL) were required to subsequently obtain a positive end result. Specific capture of *S. typhimurium* in the presence of equal numbers of competing Enterobacteriaceae using the microtitre plate test format was also a viable option, but again, high numbers of cells were required for a positive result. While these results were encouraging, a number of problems were encountered in this assay. Most importantly, *S. ealing* and *S. arizonae* were not detected by this capture method, and the 'Sapphire' phage was subsequently shown not to infect these particular organisms. Second, phage capture was very inefficient and it is likely that some phage particles were binding to the solid phase via the tail rather than the head, thus rendering them useless in the cell capture process. The small surface area of the microtitre plates may also have contributed to the poor capture rate. In addition, unusually high cell counts obtained using control (uncoated) dipsticks demonstrated that phage or cell clumping may have arisen as a result of uneven surface chemistry. A solution to the surface area issue is the use of magnetic spheres as a solid phase, but further work in this area was not reported by these or other authors.

10.7253 Phage-linked immunosorbant assays. The use of phages as reporter molecules has also been extended to the development of phage-linked immunosorbant assays (PHALISA), similar to the ELISA method, where the amount of phage bound by immobilised antigen-antibody complexes can be related back to the number of target cells present by its subsequent infection of susceptible bacterial cells. Block et al.[14] employed bacteriophage M13, engineered to contain the *E. coli lacZ* gene, as a 'bio-amplifiable tag' for the detection of herpes simplex virus (HSV). HSV-infected cells were incubated with HSV-specific monoclonal antibodies raised in mice, followed by rabbit anti-mouse serum and mouse anti-M13 serum. The resulting complexes were incubated with phage M13. The bound phage was eluted and then quantified by plaque assay and acquired beta-galactosidase activity in phage-infected *E. coli* cells. This PHALISA assay was more sensitive than an ELISA method under similar test conditions and demonstrated the potential for detecting bacterial and viral antigens in foods.

10.7254 Phage amplification assays. A similar role for phage has been exploited in the development of methods to detect *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*.[136] Known as the 'phage amplification assay', it exploits the lytic cycle of bacteriophage to indicate the presence of target bacterial cells. Following infection of cells, samples are first treated with a special virucidal reagent to destroy external virus particles not already involved in the infection process, and then the reagent is neutralized to prevent later destruction of phage progeny. If susceptible target cells are not present in the sample, then all bacteriophage will be destroyed. Otherwise, the bacteriophage replicates

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normally within the host cell and eventually lyses the cell wall, releasing its progeny into the external medium. Resulting virus particles can then be amplified further using a non-pathogenic host and detected by plaque assay. This method has been reported to have a detection limit of about 100 mycobacteria per mL of sample, detectable within 10 hours, and offers the advantages of destroying virulent *M. tuberculosis* cells during testing if they are indeed present in the clinical sample, simplicity of use with no expensive instrumentation required, and a less expensive application of phages as opposed to more costly cloning techniques mentioned previously. As with other phage-based detection methods, resistance to a particular bacteriophage is the most likely problem associated with this approach. However the use of phage cocktails could be a means of overcoming this shortcoming.

10.73 Conclusions

Bacteriophages are versatile biological particles, capable of identifying host bacteria to levels well beyond the capabilities of many conventional microbiological methods. Their demonstrated suitability for bacterial typing and cloning has resulted in their successful application in a variety of molecular-based and/or 'rapid' methodologies, particularly for the detection of pathogenic bacteria. Regardless, the limiting factor in the advancement and application of phage-based detection systems will always be the bacteriophages themselves. For example, the generic detection of *Salmonella* spp. using the broad-spectrum phage Felix O-1 has been extensively documented, but it is not a viable option for more specific detection to the serovar level. Continuing phage isolation and characterisation still remains the best way of identifying potentially useful bacterial viruses, and this avenue, in combination with more novel molecular approaches to the question of phage specificity, should ensure continued research interest in bacteriophage-based detection methods.

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Exhibit W

COMPENDIUM OF METHODS FOR THE

MICROBIOLOGICAL EXAMINATION OF FOODS

FOURTH EDITION

EDITED BY
FRANCES POUCH DOWNES
KEITH ITO



AMERICAN PUBLIC HEALTH ASSOCIATION

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Chapter 13

Psychrotrophic Microorganisms

M. A. Cousin, J. M. Jay, and P. C. Vasavada

13.1 INTRODUCTION

13.11 History and Definition of Terms

In 1887 Forster observed microbial growth at 0°C, but it was not until 1902 that the term "psychrophile" was used.[58] Psychrophiles have been defined based on growth at low temperature, optimum temperature of growth, temperature of enumeration, and other criteria not related to temperature such as only including gram-negative rods or only bacteria that do not survive pasteurization.[103] Mossel and Zwart[83] and Eddy[24] proposed the term "psychrotrophs" for microorganisms that grow at low temperatures but have higher temperature optima. Morita[82] suggested that the mesophilic microorganisms that grow at 0°C be called either psychrotolerant or psychrotrophic to contrast with psychrophilic microorganisms, which have a temperature optimum of 15°C, a maximum of 20°C, and a minimum of 0°C or below. In a recent review, Brenchley [14] referred to psychrophiles as microorganisms that grow at 5°C or below regardless of the maximum growth temperature; therefore, there is still not consensus on what to call these microorganisms that grow at low temperatures.

Microorganisms that grow in foods at refrigeration temperatures (0 to 7°C) but have temperature optima above 20°C are called psychrotrophs. Psychrotrophs are defined as microorganisms that produce visible growth at 7° ± 1°C within 7 to 10 days, regardless of their optimum growth temperatures.[120] This definition honors the long-standing practice of classifying microorganisms into three temperature groups, namely thermophiles, mesophiles, and psychrophiles, with psychrotrophs being a subgroup of the mesophiles. From a practical standpoint, the microorganisms that are most commonly associated with refrigerated foods and cause food spoilage are psychrotrophs and not psychrophiles because the latter usually die at room temperature or above. Psychrotrophs grow and spoil foods that are refrigerated, but they grow better at higher temperatures in the mesophilic range.

13.12 Growth of Psychrophiles and Psychrotrophs

If a microorganism is to grow at low temperatures, then the substrate uptake, cell permeability, enzymatic systems, and synthetic pathways must all function at low temperatures. Some theories concerning the mechanism of growth of psychrophiles and psychrotrophs focus upon the generation of low activation energy for enzymes, presence of unsaturated fatty acids in the cell membranes and subsequent fluidity, conformational changes in the ribosomal proteins, and regulatory enzymes, presence of cold shock proteins, alterations in substrate uptake, and cell permeability.[19,28,41,42,50-52,82,97,99,103,115]

13.13 Psychrophiles Involved in Food Spoilage

Psychrophilic bacteria are mainly gram negative and are found in environments where temperatures are constantly below 15° to 20°C.[52] Psychrophiles grow in environments where temperatures are fairly constant; whereas, psychrotrophs grow in environments where temperatures fluctuate.[103] Most psychrophiles found in foods are species of *Aeromonas, Alcaligenes, Cytophaga, Flavobacterium, Pseudomonas, Serratia,* and *Vibrio*. Some gram positive genera that have been isolated from arctic waters, soils, and foods include species of *Arthrobacter, Bacillus, Clostridium,* and *Micrococcus*. Psychrophilic yeasts, molds, and algae have also been identified. *Cryptococcus, Leucosporidium,* and *Torulopsis* are psychrophilic genera of yeasts. Although Makarios-Laham and Levin[75,76] isolated psychrophilic *Vibrio* species from haddock, their significance in fish spoilage is unknown. Whether psychrophiles are involved in food spoilage has not been determined.

13.14 Psychrotrophs Involved in Food Spoilage

Psychrotrophic bacteria have been studied more than either yeasts or molds. These bacteria include rods and cocci; sporeformers and non-sporeformers; gram negative and gram positive bacteria; and aerobes, facultative anaerobes, and anaerobes. The major psychrotrophic bacteria found in milk and dairy products,[17,19,62,79] meats and poultry,[10,62,67,79,80] and fish and seafood[43,55,56,62,79,124] include species of *Acinetobacter, Aeromonas, Alcaligenes, Arthrobacter, Bacillus, Brochothrix, Carnobacterium, Chromobacterium, Citrobacter, Clostridium, Corynebacterium, Enterobacter, Escherichia, Flavobacterium, Klebsiella, Lactobacillus, Leuconostoc, Listeria, Microbacterium, Micrococcus, Moraxella, Pseudomonas, Psychrobacter, Serratia, Shewanella, Streptococcus,* and *Weissella*. In addition, species of *Alteromonas* (formerly *Pseudomonas putrefaciens*), *Photobacterium,* and *Vibrio* are also important in fish spoilage.[35,80,124] Species of *Bacillus, Clostridium, Enterobacter, Erwinia, Flavobacterium, Pseudomonas,* and *Yersinia* cause soft-rotting of refrigerated vegetabes.[13,15,74]

Psychrotrophic fungi have been isolated from refrigerated fresh animal and marine products and from fruits and vegetables.

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Among the yeast genera involved are *Candida, Cryptococcus, Debaryomyces, Hansenula, Kluveromyces, Pichia, Saccharomyces, Rhodotorula, Torulopsis,* and *Trichosporon.*[13,19,20,57,60] Mold genera that have psychrotrophic species include *Alternaria, Aspergillus, Botrytis, Cladosporium, Colletotrichum, Fusarium, Geotrichum, Monascus, Mucor, Penicillium, Rhizopus, Sporotrichum, Thamnidium,* and *Trichothecium.*[19,57,60,67,111] Fungi predominate in refrigerated food spoilage when low water activity, high acidity or packaging conditions select for their growth over bacteria in foods, such as many fruits, jams, dried fruits, and fermented foods (cheese, sausages, yogurt, etc.).

The use of vacuum or modified atmosphere packaging of raw and processed meat, fish, and other foods favors the growth of both facultative anaerobes and true anaerobes in the oxygen-reduced environment. The major bacterial genera found in vacuum or modified atmosphere- packaged foods include psychrotrophic species and strains of *Brochothrix, Lactobacillus, Leuconostoc,* and members of the *Enterobacteriaceae* with lower populations of *Carnobacterium* spp. and *Weissella viridescens.*[10,71,100]

13.15 Psychrotrophic Pathogens

The emergence of psychrotrophic foodborne pathogens in recent years has raised new concerns about the safety of refrigerated foods. Pathogenic psychrotrophs that grow at or below 5°C include *Aeromonas hydrophila,* some strains of *Bacillus cereus, Clostridium botulinum* type E and non-proteolytic types B and F, *Listeria monocytogenes, Vibrio cholera, Yersinia enterocolitica,* and some strains of enteropathogenic *Escherichia coli.*[4,72,89,91,126] Further information on these pathogenic psychrotrophs can be obtained in their respective chapters. Several reviews on the role of psychrotrophic pathogens in vacuum or modified atmosphere-packaged foods have been published.[18,33,54,66,91,102,125,126]

Foodborne pathogens such as strains of *B. cereus, C. perfringens,* the proteolytic strains of *C. botulinum, Salmonella* serotypes, and *Staphylococcus aureus*[33,89] have minimal growth temperatures between 7° and 15°C; therefore, temperature abuse of refrigerated foods may allow these mesophilic pathogens to resume growth once temperatures rise above 10 to 15°C.

13.16 Significance of the Presence and Growth of Psychrotrophs in Foods

Psychrotrophs metabolize carbohydrates, proteins, and lipids across the range of temperatures at which foods are stored, but reaction rates are slower at temperatures at and below 7°C. Minor biochemical changes in the food may occur early during the growth phase of some psychrotrophs, but several days to weeks of refrigeration may be necessary for changes to become organoleptically apparent.[19]

Information about the spoilage of specific food commodities can be found in their respective chapters. Some general reviews are available for the spoilage of milk and dairy products,[19,26,79,109,112] meat and poultry,[7,67,79,80] fish and seafoods,[53,56,76,78] and fruits and vegetables.[13,57,74,111]

There is a trend in the United States, Europe, and Japan to market "minimally processed" refrigerated foods that range from deli-type salads to complete dinners.[15,72,105] Minimal processing uses procedures such as low heat instead of sterilization; and cleaning, peeling and cutting of fresh produce instead of leaving it whole.[1] Minimal processing includes various heat treatments, vacuum or modified atmosphere-packaging, conventional or microwave heating after product-package assembly, and strict refrigerated distribution systems.[72] Two methods, "sous-vide," in which the food is placed in an oxygen-impermeable bag, and "nouvelle carte," in which the food is packaged on a plate and placed in a vacuum pouch, involve minimal heat processing in the vacuum package and refrigerated distribution at 2° to 4°C.[72,105] These processes for refrigerated foods create new microbiological concerns for both safety and expected shelf life. Packaging in vacuum and modified gaseous atmospheres selects for facultative anaerobes and anaerobes. The minimal heating kills vegetative cells, but not spores. Since these processing and packaging methods are intended only to extend the shelf life, surviving psychrotrophic spoilage and pathogenic microorganisms can grow and dominate the microbiota of these products. To avoid this situation, proper thermal processing, vacuum packaging plus storage and distribution at 7°C or below must be maintained.

13.2 REVIEW OF METHODS USED TO ENUMERATE PSYCHROTROPHS IN FOODS

13.21 Cultural and Microscopic Methods

General reviews of the methods used to enumerate psychrotrophs have been published for dairy products,[19] fish,[56] and meats.[67] The traditional methods for enumerating psychrotrophs have involved either plate counting methods or the use of microscopy.[34,35] Examples of the time and temperatures of incubation for psychrotrophic plate counts are 10 days at 7°C for pour plates or 7 to 8 days at 7°C for spread plates and 16 hr at 17°C followed by 3 days at 7°C. Incubation conditions using shorter times and higher temperatures have included 25 hr at 21°C for milk and cream,[46,88,96,110,118] 45 hr at 18°C for milk,[86] and 24 hr at 25°C for meat.[45]

Several variations of the plate count procedure provide equivalent accuracy for the enumeration of psychrotrophs. These methods (Chapters 6 and 10) include spiral plating[59,99,109] dry rehydratable film like Petrifilm™,[3,36,78] and hydrophobic grid-membrane filter (HGMF).[16,107]

The composition of some selective media used for psychrotrophs is based on the assumption that most psychrotrophs are gram negative bacteria. In the first two editions of this book,[34,35] a selective medium was recommended for the enumeration of psychrotrophs that contained crystal violet and triphenyl tetrazolium chloride (CVT) and was incubated for either 48 hr at 30°C or 5 days at 22°C. Jay and Bue[63] found that the use of CVT agar with incubation for 48 hr at 30°C was not suitable for the enumeration of gram-negative psychrotrophs since many non-psychrotrophic mesophiles grew well under these conditions. While crystal violet at 2 ppm inhibited three gram positive bacteria but not three *Pseudomonas* species,[106] crystal violet at the recommended usage of 1 ppm[34,35] in CVT agar did not inhibit 41/44 non-psychrotrophs, nor 3/45 psychrotrophs evaluated.[68] Although some investigators have found media containing these inhibitors to be suitable for assessing gram negative psychrotrophs at temperatures that permit the growth of many non-psychrotrophic mesophiles, this is a reflection of the relatively large number of psychrotrophs in the products examined. Most non-psychrotrophic mesophiles will proliferate at temperatures between 22° and 40°C when incubated for 48 hr or more.

The direct microscopic count (DMC) where a specific sample size is placed on a defined area of a microscopic slide that is stained before counting the cells in a set number of fields and the microscopic colony count (MCC) where a specific amount of agar containing the food sample is incubated on a slide and the

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accuracy needed, type of refrigerated food, and degree of processing. When an accurate number of psychrotrophs is needed, plate count methods must be used. It may be necessary to choose time and temperature conditions that simulate either the storage conditions of the food or the possible abuse conditions. Selection of the method for enumerating psychrotrophs must involve consideration of sublethally injured or stressed cells. Absolute conditions cannot be given for every food or every situation.

13.32 Media Selection

The selection of media for enumeration will depend on the food, types of psychrotrophs expected, recommendations of equipment manufacturers, length of incubation, reactions expected, and other relevant factors. Media other than those listed below may prove valuable.

13.4 SAMPLE PREPARATION

13.41 Sample Collection

Samples must be collected aseptically (Chapter 2) and analyzed promptly. Refrigerated storage could allow psychrotrophs to increase in numbers because some psychrotrophs have generation times as low as 6 hr. Ideally, refrigerated foods should be analyzed within 6 hr of their collection. Refrigerated samples should not be frozen because some psychrotrophs are sensitive to freezing and can be injured or killed. If samples must be frozen for shipment, then the possibility of some death must be considered when evaluating the results.

13.42 Sample Homogenization

Samples should be homogenized with diluent in a blender for 2 min or in a stomacher. Because psychrotrophs are sensitive to heat, blending for more than 2 min is discouraged to prevent the generation of heat that can result in cell injury or death. In addition, excessive blending of molds can cause fragmentation of the mycelia, depending on blade sharpness, volume, speed, and time. The use of a stomacher in preference to the blender lessens the likelihood of these problems.

13.5 EQUIPMENT, MEDIA, MATERIALS, AND REAGENTS

Refer to the specific section in recommended methods for equipment that is needed for each method. Incubators that can be maintained at 7° ± 1°C for the traditional psychrotrophic count and 17–21°C ± 1°C for rapid methods that require incubation are necessary.

13.51 Media

1. Non-selective plate media: standard methods (plate count) agar or trypticase soy agar for bacteria; and dichloran rose bengal chloramphenicol agar (DRBC) or plate count agar plus chloramphenicol or dichloran 18% glycerol (DG18) for yeasts and molds (Chapter 20).
2. Media and reagents for the rapid methods can be obtained from the manufacturers (Chapter 63).

13.6 PROCEDURES FOR ENUMERATION OF PSYCHROTROPHS

13.61 Plate Count Method

A plate count method using plate count or trypticase soy agar, or dry rehydratable film such as Petrifilm™, is recommended for general enumeration of bacteria (Chapter 7). Enumeration procedures for yeasts and molds can be found in Chapter 20. Incubate plates for 10 days at 7° ± 1°C since this is the reference definition for psychrotrophs. Alternatively, incubation for 16 hr at 17°C followed by incubation for 3 days at 7°C can be used when results are needed in less than 10 days.[120]

Count the colonies as described in Chapter 7. Record all counts as number of psychrotrophs/milliliter, gram, or square centimeter depending upon the method of sampling.

13.7 PRECAUTIONS

13.71 Incubation Temperatures

Different types of refrigerated foods are normally processed and held at temperatures of refrigeration that are specific for the food commodity. The incubation temperature used for the enumeration may not lead to adequate assessment of the psychrotrophic population that will grow in the food because microorganisms may grow in laboratory media but not in the food and vice versa. Therefore, caution must be used when interpreting results of the enumeration of psychrotrophic populations.

Sublethally injured psychrotrophs may not be detected analytically but may cause food spoilage or foodborne illness. Therefore, steps to recover sublethally injured cells should be included in enumeration and detection methods.[84] See Chapter 5 for suggestions on methods.

13.72 Pour Plate Versus Spread Plate Techniques

Psychrotrophs are especially susceptible to injury or death when agar that is held above 45°C is used for pour plating.[122] Hence, a spread/surface plate or spiral plater technique (Chapter 12) should be used whenever possible. Plates can be pre-poured and stored at 5°C for several days or weeks before use. Dry rehydratable film such as Petrifilm™ may also be used in place of the traditional plating technique.

13.8 RAPID DETECTION

Obtaining results sooner than 10 days is pragmatically desirable, and efforts must continue to find methods that are more rapid for the enumeration of psychrotrophs in foods. Rapid detection of psychrotrophs in raw ingredients, on-line quality control samples, and shelf life samples are important. Three useful rapid methods are impedance, LAL assays, and enzymatic assays.

13.81 Impedance Determination

The equipment manufacturers generally recommend media and incubation conditions that should be followed for the specific foods being analyzed because some media can interfere with the detection time. Preliminary work to determine what correlations between impedance readings and psychrotrophic counts apply to the food usually are necessary before impedance can be used for routine analysis. An example of how the method is done is as follows. Samples of solid foods (meats, poultry, fish, vegetables, etc.) are blended for 1 min in 0.1% peptone water to make a 1:10 dilution. As little as a 1.0-mL aliquot of the 1:10 dilution can be added to recommended broth in the sterile well or tube of the impedance detection instrument. Samples are incubated at 21°C and continuously monitored for up to 24 hr depending on the sample and the degree of contamination. Preliminary incubation for 15 to 24 hr at 18° to 21°C in broth may be used to obtain early detection and shelf life predictions because foods that have un-

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dergone processing normally will have lower counts than raw foods. After preincubation, 1 or more milliliters of the mixed sample is placed in the well or tube of the impedance detection instrument and incubated at 21°C; thereafter, measurements are recorded for up to 24 hr. The impedance detection time is recorded and used to estimate the number of psychrotrophs in the sample.

Liquid foods such as milk and juice can be added directly to either broth or plated media in the wells of the impedance detection instrument. Specific media developed by the instrument suppliers can also be used to detect bacteria, yeasts, and molds in liquid foods. A sample of the liquid food (amount determined by instrument used) is added to the media in the well and incubated at 21°C for up to 24 hr for bacteria and 25° to 28°C for 1 to 2 days for yeasts and molds. Preincubation in plate count broth at 18°C for 18 hr can be done to assess the keeping quality of heat-processed milk and dairy products. The impedance detection time is recorded and the estimation of microbial numbers made.

13.82 *Limulus Amoebocyte Lysate (LAL) Assay*

The tube gelation method has been used more extensively than either the turbidity or the chromogenic substrate methods, and its use is described below. However, the chromogenic substrate is the newer of the three basic methods, provides results in about 30 min, is more sensitive (LPS detected from 1 to < 5 pg/mL), and is becoming more widely accepted. The basic operation and automation of the chromogenic substrate has been described by Tsuji et al.[121] and reviewed by Jay.[61]

The most important considerations in the use of the tube gelation method are (a) source and sensitivity of LAL reagent, (b) whether single- or multi-test vials or reagent are to be used, and (c) the choice of endotoxin or LPS standard. Suppliers of freeze-dried LAL reagents, such as Associates of Cape Cod (Woods Hole, MA) and Sigma Chemical Co. (St. Louis, MO) provide complete instructions for the proper use of their reagents, and these should be followed carefully. LAL reagents can be obtained with different levels of sensitivity usually expressed in endotoxin units (EU).

Since LPS from different gram negative bacteria varies in its reactivity to the LAL reagent, it is essential that a standard reference endotoxin preparation be used. These are available with complete instructions for use from LAL reagent suppliers. The two reference endotoxins of choice are those prepared from *Escherichia coli* O113:H10 (EC-2) or *E. coli* O55:B5.

The tube gelation method described below is taken from the review by Jay.[61] LAL assay methods require that all utensils and glassware be pyrogen free. Glassware can be depyrogenated by heating in a dry-air oven at temperatures above 180°C for about 3 hr. Sterile pipettes and disposable tubes are generally free of pyrogens before use, and pyrogen-free water should be purchased from a firm that supplies parenteral products. Specific instructions for conducting a tube gelation test are usually provided by the LAL reagent manufacturer.

LAL reagent is supplied either in ready-to-use or single-test vials, or in multi-use vials. Follow the preparation and storage directions that come with the reagents. It is generally a good idea to cover the tubes with aluminum foil until used. The tubes should be used the same day although some manufacturers indicate that the tubes may be frozen and thawed once. Quality control procedures and both negative and positive controls are supplied by the manufacturers.

Food samples should be serially diluted, using pyrogen-free water or suitable buffer, to provide dilutions that will produce negative results. Since two-fold dilutions were not significantly different than ten-fold dilutions, use of two-fold dilutions will save on both reagents and labor.[61] Beginning with the highest dilution (lowest endotoxin concentration), the same pipette tip can be used to transfer either 0.1 or 0.2 mL of diluted sample to separate LAL reagent tubes. The tubes are vortexed gently, incubated in a waterbath at 37°C for 1 hr, and read by inverting 180° and noting gelation. Simultaneously, an endotoxin standard should be included using an appropriate reference endotoxin. The two-fold diluted endotoxin standard should be treated in the same way as the diluted test preparation; the sensitivity of the LAL preparation to LPS is determined by using this standard to define the lowest quantity that produces a gel.

The quantity of endotoxin or LPS in test samples is determined by multiplying the reciprocal of the highest sample dilution by the LAL-determined sensitivity value. For example, if the highest dilution of endotoxin standard that produces a firm gel in the LAL reagent is 0.1 ng, the sensitivity of the LAL reagent is, thus, 0.1 ng. If using the above LAL reagent and the highest dilution of food that produces a firm gel is 10^3, then the total endotoxin or LPS in food is 0.1 ng × 1000 = 100 ng/mL.

13.83 *Enzymatic Methods: Catalase and Cytochrome Oxidase*

Enzymatic methods are not sensitive enough to detect microbial populations below 10^4 cells/mL or g; therefore, use is restricted to foods with high microbial loads. A preincubation of 4 to 6 hr that may or may not involve selective enrichment media can improve both the selectivity and the sensitivity of these methods. Use of these methods for solid foods needs further refinement for the extraction of the enzymes from foods.

The catalase test can be done by using instruments that measure oxygen release, e.g., BioTech International (SugarLand, TX). Catalase that is naturally present in the food may need to be inactivated by heating the sample at 50°C for 10 min before doing the test for microbial catalase, which has a higher heat stability.[9] The amount of oxygen produced in the catalase test is proportional to the number of microorganisms in the food.

The oxidase test is performed by treating 4 mL of sample with 1 mL of a freshly prepared 1% *N,N,N',N'*-tetramethyl-*p*-phenylene-diamine dihydrochloride followed by incubation at 25°C for 5 min. If sample contains particulate matter, then it can be centrifuged at 7,000 × g for 10 min before taking the reading. Blue color can be evaluated either visually against reference color standards or spectrophotometrically. The intensity of the blue color is proportional to the concentration of microorganisms in the food.

13.9 INTERPRETATION

The enumeration of psychrotrophs in refrigerated foods gives an indication of the potential spoilage, keeping quality, or safety of the food. However, caution should be exercised when trying to make absolute predictions about a food based on these results. Some temperatures of refrigeration may be close to the minimal growth temperature, and the enumeration temperature may be closer to the optimum, particularly in rapid methods that require an incubation period. The temperatures used for food storage and for detection should be closely comparable to achieve meaningful results. In addition, the nature of the food is important. If the food has been refrigerated for some time, the numbers can represent a normal increase in psychrotrophs rather than a poor quality product.

Processing can kill or injure psychrotrophs, and analyzing foods immediately after processing may not allow time for injured

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cells to recover. If processed foods are stored in the refrigerator for extended periods, even a few cells can grow to large enough numbers to cause eventual spoilage in a few days or weeks.

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Exhibit X

US006080437A

United States Patent [19]

Mauer et al.

[11] Patent Number: 6,080,437

[45] Date of Patent: Jun. 27, 2000

[54] METHOD OF PASTEURIZING MEAT PRODUCTS

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[73] Assignee: James E. Mauer, Rome, Ga.

[21] Appl. No.: 08/985,979

[22] Filed: Dec. 5, 1997

Related U.S. Application Data

[62] Division of application No. 08/677,590, Jul. 9, 1996, Pat. No. 5,741,536.

[51] Int. Cl.[7] A23B 4/005

[52] U.S. Cl. 426/392; 426/106; 426/129; 426/393; 426/412; 426/521; 426/524

[58] Field of Search 426/106, 113, 426/129, 392, 393, 412, 520, 521, 524, 407

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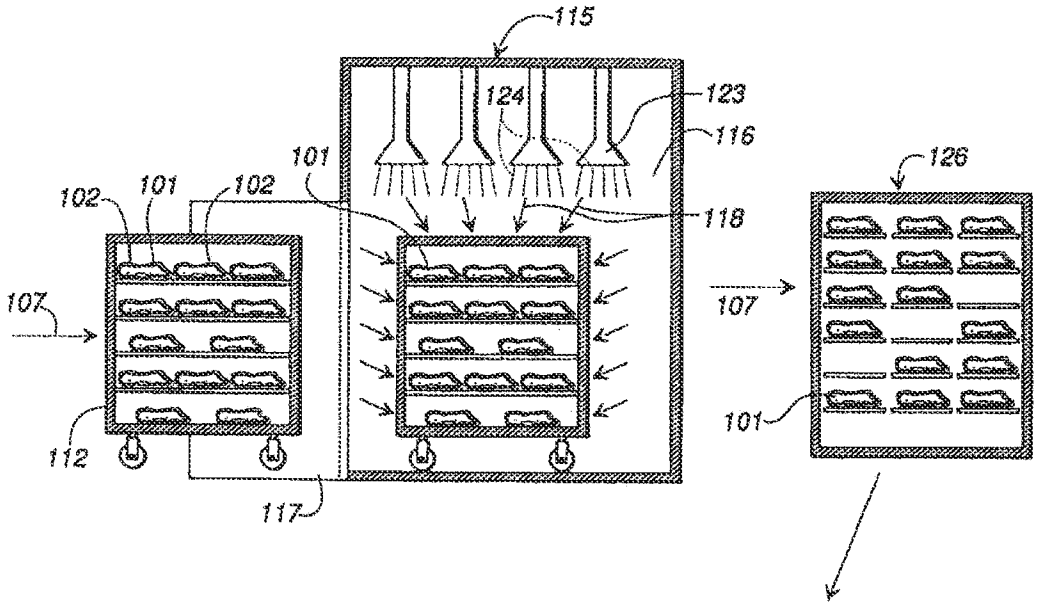
Primary Examiner—Milton Cano

Attorney, Agent, or Firm—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

[57] ABSTRACT

A process for preparing prepackaged pasteurized meat parts in which the parts are pasteurized at a low temperature for an extended period of time so as to enable thorough and even heating of the parts to eliminate bacterial contamination within the parts. After the parts have been pasteurized, the pasteurized parts are cooled and then frozen. The frozen parts are then packaged for shipment and sale.

4 Claims, 4 Drawing Sheets



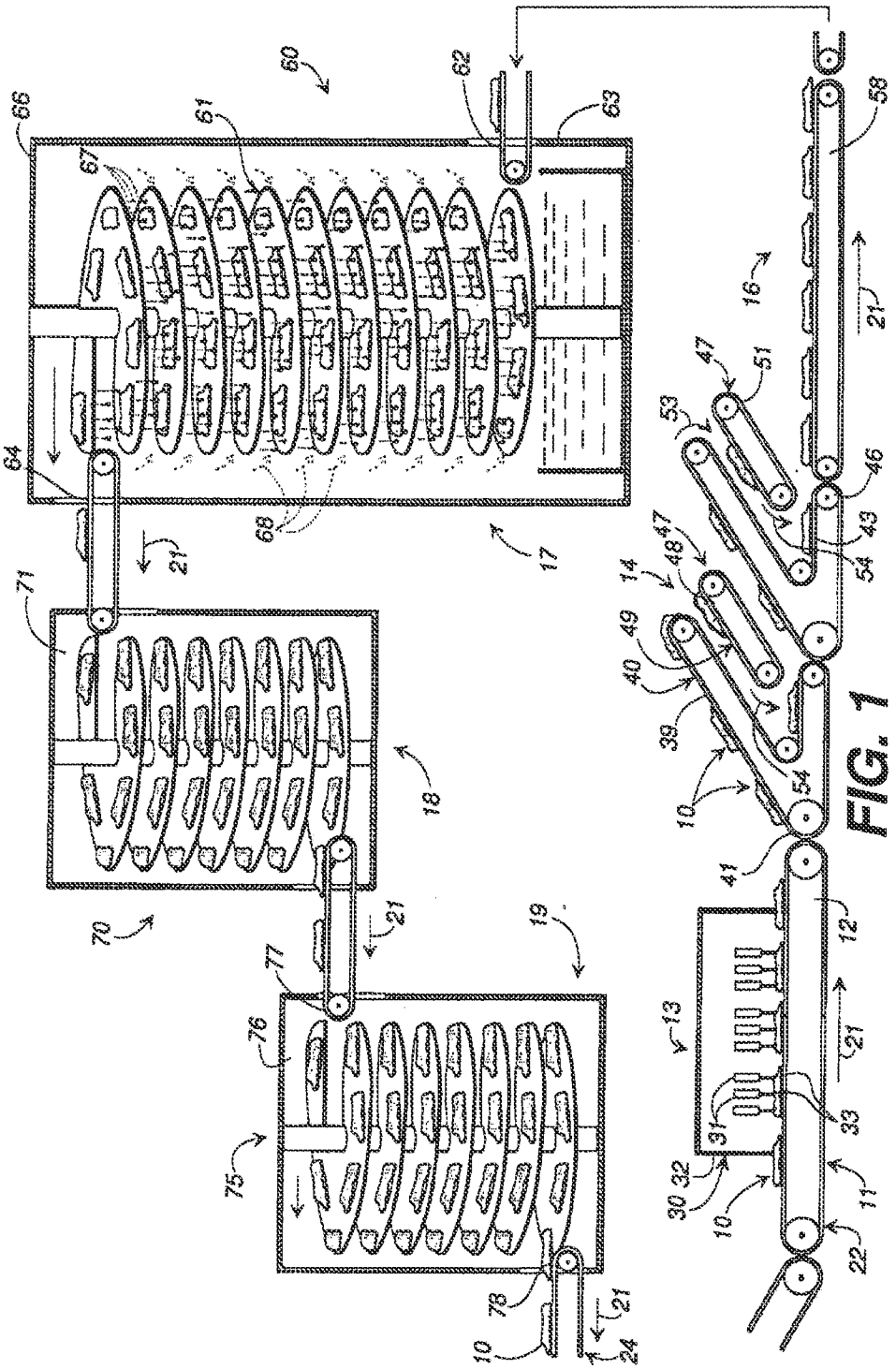

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FIG. 1

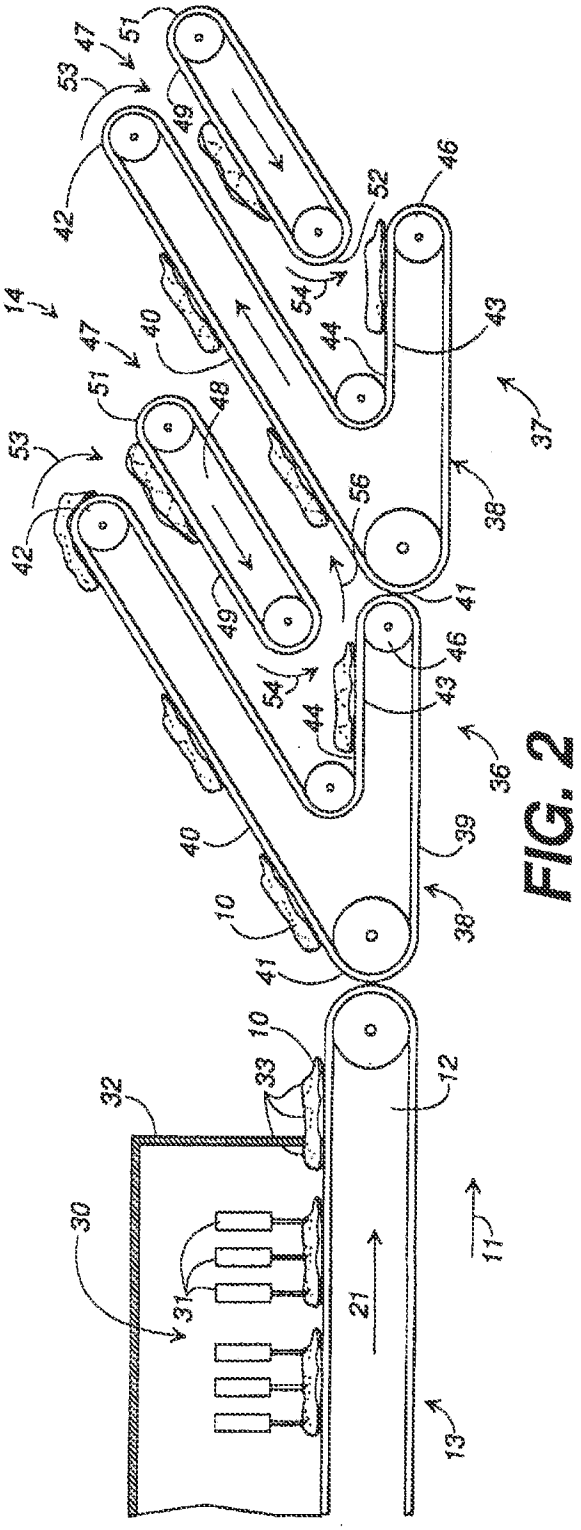

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FIG. 2

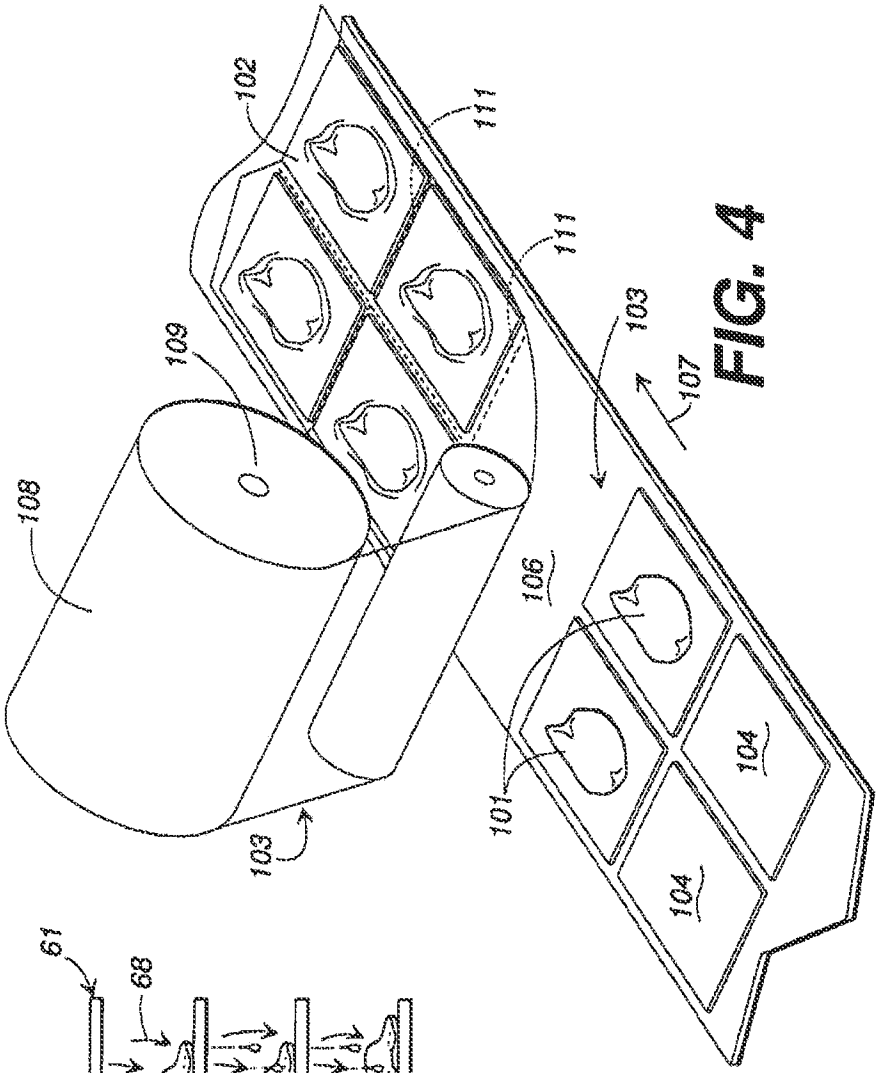

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FIG. 4

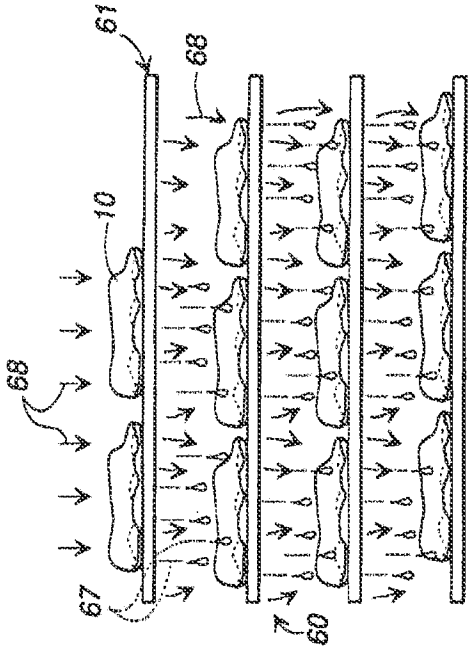

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FIG. 3

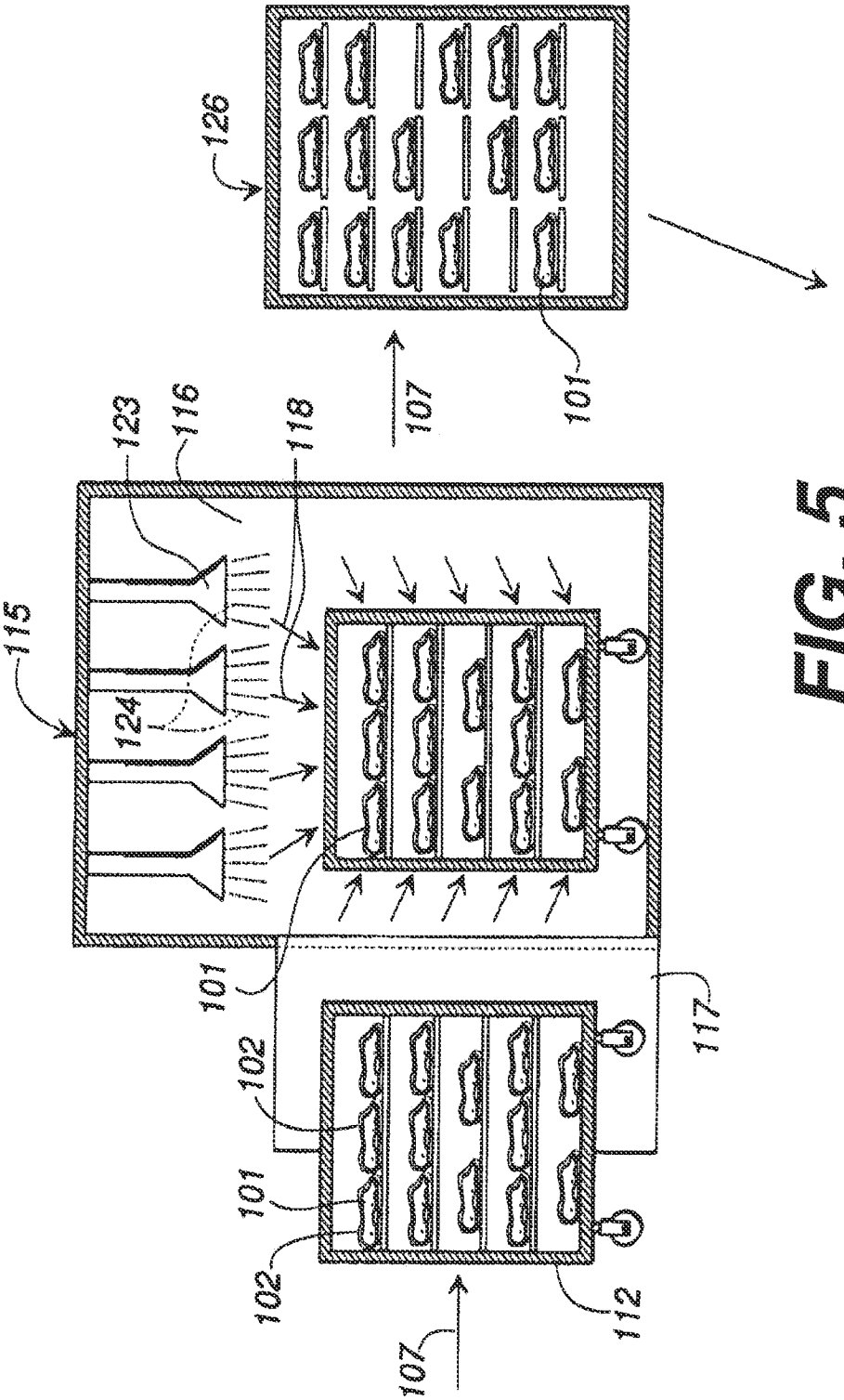


FIG. 5

METHOD OF PASTEURIZING MEAT PRODUCTS

This is a division of application Ser. No. 08/677,590, filed Jul. 9, 1996 now U.S. Pat. No. 5,741,536 which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates in general to a method of processing meat products for packaging and sale. The term "meat products" as used herein means beef, pork, poultry and other fowl and veal. In particular, the present invention relates to a method of pasteurizing cut meat products as they are moved along the processing path to essentially eliminate bacterial contamination within the parts while retaining moistness and quality of taste and appearance of the parts, and thereafter cooling and freezing the parts so as to prepare the parts for packaging for sale.

BACKGROUND OF THE INVENTION

One of the fastest growing and most profitable segments of the food service industry involves the preparation of precooked, packaged meat products such as chicken parts, beef and pork products for sale on retail and institutional levels. The market for such products has increased dramatically in the areas of home retail sales and restaurant sales as more and more people have less time to go to the trouble of preparing and cooking fresh meats, and thus find it much easier and faster to use precooked foods which require substantially less cooking/preparation/heating time. Additionally, for restaurants, the use of such precooked meat products yields faster service of orders, and the uniformity of these products enables restaurants to serve generally uniform sized portions. Also, since at the consumer level, the cooking time of precooked meat products is less than that for fresh or uncooked products, restaurants do not need as extensive cooking equipment to prepare and serve a significant volume of meals.

A significant problem that arises with packaged meat products, however, is the danger of contamination of such meat products due to bacteria. If not properly prepared and adequately cooked before being packaged, such food products can pass bacterial contaminates onto the end users, posing a serious health risk to the end users. This health risk has become especially prominent in the last several years in light of recent well publicized instances of "*E-coli*" and salmonella poisoning, etc. due to contaminated meat products being served at restaurants across the United States.

Conventional industrial food cooking processes have focused on the cooking of such meat products at relatively high temperatures in order to speed the cooking time required to cook the products. Such rapid cooking of the meat products under relatively high heat, however, creates much less attractive products often having blood spots or other discolorations therein. Further, when cooking the products rapidly, there is still a danger of the products not being thoroughly cooked, especially through the bones of the product, which contain blood, marrow, etc., where bacteria can take hold. In addition, rapid cooking of the products at high heat further tends to dry out the products making the products tougher and adversely affects their taste.

An example of a conventional cooking system for meat parts, is taught by U.S. Reissue Patent No. 33,510 of Williams. Williams teaches a steam cooking system in which products of substantially uniform size and weight, such as chicken parts, are sprayed with a water spray and thereafter are passed into a steam chamber in which the parts are exposed to an environment of 100% humidity at approximately 100° C. (212° F.). The principal purpose of the system of Williams is to cook the uniformly sized meat parts at an increased heat and moisture while attempting to retain the juices and flavor of the product. Williams, however, does not appear to address the problem of reducing the contamination of the products as they are moved through the cooking apparatus. In fact, Williams uses steam partially generated from the heating of water collected at the bottom of the cooking apparatus. Such collected water contains collected drippings and bacterial contaminates secreted from the parts as they are cooked. As this water is heated and becomes steam that is directed throughout the cooking apparatus, the parts are continually exposed to potentially contaminating steam.

In addition, the products or parts enter the cooking apparatus of Williams at the top thereof so that the raw parts are at the top of the cooking apparatus, which raw parts tend to secrete and drip matter, including contaminating matter, downwardly onto the previously cooked products, at the lower areas of the cooking apparatus. The system shown in Williams also requires an additional processing step of segregating the meat parts to parts of relatively uniform size and shape before such parts can be moved through and cooked in the cooking apparatus of Williams. After the products have been cooked, the cooked products apparently then must be desegregated prior to packaging, for example for the packaging of whole birds in "eight piece packs".

Systems such as Williams, which are designed to provide faster cooking of the meat parts in the processing plant, thus tend to require additional processing steps and cannot uniformly cook a variety of different size and weight products at the same time. Additionally, most conventional cooking processes are not specifically directed or designed for ensuring that bacterial contaminates contained in the meat parts are substantially reduced or eliminated prior to packaging and sale to consumers. As a result, while cooking times for such precooked, prepackaged meat products are generally reduced over the cooking times for fresh products, it typically is still necessary for the end consumer to cook the products for a significantly long time to ensure that the products are thoroughly cooked throughout to reduce or eliminate any contamination therein.

Accordingly, it can be seen that beyond traditional methods of precooking product, a need exists for a process of pasteurizing and prepackaging meat products, in which the potential for bacterial contamination has been substantially reduced and/or eliminated and which provides a safe, healthy meat product that can be rapidly cooked by an end consumer and which, unlike precooked product, retains moisture and quality of flavor and appearance.

SUMMARY OF THE INVENTION

Briefly described, the present invention comprises a method for heat treating, or heat processing and thereby pasteurizing cut meat parts or products of varying sizes for packaging and sale, in which bacterial contamination has been substantially reduced and/or eliminated, but which retains a fresh, moist taste and appearance, and which can be rapidly and easily prepared by the end consumer.

In a first embodiment of the present invention, the cut meat parts, such as the breasts, backs, legs, thighs, etc . . . of poultry carcasses, are moved from a cut-up line and are deposited on a pasteurizing line. The parts are moved along

the pasteurizing line along a processing path through a series of stations for the pasteurizing and freezing of the parts. The first parts are passed through a marinating station, wherein the parts are marinated. Typically the parts are marinated by being injected with a marinade solution to improve the moistness and flavor of the products as they are pasteurized. Thereafter, the parts are massaged at a second station by being moved through a series of flips, wherein the products are reoriented by dropping the parts onto a series of conveyor belts. As the parts are flipped back and forth, the marinade solution, which generally has tended to be concentrated in pockets adjacent the skin of the part where it is injected, is distributed substantially evenly throughout the parts.

Following marination and massaging of the parts, the parts generally are reoriented so that their skin sides are facing upwardly as the parts enter a heating unit. The parts enter the heating unit at a low elevation and are received on a spiral conveyor that progressively winds in a helical fashion upwardly through the heating unit toward the top thereof. The spiral conveyor further is perforated to allow juices secreted by the parts at higher elevations within the heating unit to drip downwardly onto the parts at the lower elevations. This provides a natural, automatic basting of the parts as they proceed through the heating unit. The parts generally are exposed to relatively lower heats of less then 200° F. and are heat treated for approximately 40–50 minutes or longer, until pasteurized. Further, the parts are subjected to forced streams or flows of heated air that are dry or mixed with steam up to 100% and which are directed against the parts as the parts are pasteurized in the heating unit. Such increased pasteurizing times of the parts at relatively lower temperatures than those used by most conventional industrial cooking systems together with the impingement of the parts with heated, forced air flows, tends to create an even, deep penetration of heat all the way through the parts to the bones thereof. As a result, the parts are evenly and thoroughly pasteurized to kill any bacteria contamination therein. The bones of the parts also are uniformly heated so that the marrow and blood therein is denatured, and thus any bacteria within the bones is generally killed to substantially eliminate any contamination through the bacteria within the parts.

After the parts have been pasteurized in the heating unit, the parts thereafter are moved into a precooling station positioned immediately downstream from the outlet end of the heating unit. In the precooling station, the parts are subjected to temperatures of between approximately –20° F. to 0° F. for approximately 30–40 minutes to cool the hot, pasteurized parts to a temperature of between 40° F. to 50° F. Thereafter, the parts are moved into a freezer where they are flash frozen by exposure to liquid nitrogen for approximately 20 minutes. The precooling of the parts prior to freezing helps reduce the incidence of moisture within the parts being drawn to the surface, as generally happens by simply flash freezing the parts, and thus, drying out the parts. The products are maintained in the freezer for a time sufficient to lower their temperature to 0° F. or less so that the parts are frozen solid. Thereafter, the frozen parts are packaged for shipment and sale to end consumers for both institutional and retail markets.

In a second embodiment of the present invention, cut poultry breasts are processed. Typically, the poultry breasts will have been deboned prior to being deposited on the pasteurizing line. The breasts initially are placed in pouches or pockets formed between sheets of a thermoplastic material. The sealed packages thereafter are moved into a heating unit, wherein the packages are exposed to relatively lower heats of less than 200° F. for approximately 15–20 minutes. This enables heat to thoroughly penetrate the breasts to pasteurize the breasts and eliminate any bacterial contamination therein. The packages also retain the natural juices and/or any marinade solution injected into the parts within the packages to maintain the moistness and flavor of the breasts.

After being pasteurized the pouches are doused with cool water sprays of approximately 60° F. to 70° F. for approximately 5–10 minutes to reduce the temperature of the pouches to approximately 60° F. to 70° F. Thereafter, the pouches are moved into a mechanical freezing station where the breasts are frozen to approximately 0° F. Thereafter, the pouches are packed for shipment for sale through institutional and retail channels.

The resultant pasteurized, packaged breast products require much less preparation and serve time, as it can typically be prepared and served in approximately 7–8 minutes and can be cooked by simply placing the pouch in hot water so that extensive cooking equipment is not needed. Thus, the packaged breast products formed by the second embodiment of the present invention are safe and versatile to enable them to be cooked and served without requiring a full kitchen set-up for the preparation of the breast product.

Accordingly, it is an object of the present invention to provide a method of pasteurizing meat products without adversely affecting their appearance, taste, texture and quality.

Another object of this invention is to provide a method of preparing prepackaged pasteurized meat products in which contamination due to bacteria within the meat products has been essentially eliminated to provide a safer product that is easier to prepare and serve to the end consumer.

Another object of this invention is to provide an improved method of preparing prepackaged meat products, requiring substantially less preparation and serve time by the end consumer.

Another object of this invention is to provide a method of preparing prepackaged meat products which are pasteurized to essentially eliminate bacterial contamination therein, while retaining moistness and flavor of the products.

Still another object of the present invention is to provide a method of preparing prepackaged meat parts, in which meat parts of substantially different sizes and weights can be pasteurized together, with each part being substantially evenly heated, so as to not require segregation of the parts by size and weight prior to pasteurizing.

Still another object of the present invention is to provide a process for preparing pasteurized, prepackaged meat products having a more attractive appearance and an improved taste and texture.

Various other objects, features and advantages of the present invention will become apparent to those skilled in the art upon reading the following detailed description when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of a first embodiment of the process for pasteurizing meat products according to the present invention.

FIG. 2 is a side elevational view schematically illustrating the marination and massaging of the parts to distribute the marinade solution throughout the parts.

FIG. 3 is a side elevational view schematically illustrating the forced airstreams of the heating unit impinging against

and passing the meat parts about as well as the leakage of juices, etc. from the parts at higher elevation basting the parts at lower elevations.

FIG. 4 is respective illustration of the formation of the pouches in which poultry breasts are placed and sealed for pasteurizing according to a second embodiment present invention.

FIG. 5 is a schematic illustration of the second embodiment of the process for pasteurizing cut meat parts according to the present invention.

DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

Referring now in greater detail to the drawings in which like numerals indicate like parts throughout the several views, FIG. 1 schematically illustrates a first embodiment of the process according to the present invention for heat treating, or heat processing and thereby pasteurizing cut meat products or parts 10. In general, the meat parts 10 comprise cut sections or segments or carcasses such as poultry carcasses, beef or pork. For example, the process of the present invention can be utilized for heat treating, heat processing and thereby pasteurizing poultry parts for packaging, shipment and sale to retail and institutional buyers. Thus, the parts would include breasts, thighs, drumsticks, backs and fillets or tenders of meat all being processed together.

In the first embodiment of the present invention shown in FIG. 1, the parts 10 generally will be transported directly from a cut-up line (not shown) to a pasteurizing line 11. The parts need not be segregated by size or weight prior to being placed on the pasteurizing line. Instead, the present invention enables parts of different sizes, and irregular shapes, i.e. the legs, thighs, breasts, etc of poultry carcasses to be processed together without regard to differences in the size or shapes of the parts. As shown in FIG. 1, the pasteurizing line 11 includes a series of conveyor line 12 which carry the parts 10 through a series of processing stations 13–19 as the parts are moved along a processing path indicated by arrows 21. The parts are fed onto the pasteurizing line 11 at an inlet end 22 of the pasteurizing line by transfer means such as a conveyor or slide 23. The parts proceed through their various processing stations 13–19 for marination, pasteurizing and freezing of the parts after which the parts are discharged from the pasteurizing line at a discharge area 24 thereof for packaging of the frozen parts for shipment and sale.

As shown in FIG. 1, as the meat parts 10 begin their movement along the pasteurizing line 11 and along the processing path 21, they initially are moved into the first processing station 13 wherein the parts are marinated. Typically, the first processing station 13 will comprise a injection type marinating system 30 having a series of injection needles 31 mounted within a housing 32. A series of parts 10 are moved into the housing 32 whereupon the needles 31 are moved downwardly into engagement with the parts, piercing the skin and meat thereof. The needles inject a solution of water, salt and phosphates designed to maintain moistness or juiciness of the product during pasteurizing. The marinade solution is injected into the tissues of the parts, with the solution typically pooling in the pockets 33 formed adjacent the points of entry of the needles. It will also be understood by those skilled in the art that other blends of marinating solutions can be used with the present invention to marinate and provide moistness/juiciness to the meat parts. Further, other methods of marinating the parts such as emersion of the parts in marinating solutions can be used in place of the injection marinade system of the present invention.

As FIGS. 1 & 2 illustrate, once the parts have been injected with the desired amount of the marinade solution, the parts thereafter are moved into the second processing station 14 in which the parts are massaged. The second processing station 14 is a flipping station that includes at least two sets of flipping conveyor assemblies 36 & 37. Each of the flipping conveyor assemblies generally includes a substantially C-shaped first flipping conveyor 38 having an endless moving conveyor belt 39, and including an inclined upper run portion 40 with a proximal end 41 and a distal end 42, and a lower run 43 that extends substantially horizontally and has a proximal end 44 and a distal end 46. A second flipping conveyor 47 is received between the upper and lower runs 40 & 43 of each of the first flipping conveyors 38, extending downwardly at an angle substantially parallel to the upper run 40 of conveyor 38. Each second flipping conveyor 47, like each first flipping conveyor 38, includes an endless moving conveyor belt 48 and has an upper run 49 on which the parts are received and moved downwardly and rearwardly along the processing path 21 toward the proximal ends 44 of the lower runs 43 of the first flipping conveyors 38, and proximal and distal ends 51 and 52.

As illustrated in FIGS. 1 & 2, as the meat parts 10 move along their processing path 21 through the massaging station 14, the parts are moved through a series of flips. Generally, the parts are moved through four (4) flips as the parts are passed between the first and second flipping conveyors of the flipping conveyor assemblies 36 & 37. Initially, the parts are moved through the first flipping conveyor assembly 36 which tends to flip the parts onto their top from their bottom sides as the parts are moved off of the distal end 42 of the upper run 40 of the first flipping conveyor 38 onto the second flipping conveyor 47 in the direction of the arrows 53. Thereafter, the inverted parts are moved downwardly along the upper run 49 of the second flipping conveyor 47 toward its distal end 52 whereat the parts are flipped over and downwardly onto the lower run 43 of the first flipping conveyor 38 in the direction of arrow 54.

The parts thereafter are transferred from the distal end 46 of the lower run 43 of the first flipping conveyor 38 of flipping conveyor assembly 36 onto the proximal end 41 of the upper run 39 of the first flipping conveyor or flipping conveyor assembly 37, and again are moved upwardly at an angle and are flipped over and downwardly onto the upper surface of the second flipping conveyor of flipping conveyor assembly 37 in the direction of arrow 56. The parts then are moved off of the distal end of the second flipping conveyor of flipping conveyor assembly 37 flipping over and downwardly in the direction of arrow 57 onto the lower run 43 of the first flipping conveyor 38 of the flipping conveyor assembly 37. Such flipping motion tends to massage the meat parts 10 so as to cause the pockets 33 of marinade solution to become dispersed throughout the meat parts as the meat parts are flipped from side to side as indicated in FIG. 2. It further will be understood that other means for massaging or tumbling the meat parts can be utilized as well for applying pressure and causing the distribution of the marinade solution through the meat parts.

After the marinade solution has been distributed throughout the meat parts, the parts are moved from flipping conveyor assembly 37 through a third processing station 16 (FIG. 1). The third station 16 comprises an inspection station for the parts and includes an elongated conveyor 58 along which the parts are conveyed for inspection. The meat parts

also generally are reoriented in the third station **16** so that their skin sides are facing upwardly. This ensures that the skin sides of the parts will be facing upwardly as the parts are moved into the fourth processing station which comprises a heating unit **60** so that as the parts are moved through the heating unit, the juices that sweat or weep out of the parts as the parts are cooked fall downwardly onto the uncooked products below and tend to baste the skin and meat of the meat parts for moistness and natural flavoring.

As FIG. **1** illustrates, after being inspected and reoriented with their skin sides facing upwardly, the meat parts are moved into a heating unit which comprises the fourth processing station **17**. In general, the heating unit **60** comprises a large industrial heating unit such as, for example, a heating unit model GC076 manufactured by the Stein Company of Sandusky, Ohio. The heating unit includes a spiral flighted conveyor **61** that is substantially helically shaped and extends upwardly in a helical fashion from a lower inlet end **62** at the lower end **64** of the heating unit **60** upwardly to an outlet **64** at the top or upper end **66** of the heating unit **60**. The spiral conveyor typically is formed from a metal mesh or similar material so as to be capable of withstanding high temperatures without buckling or being destroyed. The conveyor further is perforated to enable juices, etc. which are excreted from the meat parts to pass through the conveyor and drip downwardly onto the meat parts at the lower elevations as indicated in by the dashed lines **67** in FIG. **1**.

As shown, the meat parts enter the heating unit **60** at the lower end **63** or bottom thereof, and are progressively moved upwardly in a spiral path toward the top **66** of the heating unit. The heating unit generally is heated to temperatures of 200° F. or less, and preferably in range of between 195° F. to 200° F., to heat treat or processes and thereby pasteurize the parts as they are moved through the heating unit. The parts can be heat treated or processed using dry air, 100% steam, or a mixture of dry air and steam as desired for maintaining the moistness etc. of the parts. The parts generally are so treated for approximately 45 to 50 minutes until pasteurized.

In order to pasteurize the parts thoroughly, the parts further are subjected to heated airflows or airstreams, indicated by arrows **68**, that are directed against and around the parts. Such impingement of the parts with the heated airflows further contributes to the deep, even penetration of heat through the parts. As a result of such increased pasteurizing times at lower heating temperatures, the meat parts are pasteurized throughout so as to cause the entire parts, including the meat and bones thereof, to be deeply and evenly heated. Accordingly, any bacterial contamination within the meat and bones of the meat parts is essentially eliminated. Further, the blood and marrow within the bones of the parts is denatured and the bones are caused to turn white so as to further reduce the chances of bacterial contamination and to provide the parts with a more pleasing, appetizing appearance.

At the same time, moving the parts from the lower elevations of the heating unit upwardly to the upper elevations of the heating unit for cooking further tends to reduce the chances of the parts being exposed to bacterial contamination within the heating unit itself due to leakage of juices, which might include some contaminating material, from the raw parts as they are initially treated or processed. The leakage of juices from the pasteurized parts, however, tends to baste the raw or unpasteurized parts without the danger of contaminating parts so as to further flavor and maintain moistness and juiciness of the parts. This results in a safe, easy to handle product that retains a pleasing appearance and taste and in which the moistness and juiciness of the parts is maintained.

After being pasteurized, the meat parts **10** are moved from the heating unit **60** into a precooler **70** that forms the fifth processing station **18**. The precooler generally comprises a chilling or cooling chamber **71** through which the parts are moved and cooled from their heated pasteurizing temperatures of between 195° F. to 200° F. The parts are exposed to temperatures typically in the range of −20° F. to 0° F. or less for approximately 30 to 40 minutes. As a result, the internal temperature of the parts is lowered to approximately 40° F. to 50° F. to help seal in the moisture and thus the natural flavoring of the parts.

Once the parts have been substantially cooled to approximately 40° F. to 50° F., the parts are introduced into the final processing station **19**, which comprises a freezing unit **75**. The freezing unit **75** is an open ended unit having a cooling chamber **76** with open inlet and outlet ends **77** and **78** and through which the parts pass in a substantially serpentine path. In the freezing unit, the parts are exposed to liquid nitrogen for approximately 20 to 30 minutes. Such exposure to liquid nitrogen reduces the core temperatures of the parts to approximately 0° F. or less. The parts thus are frozen solid as they move through the freezer to prepare the parts for packaging for shipment and later sale.

The carrying out of the freezing process in two steps wherein the parts are first precooled to approximately 40° F. to 50° F. prior to being frozen solid prevents freezer burn and advantageously enables the moistness and juiciness of the parts to be retained within the parts after being frozen. Typically, as freshly pasteurized parts, which generally are of the temperature of approximately 200° F. or greater in conventional processes, are exposed to a liquid nitrogen environment, such as in a freezer, the parts tend to become frozen very quickly. However, such extreme low temperatures further tend to cause the juices within the parts to leak or migrate towards the skin, tending to dry out the products. This affects the taste and consistency of the products when cooked and served by the end user. Progressively lowering the temperature of the parts prior to freezing avoids the potential for the marinade and juices within the parts to be leached out of the parts or drawn to the skin as occurs when hot, just cooled products are immediately frozen. The precooling thus avoids the parts becoming dried out and affecting their taste and texture as they are thereafter moved through the final processing station **19** for freezing.

The resultant product formed by the process of the present invention thus is substantially cleaned of bacterial contaminants so as to make the product much safer to handle and consume by the end consumer. In addition, the product formed by the present invention can be prepared and served by the end consumer in approximately 7 to 10 minutes, and provides a moist, flavorful product that has a very pleasing, appetizing appearance with the bones of the product being white and clean looking in appearance. The product can be served on a large institutional basis in a much more rapid and efficient manner than conventional quick frozen products, without sacrificing any of the taste, moistness, or appearance of the products, and which further provides a much safer product, in which the dangers of bacterial contamination thereof have been substantially eliminated.

DESCRIPTION OF SECOND PREFERRED EMBODIMENT

FIGS. **4** and **5** illustrate a second embodiment **100** of the present invention in which poultry breasts **101** are sealed and pasteurized in sterile pouches or packages **102**. As shown in FIG. **4**, the pouches **102** are formed from sheets of

a thermoplastic film **103** such as a 0.5 mil polypropylene/2 mil Surlyn, or either thermoplastic resin to 1.5 mil polypropylene/3 mil extended film such as is sold under the trade name Winpak OPSU 12503 or PSU 3875, which is flexible and can be sealed substantially air and moisture tight. The pouches generally are formed by first drawing pockets **104** within a first or lower sheet **106** of the thermoplastic film material. The pockets are formed, for example, by the application of a vacuum (not shown) or by drawing the pockets into a tray or a mold (not shown) over which the lower sheet of film **106** is being passed as it is moved along a processing path in the direction of arrow **107**.

The pockets **104** formed in the lower sheet of film **106** generally are sized and shaped so as to fit the poultry breasts therewithin and to accommodate poultry breasts of varying sizes. Typically, the breasts previously will have been deboned prior to being placed within the pockets as illustrated in FIG. 4. However, it will be understood by those skilled in the art that bone-in breasts additionally can be used within the pockets for processing in the method of the present invention. After the breasts have been placed in the pockets **104** formed in the lower sheet of film **106**, an upper sheet of thermoplastic film **108** is placed over the lower sheet of film, covering the breasts as shown in FIG. 4. The upper sheet of film is fed from a supply **109** downwardly over the top of the lower sheet of film and is sealed against the lower sheet of film such as by a vacuum means to form the sterile, sealed, pouches or packages **102**. The sealed sheets of thermoplastic film **106** and **108** thereafter generally are cut such as along lines **111** to separate the pouches from one another. The segmented pouches are then placed on racks **112** (FIG. 5) for pasteurizing.

As shown in FIG. 5, the racks **112** with pouches **102** containing poultry breast **101** are placed in a heating unit **115** for pasteurizing the poultry breasts. The heating unit **115** typically is a smokehouse type heating unit having a large pasteurizing chamber **116** in which the racks **112** can be placed, or which as an alternative can include internal racks on which trays of the poultry breast containing pouches can be placed for pasteurizing. Once the pouches have been placed within the pasteurizing chamber **116** of the heating unit **115**, the doors **117** of the heating unit are sealed and the heating unit is heated to approximately 190° F. to 200° F. The heat further is typically directed about the pouches in heated, forced airflows or streams of dry air, 100% steam or a mixture of dry air and steam, as indicated by arrows **118**. The pouches are maintained in the heating unit for approximately 15 to 20 minutes.

Conventional cooking methods for poultry breasts typically cook the poultry breasts at temperatures significantly greater than 200° F. for less than half the time of the pasteurizing process of the present invention. By pasteurizing at a lower heat of below 200° F. for an increased time of 15 to 20 minutes the present invention enables a thorough and even penetration of heat throughout the poultry breasts. This ensures that the poultry breasts are evenly and completely pasteurized to substantially eliminate any bacterial contamination therein. Additionally, the sealing of the poultry breasts within the pouches further tends to seal any natural juices and/or marinade solution within the pouches so that as the breasts are pasteurized, they tend to marinate themselves in their own natural juices so that they are naturally basted and thus their natural flavoring is sealed therein.

After the poultry breasts have been pasteurized, they are then precooled in the heating unit **115**. As shown in FIG. 5, a series of showers **123** are mounted within the pasteurizing chamber **116**. The showers spray the heated pouches **102** with chilled water sprays, indicated by dashed lines **124** for approximately 5 to 10 minutes. The water sprays chill the poultry breasts to approximately 60° F. to 70° F. prior to freezing of the pouches and their breasts. The pouches **102** containing the pasteurized poultry breasts **101** thereafter are moved from the heating unit **115** into a freezer **126**. The freezer **126** generally is a mechanical blast freezer in which the pouches are received and are exposed to temperatures of 0° F. or less for approximately 4 to 5 hours. As a result, the poultry breasts are frozen solid in a slow freezing process so as to seal the natural juices and flavoring within the poultry breast while avoiding freezer burn and loss of moistness and taste. Thereafter, the frozen pouches and their poultry breasts are packaged for shipment and sale.

The resultant pasteurized poultry breast product formed by the process of the second embodiment of the present invention thus is safer and easier to handle and prepare by the end consumer than products prepared by conventional cooking processes as any bacterial contamination therewithin essentially has been removed during the pasteurization of the poultry breasts. Additionally, the pouch sealed poultry breast products formed by the process of the present invention are extremely versatile in that the cooking and preparation of these products can be done by the end user either on a grill or in an oven, or even can be cooked by emersion of the pouch in hot water with the time to reconstitute the poultry breasts generally being less than 7 to 10 minutes. Thus, the poultry breast products formed by the process of the present invention can be quickly and easily prepared and served to a consumer without requiring a full kitchen set up of a grill, etc. to cook the parts. This significantly reduces the amount of cooking equipment need by restaurants, etc by reducing preparation and service times for the product.

Various features, modifications and advantages of the present invention will become apparent to those skilled in the art upon reading the following detailed description when taken in conjunction with the accompanying drawings.

What is claimed is:

1. A method of preparing packaged, pasteurized meat parts comprising:

placing the meat parts on a first sheet of thermoplastic material;

placing a second sheet of thermoplastic material against the first sheet to secure the meat parts within pouches of thermoplastic material;

heating the pouches with the meat parts therein for 15 to 20 minutes at temperatures of less than 200° F. to pasteurize the parts throughout to substantially eliminate any bacterial contamination within the parts to provide a safe to handle and conserve product;

precooling the pouches with the parts therein; and

freezing the parts in their pouches.

2. The method of claim 1 and wherein the steps of precooling comprises spraying the pouches with water sprays.

3. The method of claim 1 and wherein the parts include poultry breasts and the step of heating the pouches comprises placing the pouches with the breasts therein in an oven for approximately 15 to 20 minutes.

4. The method claim 1 and further including the steps of forming pockets within the first sheet of thermoplastic material in which the meat parts are received.

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ORIGINAL ARTICLE

On the compositing of samples for qualitative microbiological testing

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Summary

The introduction of legislative microbiological criteria for foods [Official *J L338* (2005) 1] has increased the exposure of enforcement and industrial laboratories to the need to test multiple food samples for organisms such as salmonellae. A consequence has been an increase in the number of organizations, both official and commercial, considering whether it is permissible to composite replicate sample units for the purposes of assessing compliance with the criteria. This note summarizes the statistical and practical aspects of compositing sample units for compliance testing. Provided that the method of choice is 'fit for purpose', then there is no statistical difference between testing, say, 30×25 g sample units or 3×250 g sample units. However, compositing of sample units should be done only when the method has been demonstrated unequivocally to be sufficiently sensitive to detect potentially lower numbers of target organism(s) in the quantity of composited sample under the conditions of test. Different approaches to compositing of samples are considered.

Introduction

Microbiological criteria (e.g. those defined in Anon. 2005) in some instances impose a need to test a significant number of sample units from individual production lots for the presence of pathogens such as salmonellae. Examples of such criteria are given in Table 1.

Official agencies and manufacturers seeking to confirm that commercial products comply with the legislative criteria need to obtain an adequate number of representative samples of the manufacturing batch, or 'lot'. They need also to instruct a laboratory to undertake appropriate testing, which has significant cost and workload implications with large numbers of samples.

A consequence is that both official agencies and industrial organizations are unsure regarding the validity of compositing sample units and some have used approaches that are neither statistically nor technically valid. It seemed appropriate therefore to provide a brief account of the issues to be considered in respect of compositing.

Background

The concept of compositing of sample units is not new. It is widely used in the preparation of analytical samples for chemical and physical analyses because by efficient blending of a number of sample units, an analytical sample can be taken that is more truly 'representative' of a 'lot' (Patil 2002; Anon. 2003). The effect of compositing is to generate a sample that will be more 'typical' of a batch than will individual samples tested separately as the 'between sample' variance is reduced and the analytical result reflects more closely the true composition of the lot. Such procedures have been used widely in chemical analyses including those for trace contaminants such as mycotoxins in foods, for generic assessment of microbial populations in soil and water, for environmental studies of avian faecal microflora, etc. Note, however, that these analyses are quantitative in nature.

When a quantal analysis is used, i.e. one that is based on the presence or absence of a specific chemical or microbial contaminant, the ability of the test procedure

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Table 1 Some European legislative food safety criteria (Anon. 2005)

Micro-organism	Sampling plan		Limits	Test method	Comment
	n	c			
Salmonella spp*	30	0	Absence in 25 g	Anon. (2002)	Products placed on the market during their shelf-life
*Enterobacter sakazakii**	30	0	Absence in 25 g	Anon. (2006)	
Listeria monocytogenes†	10	0	Absence in 25 g	Anon. (1996)	

n, number of sample units; c, number of sample units permitted to exceed the defined limit.
*Dried infant formulae.
†Ready to eat infant foods.

to detect that contaminant at a meaningful level is paramount. When sample units have been composited, this issue becomes even more critical. For instance, if a 25-g sample unit contains a single viable target organism and that sample unit is composited with nine further sample units each of which is free from the target organism, then the overall incidence of contamination is reduced from one organism in 25 g to one organism in 250 g. So the critical issue is whether or not the test methodology can detect such a low level of contamination.

The work of Silliker and Gabis (1973) was quoted by ICMSF (1978) who claimed, 'multiple analytical units may be combined or "lumped" to provide larger units for testing purposes'. Furthermore, they stated, '60 25-g units selected from a particular product lot may be combined ... to produce three 500-g samples for analysis. If all three 500-g samples are ... negative ... then the inference is that the level of *Salmonella* is no greater than one in 500 g'. This statement is at best an over-simplification. Silliker and Gabis (1973) actually showed that if the test material has a high level of contamination, then compositing made no difference to the results obtained, but at lower levels of contamination the use of composited samples could give rise to false-negative results. More recently, ICMSF (2001) withdrew somewhat from their earlier position and stated, 'Numerous studies since 1974 have continued to demonstrate the merits and limitations of compositing. Experience may vary for different food–pathogen combinations and analytical methods, indicating a need for caution and validation that compositing is acceptable'.

Quantal analysis for the presence or absence of a specific organism is predicated on the ability of the test procedure to detect a very small number of the target organisms in a defined quantity of sample. Usually, such tests involve nonselective pre-enrichment culture followed sequentially by transfer of an inoculum to a selective enrichment medium, plating onto selective/diagnostic agars and, finally, purification of organisms giving typical reactions and their subsequent identification. Whether or not a specific organism is transferred from the pre-enrichment stage, or indeed from the enrichment stage, will depend on:

i. The initial level of contamination of the test sample unit by both the target organism and competitive organisms;
ii. The initial condition (e.g. sublethal damage) of the target organism and the competitors; and
iii. The growth kinetics (length of lag phase, specific growth rate, etc.) of target and competitive organisms in the conditions of the test (time, temperature, presence of inhibitors, etc.)

Possibly, the most important practical issue is the period of incubation of the pre-enrichment culture. Short incubation periods may not provide sufficient time for adequate recovery and growth of sublethally damaged target organisms (Jarvis 1989). Growth of the target organism must reach a critical level such that it is 'beyond reasonable doubt' that the organism will be transferred from the pre-enrichment stage to the enrichment medium. In my view, this requires at least a 99.9% probability that one or more viable organisms will be transferred in the inoculum. Assuming random distribution of organisms in the pre-enrichment culture, the probability of not transferring any organisms ($P_{x=0}$) in an inoculum of 1 ml can be derived from the Poisson distribution. Table 2 gives the probabilities for $P_{x=0}$ and $P_{x\geq 1}$ for different levels of viable organisms in a culture. To be certain of transferring at least one organism in 1 ml of the pre-enrichment culture requires an average level of occurrence of at least seven viable organisms per ml of the culture. Similar considerations apply also to the transfer of organisms from enrichment culture to a diagnostic agar plate.

Suppose there is one sublethally damaged target organism per 25 g of sample diluted into 225 ml medium; to obtain at least seven target organisms per ml of incubated pre-enrichment culture requires a total population of 1750 organisms per 250 ml culture. This will require at least 11 generations ($2^{11} = 2048 > 1750$) of the organism once the organism passes from the lag phase into the exponential growth phase. Depending upon the incuba-

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Table 2 Statistical probability of transferring less than, or greater than, one viable organism in an inoculum of 1 ml, assuming random distribution of the target organism in a pre-enrichment culture

Average level of target organisms per ml of pre-enrichment culture	Probability that 1 ml of inoculum will contain	
	0 organisms	1 or more organisms
1	0·368	0·632
2	0·135	0·865
3	0·050	0·950
4	0·018	0·982
5	0·006	0·994
7	0·001	0·999
10	<0·001	>0·999
20	<<0·001	>>0·999

tion temperature and the possible inhibitory effects of competitive organisms, this may require more than 12-h incubation of the pre-enrichment medium (Jarvis 1989). If the inoculum were smaller (e.g. one viable organism in 250 g of composited sample), the minimum incubation time could be longer than 12 h.

Similar constraints apply also to newer developments in microbiological test procedures, e.g. the use of immuno-magnetic beads as an aid to more rapid isolation of target species and even PCR techniques require a minimal amount of recoverable DNA to detect an organism.

Microbiological test procedures

Two key issues need to be considered: the sensitivity (i.e. the level at which a target organism can be reliably detected) and the specificity of the procedure. For quantal tests, both sensitivity and specificity are critical. A procedure that is capable of detecting, say, one viable cell of a specific organism in a defined quantity of a food material may not be capable to detecting one viable organism if the quantity of the test material is increased 10- or 20-fold through compositing of sample units. The more sensitive the test, the lower the level of organisms that is likely to be detected under the conditions of the test.

A test that is highly specific will detect only the chosen species or strains of an organism and will not detect closely related organisms that may be of equal importance in relation to food safety. Such a test procedure may therefore be unreliable as it is likely to lead to genuine 'false-negative' results. A procedure that has low specificity may detect related strains of organism that are not of public health concern, creating 'false-positive' results. To be of practical value in food microbiology, the method must be capable of detecting the 'most significant' strains of the target organism at a low level of contamination. A method designed to detect the presence of high levels of specific pathogens in heavily contaminated material, e.g. faeces, may be of limited value in seeking to detect very low, but possibly significant, levels of the organisms in foods.

Unfortunately, practitioners sometimes ignore such limitations and assume that a standard procedure will always detect the target organism if it is present in a sample.

Microbiological sampling plans and criteria

Criteria, such as those defined by Anon. (2005) using presence or absence tests for specific organisms, are based on two-class sampling plans. In general, sampling plans for pathogenic organisms such as salmonellae require that no target organisms should be detected in a given quantity of each of several sample units of product drawn from a lot. In a few cases (e.g. for coagulase-positive staphylococci), a less stringent plan may be used.

The stringency of the sampling plan is dependent on the number (n) of sample units of a given size (weight, volume) to be tested and on whether one or more samples (c) can be accepted if a positive result is found. A sample plan is designated as e.g. $n = 5$ (or more), $c = 0$ if no positive results are acceptable. However, there are two primary considerations that need to be understood:

i. Any result that can be classified on the basis of a presence or absence result is governed by the binomial distribution; hence, the stringency of the sampling plan will be increased if n is large (Table 1) but no matter how many sample units are tested with negative results, it can never be stated unequivocally that the lot from which the samples were drawn is totally free from contamination by the target organism, although confidence limits (CLs) can be established on the 'zero' result (see below).

ii. The typical application of the binomial distribution is predicated on the assumption that the target organisms will be distributed homogeneously (i.e. randomly) throughout the sample and that there is an equal opportunity for any sample unit to contain the target organism. In practice, such even distribution of low numbers of pathogenic organisms in food products occurs only rarely. Dried milk powders, infant formulae and chocolate are but some of the foodstuffs where there is evidence of microbial clumping in a small proportion of the lot. Thus, more accurate models that account for the non-homogenous distribution would depend on having some knowledge or understanding of the clustering, which, in most applications, is not available. However, if a homogeneous distribution of organisms throughout the material being tested is assumed, then it is possible to express the probability in terms of the assumed constant organism density, using the Poisson distribution.

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The stringency of sampling plans

The data in Fig. 1 illustrate the statistical probability for acceptance of a product (i.e. the probability that no positive tests are obtained) for different levels of 'true' prevalence of defective units in the lot (i.e. prevalence of contamination) and the number of sample units (n) tested. If negative results are obtained on 30 samples and the 'true' prevalence is 2%, the likelihood of accepting the product is 55% (conversely, the probability of rejection is 45%), but if only five samples are tested the probability of accepting the product is 90%. Similarly, if the lot contains 10% 'true' defective units, then the likelihood of product acceptance after testing 30 sample units with negative results will be only 4%, but if five samples are tested the likelihood of acceptance is 59% and if only one sample is tested, with a negative result, there is a 90% chance of accepting the lot with 10% 'true' defectives. These data are obtained from a rearrangement of the binomial distribution equation:

$$P_{(x>0)} = 1 - (1 - d)^n \qquad (1)$$

where d is the true prevalence of contamination of the lot and n is the number of sample units.

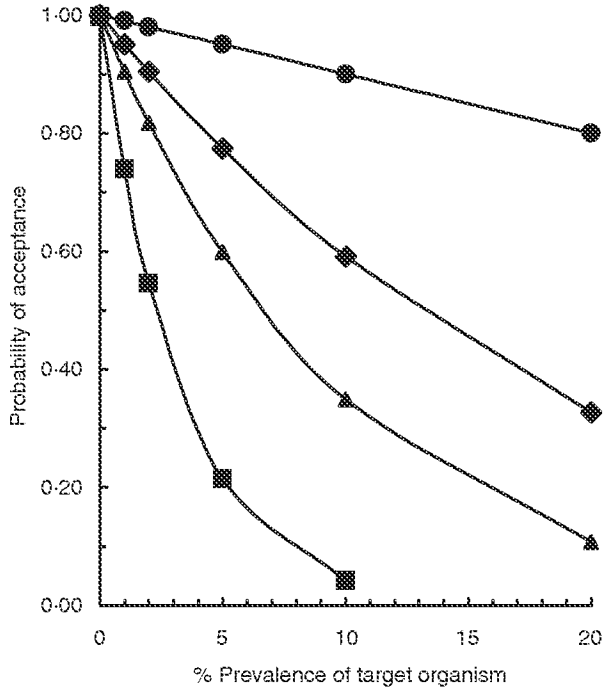


Figure 1 Operating characteristics curves for probability for acceptance of samples having a prevalence of contamination from 0% to 20% and various numbers (n) of samples examined from $n = 1$ (●), $n = 5$ (◆), $n = 10$ (▲) and $n = 30$ (■).

In setting a criterion for the number of samples to be tested, an assumption is made about the incidence of 'true' defective (i.e. contaminated) sample units that are acceptable in relation to the number of replicate tests that can reasonably be performed. For instance, a criterion having a sampling plan of $n = 30$, $c = 0$ would be likely to reject a product having 10% or more defective units with a better than 95% probability. For any given true prevalence of salmonellae in a lot and assuming the distribution of contaminated units amongst a lot is random and then by rearranging eqn (1), we can determine the number of sample units (n) that it would be necessary to test for a defined incidence of contamination and a given probability:

$$n = \log_{10}(1 - P_{(x>0)}) / \log_{10}(1 - d) \qquad (2)$$

If a lot contains 1% defectives ($d = 0{\cdot}01$) and $P_{(x>0)} = 0{\cdot}95$, we can use eqn (2) to calculate the number of sample units (n) that must be tested to have a 95% chance of obtaining at least one positive result:

$$n = \log(1 - 0{\cdot}95) / \log(1 - 0{\cdot}01) = \log 0{\cdot}05 / \log 0{\cdot}99 = 298$$

Table 3 summarizes the minimum number of samples to be tested for different levels of 'true' contamination to be able to give at least a 90% assurance that the sample units tested are not contaminated. Even if all the test results are negative, little statistical assurance of freedom from contamination can be given for small numbers of sample units tested.

If all sample units test negative, then it is possible to assign CLs for the true prevalence of contamination, reflecting the upper bound of the contamination prevalence at a given confidence level $(1 - \alpha)$. These CLs are bounded by 0 and P_u, where P_u is chosen so that if the

Table 3 Number of sample units to be tested with negative results in order to be able to give a 90%, 95% or 99% assurance that the sample units comply with a criterion for freedom from a defined target organism in a given number of sample units [based on eqn (2)]

True incidence of defective sample units (%)	Number (n) of sample units required to be tested		
	$P_{(x=0)} = 0{\cdot}90$	$P_{(x=0)} = 0{\cdot}95$	$P_{(x=0)} = 0{\cdot}99$
10	22	28	44
5	45	58	90
2	114	148	228
1	229	298	458
0·5	459	598	919
0·1	2301	2994	4603
0·05	4604	5990	9208
0·01	23 025	29 956	46 049

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true prevalence were P_u, then the probability of the observation of no positive results in n samples is equal to α. Thus, P_u satisfies

$$(1 - P_u)^n = \alpha \quad (3)$$

We can solve this equation for P_u by rearrangement:

$$P_u\,(\%) = 100(1 - \sqrt[n]{\alpha}) \quad (4)$$

For example, with $n = 30$ and $\alpha = 0{\cdot}05$, we obtain

$$P_u\,(\%) = 100(1 - \sqrt[30]{0.05}) = 9{\cdot}5\%$$

For this example, the CLs of 0–9·5% provide a 95% confidence of enclosing the true prevalence of contamination (τ%). In other words, if the same sampling and testing procedure was repeated a very large number of times, then this interval would be expected to enclose τ on 95% of occasions; but on 5% of occasions the value of τ would exceed 9·5% (by an unknown amount).

Hence, if we test 30×25 g sample units with negative results, we have a 95% confidence that the true incidence of defectives would not exceed 9·5%; for 25-g sample units, this is equivalent to a prevalence level of four organisms kg^{-1}. However, if we test only 1×25 g sample unit, then the 95% upper CL would give a possible prevalence of 120 organisms per kg. Further examples of 95% and 99% CLs are shown in Table 4.

Table 4 Percentage defective sample units and maximum contamination levels assuming random distribution of target organism and assuming 100% test effectiveness (i.e. any target organism in the sample would be detected) (modified from Jarvis 1989)

No. of sample units tested (n)	% Defectives (τ%)* at		No. of organisms per kg*† at	
	$P_U = 0{\cdot}95$	$P_U = 0{\cdot}99$	$P_U = 0{\cdot}95$	$P_U = 0{\cdot}99$
1	95·0	99·0	120	184
2	77·6	90·0	60	92
3	63·2	78·5	40	61
4	52·7	68·4	30	46
5	45·1	60·2	24	37
10	25·9	36·9	12	18
20	13·9	20·6	6	9
30	9·5	14·2	4	6
50	5·8	8·8	2	4
100	3·0	4·5	1	2

*Assuming no positives detected, the true incidence will not be more than the values shown with indicated confidence.
†Rounded to the nearest whole number and assuming sample size = 25 g.

To composite or not to composite?

Suppose the criterion requires that 30 sample units each of 25 g should be tested for the absence of salmonellae and the test procedure is capable of detecting one viable *Salmonella* in a 25-g sample unit. To carry out the test, it would be necessary to inoculate each of the 30×25 g sample units into a 225 ml volume of buffered peptone pre-enrichment medium and after incubation for 18 h at 37°C, to subculture 1 ml of the pre-enrichment culture into 10 ml of each of two enrichment media which are then incubated for 24 h, before subculturing onto each of two diagnostic media. After incubation, colonies showing typical characteristics need to be purified and tested to confirm, or rule out, that they are salmonellae. Hence, for a single set of 30 samples, the laboratory would need to set up 30 pre-enrichment cultures, 60 enrichment cultures, 120 diagnostic plates and possibly as many as 600 identification cultures. This is an enormous workload to provide evidence of compliance with a defined criterion for just one set of samples.

To reduce this workload, it is sometimes recommended that the initial sample units should be composited into a smaller number of larger test samples. The following scenarios describe the practical implications of compositing:

i. *An invalid approach*: Composite the 30×25 g sample units (i.e. 750 g) into a single well-mixed sample and test one analytical sample unit of 25 g. With this option, only one representative sample unit of 25 g is tested and a negative result is meaningless as the test has not complied with the requirements of the criterion (i.e. to test 30×25 g sample units). At best, the statistics of this test would merely indicate, with 95% confidence, that the average prevalence of salmonellae would likely not exceed 120 salmonellae per kg (whereas negative results from all tests on 30 samples indicate, with 95% confidence, a likely average density of no more than four salmonellae per kg).

ii. *A compromise approach*: Composite the 30×25 g sample units randomly into three sets each of 10×25 g sample units and inoculate each 250 g composite sample into a volume of 2·25 l of pre-enrichment medium. After incubation, carry out the subsequent cultivation procedure based on three pre-enrichment cultures. This approach is valid provided the ability of the method to detect one viable *Salmonella* in 250 g of product has been validated; if not, then this compromise approach is not permissible.

iii. *My preferred approach ('wet' compositing)*: Set up 30 individual pre-enrichment cultures and after incubation transfer 1 ml of each of the 30 pre-enrichment cultures into 300 ml of each of two enrichment media. This would then provide composite enrichment cultures on which further work can be done. The original pre-enrichment media can be stored, pending the outcome of the tests,

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such that if a positive result is obtained it is possible to go back to the original pre-enrichment cultures to assess by further testing how many contaminated samples are present. The benefit of this approach is that the concept of testing 30 samples has not been compromised, as compositing has taken place only after the first culture stage

Statistical considerations

Provided that the test procedure is sufficiently sensitive to detect a single *Salmonella* in the larger quantity of sample, then from a statistical perspective there is no difference in testing 1×750 p_g, 3×250 p_g or 30×25 p_g samples. But if there is any doubt as to the level of sensitivity of the test procedure, then compositing should not be done, except using the 'wet' compositing approach where each individual sample unit is pre-enriched separately.

Regarding the contention, given above: assume n sample units, each unit (i) being of size k_i grams with an organism density of δ_i organisms per g. Then, provided that the test procedure is sufficiently sensitive to detect the organism if present, the probability ($P_{x=0}$) of a negative result on any sample i is given by the Poisson probability of finding zero organisms:

$$P_{(x_i=0)} = e^{-\delta_i k_i} \tag{5}$$

where δ_i is the expected prevalence of contamination and k_i is the size of sample. If $\delta_i = 0{\cdot}1$ organisms per g and $k_i = 25$ g, then the probability of not detecting any organism in the 25-g sample (assuming the test protocol is 100% effective) is given by

$$P_{(x_i=0)} = e^{-0{\cdot}1\times 25} = e^{-2{\cdot}5} = 0{\cdot}082$$

When the probability of detecting zero organisms is 5%, the product of the density (organisms per g) and sample size (g) is equal to about three organisms. Thus, the upper 95% CL of the density, given a negative result, is 3 divided by the sample size. For example, with 25 g tested and a negative result, the upper 95% limit density is $3/25$ g $= 0{\cdot}12$ g^{-1} $= 120$ organisms per kg (Table 4).

The probability of finding a negative result on all n units is the product of the individual probabilities for a negative result in each unit:

$$P_{(x_1=0)(x_2=0)(x_3=0)\cdots(x_n=0)} = e^{-\delta_1 k_1} \times e^{-\delta_2 k_2} \times e^{-\delta_3 k_3} \times \cdots \times e^{-\delta_n k_n} = e^{-\sum \delta_i k_i} \tag{6}$$

To illustrate this effect, suppose that we have 3×250 g sample units with levels of 0·01, 0·00 and 0·02 viable organisms per g, respectively, then the total organism count in the three sample units will be 2·5, 0·0 and 5·0 organisms per 250 g, respectively. The probability of not detecting a positive result in the individual sample units will be 8·2, 100 and 0·7%, respectively. The combined probability of a negative result on the three sample units will be given by:

$$P_{(x_1=0)(x_2=0)(x_3=0)} = e^{-\sum \delta_i k_i} = e^{-\sum (2{\cdot}5+0+5{\cdot}0)} = e^{-7{\cdot}5} = 0{\cdot}00055$$

This is the same probability (0·055%) that would be found by using the mean organism count (0·01 organisms per g) and multiplying by a k value of 750 g. What these formulae show is that, when the testing is 100% effective, the probability of detection just depends on the total quantity of sample tested, not on the number of individual tests.

However, if the test procedure cannot detect the target organism at such a low level (i.e. 0·009 organisms per g in a combined 750-g sample unit) then a different situation pertains. Equation (5) is valid only if it is assumed that the viable organisms in the k grams would be recovered. If this were not the case, then another factor is needed to account for the possibility that organisms would not be recovered. For example, if there were one viable organism in the k grams, we could assume the probability of its not being recovered is r_1; if there were two organisms, we could assume that the probability of not recovering any organism is r_2; and so forth, if there were g organisms, the probability of not detecting any of them would be r_g. A simplifying assumption (just to demonstrate the mathematics) would be to assume that $r_g = r^g$, for g organisms. With this simplifying assumption, it can be shown that the probability of not detecting any organism would be

$$P_{(x=0)} = e^{-dk(1-r)} \tag{7}$$

where r is the probability of not detecting a single organism if present in the k grams. In the example given above, it was assumed that $r = 0$, i.e. that any organism would be detected if present in 25 g. However, for 750 g, r might no longer be zero, but rather some positive value. If this were the case, the probability of a negative could increase quite substantially. For example, if the density was 0·01 organisms per g and $r = 0{\cdot}25$, then the probability of a negative result is 0·361% compared with 0·055% when $r = 0$ was assumed. Hence, there would be an increased factor of 6·5 of getting a negative result.

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Hence, unless the recovery of organisms is independent of the volume being tested, testing larger quantities of sample will be less sensitive than testing individually smaller quantities of samples. This condition does not seem likely to be satisfied, particularly for large volume composite samples. Thus, it would be incorrect to indicate a possible level of contamination when the sample units are composites assuming 100% testing effectiveness; negative results might be due to the inability of the method to detect the target organism, and thus such conclusions would be erroneous.

Conclusion

Before compositing samples for testing, it is essential to validate the test procedure for each food matrix to ensure that the method sensitivity is sufficiently high to detect the presence of the target organism in the increased quantity of sample. Validation requires that the sensitivity of the method must be determined, ideally using naturally contaminated food matrices, for the lowest possible level of contamination that might be encountered in composited samples. In practice, this requires that the method is sufficiently sensitive to detect one viable *Salmonella* in the largest quantity (e.g. 250 or 500 g) of the food matrix likely to be examined. If such a level of contamination cannot be detected reliably, even though one organism in 25 g can be detected, then no certainty exists that the method is suitable for used with composited sample units. Without such certainty, the test result may not indicate the presence of contaminants even if target organisms are present in one or more of the original sample units. Such false-negative results will be grossly misleading and the test protocol will not comply with the requirements of the microbiological criterion. In such circumstances, the only reliable method is to 'wet' composite, i.e. to composite aliquots of incubated pre-enrichment cultures prepared form each individual sample unit.

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