1

Food Microbiology: Fundamentals and Techniques

Raina Jain, Prashant Bagade*, Kalpana Patil-Doke and Ganesh Ramamurthi

National Commodities Management Services Limited, Hyderabad, India

Abstract

Food microbiology is a broad subject encompassing study of both beneficial and harmful microorganisms in food, and their effects on the quality and safety of food. Beneficial microbes present in food offer an array of health benefits to humans and are important sources for fermentation, probiotics and bio-preservatives. Contrarily, harmful bacteria lead to food spoilage and a countless number of foodborne diseases which may even prove to be lethal, if uncontrolled. Food microbiology uses a number of testing methods to detect, enumerate and identify the microorganisms present in food. Conventionally, it involved culturing of microbes on suitable media and analyzing the results on the basis of physical or biochemical tests. However, such techniques are time-consuming and laborious. As a result, rapid and high-throughput techniques with use of advanced equipment and strategies have been developed to ensure quality and safety of food in real time. The chapter presents the long history of the development of Food Microbiology as a subject, along with classical and advanced techniques used to identify and quantitate foodborne microorganisms.

***Keywords*:** Food microbiology, food spoilage, food microbes, food regulations, microbiological techniques, probiotics, AI and ML in food, biosensors

1.1 Introduction

Food microbiology is the study of microorganisms that colonize, modify, process or spoil food. It deals with foods and beverages of diverse composition, combining a broad spectrum of environmental factors, which may

**Corresponding author*: prashant.bg@ncml.com

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influence microbial survival and growth. A variety of microorganisms having beneficial or harmful effects on food quality and safety are studied in food microbiology. This includes spoilage, pathogenic, fermentative, and probiotic bacteria; molds and yeasts; viruses, prions, and parasites.

Microorganisms, viz., bacteria, molds and viruses can contaminate foods across the food value chain. Consumption of such foods can cause foodborne diseases. Effective intervention technologies are being developed and implemented to ensure safety of consumers against foodborne diseases. Food spoilage occurs due to growth of microorganisms in food or due to the action of microbial enzymes. Incidences of food contamination and spoilage are very frequent, and are being reported on a regular basis. Such incidences are partly due to consumers' changing palate in desiring undercooked, minimally processed, unpreserved foods, etc. Additionally, inadequate infrastructure along the supply chain, especially for temperature-sensitive foods, also plays a major role in food spoilage leading to economic losses.

Food bioprocessing is a technique of food and ingredient extraction, purification and production using processes that involve the application of enzymes and/or microorganisms. It is one the most primitive forms of food processing method, used by early Egyptians for production of wine, beer, and bread. Additionally, microbial enzymes were also being used to produce food and food additives. Since the past few years, by adoption of genetic recombination techniques and use of different microbial sources, enzymes of higher purity and activity are obtained. Nowadays, many types of additives from microbial sources are being developed and utilized in food processing. Some of these include single-cell proteins, essential amino acids, colour and flavour compounds, stabilizers, and organic acids [1]. Food bio-preservation through anti-microbial metabolites such as bacteriocins and organic acids like acetic, propionic and lactic acids are being developed and used, replacing preservatives of non-food origin. Probiotics – a rapidly emerging health food – contain live cells of bacteria that have apparent health benefits. The role of these bacteria for health and bacterial efficacy benefits are being researched upon.

1.2 Food Microbiology: A Historical Perspective

Foodborne disease and food spoilage have been part of the human experience since the dawn of our race. Several events spanning centuries led to the recognition of the role of microorganisms in foods. We are aware that early civilizations discovered and applied effective methods to preserve and protect their food. As far back as 7000 BC, Babylonians manufactured beer and wine.

Egyptians, in 3000 BC, manufactured cheese and butter. Around the same time, use of salt to preserve meat and other foods became popular. In 1000 BC, the Romans discovered fermentation, salt, ice, drying and smoking to preserve shrimp and meat, though they did not know how these practices inhibited food spoilage or caused foodborne diseases. This was compounded by their belief that living things formed spontaneously from non-living matter.

In 1665, Francesco Redi demonstrated that maggots on putrefying meat did not arise spontaneously but were instead the larval stages of flies. This was the first effort opposing the doctrine of spontaneous generation. In 1765, Spallanzani disproved the theory of spontaneous generation of life by demonstrating that beef broth which was boiled and then sealed remained sterile. The French government offered 12,000 francs to anyone who could develop a practical way to preserve food in 1795. Nicholas Appert showed that meat could be preserved when it was placed in glass bottles and boiled. This was the beginning of food preservation by canning. Later, Schwann demonstrated that heated infusions remain sterile in the presence of air, again to disprove spontaneous generation. It is interesting to note that although Spallanzani and Schwann each used heat to preserve food, neither apparently realized the value of turning these observations into a commercial method for food preservation [2].

The first person to really appreciate and understand the cause-effect relationship between microorganisms in infusions and the chemical changes that took place in those infusions was Louis Pasteur. He convinced the scientific world through his experiments that all fermentative processes were caused by microorganisms. Later, he showed that souring of milk was caused by microbes and heat destroyed undesirable microbes in wine and beer. The later process is now used for a variety of foods and is called pasteurization. Because of the importance of his work, Louis Pasteur is known as the founder of food microbiology. Using his famous swan-necked flasks, he even demonstrated that air does not have to be heated to remain sterile, and this finally put an end to the theory of spontaneous generation. The knowledge that microbes were responsible for fermentation and putrefaction led Pasteur to argue that microbes were also causative agents in disease. These arguments eventually helped Joseph Lister to develop the first aseptic surgical procedures. Since that time, microbiological discoveries and developments began to proceed more rapidly, leading to implications of microbes in several diseases. This led governments to enact legislation to protect the quality of food.

Most of the food industries hesitated in adopting microbiological food safety norms in their routine procedures until they were economically affected by outbreaks of foodborne diseases in their products. One similar

case occurred in 1920s with the outbreak of Botulism, which affected food canning industries. This resulted in adoption of the 12D process for heat treatment of *C.botulinum*. At about the same time, the dairy industry was driven to implement microbiological control over safety in milk production, because of several nasty outbreaks of milk-borne diphtheria, tuberculosis, typhoid fever, and brucellosis. Regulatory bodies made it compulsory to address the risks with focus on animal health, sanitation, and pasteurization – which had an immediate and very effective impact on the problems.

In one of the cases of early food microbiology, the US government had institutionalized a woman who came to be known as "Typhoid Mary". She was an asymptomatic typhoid carrier who worked as a cook for several families. Over 10 years, seven outbreaks of typhoid were directly traced to her and estimates suggest that she may have been responsible for 120 cases [3] of typhoid fever. New York authorities arrested her but eventually released her when she agreed never to work as a cook again. When another outbreak was traced to her a few years later, she was arrested as a threat to public safety and institutionalized until her death in 1938.

1.3 Beneficial Microbes in Food

The role of beneficial microbes is not given due recognition since it is a common perception to think of microbes only as harmful. Their presence in human gut play a significant role in maintaining human health by ensuring proper digestion apart from a range of benefits. The number of microorganisms that are present in the human GI tract is estimated to be over 10^{14} [4]. A recent study [5] has identified about 2,000 bacterial species in the human gut by using computational methods. However, these species are yet to be cultured in the lab. Beneficial microbes are used in the food industry for a variety of applications with simplest being in fermentation, which has been used since ancient times for production of wine, bread, cheese, etc., apart from a host of traditional dishes. Fermented foods are considered healthy due to the presence of various health promoting microorganisms. Based on the concept, a new trend that has taken over and is gaining popularity is probiotic foods. These are foods to which health-promoting microorganisms are added and are generally considered as "super foods". A few such foods include Yogurt, Kefir (made by adding kefir grains to cow's or goat's milk), Sauerkraut (finely shredded cabbage fermented by lactic acid bacteria), Miso (made by fermenting soybeans with salt and koji, a fungus), etc. Furthermore, some microbes have been found to retard spoilage of food products when added in the appropriate

proportion; they are known as microbial bio-preservatives. Though certain examples exist of such organisms, it is a field that is still in its infancy and needs more research and acceptance by consumers.

1.3.1 Factors Influencing Microbial Growth in Food

Ecology helps us learn the factors and their interactions determining growth of an organism in a given environment. Microbial growth in food is a complex process, and there are multiple genetic, biochemical and environmental factors affecting this [6]. They can be classified into four major classes:

i. Intrinsic factors – Characteristics of food itself, including naturally occurring compounds, added preservatives, nutrient content, water activity, pH, and the oxidation-reduction potential, are called intrinsic factors.
ii. Extrinsic factors – They are related to the environment in which food is stored – relative humidity and temperature of atmosphere of food storage, composition of gases. Environmental temperature plays a critical role in influencing microbial growth, e.g., in a refrigerator, microbial cells grow at a much lower rate compared to room temperature.
iii. Implicit factors – These include interactions between microbes contaminating the food and between these organisms and food itself. For example, a microorganism's inner ability to utilize different nutrient sources and tolerate stress will define its growth in a particular food.
iv. Processing factors – Food treatments, such as heating, cooling, and drying, which affect composition of food, also affect the microorganisms available in treated food.

Moreover, the combined effect of the four factors described above influence the microbial growth in food, in a more or less synergistic manner [7].

1.3.2 Food Fermentation

The science of fermentation is known as zymology or zymurgy. In the simplest terms, fermentation is the process of breaking down of carbohydrates such as starch and sugars to alcohols by either inherently present or externally added microbes. Despite being one of the most ancient techniques of

food preservation, it is still in use for production of a variety of products apart from preservation. Countries with rich ancient civilizations have a host of traditional fermented food as part of their diet. Such foods and beverages serve as a rich source of nutrition apart from being capable of maintaining human health and preventing diseases. Though such foods have seen a decline in their consumption in the past, recently their consumption has increased owing to their health benefits. They are generally produced naturally or by addition of a starter culture resulting in food products that are considered superior nutritionally and/or organoleptically. However, such foods have limited appeal since culture plays a significant role in their consumption. Hence, most such foods are restricted geographically and culturally.

A variety of bacteria, fungi and yeast are involved in food preservation by using food as the substrate and by producing citric acid, lactic acid and/or acetic acid. Apart, they are also responsible for production of aroma in foods by metabolising foods into amino acids, fatty acids, and nucleotides [8]. Cheese, a globally consumed product, derives its flavor depending upon the variety, microflora and ripening conditions resulting in formation of free fatty acids [9]. Fermentation is also known to reduce the amount of anti-nutritional factors such as phytates, thereby improving the availability of nutrients present in food. Phytates are known to bind with certain important micronutrients such as iron and zinc, limiting their bioavailability. In a study [10] using Pearl millet, fermented dough was found to contain lower levels of phytate compared to flour. Apart, the process has resulted in increased content of polyphenols which are known to have higher antioxidant activity and are beneficial to human health.

1.3.3 Probiotics

Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body. There are a wide range of natural probiotics foods which are being manufactured to cater to the needs of changing lifestyles. Such foods are manufactured by adding live microorganisms that improve gut health resulting in a wide range of benefits to the consumers. Though they may contain a variety of microorganisms, most of the bacteria belong to two groups – Lactobacillus and Bifidobacterium. The most common yeast used in probiotics is *Saccharomyces boulardii*. Research results of the Human Microbiome Project enabled the study of microbial communities that live in and on our bodies and the roles they play in human health and diseases. It has helped in mapping the normal gut microbiome forming a basis for establishing linkages between diseases

and changing microbiome. Such information can be exploited to develop tailored probiotic foods which can address specific health disorders.

1.3.4 Microbial Bio-Preservatives

All foods have a shelf life beyond which they will be considered unfit for consumption due to various reasons such as development of unpalatable flavour, taste, texture, microbial growth, etc. To extend product shelf life, a wide variety of preservatives are added – chemical or otherwise. Considering the undesirable nature of chemical preservatives, a new trend in the form of bio-preservatives has taken root and is gaining attention. Bio-preservation is the use of antimicrobial active substances extracted from food or obtained by food-grade microbial fermentation to enhance safety and food quality [11]. The most commonly used bio-preservatives are Lactic acid bacteria (LAB) belonging to the order, Lactobacillales, which includes more than 200 species under 36 genera [12]. The most common genera to which LAB species belong are Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Enterococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus, and Weissella. LAB species exert their effect by producing certain metabolites such as lactic acid, acetic acid, bacteriocins, etc. Their antagonistic effect is based on the principle that the majority of bacteria will stop multiplying when the pH reaches below 4. Apart from LAB, dairy propionibacteria (PAB) and Bacillus species have also been found to possess antifungal properties. Mycocins, also known as "killer proteins", produced by yeasts have also been documented to produce antimicrobial compounds [13].

In a study [14] on seafood products, certain LAB species were found to be suitable as bioprotective agents in a hurdle technology strategy applied to cod and salmon-based products. In another study [15] on beef and lamb meat, microbial bio-preservatives were found to retard the growth of the major meat spoilage bacteria – *Brochothrixthermosphacta*, Pseudomonas species, and Enterobacteriaceae. Apart from being able to reduce the bacterial spoilage, they did not have any noticeable negative impact on the sensory properties of meat. Bacteriocins, ribosomally synthesized antimicrobial peptides, are produced by bacteria. These have been reported [16] to not only preserve food but also retain organoleptic and nutritional properties.

Though few such bio-preservatives are commercially available, their numbers have been significantly lower than the vast majority of strains described in literature. A few of the possible reason include mismatch in efficiency of such microbes under research conditions as compared to

field applications. Consumer acceptance is another major constraint since microbes are generally considered as "harmful" and their intentional addition to food is not easily accepted by consumers. Also, regulatory aspects must be kept in mind since country-to-country requirements would vary.

1.4 Harmful Microbes in Food

Food is the most susceptible commodity targeted by microorganisms to grow and spoil. Raw food is a good home to all sort of microorganisms, may it be in the form of raw meat, milk or fresh fruits. The number of microbes existing on raw food decreases upon washing or processing or cooking or adding preservatives. However, adequate process efficiency and its packaging and storage are equally crucial in maintaining safety of the food. The cooked food, containing all nutrients required by microorganisms, easily attracts bacteria, yeasts & molds within a few hours after preparation, if left open [17].

On the other hand, unlike spoiled food, food contaminated with pathogens cannot be identified superficially by a general sense of appearance and smell. Consumption of such foods causes serious health hazards, known as foodborne diseases or foodborne illness. Overall, all the microorganisms which spoil the food to make it unacceptable for human consumption and/or lead to development of diseases are classified as harmful microbes.

1.4.1 Factors Influencing Food Spoilage

As we have described in the previous section as well, the growth of microbes in food essentially depends on the extrinsic, intrinsic and implicit factors, which together decide the fate of food. Harmful bacteria challenge the shelf life of particular food. However, not all foods are susceptible to microorganisms; it mainly depends on the nature of food, its processing or preservative activities. Perishable foods, like dairy products, meat, poultry, fish etc., can be spoiled quickly by activity of microbes within a few days. Meat putrefies and sours because of bacterial growth on it and milk products are spoiled either by acid or mold growing in it. Similarly, fresh fruits and vegetables are spoiled because of degradation of pectin in them by pectin-degrading bacteria. Unlike perishables, semi-perishable foods have a relatively long shelf life, a few weeks or months, and include bread, butter, cake, canned fruits, pickle, jam juices, etc. Further, non-perishables having a very long shelf life of months or years include dried fruits and vegetables, peanut butter, etc.

Moreover, the food environment itself sometimes favors or restricts the growth of harmful microbes. pH, water activity (Aw), oxidation-reduction potential, nutrients and inhibitory agents are the key factors for controlling spoilage. Furthermore, microorganisms used in food fermentation and pathogens can multiply in a food to reach the spoilage detection level and are capable of causing food spoilage. The best example is curd or yoghurt, which spoils because of overgrowth of its own organisms.

Apart from inherent properties of food responsible for spoilage, factors during processing are also a part of risk of spoilage. For microbial food spoilage to occur, microorganisms have to get into the food from one or more sources. It can be through a low-quality ingredient, unhygienic environment and handling at any stage, inadequate thermal processing or preservative dosages and many more. Packaging integrity, time of storage and any deviation in storage conditions are some more causes responsible for spoilage.

Different categories of bacteria thrive in varied food environments, such as raw meat, which is liked by Salmonella and Campylobacter, whereas fresh fruits and vegetables are liked by *E. coli* and dairy products are home for *Listeria monocytogens*. *Clostridium perfringens* is better known as "buffet germ" because it is found in foods which are made in larger batches and get inadequate heat treatments. Home canned foods are always threatened by anaerobic bacteria, *Clostridium perfringens* and *Clostridium botulinum*, which can cause gas formation and bulging of the can.

It is the nature of food-spoiling bacteria which affect different categories of food, as given below:

Psychrotrophic Bacteria – As the name indicates, these bacteria are capable of growing at 5°C and below, but multiply rapidly at 10 – 25°C. These bacteria grow on many foods, stored on ice and in a refrigerator and some are expected to have a long shelf life of 50 days or more. Examples are *Pseudomonas fluorescens*, *Pseudomonas fragi*, Acinetobacter, Moraxella, Flavobacterium and some molds and yeasts.

Anaerobic Bacteria – These bacteria grow in the absence of oxygen in foods stored anaerobically, also in the interior of prepared food. Examples are *Lactobacillus viridescens*, *Lactobacillus sake*, *Lactobacillus curvatus*, *Leuconostoccarnosum*, *Leuconostocgelidum*, some Enterococcus Spp., Alcaligenes Spp., Enterobacter Spp. and some Microaerophilic yeasts.

Thermoduric Psychrotrophs – include facultative anaerobes such as spores of *Bacillus coagulans*, *Bacillus megaterium*, *Lactobacillus viridescens.*

Mesophiles – grow in moderate temperature range of 20 to 45°C and include *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli*.

Thermophilic Bacteria are – a group of bacteria which grow between 40 – 90°C, with optimum temperature at 55 – 65°C. Spores of these bacteria also germinate and start spoilage. Examples are spores of some thermophilic Bacillus and Clostridium Spp.

Thermoduric vegetative bacteria surviving low heat processing (such as pasteurization) or thermophiles can also multiply in these warm foods especially if the temperature is close to 50°C. These include some lactic acid bacteria such as Pediococcus Spp. and Streptococcus Spp., Bacillus and Clostridium Spp.

Aciduric Bacteria are the bacteria that can grow in food at pH 4.6 or below. They are associated with spoilage of acidic food products such as fruit juices, pickles, salsa, salad dressing and fermented sausages. Heterofermentative and homofermentative lactic acid bacteria have been associated with such spoilage. Yeasts and molds are aciduric and are also associated with spoilage of such foods.

1.4.2 Indicators of Food Spoilage

Once these huge numbers of microorganisms start releasing the extracellular and intracellular enzymes in the food environment, their effects are visible in the form of colour change, odour, texture changes with slime formation or gas accumulation, foam accumulation and many more. Molds form visible growth on surfaces; and affected food appears softened and rotten to the naked eye. Slime formation and Ropiness is formed after U-ring growth of *Pediococcusdamnosus* and *Lactobacillus brevis.* Some spoilages are associated with formation of hydrogen sulfite gas, giving a rotten egg smell to food. Other end products released from microbial metabolism of food nutrients include carbohydrates, CO_2, H_2S, H_2O_2, lactate, acetate, formate, succinate, butyrate, ethanol, propanol, butanol, diacetyl, dextran proteins and non-protein nitrogenous compounds, amines, ketoacids, putrescines, lipids, fatty acids, glycerol, hydroperoxides, aldehydes, ketones.

To identify spoilage of food, microbiological analysis is the first tool where enumeration techniques reveal the status of deterioration. Aerobic plate count indicates the effectiveness of sanitary procedures used during processing and handling and before storage of a product. A similar conclusion is drawn by yeast & mold count. But the major disadvantage of microbiological enumeration methods is that it takes several days. To overcome this problem, other indirect methods have been used [18]. Examples of such methods are determination of lipopolysaccharides (LPS) in a food

(for Gram-negative bacteria), measurement of ATP as its concentration is increased with high numbers of viable cells. Chemical indicators of food spoilage are also useful in determining food spoilage. Pasteurization is done to ensure effective treatment of milk. A quick indicator of pasteurization is use of Alkaline Phosphatase (ALP), an enzyme naturally present in all raw milks, which is inactivated upon complete pasteurization. Heat stability of ALP is greater than that of pathogens which may be present in milk; the enzyme serves as an indicator of product safety. MBRT and resazurin tests are similar tabletop tests in the dairy industry which indicate bacterial population [19].

1.4.3 Foodborne Infections and Intoxications

Food infection is caused by consumption of a food that contains viable pathogenic cells, present in large numbers. Food pathogens can be bacteria and viruses like hepatitis A & E. Depending upon the origin of the food and extent of process of preservation, pathogenic bacteria remain viable on food and get into the human body. Upon consumption, within 24 hours till 3 days, bacteria colonize and grow on the gastrointestinal tract of the host and then lead to localized infections, tissue damage and deeper infections to cause systemic infections [20]. Visible effects of such infections are mostly nausea, vomiting, diarrhea, abdominal cramps and fever. Further typical infections are bacteria-specific – Hepatitis, Salmonellosis, vibriosis, yersiniosis, campylobacteriosis, listeriosis, etc. As per the type of infection, food poisoning can be categorized as non-inflammatory, inflammatory and systemic/penetrative (Table 1.1).

Food intoxication is another type of foodborne disease; however, unlike food infection, intoxication is caused due to toxins released by bacteria in food. Actual bacterial cells need not be present at the time of consumption but the toxins produced by them are ingested and cause symptoms. Staphylococcal enterotoxin, botulinum toxin, and *Bacillus cereus* toxin are a few of the examples which cause botulism, staphylococcal poisoning, and *Bacillus cereus* poisoning, respectively. Botulinum is believed to be lethargic since it is a kind of neurotoxin and a very little dose is also enough to cause severe effects on health. Additionally, mycotoxins – aflatoxins, and ochratoxins – are also part of food toxins which are produced by fungi. Foodborne toxico-infection, the combination of food intoxication and infection, is caused when a pathogen is consumed and then it releases toxins in the GI tract, resulting in illnesses.

Table 1.1 Different types of food poisoning caused by food pathogen infections.

Mechanism	Pathogens	Illness
Non-inflammatory	*Vibrio cholerae*, Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli*, *C. perfringens*, *Bacillus cereus*, *S. aureus*, Rotavirus, norovirus, Enteric adenoviruses, *Giardia lamblia*, Microsporidia	Watery diarrhea
Inflammatory	Shigella, Salmonella, *C. jejuni*, Shigatoxigenic verotoxigenic *E. coli* (EHEC), *Enterocolitica*, *Vibrio parahaemolyticus*, *C. difficile*, *E. histolytica*	Dysentery/ Inflammatory diarrhea
Penetrating	*Salmonella typhi*, *Yersinia enterocolitica*, *Campylobacter fetus*	Enteric fever
Neurological	*C. botulinum*, Ciguatera toxin, Combroid neurotoxic shellfish poisons, mushroom toxins	Giddiness
Miscellaneous	*Listeria monocytogenes*, Group A streptococci, Hepatitis A virus, *Brucella* spp. toxin, *Trichinella spirali*	Stillbirth, fever

1.4.4 Food Preservation to Control Spoilage

Food Preservation is equally important as food preparation or manufacturing to assure long-term storage and complete food safety upon consumption. Food preservation has been practiced since ancient times; pickles, jams, jellies, and alcoholic drinks maturing for years are quick examples of the understanding of preservation techniques by our ancestors. With the development of urbanization and industrialization, more techniques are being invented and practiced. Still, perfect preservation is an everyday challenge for the entire food industry.

Generally, it is believed that temperature and chemical preservatives can prevent food spoilage. But in actuality, there are many more ways of control including selection of the appropriate nature and quality of ingredients, quality of water being used for washing and preparation, efficiency of thermal processing/drying, effectiveness of food preservatives, hygienic handling, health status of the food handlers, hygienic premises

and hygienic processing facilities, hygienic packaging, integrity of packaging materials, storage conditions and well-established shelf life period. All these factors are considered in food safety management systems and HACCP while manufacturing the food on industrial scale, and hence food safety is assured through adopting the standards like FSSC 22000 or BRC or similar standards globally [21].

Major preservation techniques include heat processing, low-temperature storage, control of water activity, chemical preservatives, modification of atmosphere and irradiation [22]. Heat processing is probably the best way to eliminate microorganisms from a food. It has the capacity to kill microorganisms, their spores as well as denature their toxins. Nevertheless, during heat treatment of food it is also important to retain its nutritive, organoleptic and texture properties. Generally, the process of heating validation involves a thermal death curve, where a destruction pattern of microorganisms is plotted in the form of a graph or in the form of a formula and studied how much reduction occurs at what temperature and time stage. It is also calculated as D-value and Z-value. D value is the indicator of resistance of a microorganism to the heat. It is the time in minutes at a given temperature required to destroy 1 log cycle (90% population or 100 bacteria reducing to 10). On the other hand, Z value reflects the temperature dependence of the reaction. It is defined as the temperature change required changing the D value by a factor of 10. Retort technology, also called as autoclave, is a kind of a pressure vessel where sealed food is kept in and heated for 110 to 135°C. This is as good as commercial sterilization. Ready meals or low acid foods are preferably treated with retort technology to extend the shelf life and ensuring food safety.

Foods where high heating procedures are not suitable are subjected to low-temperature methods. This includes ice cooling, chill storage, freezing, freeze drying, etc. Low temperatures prevent and reduce growth and catalytic activities of microorganisms. It is a preferred method for meat, fish and seafoods. General cooling is achieved by use of ice, essentially made by potable water. But it is a very primary means of preservation and also poses a risk of heterogeneous chilling, cross-contamination and ice melting risk. Chilling storage for fruits and vegetables is used at 10 – 20°C with controlled humidity. Highly perishable foods are stored at 1 – 4°C, whereas other perishable foods are stored at 4 – 5°C. Freezing is done either slowly at -20 – -30°C or rapidly at -70°C. Nonetheless, there are many drawbacks of low-temperature treatments – microbial death is not assured, rate of death is unpredictable, does not affect endospore viability and cannot prevent germination. Moreover, these techniques are useful to preserve a food which is already having less microbial load.

Individually Quick Frozen (IQF), is a promising technology widely being used as flash-freezing [23]. In this technique, literally every individual food item is placed on a kind of conveyor belt that speedily moves and enters into a blast chiller that freezes the item very quickly. The temperature of a blast chiller is 0.5 to -4°C and the time is short; this forms smaller ice crystals unlike a slow freezing process where bigger ice crystals are formed that can damage the fibres of foods, squeeze the components of food and water outside and rupture cell walls. IQF saves all these reductions in ice crystallization and saves time as well. Fruits like berries, vegetables like corn and peas, fish, and prawns are processed using IQF techniques.

Control of water activity is one more means of achieving freedom from spoilage microorganisms. Microorganisms need moisture to grow. Most of the bacteria require water activity of more than 0.91, whereas molds need more than 0.80, and a few can grow at 0.75 too. But 0.60 is the activity where almost no microorganism can survive. Hence reducing water activity of a food till lowest is a promising way to control microorganisms and their spoilage. It is achieved by lesser additions of salt and sugar, which binds the water and makes it less accessible to microbes. This is the secret to pickles and jams preservation! An alternate way to reduce water activity is physical removal of water by means of drying. Drying can be natural dehydration by solar heat. It can be mechanical drying too, where drying ovens or drying tunnels or fluid beds, hot rolls are used [24]. Freeze drying or lyophilization is also an advanced technique of drying, where food is subjected to a process that involves freezing a substance at very low temperatures and then extracting liquid through sublimation, converting water from a solid to a gaseous state. Water extracted in such a way passes directly from solid to gas without going through the liquid phase and hence product integrity remains unchanged. This kind of preservation provides very much longer shelf life, till years also.

There are many chemicals which can kill or restrict and delay the growth of microorganisms, essentially classified as chemical preservatives. But not all chemicals can be used in food because either they are toxic for humans or it may affect the narcoleptic properties of the food. Hence in commercial food preparation, one cannot use any chemical without legislative norms. National and international food authorities have laid down a list of approved additives and preservatives and their dosage. Most of the preservatives are used in concentration of less than 0.2%. Commonly used chemical preservatives include salt, sugar & saccharides, acids like vinegar (acetic acid), lemon juice (citric acid), etc. Spices like cinnamon & cloves also impart antimicrobial properties to food along with the flavors. No limit is set for these preservatives by any regulatory food authority.

Acidulant preservatives include benzoic acid, sorbic acid, lactic acid, malic acid, tartaric acid. Where weak acids are used as preservative, they inhibit the outgrowth of both bacterial and fungal cells. Acids like sorbic acid inhibit germination and outgrowth of bacterial spores. Sodium benzoate is one of the most commonly used preservatives to inhibit yeast growth in acid or acidified foods like fruit juices, pickles, jams, etc. Sorbate is another popular preservative, since it is tasteless and odorless and nontoxic; it is an approved preservative by WHO with highest daily intake dosage.

Smoking of a few food types is also a traditional way of food preservation, which involves heating, drying and adding antimicrobial preservatives.

Preservation using gaseous chemicals like Sulphur dioxide is another example of a food preservative. CO_2, a colorless, odorless non-combustible gas, is also sometimes used as a food preservative. It inhibits the growth of many psychotropic bacteria and is introduced as a direct additive in storage of fruit and vegetables. Additionally, if CO_2 is introduced in packaging environment of food along with other gases like nitrogen, it can prevent further spoilage of food. The technique is called as modification of atmosphere and is employed for a few food types, where it is used to replace oxygen in packaged conditions. Unavailability of oxygen discourages microbial growth; additionally CO_2 acts as an inhibiting agent too. When CO_2 is compressed, it results in dry ice, a very effective way to maintain chilling conditions in sealed containers. Hence it is used for transport of food samples.

Irradiation is a modern technique of food preservation, which can be called as cold sterilization. Foods are exposed to ionizing radiations, including gamma rays, X-rays or electron beams, which eliminate microorganisms by destroying them and provide spoilage protection. Also, it destroys insects and prevents food from sprouting and ripening. Radiations create energy waves in the product and collide with other particles on a targeted surface. Chemical bonds between these particles are broken and generate short-lived radicals leading to disruption of microorganisms' structures, nuclear materials and hence growth is altered or stopped. Visually, the product appears the same as before; there is no change in appearance, taste, or nutritive quality of food after irradiation. Norms of dosage are to be followed as laid out by a regulatory body while using irradiation technique.

Different technologies are being used for food preservation based on different principles. Every method has its own advantages and disadvantages. A thought was put out in 1976 by Leistner for combining two or more technologies for better preservation of food and to achieve a total quality, known as hurdle technology. The principle of hurdle technology

indicates that hurdles are created to disturb microbial well-being or homeostasis, and multiple challenges are in the way of microbial growth, where microbes cannot jump over all the hurdles [25]. By combining hurdles to address multiple targets, individual techniques, e.g., removing moisture and lowering water activities, maintaining pH, addition of preservatives, disadvantages of individual techniques are overlapped or minimized. This can fulfill the demand of next-generation consumers to get a minimally processed "as natural as original food" as can be possible.

1.5 Classical Food Microbiological Techniques

Classical techniques of microbial analysis originated with the invention of the microscope by Leeuwenhoek in 1674. These are mainly observational techniques for detection of microbes. Notably, less than 5% and perhaps as little as 1% of all bacteria can be cultured in the laboratory. If one includes all viruses infecting all species on earth, the number becomes lower by several orders of magnitude. Hence, one caveat of classical microbial methods is that the tools used to describe microbial development and provide systematic organization are based on a statistically minor portion of all bacteria [26, 27]. Still, these techniques have been routinely utilized for a long time in major food microbiology laboratories throughout the world, despite development of advanced techniques, and are biased for detection of medically important species infecting humans.

The design of the laboratory plays a pivotal role in any food microbiological analysis [28]. Laboratories must be constructed with materials that will restrict microbial growth and shall have a unidirectional flow, with a separate area for each activity. It is imperative that the work environment is maintained in a hygienic condition by rigorous housekeeping procedures and regular fumigation. During each step of analysis, appropriate temperature and humidity levels are maintained to discourage colonization of bacteria and mold. Such setup will result in minimizing false negatives and false positives resulting in accuracy of results.

Microbial media plays an important role and is crucial for obtaining good results. It acts as "food" for microorganisms. All growth media consists of peptones – extracts of protein that are obtained from animal tissues, milk casein and various plant sources like soybean, which serve as basic building material in the growth of microorganisms. Apart from peptone, they also contain yeast extract as nitrogen source and other growth supporters such as minerals, vitamins and growth factors. A media with such composition is known as primary enrichment media, supportive to almost

all types of bacteria in food samples. Upon addition of certain inhibitory components, primary enrichment media becomes selective for growth of select microorganisms. Such media are known as secondary or selective enrichment media. A differential media consists of certain dye indicators which result in color changes upon encountering specific conditions such as acidic conditions. Bacterial colonies grown on such media can be visually differentiated based on their color [29]. Typical colonies of select microorganisms are shown in Figure 1.1. It is vital to select appropriate media depending upon the organism of interest. All prepared media are subjected to autoclave sterilization prior to their use in analysis. However, it is important to note that certain media contain heat labile compounds which can degrade during autoclave sterilization. Such media must be sterilized by boiling.

Microbial analysis is performed either qualitatively (detection) or quantitatively (enumeration) [30]. Qualitative techniques are used mainly for detection of pathogens, whereas quantitative techniques are utilized for analysis of bioburden or hygiene parameters, for example, total bacterial count, total yeast & mold count, Enterobacteria count, etc. Enumeration of microbes is normally performed using one of the following three inoculation techniques:

- Pour plate technique: Measured sample volume is transferred to a diluent and serial dilutions (Figure 1.2a) are made further. All the dilutions are transferred to sterile petri

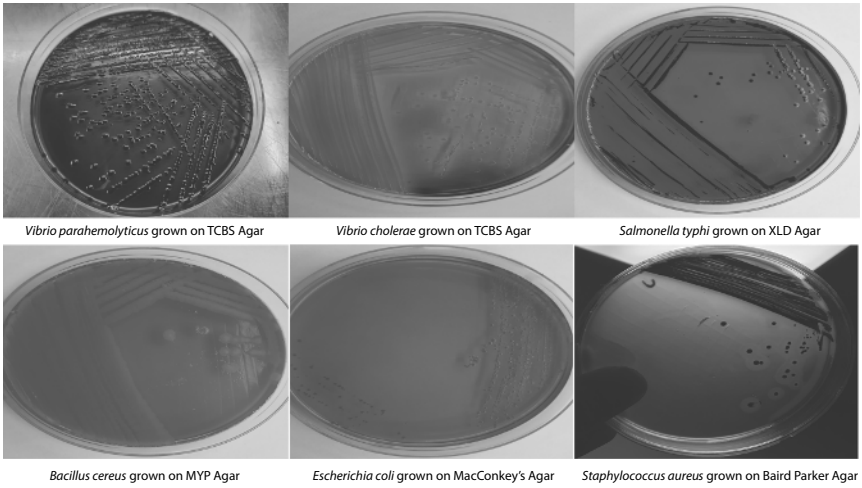

Figure 1.1 Colonies of select microorganisms when grown on selective agar media.

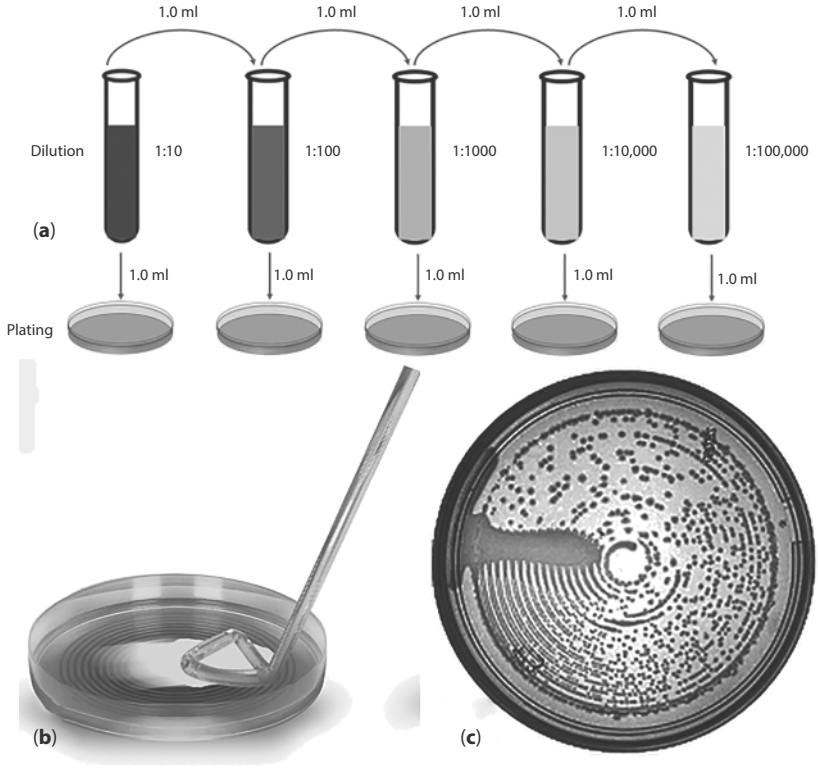

Figure 1.2 Common plating techniques – (a) pour plate, (b) spread plate, (c) spiral plate.

plates, molten agar medium is poured over it and immediately mixed to uniformly distribute the inoculum throughout the plate. The plates are allowed to solidify and incubated at appropriate conditions, depending upon the organism of interest.

- Spread plate technique: This technique is followed where the growth of bacterial population is expected to get restricted by being embedded into agar. Such samples are spread (Figure 1.2b) over the surface of agar plates and then incubated at a desired temperature and time.
- Spiral plate technique: In this technique, the activity of pipetting and plating is performed by an instrument – Spiral plater. A plate kept on the plater rotates and at the same time the dispenser tip releases the inoculum onto the plate in a uniform spiral pattern (Figure 1.2c). The movements are adjusted to occur at a speed such that inside the spiral

a higher concentration of inoculum is exposed and gradually it gets diluted as the dispenser moves to outside spiral. These movements are adjustable as per the requirements. This technique can save resources like petri plates, other consumables, time and effort, apart from eliminating the risks of manual errors, thereby adding more accuracy to the process [31].

After inoculation, petri plates are incubated for the appropriate time and temperature, depending on the organism in question (Table 1.2). At the end of the incubation period, appearing colonies are counted and where required, further confirmatory tests are undertaken.

Table 1.2 Incubation conditions and most commonly used growth media for select microorganisms.

Microorganism	**Most commonly used media**	**Incubation conditions**
Total Plate Count	Plate Count Agar	30°C for 72 hrs
Yeast and Mold Count	Dichloran Rose Bengal Agar	25°C for 5 days
Coliform	Violet Red Bile Agar	37°C for 24 hrs
Enterobacteria	Violet Red Bile Glucose Agar	37°C for 24 hrs
Sulphite Reducing Clostridia	Iron sulphite Agar	37°C for 48 hrs anaerobically
E.coli	MacConkey Agar	37°C for 24 hrs
Staphylococcus aureus	Baird Parker Agar	37°C for 48 hrs
Salmonella	XLD Agar	37°C for 24 hrs
Clostridium perfringens	Iron sulphite Agar	37°C for 24 hrs anaerobically
Vibrio chloeare	TCBS Agar	37°C for 24 hrs
Vibrio parahemolyticus	TCBS Agar	37°C for 24 hrs
Shigella	Deoxycholate citrate agar	37°C for 24 hrs
Listeria monocytogens	PALCAM Agar	37°C for 48 hrs
Bacillus cereus	MYP Agar	30°C for 24-48 hrs

While the aim of enumeration is to assess the sample hygiene status "as is", detection techniques work on a different concept. It allows growth of target microorganisms in an enrichment media which minimizes chances of false negatives. When pathogen cells arepresent in extremely low numbers and/or when the cells are damaged due to certain processes such as drying or thermal processing, there is a possibility that they may not get detected. Then, enrichment steps would help to overcome such a limitation by allowing cells to grow. Such a step is necessary considering that detection techniques are used in case of pathogenic organisms and it is vital to ensure that food is devoid of such organisms. Hence, the sample is suspended in an enrichment media first, followed by growing on selective or secondary enrichment media which supports growth of selective bacteria while inhibiting other flora. It is achieved by addition of inhibitory components to the media, temperature elevation or lowering pH. Further to secondary enrichment, a loopful of enriched sample is streaked onto the surface of agar plate to obtain well-isolated colonies. Streaking is performed with nichrome wire loop in such a way that individual colonies appear after incubation. Upon development of colonies, they are observed for their characteristic features using a microscope (Table 1.3). When in doubt, such colonies are purified further by streaking each colony onto a non-selective agar plate. They are further subjected to biochemical confirmatory tests and in certain cases to serological confirmatory tests to prevent reporting of false positives.

Each type of microorganism produces unique metabolic by-products either by reducing, fermenting or oxidising certain compounds. Biochemical confirmatory tests identify those by-products through either color reaction or chemical reaction. These tests are performed as a mandatory part of all confirmatory tests. Apart, serological confirmatory tests are available for certain bacteria such as Salmonella, Vibrio, *E.coli* O157: H7. In these methods, an antibody solution is mixed with suspected colony and allowed to conjugate with the antigens. The resulting conjugation is visible as agglutination or clump formation and is considered highly reliable due to specificity of antigen – antibody reaction.

Overall the classical techniques are relatively laborious and time-consuming, due to the time required for pre-enrichment, incubation, and execution of biochemical tests. Nevertheless, these techniques are still regarded as the gold standard due to their reliability, efficiency, sensitivity, and range of applications, remaining a necessary step for detection and enumeration, to determine viability, and to validate phenotypic predictions based on genomic analysis. Moreover, most of the international standards of microbiological detection and/or enumeration are based on these

Table 1.3 Typical appearance of select microorganism colonies.

Microorganism	Appearance
E. coli	Pink colonies with metallic sheen
Staphylococcus aureus	Jet black colonies with zone of precipitation
Salmonella	Red transparent colonies with or without black centers
Clostridium perfringens	Black colonies with zone of precipitation
Vibrio choleare	Yellow colonies
Vibrio parahemolyticus	Green colonies
Shigella	Colorless to pink colonies
Listeria monocytogens	Greyish colonies with a central depression and blackening around the colony
Bacillus cereus	Pink colored flat colonies with zone of precipitation

classical techniques. For example, techniques for enumeration of safety and hygiene indicators (*Enterobacteriaceae*, yeasts and molds, and lactic acid bacteria); detection of pathogens (*Listeria, Salmonella, Staphylococcus*, *E. coli* etc.), etc. [32].

1.6 Advances in Food Microbiological Techniques

In the previous section, we have seen that traditionally foodborne pathogens were monitored and characterized by laboratories using standard culture methods, which require 5 to 7 days to obtain presumptive results. As an alternative to these classical methods, molecular techniques have revolutionized microbial detection owing to shorter duration and higher performance. There are multiple advanced techniques for rapid detection/identification of microorganisms, as given in the subsections below.

1.6.1 Protein-Based Methods

Protein recognition strategies depend on immunoassays for discovery and evaluation of proteins present in specific microbes. Immunoassays involve reaction between an antigen and a counteracting agent, mostly

an antibody. Thus, the availability of antibodies with the desired affinity and specificity is the most important factor for setting up an immunoassay system. ELISA is the most commonly used protein-based method for detection of microorganisms in food. In ELISA, the antigen-antibody reaction takes place on a solid phase, generally a microtiter plate. The target antigen (bacterial cells or toxins from the food sample) binds to the immobilized primary antibody and the remaining unbound antigens are removed. After that, an enzyme-conjugated secondary antibody is added, which binds to the antigen and the remaining unbound antibodies are removed. The complex consisting antigen sandwiched between two antibodies is formed, which can be detected by adding a colorless substrate which will be converted into a colored form in the presence of the enzyme. ELISA has proven to be a rapid and specific detection technique and is routinely applied for qualitative and/or quantitative detection of *E. coli*, Salmonella species, *L. monocytogenes*, *Bacillus cereus*, Campylobacter species, and other pathogens [33]. The limit of detection (LOD) for pathogens are normally in the range of 10^4 and 10^5 CFU ml^{-1} and the assay time can take around 48 h, since a pre-enrichment step is commonly required in order to achieve the threshold limits for presence of bacteria on food samples. Nevertheless, some companies have recently developed fully automatic and quicker ELISA systems – VIDAS (VITEK immunodiagnostic assay system by Biomerieux) and Assurance® EIA (Assurance Enzyme Immunoassay by BioControl). VIDAS uses sensitive, fluorescence-based method for detection of foodborne pathogens in a variety of food matrices. The assay can be completed in 45 min to 2 h time and is recognized equivalent to ISO method for pathogen detection. Likewise, Assurance® EIA ELISA-based test kits are commercially available and allow for automation and high-throughput testing for Salmonella, *E. coli* O157:H7, *L. monocytogenes*, and Campylobacter [34].

In addition to ELISA, lateral flow immunoassays, also known as immunostrips or dipsticks, are used for rapid on-site detection of pathogens. A range of paper-based immunostrips for detecting foodborne pathogens have been commercialized and used in the food industry. Colloidal gold-based lateral flow strips are the most widely used immunostrips to detect foodborne pathogens. Ligand-based colloidal gold elements form spherical particles to which antibodies (biomolecules) bind. It results in formation of highly stable complex with retained biochemical activities of coupled biomolecules. These devices have been commercialized for detection of *E. coli*, *L. monocytogenes*, *S. aureus*, etc., in multiple food matrices, with detection limits as low as 10^6 CFU ml^{-1} [35]. Additionally, immunostrips can also be used to detect toxins which may cause foodborne diseases such

as botulinum, shiga, staphylococcal enterotoxin, etc. [36]. Although lateral flow immunoassays are simple and fast, a major limitation to their application is that most of these assays are designed for individual tests rather than high-throughput screening.

1.6.2 DNA-Based Methods

DNA-based methods such as polymerase chain reaction (PCR) and real-time PCR (qPCR) are rapid, versatile and sensitive, and allow both specific and quantitative detection of microorganisms from a variety of origins [37]. PCR works on the principle of enzymatic amplification of nucleic acid sequence in a three-step cyclic process – denaturation, annealing and extension. This allows for production of multiple copies of DNA of choice from a single or a few copies of DNA template. PCR amplification products are visualized using electrophoresis after staining with Ethidium bromide. Different ISO standards for foodborne pathogens are used by laboratories, which combines enrichment and PCR screening, resulting in reduced number of samples, increased testing sensitivity and quicker results. Moreover, PCR reactions are highly affected by inhibitors present in different food matrices, therefore it is critical to include internal amplification controls during assay. Furthermore, standard End-point PCR methods alone could not discriminate between DNA derived from live (viable) and dead (non-viable) cells. To overcome this limitation, the use of cell viability dyes in combination with DNA amplification methods, sometimes termed viability PCR, is explored. Ethidium monoazide (EMA) or propidium monoazide (PMA) are the two most common dyes, used to stain cells before any DNA amplification. This step leads to irreversible damage of nucleic acids in cell membranes where dyes have perforated, thereby leading to amplification of DNA only from cells with intact membranes. The major limitation of viability PCR is the occurrence of false positive results, since some cells might remain intact without any metabolic activity. In such cases, detection of mRNA is considered a better indicator of cell viability than DNA, since mRNA molecule is only present in metabolically active cells. Reverse-transcriptase PCR (RT-PCR) is used in such cases, which convert mRNA to cDNA before its amplification [38]. It has been demonstrated that End-point PCR is capable for detection of microbes such as *E. coli* O157:H7, *Staphylococcus aureus*, *Campylobacter jejuni* and *Shigella* spp.

qPCR is an advance form of PCR which monitors the exponential increase in amplicons during each cycle using fluorescent reporter. The increase in fluorescence is plotted against number of cycles to obtain an amplification

curve from which quantification cycle (Cq) is determined, which can be linked to initial concentration of target nucleic acid. Two of the most common chemistries employed in qPCR are based on DNA intercalating dyes (SYBR® green chemistry) or hydrolysis probes (TaqMan chemistry). SYBR® green chemistry is used when opting for a low-cost routine analysis of pathogens, whereas TaqMan chemistry is helpful for simultaneous detection of multiple pathogens in short time. qPCR is capable of detection of subdominant bacterial populations, even in the absence of selective enrichment medium and in the presence of other dominant populations [39]. Application of these rapid and accurate methods for detection of bacterial pathogens in food products has proven to be of great value to the food industry. Although it is a robust technique, due to its high sensitivity even small variations can induce non-negligible difference in results [40]. Still the advantages of this technique have led to the development of several commercial qPCR kits for foodborne pathogen detection, for example Romer Lab's RapidChek® series of kits for *E. coli* O157, Salmonella, Listeria and Campylobacter; Thermo Scientific's SureTect™ kit for Salmonella, Listeria, *E. coli* O157:H7 and Cronobacter; Biotecon Diagnostic's foodproof® series of kits for Aspergillus, Campylobacter, Clostridium, *E. coli*, Enterobacteraceae, Salmonella and many more pathogens.

Both End-point PCR and qPCR technologies have also been widely utilized for simultaneous detection of more than one pathogen in a single reaction, using a set of microbe specific primers. This is known as multiplexing and is able to detect organisms with different species or genera simultaneously from a single food sample. Most critical factor for a designing of a successful multiplex assay is primer design, as primer sets should have similar annealing temperature and non-complementary regions. Some other important factors to be considered are concentrations of primers, PCR buffer, magnesium chloride and deoxynucleotides. One can successfully analyze two to five different microbes, or even six in some cases, if all these factors are taken care of. For instance, *E. coli* O157:H7, Salmonella spp. and *L. monocytogenes* were detected at an LOD of 10 CFU in 25 g sample in a complex food matrix using End-point PCR [41], while the same three pathogens were detected in milk at an LOD of 1 CFU in 25 ml aliquots [42]. Likewise, six different *Listeria* spp. – *L. monocytogenes*, *L. grayi*, *L. ivanovii*, *L. innocua*, *L. welshimeri* and *L. seeligeri* – were detected in processed meat samples using multiplex PCR [43].

Reverse trascriptase PCR performed on a qPCR machine to analyze amplicons in real time is better known as RT-qPCR. It has been widely applied to environmental samples to analyze the functioning of target genes in varied conditions. Additionally, RT-qPCR has also been utilized

to carry out microbiological risk assessment analysis. In a study, it was applied to determine the expression level of Staphylococcal enterotoxin genes from *S. aureus* obtained from cheese [44]. Moreover, the most prominent application of the RT-qPCR is observed for rapid detection of viral foodborne pathogens, since it does not require post-amplification manipulations thereby limiting chances of contamination. RT-qPCR was capable of discriminating the infectivity of hepatitis A virus and norovirus in multiple food samples [45, 46].

Loop-mediated isothermal amplification (LAMP) is a recently developed nucleic acid amplification method by Notomi and his co-workers [47]. It is carried out in a single tube at constant temperature using two outer and two inner primers to target six specific regions of DNA. *Bst* DNA polymerase, which leads to auto-cycling strand displacement DNA synthesis, is used and final products of reaction are stem-loop DNAs of different sizes. Typically, a LAMP reaction is completed in an hour and results are analyzed using agarose gel electrophoresis or SYBR green dye. It is more sensitive and specific compared to PCR assays for foodborne pathogen detection, and the technique has already been commercialized for *Salmonella*, *Listeria*, *E. coli* and many other foodborne pathogens. Recently, a multiplex LAMP assay has been developed for simultaneous detection of *S. aureus*nuc, Salmonella fimY, and ShigellaipaH in fresh fruit juice using three sets of primers, with LOD of 2 CFU/10 mL [48]. Additionally, real-time monitoring of LAMP amplification products has been demonstrated successfully for detection of viable *E. coli* O157:H7 and *Vibrio parahaemolyticus* with utmost sensitivity and specificity.

Nucleic acid sequence-based amplification (NASBA) is another technique which operates under isothermal conditions for nucleic acid amplification, without the need of thermal cycling system. It was developed by Compton to amplify RNA by converting it to cDNA. The reaction occurs at around 40 °C, using two target specific primers and three enzymes – AMV Reverse transcriptase, T7 RNA polymerase and RNase H [49]. Similar to LAMP, products of NASBA are detected using agarose gel electrophoresis. It is a high throughput method and is widely used for detection of RNA viruses in complex matrices. Real-time NASBA monitoring using fluorescent molecular beacons is capable of detecting viable microorganisms present in food samples.

1.6.3 Biosensor-Based Methods

Biosensors are defined by the International Union of Pure and Applied Chemistry as "an integrated receptor ± transducer device, capable of

providing selective analytical information using a biological recognition element" [50]. In brief, a biosensor is an analytical device consisting of two parts – a biologically sensitive recognition element (whole cell, nucleic acid, enzyme or antibodies) and a physicochemical transducer connected to a detector. This technology has revolutionized food safety research and is available in a range of readout platforms on the basis of transducing element – electrochemical, piezoelectric, potentiometric, amperometric, calorimetric, optical, acoustic, and immunosensors. Moreover, the key to success of biosensors is their bio-recognition element, which imparts superior level of specificity and binding affinity with the target molecule, thereby enabling precise interactions in complex matrices. Earlier research and development of biosensors was focused mainly in the field of clinical research; however, since the last decade the focus towards use of biosensors in food analysis has increased sharply, mainly due to improved accuracy in target pursuit and higher demand of quality food from stakeholders [51]. Biosensors are particularly of interest for food microbiological analysis as they are easy to operate and do not require sample pre-enrichment step, unlike protein and DNA-based methods described above.

Immunosensors are one of the most widely developed biosensors for detection of pathogenic bacteria. They are based on the specific antigen–antibody binding reactions, where the antibody is immobilized on the sensor platform to capture the bacteria that are of interest. Then, the bacteria detection is measured through a transducing element – mainly electrochemical, optical, or piezoelectric readouts. The transducers chosen are directly related to the labelling, enzymatic or not, performed on the antigen or on the antibody. Piezoelectric sensors (quartz crystal microbalance, QCM) and modern optical sensors based on surface plasma resonance (SPR), allow the label-free detection with a direct quantification of the immunocomplex (Ab–Ag). Most of the developed immunosensors for foodborne pathogens are based on sandwich assay similar to ELISA technique [52]. Detection of *E. coli*, *L. monocytogenes* and *C. jejuni* was demonstrated using sandwich amperometric assay system, with detection limits of 50, 10 and 50 CFU/mL, respectively, in overall time of 30 min [53]. This system was later applied to milk and chicken extract samples as well. An alternative fibre-optics biosensor-based sandwich immunoassay system was developed to detect *L. monocytogenes*, with threshold levels of 4.3×10^3 CFU/ml [54]. Delibato *et al.* [55] developed a simple and rapid multichannel electrochemical immunosensor (MEI) based on the use of a 96-well plate and a "sandwitch" format for the detection of *Salmonella*

enterica, with LOD calculated to be 2×10^6 CFU/mL, with total analysis time of about 3 h.

Recently, there has been a surge in research based on use of genetic and bacteriophage biorecognition elements in biosensing. DNA biosensors are created by immobilization of an oligonucleotide sequence (probe) onto a transducer element, which converts the signal generated in response to hybridization of probe and target sequence to optical, electrochemical or piezoelectrical element, detected using suitable devices. Conducting polymers (mostly polyaniline and polypyrrole) are used to immobilize DNA onto a transducer since they offer better signal transduction, enhanced sensitivity, selectivity, durability, biocompatibility, direct electrochemical synthesis, and flexibility for the immobilization of biomolecules, including DNA [56]. Detection of different pathogenic bacteria is performed using disposable low-density genosensor array, fabricated using gold electrodes having immobilized thiol-tethered oligonucletide and biotinylated signalling probes for the detection of sequence complementarity. Primary criteria for selection are based on strain specific toxin produced by particular pathogen, as the encoded genes frequently express the toxins in food [57]. A promising tool for simultaneous detection of *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* based on multiplex PCR and electrochemical magneto-genosensing on silica magnetic particles was reported in two separate studies [58, 59], proving the rapidity and sensitivity of this technique. A PCR-free method, using an electrical 16S rRNA specific oligonucleotide microarray and automated analysis system was devised for *E. coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *S. aureus* and *Staphylococcus epidermidis* detection. Thiol-modified oligonucleotides for specific recognition of 16S rRNA were immobilized on interdigitated gold array electrodes to which biotin labelled detector oligonucleotide were hybridized, enabling the binding of avidin alkaline phophatase conjugates. The phosphatase liberates the electrochemical mediator p-aminophenol from its electrically inactive phosphate derivative, resulting in generation of electrical signals by amperometric redox cycling, detected by a unique multipotentiostat [60].

Bacteriophages are ubiquitous virus present in almost all environmental conditions, soil, food, ground water, surface water, etc. They specifically bind the host bacteria and inject their DNA to begin multiplication and propagation of mature virions. Use of these bacteriophages as bio-probes in biosensor devices offer multiple benefits – have shorter replication time so it's easy to obtain their mass production; specific to host bacteria enabling bacterial screening; resistant to critical conditions

including organic solvents, pH and temperature ranges. Amine and thiol groups present in phages are allowed to bind to the gold surface through Van der Waals, hydrophobic and covalent bonding. Additionally, many phage-based optical biosensors are available commercially for foodborne pathogen detection – BIOCORE 300 for *Salmonella enteritidis* and *E. coli* O157:H7 and SPREETA for *S. typhimurium* and *S. enteritidis*. Optical biosensor techniques are classified into two major subclasses, fluorescence and label-free, based on their working platform. Although use of bacteriophage-based biosensors for direct diagnosis of pathogens in fresh foods such as milk, water and complex food matrices, is very common, some of the major challenges faced during development of phage-based biosensors are the optimization of phage size and expression of binding units on phage's surface for specific binding to bacteria. With advances in methods of genetic engineering, novel phage probes are being developed to increase capability and sensitivity [61].

Cell-based biosensors are another widely used device for detection of foodborne pathogens. They use electrical properties of cells to analyze changes in cell's vicinity. Cell membrane functions as capacitor while the fluid acts as resistance element. Enzymes and co-factors, which release a large amount of chemical compounds and are present in a cell's metabolic system are used for detection of pathogens. Mostly mammalian cells, with specific sensitivities are used for discriminating pathogenic and non-pathogenic bacteria [62]. Cytotoxicity is assessed by measuring the colour change caused by alkaline phosphatase activity using optical-fibre devices. Application of this technique was demonstrated for detection of L. monocytogenes in ready-to-eat meats and rice at 10^3 – 10^4 CFU/ml following enrichment for 4–6 h [63].

Thus, we have seen that advanced techniques offer various advantages for detection of foodborne pathogens and are generally more sensitive, specific, time-efficient, and reliable compared to conventional methods. All the techniques have their own pros and cons. For example, PCR and qPCR-based techniques are high-throughput but require trained personnel and high-end equipments, at the same time NASBA and LAMP are comparatively easy to perform without the need for specialized equipments. Protein-based methods, ELISA and immunostrips, give best results in the absence of interfering molecules. At the same time, biosensors are relatively easy to operate and do not require trained personnel; also it can be used without a pre-enrichment step, but its on-site application is still limited. To overcome these limitations, use of a combination of these advanced methods for detection of foodborne pathogens is the best alternative and further studies in regard to them is the need of the hour.

1.6.4 AI and ML Applications

A few of the unexpected techniques that have found their way into food microbiology and expected to be widely used are [64] – Artificial Intelligence, Machine Learning, Big Data, Artificial Neural Networks, Hyperspectral Imaging, etc. Though these technologies were initially not developed for application in food industry, their capabilities are being exploited to make food more safe and secure, apart from providing almost real-time data. Some of the applications of these new techniques/tools are real-time monitoring of microbial load, identifying food anomalies through scanning and/or against genome sequenced databases, modelling microbial growth considering various factors including the food product, etc. It is imperative that these tools be fine-tuned to make them more robust and be made part of food supply chain surveillance to ensure safe food.

Food processing plants have to be routinely shut down for cleaning their processing equipment to prevent microbial contamination of foods. Since the activity is taken up at defined frequency considering the worst-case scenario, it may result in "over cleaning" leading to wastage of precious natural resource – water, apart from reduced efficiency of the processing plant. However, it is also possible that the processing lines may be "under cleaned". To avoid such instances, it will be ideal to have an on-line monitoring system which can automatically determine microbial loads and initiate cleaning. One such system is AI-based SOCIP – Self-Optimising-Clean-In-Place. It uses ultrasonic sensing and optical fluorescence imaging which can measure the amount of food residue and microbial debris in an equipment based on which the cleaning process is optimized. Deployment of such systems will become more prevalent in years to come. In a study on species identification [65], *Bacillus cereus* group species were detected and identified using a machine learning approach, based on artificial neural network (ANN) assisted Fourier transform infrared (FTIR) spectroscopy. The detecting model developed was 100% accurate in detecting and identifying the group species in the training sets while the accuracy was 99.5% in overall identification. Differentiation of group species was achieved by constructing one-level ANN. Such models will be very useful during foodborne outbreaks since they are rapid and accurate.

Technological advancement has made it possible to generate a huge amount of data. With availability of techniques for high throughput data generation, it becomes imperative to develop tools for efficient utilisation of big data. This has led to development of predictive microbiological tools for assessing product shelf life, risk assessment planning, identification of critical testing areas, its frequency, etc. Such models have already been

developed for spoilage as well as pathogenic microorganisms. Their efficacy would be dependent on the parameters considered for model development and extensiveness of the database deployed. One such software tool for predictive microbiology and microbial risk assessment in foods is – "MicroHibro" [66], created by the HIBRO research Group, based on Predictive Microbiology Model Data Base (PMDB). It integrates the application of predictive microbiology models and quantitative microbiological risk assessment in foods, providing a comprehensive solution for the improvement of food quality and safety. It allows models to be entered, shared by users apart from comparing with other models leading to development of effective microbial risk assessment models. However, a few other models have also been developed with varying utility. Such tools offer an excellent system for public health monitoring agencies, food processing companies, etc., for monitoring food quality and safety by modelling the growth and development of potential pathogens and spoilage microorganisms along the food value chain. In future, it is expected that voluminous databases will be created, and a fingerprint of food products will be scanned against such databases to determine presence of "foreign" microbes and their nature. Such systems will yield results in almost real time and will help in quick decision making. It is believed that such tools and techniques will find diverse applications in years to come and will form an indispensable part of surveillance programs for monitoring food safety and quality.

1.7 Regulations Governing Food Microbiology

Food regulations have been in existence since ancient times. However, during those times, the primary purpose was to facilitate trade by preventing economic deception. There are innumerable references of the prevalence of such regulations in ancient Greek, Roman, Indian and Chinese mythologies. With time, as food trade has increased, so were the incidences of food fraud, including compromises on food quality and safety, and the dire need to bring in regulations through legislation to address such issues has arisen. Earlier, only developed nations had regulations governing food quality and safety. However, during the last two decades, the majority of countries have developed their own food safety regulations, keeping in view country-specific needs. At present, almost all the countries have regulations, with varying degree of permissible limits, to deal with food safety and quality. It is almost impossible to list all these standards in a single chapter. The Food and Agriculture Organization (FAO) maintains a

database of national legislations, policies and bilateral agreements on food, agriculture and natural resources management called Food, Agriculture and Renewable Natural Resources Legislation Database (FAOLEX) [67], which can be referred to for complete details. Despite availability of national level regulations, certain global regulations have been accepted as standard guidelines by the majority of countries and are considered as benchmarks for global trade and preparation of national-level guidelines. One of the most commonly accepted principles for ensuring food safety is implementation of Hazard Analysis and Critical Control Points (HACCP), an approach towards hazard identification, assessment of risk, and control [68]. During the 1960s, initially, it was developed by National Aeronautics and Space Administration (NASA), the Pillsbury Company and the U.S. Army Laboratories, with only three principles. In 1971, it was presented at the first National Conference on Food Protection. In 1980, the World Health Organization (WHO) and International Commission on Microbiological Specifications for Food published a report on HACCP, followed by WHO Europe recommending it three years later. Subsequently, in 1985, the National Academy of Sciences recommended HACCP, following which in 1992 the National Advisory Committee on Microbiological criteria for Foods issued revised guidelines with seven principles, which still prevails. During 1993, Codex issued the first HACCP guidelines and a year later the International HAACP alliance was formed. A few agencies that are involved in issuing globally acceptable microbial safety guidelines include the following:

- The Codex Alimentarius Commission: It was established jointly by FAO and WHO in 1963 with the objective of protecting consumers' health and ensuring fair practices in food trade. It is a collection of internationally recognized standards, codes of practice, guidelines, and other recommendations relating to various aspects of foods including production and food safety. Its international standards, guidelines and codes of practice documents are considered as a "gold standard" by countries across the world as they are referred to for ensuring food safety and quality. A Codex committee on Food Hygiene recommends microbiological test methods in order to verify hygiene requirements. Codex Alimentarius has come up with microbiological standards for foods, relevant for pathogenic and spoilage organisms, which are incorporated by 160 member countries.

- USFDA: The US Food and Drug Administration is the oldest comprehensive consumer protection agency of the US federal government. The regulatory activities that it undertakes currently began with the Pure Food and Drugs Act (1906); a law came into force to prohibit interstate commerce in adulterated and misbranded food and drugs, providing basic elements of protection to consumers. With changing times, it has evolved to address food safety challenges to ensure public health. It publishes a host of standards for regulations of various products that have a direct bearing on human health. From time to time, the Office of Food Safety recommends guidelines for food industries to ensure food safety.
- European Commission: It is an executive branch of the European Union and is confirmed by the European Parliament upon nomination by the European Council. It is responsible for proposing legislation and implementing decisions apart from other activities. EUR-Lex, through its Official Journal of the European Union, publishes various commission regulations including those related to food safety as applicable to EU countries. One such regulation is Commission Regulation (EC) No. 2073/2005 on microbiological criteria for foods, which lays down food safety criteria for relevant foodborne bacteria, their toxins and metabolites, etc., in specific foods. These criteria define the acceptability of a product. Apart, it also lays down food processing hygiene criteria to indicate safe functioning of the production process. The microbiological criteria have been developed in accordance with internationally recognized principles, such as those of Codex Alimentarius. Scientific advice on matters relating to microbiological risks in food is provided by the European Food Safety Authority (EFSA), an organization tasked with risk assessment in EU.
- The International Commission on Microbiological Specifications for Foods (ICMSF): It was formed in 1962 through the action of the International Committee on Food Microbiology and Hygiene, a committee of the International Union of Microbiological Societies (IUMS). Through the IUMS, the ICMSF is linked to the International Union of Biological Societies (IUBS) and WHO of the United Nations. Its goal is to provide timely, science-based guidance to government and industry on appraising and controlling

the microbiological safety of foods. The primary objectives of ICMSF are to provide the scientific basis for microbiological criteria and to promote principles for their establishment and application; to overcome the difficulties caused by nations' varying microbiological standards and analytical methods. It also publishes books and articles in the area of food microbiology for the benefit of food safety value chain stakeholders.

- National Advisory Committee on Microbiological Criteria for Foods (NACMCF): It was established in 1988 to provide impartial scientific advice to federal agencies to use in developing integrated food safety systems from farm to table and for ensuring food safety in domestic and imported foods. Its recommendations are utilized by various food safety agencies such as USDA, Centers for Disease Control and Prevention (CDC), Department of Defense, etc., of the U.S. federal government. The Committee covers public health issues relative to the safety and wholesomeness of the U.S. food supply, including development of microbiological criteria and review and evaluation of epidemiological and risk assessment data and methodologies for assessing microbiological hazards in foods.

1.8 Conclusions

Microorganisms are ubiquitous and food microbiology has assumed great importance over the last few decades. Food microbes certainly have certain harmful effects but at the same time they have significant benefits to human health. From the food supply chain perspective, it is important to determine various microorganisms – either quantitatively or qualitatively. Traditionally, these microorganisms were detected by tedious, time-consuming techniques. Despite limitations, such techniques are still routinely employed across the industry due to their wider acceptability. However, advanced techniques are replacing these techniques, albeit at a slower rate, primarily due to their sensitivity and rapidity. They are primarily protein, DNA or bio-sensor-based methods. However, in the recent past, machine learning, artificial intelligence and big data started finding their applications in food microbiology too, though at a very primitive level. Considering the potential applications and speed at which results can be delivered, it is expected to find wider usage across the food value chain.

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