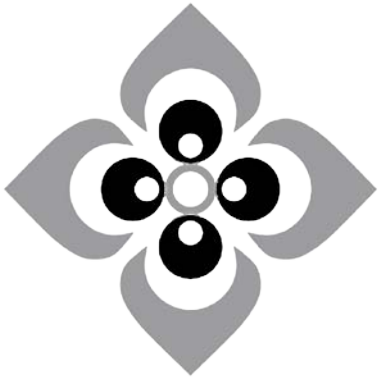

Consortium for Educational Communication

Module
on
Enumeration Of Microorganisms In Foods

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TEXT

Introduction

Microbiological assessment for quality and safety of foods traditionally relies upon the enumeration and specific detection of pathogenic and spoilage microorganisms. Conventional testing methods make use of growth media and cultivation to enumerate and isolate cells of microorganisms from food samples brought into suspension with diluents. Isolated colonies of microbes are then subjected to a series of biochemical, physiological and serological tests in order to find species identity and subtypes of the microorganisms in question. These methods can provide both quantitative and qualitative information on the importance of microorganisms present in foods, and have been verified over the years. However, testing methods relying on cultivation are generally slow and give results after a period of several days; moreover they use a lot of material and labor. Modern quality management and control systems, such as good manufacturing practice (GMP) and hazard analysis and critical control point (HACCP) systems require methods and techniques that can be used on-line and give results in real-time. Hence, food microbiologists seek more rapid, sensitive and specific methods to get adequate information in due time to monitor the safety and quality of products. In recent decades a number of improved conventional and alternative non-traditional methods and techniques have been developed that appear suitable for early detection and characterization of microorganisms significant in foods.

Significance

The total microbial population in a food varies greatly and depends on:

- The level of sanitation used at all phases.
- The degree of abuse that leads to microbial growth.
- The processing and preservation methods used to kill and prevent growth of microorganisms.

Contamination of a food by specific types or species of microorganisms depends on the presence of a source of these microorganisms, and their entrance into the food mostly due to poor sanitation during handling and processing.

Micro-organisms in Foods

Micro-organisms, in relation to food can have the following roles:

- Spoilage or saprophytic microorganism play a role in biodegradation and cause food spoilage
- Pathogenic microorganisms can cause infections or intoxications

Saprophytic microorganisms

Saprophytic microorganisms that result in food spoilage include bacteria, moulds and yeasts. Spoilage is the change of look, consistency, flavor and odor of foods.

Bacteria: Examples of action of bacteria involved in food spoilage:

Lactic acid formation: Lactobacillus, Leuconostoc

Lipolysis: Pseudomonas, Alcaligenes, Serratia, Micrococcus

Pigment formation: Flavobacterium, Serratia, Micrococcus

Gas formation: Leuconostoc, Lactobacillus, Proteus

Slime or rope formation: Enterobacter, Streptococcus

Moulds: Some strains produce mycotoxins under certain conditions

1. Aspergillus produces aflatoxin, ochrtoxin, citrinin and patulin
2. Fusarium
3. Cladosporium
4. Alternaria

Mycotoxins can penetrate into the parts of food that are not visibly mouldy as well. It is therefore necessary to throw away all of the food if any part of it is mouldy. They are also notoriously difficult to destroy as they are stable to both heat and chemicals. These toxins include:

- Hepatotoxins: aflatoxins, sporidesmins, luteoskyrin
- Nephrotoxins: ochratoxin, citrinin
- GIT toxins: trichocetens
- Neuro- and myotoxins: tremorgens, citreoviridin
- Dermatotoxins: verukarins, psoralen, sporidesmins, trichocetes
- Respiratory tract toxins: patulin

Foods most at risk for moulds:

1. Grains and grain products - many mycotoxin types

2. Peanuts, nuts and pulses - aflatoxin

3. Fruits and vegetables (raw and preserved) - patulin

4. Milk and milk products - aflatoxin

It is important to note that if any contaminated fodder is fed to animals, this is metabolized and the toxic derivatives can be found in animal products consumed by humans, e.g. milk and meat.

Pathogenic micro-organisms

Pathogenic micro-organisms cause food-borne infections or intoxication, and include bacteria, viruses, parasites and moulds **.** It is important to note that pathogenic bacteria and viruses usually do not cause food spoilage, their contamination cannot be seen nor tasted. The main factors that contribute to occurrence of food borne diseases are:

- The use of raw food and ingredients from unsafe sources
- Inadequate cooking or heat processing
- Improper cooling and storing, for example leaving cooked foods at room temperature for longer periods of time, or storing foods in large containers in the fridge
- Allowing several hours to pass between preparation and eating of food
- Inadequate reheating

- Improper hot holding, meaning below 65°C
- Food handling by infected persons or carriers of infection
- Cross contamination from raw to cooked food. For example by cutting vegetables for salad on a cutting board where you have cut raw meat before
- Inadequate cleaning of equipment and utensils

Bacteria

Campylobacter jejuni: Is a common cause of diarrhea humans as well as some animal species. The transmission can be by direct contact between humans and infected animals or their feces. More commonly, it is transmitted by the consumption of contaminated food or water, person-to-person spread. The symptoms range from mild diarrhea to sever invasive disease which can include abdominal pain, fever, and blood and mucous in stools.

Non-typhi salmonellosis: There are more than 2000 serotypes of salmonella spp, of which only a few cause Salmonella gasteroenteritis in humans. The symptoms include acute watery diarrhea accompanied by nausea, cramps and fever. Blood in stool may occur. Animals are the main reservoir, and transmission occurs by ingestion of contaminated products. Foods especially at risk are poultry, meat, eggs and milk.

Salmonella typhi and paratyphi: Cause typhoid fever and paratyphoid fever respectively. Since the reservoir for both these bacteria are usually humans, transmission occurs mainly through person-to-person contact or contamination of food by food handlers.

Staphylococcus aureus: The source of this infection are humans. The bacteria are often found in smaller amounts in the nose and on the skin of clinically healthy people. Higher amounts can be found in lesions of skin such as infected eczema, psoriasis or any other pus draining lesion. These people should therefore not be handling food. Food poisoning caused by this bacteria is caused by heat resistant staphylotoxin, resulting in diarrhea, vomiting, cramps and fever. The symptoms start suddenly and usually disappear within 24 hours.

Escherichia coli: There are several serotypes, some of which are harmless to humans whereas others can cause gastroenteritis. Enterotoxigenic E.coli is the most common cause of traveller's diarrhea. The source is humans, and transmission usually occurs through contaminated food and water.

Listeria monocytogenes: This bacterium is highly associated with food stored for long periods of time in the fridge because it is ubiquitous, and has the ability to grow slowly, even at low temperatures. It Can be fatal in immunocompromised persons, where it can cause septicemia and meningitis.

Shigella: The source is humans and primates. Because it has low infectious dose, the main mode of transmission is person-to-person contact. It can also be transmitted through infected food and water. The symptoms of shigellosis are fever and watery diarrhea. The infection can also manifest as a dysenteric syndrome which includes fever, abdominal cramps and tenesmus, and frewuent, small volume, bloody stools containing mucous.

Vibrio Cholerae: The source of this infection is humans. The main mode of transmission is through contaminated water and food, or person-to-person spread in overcrowded, unhygienic situations. It causes severe watery diarrhea, which can reach up to 20 liters per day.

Clostridium Botulinum: Its source is the intestinal tract of fish, birds, and mammals. It is also widely distributed in nature. The bacterium is a spore producing anaerobe, with a highly potent heat labile toxin that affects the nervous system.

Viruses

Viruses, unlike bacteria, cannot multiply in foods. The main mode of transmission therefore by food handlers and the use of dirty utensils, which transfer the virus to food whereupon it is ingested by humans. Rotaviruses and Norwalk virus are the major causes of gastroenteritis. Viral hepatitis A outbreaks are mainly caused by asymptomatic carriers which handle food.

Parasites

Many parasites, such as the helminths, have a complex lifecycle involving more than one host. The major route of transmission for these parasites to humans is by the route of food. The consumption of undercooked pork or beef, or the consumption of raw salads washed in contaminated water seems to be the trend.

Taenia solium and T. saginata: also called pig and beef tapeworms. Their cysts, present in the muscle of the animal are ingested and the adult worm develops in the gut. The ova may develop into larvae that may invade other tissues, such as the brain, forming cysticercosis and severe neurological disorders as a consequence.

Trichinella spiralis: is found in undercooked pork. The larvae can invade tissues and cause a febrile illness.

Giardia lambila: This infection can be foodborne, waterborne or spread by interpersonal contact. It causes acute or subacute diarrhea, with malabsorption, fatty stools, and abdominal pain and bloating.

Entamoeba histolytica: The transmission is mainly food- or waterborne. The cysts pose a major problem since they are highly resistant to chemical disinfectants, including chlorination. The infection is usually asymptomatic, but may appear as either a persistent mild diarrhea or a fulminant dysentery.

Methods used for Enumertion of microorganisms in foods

These methods are whether qualitative or quantitative.

Quantitative methods

Quantitative methods are designed to enumerate or estimate directly or indirectly the microbial load in a test material. None of the quantitative methods used now enumerate or estimate total microbes, total bacteria, or total viable population, rather each method enumerates or estimates a specific group among the total microbial population normally present in a food and that grows or multiplies preferentially under the conditions or methods of testing. These include composition of an enumeration medium, temperature, time of incubation, oxygen availability, pH, and treatments of a sample before enumeration and estimation.

Examples of quantitative methods:

Aerobic Plate Count (APCs), or standard Plate Count (SPCs) for dairy products. Anaerobic Counts, Psychrotrophic Counts, Thermoduric Counts, Coliform Counts, *S.aureus* Counts, Yeast and Mold Counts.

Qualitative methods

They determine whether a representative amount (a sample) of a food or a certain number of samples in a batch of a food contain a specific microbial species among the total microbial population. Qualitative methods are used to detect the possible presence of certain food borne pathogens, especially those capable of causing high fatality rates among consumers. Salmonella, Clostridium botulinum, Escherichia coli 0157:H7, and probably Listeria monocytogenes in

ready-to-eat food, are some that fall into this group.

Sampling Procedure

The sample should be collected by using proper sanitary measures to prevent any contamination. Following collection and until tested, the samples should not be handled to avoid growth or death of microorganisms. If the product is frozen, samples should be kept frozen until analyzed. Otherwise, they can be stored at 0-4 °C. Each sample should be labeled to identify date, time, nature of sample, and types of analysis to be conducted, and the persons who collected the sample. The samples should be transported to the laboratory under conditions that avoid microbial contamination, growth, or death. The laboratory that will test the samples should examine the conditions, such as temperature, appearance, and the sampling information, and note the time of receiving the samples. Once received in good condition, a sample should be tested as soon as possible. The unused portion of the sample should be stored under proper conditions until the results are available. The results should be recorded immediately and properly in permanent form. Standard or recommended methods should be used to prepare the sample and the procedures of testing for a specific microorganism or a Microbial group.

Quantitative Methods For Microbial Enumeration In Foods

Colony-Forming Units (CFUs) in Nonselective Agar Media:

Aliquotes from selected dilutions of a serially diluted sample are either pour plated or surface plated by using nonselective media such as plate count agar (PCA), tryptic soy agar, or nutrient agar. The temperature and time of plate incubation differ with the microbial groups. For SPCs, they are 32 °C for 48 h; APCs.35 °C for 48 h; for psychrotrophic counts, 7 °C for 10 days or 10 °C for 7 days. The same procedures with specific modifications can be used to determine thermophilic, thermoduric, and anaerobic groups present in a food sample. The specific groups to be tested depend on their relative importance in a food. For a vacuum-packaged refrigerated food, the most important groups will be psychrotrophic, anaerobic, and facultative anaerobic groups.

CFUs in Nonselective Differential Media

A nonselective medium is supplemented with an agent capable of differentiating the colonies produced by specific groups of microorganisms that differ in metabolic or physiological

characteristics from one another in the population. pH indicators are often used in the medium. Colonies of cells capable of metabolizing lactose to lactic acid are differentiated from those that do not ferment lactose by growing them in agar medium supplemented with lactose as a carbon source and a pH indicator such as bromocresol purple. The lactose fermenting colonies will be yellow, others will be white.

CFUs in Selective Agar Media

A medium can be supplemented with one or more selective or inhibitory agents and used by pour or surface plating of serially diluted samples. In the presence of such an agent, only the microorganisms resistant to it can grow. Incubation conditions to stimulate colony formation differ with the organisms being studied. Enumeration of aciduric bacteria in a medium at pH 5.0, yeasts and molds at pH 3.5, C. perfringens in the presence of cycloserine are examples of selective enumeration of specific groups in food. Halophilic bacteria can be enumerated by specific selective procedures.

CFUs in Selective-Differential Agar Media

A medium is supplemented with one or more selective agents to allow selective growth of specific resistant microbial groups while inhibiting growth of other sensitive microbes. In addition to selective agents, a medium is also supplemented with agents that enable each type among the selective microbial groups to produce colonies that differ in characteristics from one another. Violet red bile agar for coliforms, KF-azide agar for Enterococcus spp. are selective as well as differential agar media. Selective agent allows selective growth and colony formation, while differential agents help differentiate these species from one another by their specific colonies.

Indirect Estimation

Most Probable Number (MPN) in Selective Broths:

Aliquots from a serially diluted sample are inoculated in a broth (in tubes) having one or more selective agents that facilitate growth of selected microbial groups present in a food. Three or five broth tubes in each dilution and a minimum of three consecutive dilutions are used. After incubating at recommended temperature and time, the broth tubes in each dilution are scored for the presence and absence of growth. From the number of tubes

showing growth in each of the three successive dilutions, the number of viable cells of the specific microbial group can be estimated from the available statistically calculated tables. This method gives wide variation. MPN methods are used to estimate coliforms and fecal coliforms in foods and water by using brilliant green lactose bile broth and EC broth.

Dye Reduction Test

Some dyes such as methylene blue and resazurin are colored in oxidized states but colorless under reduced conditions. This change can occur because of microbial metabolism and growth. It is assumed that the rate of reduction during incubation of a specific concentration of methylene blue added to a food is directly proportional to the initial microbial load in the food. This method is generally used to determine the microbiological quality of raw milk.

Qualitative Methods

Isolation of Pathogens

The main objective of this method is to determine whether a sample contains viable cells or spores of a specific pathogen. Foods are tested for several pathogens, such as *Salmonella, E.coli 0157:H7, L. monocytogenes, Vibrio cholerae, and Shigella spp.* By the specific isolation procedure. For other pathogens, such as enteropathogenic *E. coli*, *Y. enterocolitica*, and *campylobacter jejuni*, isolation procedures are not generally used, instead, enumeration procedures are used. An isolation procedure contains several steps:

- Nonselective preenrichment
- Selective enrichment
- Testing on an agar medium containing selective and differential agents.

A food normally contains a low population of a pathogen as compared with associative microorganisms, and the pathogens could be in the injured state. The food sample (e.g., 25 g) is first pre-enriched in a nonselective broth and incubated for the injured cells to repair and then multiply in order to reach moderately high numbers. An aliquot is then transferred from the preenrichment broth to a selective enrichment broth and incubated. It is expected that

during incubation, the specific pathogen and closely related microorganisms will selectively grow to a high number, whereas many of the associated microorganisms will not grow. A small amount (0.01 ml) of the enrichment broth is streaked on the surface of a pre-poured selective-differential agar medium plate, which is then incubated for specified time for the colonies to develop. The presence of a specific pathogen can be tentatively established from the colony characteristics. This is generally considered a presumptive test. For confirmation, the cells from characteristic colonies are purified and examined for biochemical reaction profiles and serological reaction against specific antibody. Isolation of a pathogen using the conventional methods can take 10-12 days, depending on a particular species.

Test For Bacterial Toxins In Foods

S. aureus Enterotoxin

Toxin is extracted from 100 g food, then it is concentrated in 0.2 ml M saline.Toxin is detected by reaction with specific antibody on a microslide. ELISA can also be used.

Botulin Toxin

The toxin is extracted from food, then it is activated by trypsin treatment, 0.5-ml is injected into mice intraperitoneally, control (a portion is heated at 100C) is also injected into another \ mice. The mice are observed for botulism symptoms and death.

Rapid Methods for Detection of Microorganisms in Food

The conventional methods used for the quantitative or qualitative detection of microorganisms and toxins in foods take a relatively long time. Different rapid methods have been developed .To detect microbial loads, food borne pathogens, and their toxins. In addition to being rapid, they are specific, sensitive, accurate, and less labor intensive.

Immunofluorescence

Specific fluorescence-conjugated antibodies which are directed against somatic or flagellar antigens of a pathogen are mixed with an enriched medium suspected to contain the specific pathogen, such as Salmonella, on a glass slide. Following incubation and removal

of reagents, the slide is examined under a fluorescence microscope for cell showing fluorescence on the cell wall or flagella or both .

Enzyme Linked Immunosorbent Assay (ELISA)

The specific antibody is allowed to bind on a solid surface (microtitration plastic plate). The sample suspected of containing the antigen (pathogens or their toxins) is prepared and added to the well and incubated for the antibody-antigen reaction. After removing the unbound antigen(washing step), another antibody labeled with a specific enzyme(such as alkaline phophatase) is added and incubated for antigen binding, a sandwich is formed (Ab-Ag-Ab*enzyme). The unbound enzyme-linked antibody is then removed. The sandwich complex is detected by adding a chromogenic substrate specific for the enzyme (such as p-nitrophenyl phosphate), incubating for a specified time, and adding an enzyme inactivator to stop the reaction. The intensity of the color can then be measured to identify the presence of a specific pathogen or toxin.

Polymerase Chain Reaction (PCR)

The PCR technique helps amplify a segment of DNA. It is thus possible to obtain large numbers of copies of a specific DNA segment from a small sample (50 ng), which in turn facilitates its detection by gel electrophoresis. PCR is a sensitive method and may not need pre-enrichment or enrichment steps. To eliminate confusion between dead and viable cells of a pathogen as the source of DNA, a short pre-enrichment step may be necessary to increase the viable cell number of the target pathogen.

Bioluminescence

The bioluminescence method measures the ATP content in a sample as an indirect measurement of microbial load. As only viable cells retain ATP, the amount of ATP is regarded as directly related to the microbial load in the sample. Using the luciferin-luciferase (from firefly) system in the presence of Mg, the ATP concentration in the lysed cells in a sample is measured. The method is very rapid.