Microbial Determination Methods in Pharmaceutical and Food Quality Control

By

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing Master of Science in Biotechnology degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Ethics Statement

This material is an original work, which has not been previously published elsewhere. It is my own research and analysis in a truthful and complete manner. The paper properly credits all the sources used (correct citation).

Abstract

Within the realms of pharmaceutical and food production, maintaining stringent quality control measures is imperative. The determination of microbial content emerges as a pivotal component in safeguarding the integrity and safety of these products. This in-depth investigation traversed the multifaceted landscape of microbial determination methods, encompassing diverse sample types and an array of analytical techniques. The foundational section on sampling processes meticulously outlines the intricacies involved in acquiring representative samples across various matrices. It delves into the nuances of sampling methodologies tailored for liquid, solid, and environmental samples, emphasizing the pivotal role of meticulous sampling in ensuring the accuracy and reliability of subsequent microbial analyses. The exploration extended further into conventional microbial determination methodologies, elucidating the operational nuances and utility of stalwart techniques such as the bioburden test, standard plate count, most probable number and the direct epifluorescent filter technique. Emphasizing their historical significance and established application domains, this section served as a primer to contextualize the evolution of microbial determination methods. A substantial segment of this research is dedicated to rapid microbial determination methods, presenting a thorough examination of Polymerase Chain Reaction (PCR), Enzyme-Linked Immunosorbent Assay (ELISA), ATP bioluminescence assays, and flow cytometry. This detailed analysis underscores the unparalleled speed and accuracy of these contemporary techniques, revolutionizing microbial analysis across pharmaceutical and food industries. Venturing into the cutting-edge frontiers of microbial determination, this study explored the burgeoning domain of newer rapid techniques. It provided an incisive insight into miniaturized systems, biosensors, and their recent strides, notably highlighting the advancements in Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). This section encapsulates the transformative potential of these novel techniques, offering glimpses into their applications poised to redefine microbial determination across industries. In summation, this paper synthesized the intricacies and advancements discussed, underlining their profound implications in fortifying the foundations of quality control protocols. A blend of conventional, rapid and cutting-edge methods forms a robust system for precise microbe identification, bolstering safety and quality in pharmaceutical and food production.

Keywords: Microbial determination methods; Pharmaceutical quality control; Food quality control

This work is dedicated to

My Beloved Parents

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Sincerely,

ARPON KUMAR DAS

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List of Acronyms

HACCP- Hazard Analysis Critical Control Point

CFU- Colony Forming Unit

MPN- Most Probable Number 3.4

DEFT- Direct Epifluorescent Filter Techniques

PCR- Polymerase Chain Reaction

ATP- Adenosine Triphosphate

DNA- Deoxyribonucleic Acid

PDA- Parenteral Drug Association

NAT- A Network Address Translation

FDA- Food and Drug Administration

CFR- Code of Federal Regulations

qPCR- Quantitative Polymerase Chain Reaction

Cq- Quantification Cycle

ICTS- Immunochromatographic Strip

IMBs- Immunogenetic Beads

HRP- Horseradish Peroxidase

IgG- Immunoglobulin G

FITC- Fluorescein Isothiocyanate

PI- Propidium Iodide

VBNC- Viable but Non Culturable

TCC- The Total Cell Count

ICC- Intact Cell Count

FISH- Fluorescence In Situ Hybridization

miRNA- Micro Ribonucleic Acid

AChE- Acetylcholinesterase

cAMP- Cyclic Adenosine Monophosphate

cGMP- Cyclic Guanosine Monophosphate

TFA- Trifluoroacetic Acid

ZEA- Zearalenone

LAB- Lactic Acid Bacteria

Chapter 1

Introduction

Microorganisms exist and multiply, which leads to the acquisition of microbial infection. The capacity of microbes to proliferate in food, pharmaceutical, and cosmetic items has long been known and has generated discussion. From an infectious perspective, food and pharmaceutical goods are dangerous and should be avoided since they contain pathogenic germs [1]. The physicochemical and biological characteristics of active compounds can also be changed by microbial infections, potentially turning them into hazardous substances [2]. Certain illnesses, like diarrhea, acute gastroenteritis, and stomach pain, are caused by microbial toxins. However, symptoms vary from minor discomfort to gastrointestinal death depending on the individual's sensitivity to the poison [3]. The last century has seen the development of numerous methods for sensitive, accurate, and speedy microbial detection in food and pharmaceutical microbiology labs. Automation of some kind is typically used in quick procedures to gather information on the number and caliber of microorganisms in the sample [4–7]. It's vital to note, though, that the term "rapid" is occasionally used to characterize the variety of tactics deployed. To be sure, when compared to traditional procedures, some new technologies do not yield results more quickly. It would be more appropriate to refer to them as "alternatives" since they offer a more exact, detailed, or accurate outcome [4]. Businesses could save time and money by implementing quick procedures. Rapid microbiological approaches are vital for improved and expedited control over raw materials and finished goods. Better responsiveness during the production process can also be provided by these quick approaches. The conventional methods are still often employed in many microbiological investigations because of their affordability and simplicity. Nevertheless, prior to the ultimate outcome of microbial contamination, these procedures require the product to be incubated for two to seven days (in liquid or solid culture media). Due to the fact that the microorganisms present in the complex environments of pharmaceutical and food goods require several days to grow into visible colonies before they can be discovered, a lengthy incubation period is required in this case. Furthermore, this incubation period might be extended to 14 days for the release of pharmaceutical formulations under certain circumstances, such as sterility testing. Thus, the primary issue with the conventional method is the duration required to obtain microbiological outcomes. Rapid approaches have also proven beneficial to the pharmaceutical and medical industries. Rapid microbiological technologies were first introduced in the medical field in the middle of the 1960s; they picked up speed in the 1970s and have been expanding ever since [6-8]. Selecting the most effective and efficient approach can be challenging and costly due to the variety of technology on the market. Information about advancements in the quick techniques in microbiology is provided by this review. The pharmaceutical items are the primary focus.

Nonetheless, the article's general information is shared by the food, cosmetic, and pharmaceutical businesses.

Objectives: Therefore, the objective of this study is to comprehensively evaluate and compare diverse microbial determination methods utilized within the pharmaceutical and food industries. Through a systematic analysis, this research aims to assess the efficacy, accuracy, and applicability of these methods, thereby providing insights to enhance quality control measures essential for ensuring product safety and quality in these critical sectors.

Chapter 2

Sampling processes

Any product that has the potential to harbor and cultivate microorganisms must undergo routine microbiological testing as part of its examination. Microbiological testing is a crucial step in determining the efficacy and safety of these products, in fact. The sampling procedure is one of the most crucial elements in this process. The first step in a process is sample collection, which gathers information about a batch's characteristics for assessment. Since only a portion of the batch is actually sampled for testing, that portion needs to be typical of the batch being studied. Sample selection has to be considered a crucial process because the outcome of that initial sample will determine the fate of the batch [9]. The efficacy of manufacturing control methods must be verified through an appropriate sampling procedure. The primary aim of the sample preparation processes is to reduce the number of microorganisms present in the product by either killing or decreasing their growth [4, 6, 8, and 10]. However, in microbiological sampling, microorganisms may not necessarily be randomly distributed throughout the whole batch or product and random sampling scheme should not therefore work there. Sampling schemes employed in microbial quality control are based on an analysis of susceptible points in a system, known as hazard analysis and control of critical points (HACCP). The seven principles of HACCP are listed in Table 1. There are four categories into which microbiological samples can be divided: solid, liquid, surface, and air.

Step 1	• Conduct a hazard analysis
Step 2	• Determine the Critical Control Points (CCPs)
Step 3	• Establish critical limits
Step 4	• Establish monitoring procedures
Step 5	• Establish corrective actions
Step 6	• Establish verification procedures
Step 7	• Establish recordkeeping and documentaion procedures

Table 1. The seven principles of HACCP

2.1 Liquid samples

This category includes liquid pharmaceutical samples (such as syrups, aqueous preparations, suspensions, and emulsions), liquid food samples (such as water, fruit juices, milk, coffee, and teas), and liquid cosmetic samples (such as shampoos and liquid soaps). Liquid sample preparation is, as far as we know, simpler than solid sample preparation. After sample collection and transportation to the lab, the proper mixing is needed to create a uniform mix. Either an instrument or brisk hand shaking might be used for this. The next step is to undertake an aseptic dilution process using a sterile diluent (a known volume of liquid sample and a desired volume of sterile diluent) [6, 11]. To get a homogeneous sample, a surfactant like lecithin or polysorbate 80 can be added to the diluent [9].

2.2 Solid samples

This group includes solid medicinal dosage forms (such as tablets and capsules), solid foods (such as fruits, vegetables, bread, and meat), and solid cosmetic items (such as solid toilet products). Pharmaceutical dose forms that are solid are discarded due to microbial deterioration or spoiling. Solid sample preparation commonly involves several steps, such as aseptic sample collection, quick sample transit (less than 24 hours) to the laboratory, and aseptic subsample removal. The next step is dilution in an appropriate sterile diluent, which can be done in ratios of 1:10, 1:25, 1:50, and so forth. As needed, more dilutions can be made. Following dilution, the sample needs to be homogenized using a variety of techniques, including blenders and homogenizers. In an aseptic state, surfactant addition and heat treatment are additional options. When the material has reached the homogenization stage, analysis can begin [6-8].

2.3 Environmental samples

2.3.1 Surface sample

Surface sample collection is used to identify the microbial flora on the surfaces of food items, medications, cosmetics, and their surroundings [12]. The swap method is the primary standard technique for surface sampling. A sterile, moistened swab typically made of cotton which is required to gently gather microorganisms from the surface [13, 14]. Following a shake and placement of the swab in a diluent (of defined volume), the sample is plated on an agar plate. Another way for surface sampling is to use a sterile knife, especially for food ingredients or delicate tissues. It is assumed that the product is sterile and that all of the organisms are on the surface [6-8]. Surface sampling can also be done with sterile sponges, sterile gauges, and sticky adhesives. The "Hands-free, Pop-up" sticky tape technique was created by Fung and associates [15]. With this strategy, an analyst wears a tape unit on their wrist, freeing up both hands to

conduct other experimental tasks [6, 8, and 15]. The analyst should select the proper unit to provide the sampling results for surface sampling. The number of germs per inch square, per centimeter square, or in other units can be used to report these results. For surface sampling, the sample unit's shape is also crucial. Using a sterile template might make sampling easier. Unfamiliar forms are rarely used by analysts for surface sampling; therefore the computation and quantification of these areas will be difficult [8].

2.3.2 Air samples

The monitoring of product manufacturing settings is crucial because air can serve as a primary reservoir for microorganisms. Checking and controlling microorganisms and their poisons in the air is also urgently needed due to recent worries about environmental air pollution and public health [16]. In air sampling, passive and active monitoring techniques are the two primary approaches [17]. Using air plates which are just regular "Petri dishes" with culture media without a lid is the most popular passive technique for air sampling. To collect biological particles, the plates are left in the air for a predetermined amount of time. Ten, thirty, or four hours can pass while taking it. After covering and incubating, the plate is ready to count the colonies. If the colony counts surpass a particular threshold, the air quality is deemed unsatisfactory. The passive method yields information that is not overly quantitative. Colony Forming Units (CFU)/plate/time or CFU/m2/hour is the units used to report passive monitoring results [6, 8, 16, and 17]. A particle collection device or an air suction sampler can be used for the active monitoring method to draw air through. The apparatus may consist of a nitrocellulose membrane, liquid culture fluid, or solid culture media. The quantity outcome is shown in CFU/m3 of air. Nonetheless, active monitoring techniques are useful in environments with low microbiological concentrations, such as clean rooms and hospital controlled environments[6,8,16,17].

Chapter 3

Conventional Microbial Determination Methods

Conventional microbial determination methods refer to established and time-tested laboratory techniques used for the identification, quantification, and characterization of microorganisms in diverse samples. These methods encompass a range of procedures, including culturing microorganisms in specific growth media, microscopic examination for morphological analysis, biochemical assays to identify microbial metabolic characteristics and enumeration techniques to quantify microbial populations. They form the foundational toolkit in microbiology, providing reliable, validated and often standardized approaches for assessing microbial presence, load and species composition in pharmaceuticals, food production, healthcare and environmental monitoring. Despite the emergence of newer rapid techniques, conventional methods remain integral due to their established protocols, regulatory acceptance and proven track record in ensuring product safety, quality control and research integrity.

3.1 Bioburden Test

The microbiological content of a material (or on the surface) at a specific moment in time is referred to as bioburden. This could be before sterilization or during a process hold time [18]. The term "bioburden" refers to an estimate of the amount of bacteria and fungi found in a liquid sample.

Bioburden assessment is a key activity in the evaluation of microbiological control. Bioburden is used in pharmaceutical production to assess bacteria levels in intermediate product samples. This enables an evaluation to determine:

- If bioburden levels are higher at the beginning of the process (upstream samples) than later in the process (downstream samples).
- If aspects of the process that contribute to bioburden reduction are effective.
- Whether extra process steps, such as water rinses, contribute to the bioburden.
- If process additives, such as formulation buffers, contribute to the bioburden and are not filtered.

All intermediate products should be deemed non-sterile. Given the nature of the substance, microbial recovery is to be expected. Regulatory bodies on the other hand anticipate adequate bioburden management throughout the manufacturing process [19]. Furthermore, the collected bioburden levels are of interest in terms of long-term trending for:

a) The percentage of samples that surpass the warning or action level;

b) Variations in the total or mean count;

c) Variations in the profile of microorganisms collected.

As this will represent the "worst case," samples should be taken near the end of any hold time to increase the sample's meaning. Furthermore, it's critical that the sample, in terms of homogeneity, accurately reflects the stage of the process.

The Total Aerobic Microbial Count method, which can be used with either a pour plate agar method or, more ideally, membrane filtration, is frequently used for bioburden testing. There are quick and other microbiological techniques accessible. Care must be taken when using the agar methods to achieve optimal recovery, including choosing the right agar (usually tryptone soy agar) and controlling the incubation time and temperature [20]. Using low-level microbial challenges, recovery assessments are carried out. In order to slow down or stop the rate of microbial development, collected samples must be given an expiration date (which must be qualified) and stored typically at 2–8°C.

3.2 Standard Plate Count

For many years, the "Standard Plate Count" has been the primary and most widely accepted method in applied microbiology. Following the preparation and dilution steps, the sample is combined with a general agar media, incubated (at 35°C or 25°C), and the colonies are counted after 48 hours [73,83,84]. Despite being straightforward and simple to use, this system has some flaws. This labor-intensive procedure requires a lot of culture media, sterile test instruments (such tubes, pipettes, and sterile plates), as well as big incubation areas. Re-sterilization and cleaning of the recyclable glassware are also required [84-86]. Some efforts have been made to semi-automate and simplify the standard method, such as mechanically spreading the sample on the surface of an agar plate that has already been formed, [86] applying membranes to trap microorganisms and then performing a standard plate culture, [87] and using nutrient-based methods that use nutrients that are stored in films or strips [73,83]. In order to do viable cell counts in the pharmaceutical sector, these approaches have been created as alternatives.

3.3 Most Probable Number (MPN)

Since more than a century has passed, notably in the food industry, the Most Probable Number (MPN) system (typically the three or five tube) has been used as one of the most typical techniques [88]. The MPN system has undergone automation just like the traditional plate approach. In 2007 a mechanical and automated system for the viable cell count process was demonstrated. It was a hands-free 16 tube system for the MPN method that was later used in laboratories for food and medicine all around the world [73, 83, and 89].

3.4 Direct Epifluorescent Filter Techniques (DEFT)

Real-time viable cell counting-based techniques have been tested in recent years. These real-time tests rely on incredibly quick procedures that stain "live" cells with "vital" stains so that they can be counted as viable fluorescing cells under a microscope. One automated system or the human eye can complete a viable cell count in less than one hour. In Direct Epifluorescent Filter Techniques (DFET), living microorganisms stain red with acridine orange whereas non-viable organisms stain green (most of which contain DNA). This method's main drawback is that RNA may be persistent in heat-damaged or preservative-damaged cells and stain red even in non-viable cells [81]. The micro-colony technique (DEFT-MEM) method is a modified DEFT method that was reported by Newby in 1991. In the modified procedure, cells are first filtered using a membrane, then cultured to form micro-colonies, and then labeled and counted [90].

Chapter 4

Rapid Microbial Determination Methods

Rapid microbial determination methods encompass a suite of innovative, technologically advanced techniques designed to swiftly and accurately identify, quantify or characterize microorganisms within diverse samples. These methods leverage cutting-edge approaches, such as molecular biology assays e.g., PCR, ELISA, ATP Bioluminescence Assay, Flow Cytometry, DNA sequencing, Biosensors, Microfluidic systems and MALDI-TOF mass spectrometry, among others, to expedite the detection, enumeration and characterization of microbial populations. These approaches offer accelerated turnaround times, often providing results within minutes to hours, compared to conventional methods, while maintaining high specificity, sensitivity and reliability. Rapid microbial determination methods play a pivotal role in numerous sectors including healthcare, food safety, environmental monitoring and pharmaceuticals by enabling prompt decision-making, swift response to potential hazards and efficient quality control measures to ensure product safety and integrity.

4.1 Polymerase Chain Reaction (PCR)

A crucial element in ensuring the safety, stability, and potency of pharmaceutical raw materials and final products is the detection of microbial contamination. Analyses for pharmaceutical and food quality control using quick microbiological techniques, including polymerase chain reaction, are common. The use of polymerase chain reactions for pharmaceutical quality control, however, has been rare and relatively slow. Important considerations are needed to generate sensitive and reliable assays that will conform to good manufacturing procedures for the successful adoption of these technologies in pharmaceutical firms in underdeveloped nations.

In a study by Volokhov et al. [21], it was shown that PCR may be used to detect Mycoplasma in biopharmaceutical products, intermediates, and raw materials. Two tests are used in the traditional method of mycoplasma detection to distinguish between culturable and nonculturable mycoplasma: the agar/broth media assay and the indicator cell culture assay. The lengthy testing period, which could last up to 35 days, is one of the disadvantages of the conventional approach. Results of an assay may also be influenced by additional elements like sample handling, analyst mistakes, and medium components. Another significant flaw is the absence of common preparations of reference Mycoplasma strains. Although compendial laws like USP [22] [23], EP 2.6.7 [24], and Japanese Pharmacopoeia XV General Information/Chapter 9 [26] do not specifically forbid the use of PCR for the detection of Mycoplasma in cell cultures, they do permit it. Additionally, there are documents that came from organizations that

discussed this trend, such as PDA Technical Report No. 50, which illustrated alternate techniques of mycoplasma testing such NAT approaches. Primer and probe selection, sample amount, nucleic acid extraction and purification, assay setup, data interpretation, reference materials, and technique validation were among the crucial NAT-related topics covered [25]. A U.S. Food and Drug Administration (FDA) Points to consider paper [27] and Code of Federal Regulations 21 CFR 610.30 [28] both gave more talks. Other studies [21, 29-35] also showed that PCR-based approaches for the detection of Mycoplasma were validated. But with huge time and budget savings, pharmaceutical corporations adopted Mycoplasma PCR testing and regularly employed them. Commercial kits like MycoSEQ (Applied Biosystems), MycoTool (Roche), Cytocheck (Greiner Bio-ONE), and others are also available for the detection of mycoplasma.

The performance of the assay, which is dependent on the structures and sequences of the primers and template, is one of the factors that affect PCR efficiency. The efficiency of PCR is decreased by three factors: (a) the sample matrix, which may contain inhibitors and other interfering chemicals from the sample or carryovers from upstream processing steps; (b) the reagents used and their quantities. Basically, any PCR reagent, including the PCR methodology; competing reactions, might have a negative impact on rate and performance [36]. Utilizing RNA or DNA spikes makes it simple to evaluate the samples for inhibition [37], [38]. A serial dilution can also be used to observe it [39]. Since inhibition is more obvious in the most concentrated samples and causes a departure from linearity, it is frequently the root cause of inflated PCR efficiency estimates (E > 100%). These samples diminish the slope and, if disregarded or inadvertently included in the linear fit, result in estimations of PCR efficiency that are excessively high. In certain circumstances, qPCR is not affected by inhibition since it only manifests in upstream processes like reverse transcription. Both the matrix and the assessment must use a template. The estimated efficiency will represent how well the PCR test performs in the actual samples that will be examined by selecting a matrix characteristic of the field sample. However, for this to work, a pure matrix must be accessible. A new assay is often first verified in a pristine matrix devoid of interference-causing substances. High PCR efficiency indicates a robust assay that is less likely to be inhibited in complicated matrices. Because it is simple to generate, purified PCR product is frequently utilized as a template for PCR efficiency estimates. Because of its short length, it frequently causes side effects, and it does not account for the impact of surrounding sequences that could disrupt PCR by wrapping over the template [40]. When the original template is plentiful during the first few cycles of the PCR, this interference can be severe and affect the Cq value. An appropriate source of lengthy template molecules with typical secondary structures for the validation of tests for gene expression profiling is a cDNA library. The validation of DNA analysis assays can use genomic DNA or plasmids carrying the desired gene, preferably after excising a portion containing the target sequence to

eliminate interfering supercoiling [40]. Utilizing artificial templates is still another choice (examples include gBlocks - IDT and GeneArt - LT).

4.2 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is an immunological method used to detect specific microbial antigens or antibodies, which is helpful in identifying pathogens or toxins. Originally described by Peter Perlmann and Eva Engvall in 1971, the enzyme-linked immunosorbent assay (ELISA) is a frequently used analytical biochemistry test.(Source:) The assay employs an enzyme immunoassay (EIA) solid-phase type to use antibodies directed against the protein to be assessed to find the presence of a ligand, often a protein, in a liquid sample. A quality control measure in many businesses, ELISA has also been employed as a diagnostic tool in the fields of biotechnology, plant pathology, and medicine [41].

In polystyrene plates, typically 96-well plates coated to firmly bind protein, ELISA tests are carried out [42]. Depending on the ELISA type, testing calls for an analyte/antigen, coating antibody/antigen, buffer, wash, and substrate/chromogen.[43] The primary detection antibody is a particular antibody that solely connects to the target protein. A secondary detection antibody, on the other hand, is an additional enzyme-conjugated antibody that interacts with a primary antibody that has not been enzyme-conjugated [41]. An ELISA immunoassay is completed in four major general phases. They are as follows:

1. Coating (with either antigen or antibody)
2. Blocking (typically with the addition of bovine serum albumin [BSA])
3. Detection
4. Final read

By including a substrate that may produce color, detection is carried out. For ELISA detection, a variety of substrates are available. Horseradish peroxidase (HRP) and alkaline phosphatase (AP) are, however, the substrates that are employed most frequently [43]. Hydrogen peroxide serves as the HRP's substrate, which causes a blue color shift. p-Nitrophenyl-phosphate (pNPP) is typically used as the AP's substrate, and it measures the nitrophenol's yellow hue after 15 to 30 minutes of room temperature incubation [42]. In order to remove unbound material, the plate is "washed" using a buffer, such as phosphate-buffered saline (PBS) and a non-ionic detergent, between each of the aforementioned four processes. In accordance with the particular methodology, the wells are washed twice or more at each wash phase [41]. A series of concentration dilutions are added to the plate's wells in an ordinary ELISA process. After the results are measured, a standard curve derived from the serial dilutions data is shown with

concentration on the x-axis and absorbance on the y-axis using linear scales [44].In general, ELISA comes in four different forms:

- (Antigen-coated plate; antibody screening) Direct ELISA.
- Screening antigen/antibody using an indirect ELISA (antigen-coated plate).
- Sandwich ELISA (screening antigen; plate covered with an antibody).
- (Screening antibody) competitive ELISA.

ELISA tests are used to identify antigenic compounds, primarily proteins, as opposed to tiny molecules and ions like glucose and potassium. Some of these include hormones, bacterial antigens, and antibodies. The pharmaceutical sector uses ELISA techniques as key instruments for drug discovery, animal investigations, and clinical trials [45]. Food contamination can take the form of dangerous bacteria, pesticide and veterinary medicine residues, heavy metals, and other pollutants. For the identification of food pollutants, nano-ELISA is growing in popularity and offering great ease. It is common knowledge that traditional methods for identifying hazardous bacteria, such as Salmonella, Listeria monocytogenes, and Escherichia coli, relied heavily on microbiological identification. Long assay times, complicated enrichment processes, and culturing procedure were challenges for the approach. With the use of nanotechnology, ELISA can, nevertheless, achieve the sensitive, quick, and precise detection of food pollutants. In order to identify eight tested L. monocytogenes serotypes from Listeria spp., Wang et al. devised an AuNP-based ICG strip assay, which demonstrated adequate sensitivity (3.7 106 CFU/ mL) and satisfactory accuracy [46]. Following an 8-hour enrichment period, Wang et al. established a quick and sensitive Au NPs-based LFIA for the identification of Listeria spp. in milk samples [47]. Prior to being coupled with Au NPs as read-out labels, monoclonal antibodies were originally created to specifically identify the P60 protein on the surface of Listeria spp (Figure 1). A modified ELISA using Fe3O4 nanoparticle clusters (Fe3O4 NPC) was created by Zhang et al. as well for the quick detection of Listeria monocytogenes in popular foods [48]. In this study, Listeria monocytogenes was captured by first fixing vancomycin to the substrate via bovine serum albumin conjugation. As a signal label to identify the target, Fe3O4 NPC modified aptamers were then used. With a sensitivity of 5.4 103 CFU/mL, the suggested approach allows for the linear detection of Listeria monocytogenes from 5.4 103 to 108 CEU/mL. A selective and sensitive SPR-based competitive immunoassay for the detection of Cd2 in water samples was disclosed by Kang et al. for the analysis of heavy metals in food [49]. With a LOD of 1.25 ng/mL, the technique displayed linear Cd2 behavior in the range of 0 to 1000 ng/mL. For the quick and accurate detection of Hg2, Wang et al. created a chemiluminescent immunoassay strip approach [50]. To directly identify Hg2 without the use of a chelator, an anti-Hg2 monoclonal antibody was created in this study. It was then HRP-tagged and applied to the conjugate pad to serve as a signal tracer. With a detection limit of 0.2 ng/mL (S/N=3), the approach demonstrated a linear range of 1.0e100 ng mL1. Kong et al. created a multi-ICA strip method

for the simultaneous detection of 20 different mycotoxin kinds in cereal food samples for the purpose of mycotoxin detection in food [51]. Zearalenones, deoxynivalenols, T-2 toxins, aflatoxins, and fumonisin detection limits for the proposed approach were calculated to be 0.04e0.17, 0.06e49, 0.15e0.22, 0.056e0.49, and 0.53e1.05 mg/kg. Zearalenone (ZEN) detection in maize was accomplished by Zhang et al. using a fluoroimmunoassay (FLISA) and a quick immunochromatographic strip (ICTS) [52]. Quantum dots of CdTe, CdS, and ZnS were used as a fluorescent probe in the study and were tagged with an anti-ZEN monoclonal antibody. The LOD for the proposed FLISA and ICTS for ZEN determination in maize extract was 0.012 ng/mL and 1.5 ng/mL, respectively.

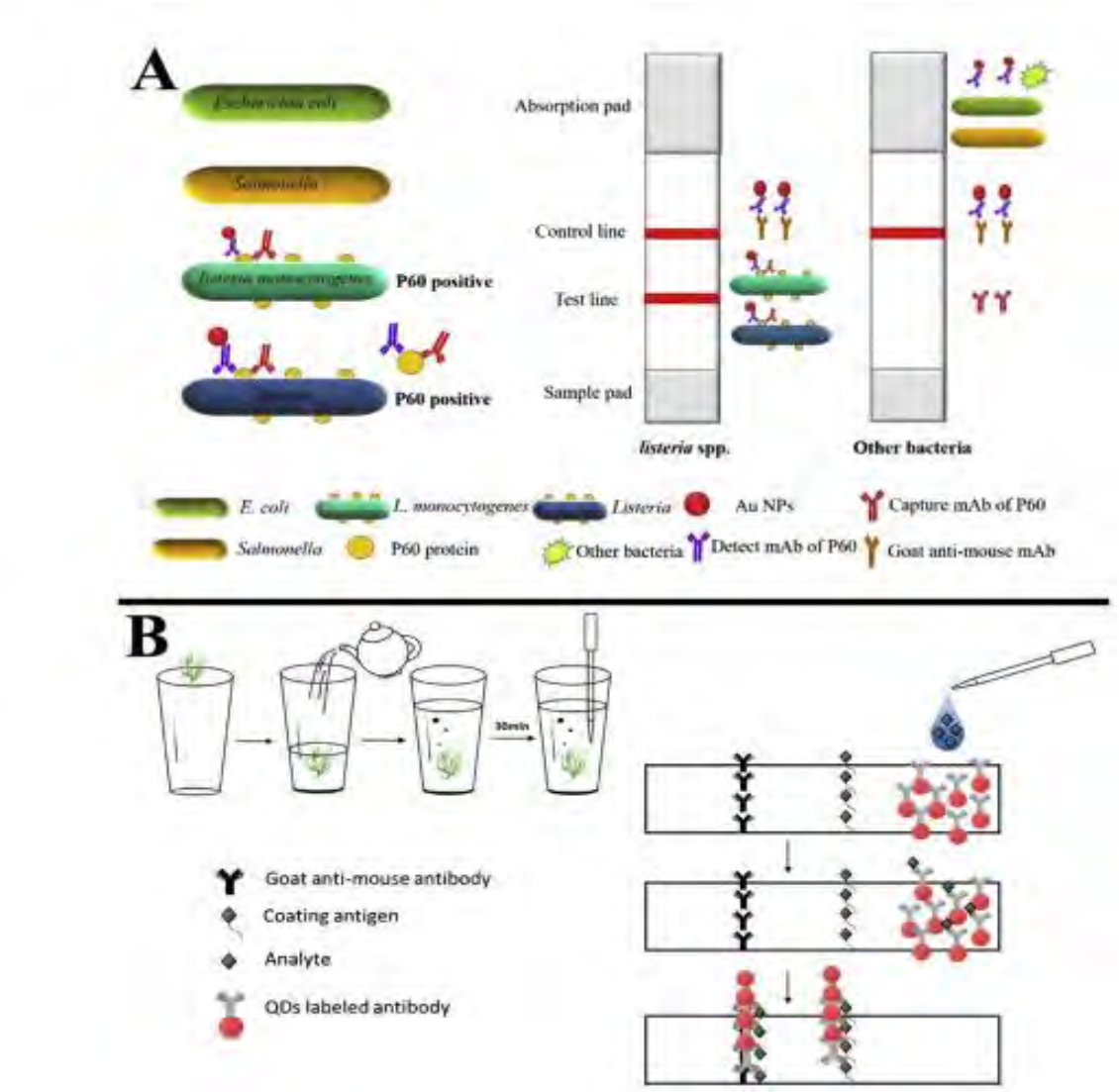

Figure 1: Diagram of a smartphone with an Au NPs-LFIS reader mounted and an LFIS in place. The assembly of silver nanoparticles (Ag NPs) and magnetic nanoparticles (MNPs) is used to create the cascade reaction-mediated magnetic relaxation switching (CRMRS) sensor shown in (B). With permission from Elsevier and the American Chemical Society, reprinted from Refs. [53, 54].

Realizing the quick determination of food's physicochemical properties during manufacturing and storage is essential since ensuring the food's safety, sensory quality, and shelf life is a vital concern [55]. Genetic or immunological approaches are typically used to identify a variety of food types, including fish, milk, foods that have been genetically modified or exposed to radiation, and other dangerous dietary ingredients [56, 57]. These conventional techniques are, however, rather pricy, labor-intensive, and difficult to carry out. Therefore, it is crucial to develop simple and reliable procedures for food risk assessment and quality control. Santaclara et al. presented a method to authenticate the gene of Thunnus species and Katsuwonus pelamis in food by PCR linked ELISA [58] for the purpose of identifying fish species. The strategy's success in commercial items, including fish species mixes that acted exclusively inclusive of all samples and inclusive of none, was confirmed by the commercial samples.

Using immunomagnetic beads (IMBs) as the basis, Zhang et al. created a direct competitive ELISA to identify total aflatoxins in samples of maize [59]. Specifically, monoclonal antibody 5H3 modified IMBs were used as the immobile phase to substitute microplates, and AFB1-CMO labeled HRP was used as the capture probe to compete with free aflatoxins. According to the approach, the LOD for detection of total aflatoxins was 0.21 ng/g, with a linear range of 0.22e19.8 ng/g. Perestam et al. reported that the PCR assay was more accurate and less expensive when compared to the ELISA technique, and ELISA was considered to be less time-consuming and easier to execute than PCR [60]. A competitive ELISA for the detection of pig adulteration in meat was established by Mandli et al. [61] by immobilizing IgG standard to compete with the extracted IgG. Using nanomaterials in various units, this technique can further discriminate between 0.1% of adulterated pork in 45 minutes. In addition, He et al. developed a method for the colorimetric detection of pork using Au NPs, with swine-specific DNA as the target and two other complimentary DNA sequences as probes [62]. Using a digital camera, the technique revealed a LOD of 0.1 mM and an emiquantification of 0.001e1 mM. 2-Dodecylcyclobutanone (2-DCB) and 2-Tetradecylcyclobutanone (2-TCB) are regarded as unique radio lytic products for the screening of lipid-containing foods because they cannot develop under any other processing conditions. For the detection of irradiated food, numerous techniques have been developed, however none of them are based on the ELISA method [63-66]. The immuno-rotary biosensor developed by Zhao et al. [67] is based on 2-DCB and -subunit monoclonal antibodies and is linked with biotin-avidin-biotin. With a sensitivity of roughly 108 mg/mL, the suggested approach can identify 2-DCB in 10 minutes. Through the simple application of the antigen-antibody model to ELISA or nano-ELISA, the monoclonal antibody recognized technique can be used to identify irradiated food. Numerous biosensors and bioassays based on nanoparticles have been developed in recent years to analyze allergen components [68-70]. A useful instrument in this context is nano-ELISA. As an illustration, Wang et al. developed a quick LFIA approach using an antibody that has been tagged with Au NPs [71] to selectively identify the allergen glycinin found in soybeans. For the 10-minute detection of

glycinin, the approach demonstrated good sensitivity of 0.69 mg/kg. With RSD of less than 5.29% (intra-assay) and 6.72% (intraassay), respectively, the recoveries of the proposed approach ranged from 80.5 to 89.9%. The suppression of the formation of Ag nanosphere (Ag NPs) dimers when the appropriate antigen was present is the basis of Mercadal et al.'s suggested enzyme-free technique for gliadin detection [72]. The sensitivities of the proposed method are around 1.5 x 103 and 9 x 103 pg/mL for 58 nm Ag NPs and 78 nm Ag NPs.

4.3 ATP Bioluminescence Assay

Detecting ATP in living cells that are contained in particular membranes is another technique used in this field. Somatic ATP in food products causes some challenges in the identification of microbes in food samples. However, this technique is useful in the pharmaceutical and cosmetic industries. In between one and four hours, viable cell counts can be obtained using ATP detection techniques [73]. Generally, ATP detection devices use the amount of light released during the enzymatic reaction (bioluminescence process) to determine the level of ATP [74]. ATP bioluminescence based on the luciferine/luciferase reaction has attracted a lot of interest in the realm of rapid microorganism identification. In fact, adenosine triphosphate (ATP), which is a great indicator of vitality and cellular pollution, is present in all living things (Figure 2). For this reason, using ATP-luminescence technology to detect ATP is preferred over traditional methods since it dramatically reduces the time it takes to make a discovery without compromising accuracy [75-78].

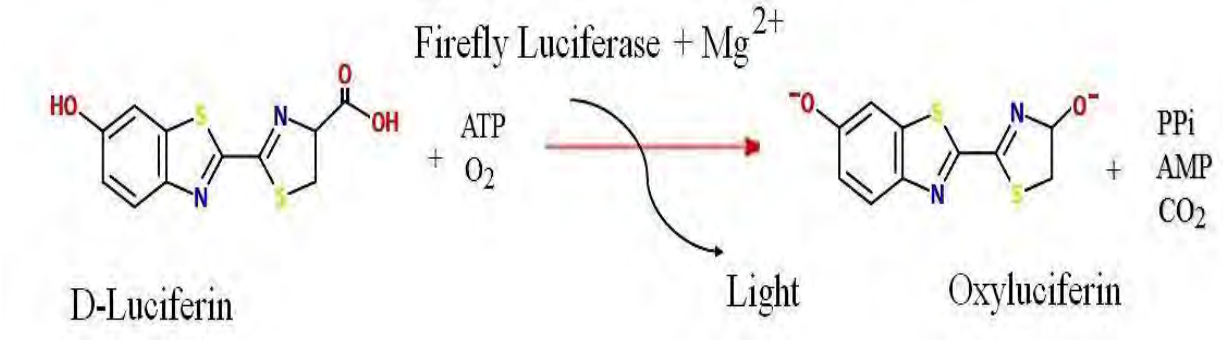

Figure 2. ATP bioluminescence based on luciferine/luciferase reaction

A key subfield of rapid methods is impedance microbiology, which measures changes in electrical conductivity to trace bacteria both qualitatively and quantitatively. Direct impedance technology and indirect impedance technology are both available. In a direct kind of impedance technology, the change in the electrical conductivity of a reaction solution is measured, whereas the change in the conductivity of a liquid culture medium serves as the measuring parameter in an indirect type [79, 80]. To combat Staphylococcus aureus, Pseudomonas aeruginosa, Aspergillus niger, and Candida albicans, Connolly et al. studied three rapid

microbiological methods, including impedance, DEFT, and ATP bioluminescence. For untreated suspensions of Candida albicans, Pseudomonas aeruginosa, and Staphylococcus aureus, they showed a strong association between these techniques and total colony counts. Only the impedance approach provided good correlation for Aspergillus niger. According to their findings, the ATP bioluminescence and DEFT methods failed to produce a good dose/response curve, however the impedance method did [81].

4.4 Flow Cytometry

Flow cytometry (FCM) is an alternate technology that is effective, quick, and highly sensitive [127, 128] for the detection, monitoring, enumeration, and characterisation of microorganisms in both specific and non-specific ways. A quantitative count of microbial cells is possible with FCM regardless of culture. The physiological, structural, and viability features of microbial cells are also disclosed by flow cytometry, which makes it a useful technique for additional characterisation. To reduce foodborne infections, the food sector is very interested in the rapid and accurate detection, quantification, and characterization of foodborne pathogens [129]. Methods based on immunology, biosensors, and nucleic acids can be utilized to quickly identify foodborne pathogens [130]. A quick measurement of the light scattered and fluorescence emission produced by properly illuminated cells is possible with the use of the analytical technique known as FCM. When the cells, or particles, individually pass through a light beam while suspended in liquid, they emit signals (Fig. 3). Since measurements are done separately for each particle or cell, the outcomes represent the totality of the individual cytometric features. Multiple cellular parameters can be measured by analytical flow cytometers, which is an important analytical characteristic. Depending on their cytometric properties, some flow cytometers can physically separate cell subsets (sorting) (Fig. 4). Detectors gather the scattered light (intrinsic parameters) and fluorescence emissions of each particle, which are then transmitted to a computer where the population's distribution with respect to the various parameters is displayed. Cell size can be determined from scattered light collected in the same direction as the incident light, and particle complexity can be inferred from scattered light collected at a 90° angle. The number of organelles present in the cell and the roughness of the cell surface are associated by this characteristic. Since they may be derived without staining the material, size and complexity are regarded as intrinsic factors. Samples can be stained with various fluorochromes to gather more information. According to their mode of action, fluorochromes can be divided into three categories [131]: those whose fluorescence increases upon binding to particular cell components, such as proteins (fluorescein isothiocyanate [FITC]), nucleic acids (propidium iodide [PI], and lipids (Nile Red); those whose fluorescence is influenced by cellular physiological factors (pH, membrane potential, etc.); and those whose fluorescence is For the purpose of directly detecting microbial antigens or DNA and RNA sequences, fluorochromes can also be coupled to antibodies or nucleotide probes.

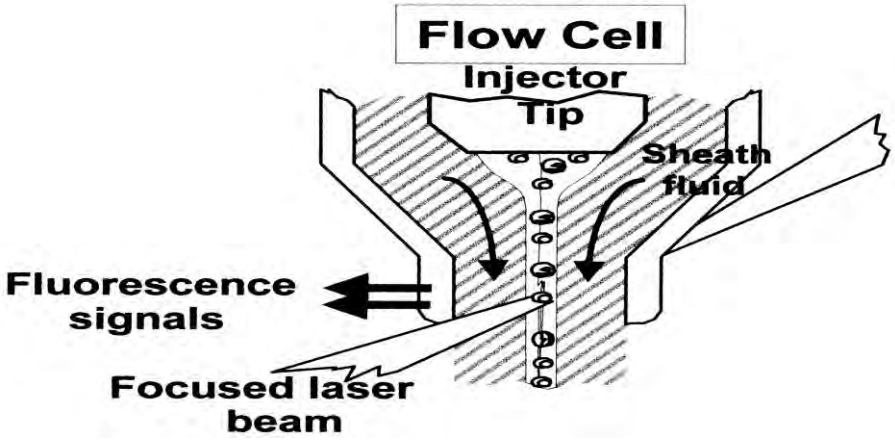

Figure 3. Fluorescence signal generation and light scattering at the flow cytometer's analysis site for flow cells, taken from Purdue Cytometry CD-ROM

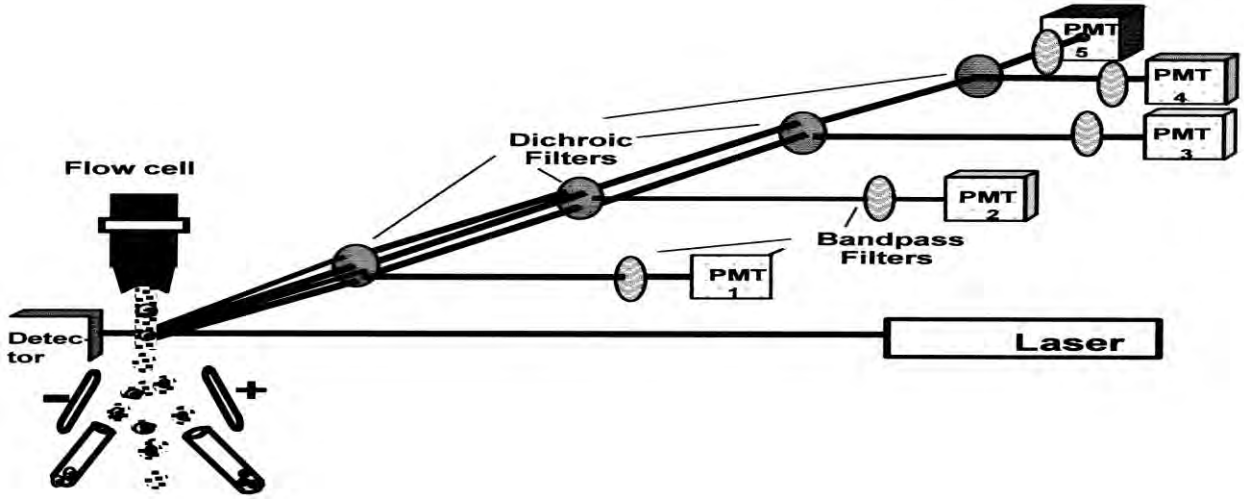

Figure 4. Scheme of optic (dichroic mirrors and bandpass filters) and illumination (laser) systems of a flow cytometer with six parameters detected (size, granularity, and four fluorescences) by separate photomultiplier tubes (except size, which can be detected by photodiode or a PMT tube) and sorting capacity. From Purdue Cytometry CD-ROM

FCM is primarily used in food-related research for the performance testing of food preservation or disinfection approaches, such as sodium hypochlorite or peracetic acid disinfection, ultraviolet light (UV-C), supercritical CO2 pasteurization, ohmic heating applications, non-thermal inactivation technologies, such as pulsed electric fields and cold atmospheric pressure plasma treatment, as well as natural preservatives like essential oils [132,133-141]. *E. Coli* was the microbe that was most frequently studied [142,134–136,138–140,143–146]. FCM is a good method for spotting viable but non-culturable (VBNC) cells in addition to sublethally wounded cells. This is crucial because environmental disturbances like temperature changes, changes in pH, or a lack of nutrients during food processing can cause cells to enter the VBNC state. Only culturable and live cells are found using culture-based methods. However, VBNC cells have the capacity to revive and recover their culturability [147]. Consequently, FCM viability staining and plate counting can be used to identify cells that are in the VBNC condition. Khan et al.'s [144] adjusted cell concentration to 10^4 cells mL-1 and optimized staining techniques for VBNC enumeration by removing interference from other particles. The sensitivity was raised by Yu et al.'s subsequent research [146]. In order to find bacteria in the VBNC state, it used a high-sensitivity flow cytometer, which dramatically reduced the background signals of impurity particles. In order to remove influence from other particles, preliminary washing steps had to be incorporated when measuring the viability and growth dynamics of yeasts and bacteria in wine samples [148]. Viability assessment was still achievable with the stain ChemChrome even when there were natural particles in the sample [149]. Bunthof and Abee [150] assert that FCM is more accurate and extremely sensitive than plate counts. For liquid samples, including juice, tea, water, wine, probiotic products, and milk, to name a few, most of the research that are currently accessible on food use FCM. FCM was employed by Fröhling, Durek, and colleagues [151] to assess indirect plasma treatment of fresh hog flesh. To get rid of the residual meat debris for their investigation, the meat samples were homogenized and centrifuged (Table 1). After plasma treatment, cFDA and PI staining was successfully carried out to analyze the viability of any residual bacterial cells. In a different study, Juzwa et al. [142] combined FCM with cell sorting to enhance the separation of microbial strains from stainless steel surfaces in a fruit and vegetable processing facility. In order to conduct additional FCM analysis, surfaces were swabbed with sterile cotton swabs and then quickly redissolved in buffer solution. For the microbiological study of aquatic milieus, FCM has been employed extensively. The total cell count (TCC) analysis uses only one nucleic acid binding stain and is arguably the simplest FCM procedure available. The Swiss recommendations for drinking water analysis introduced TCC measurement using FCM in 2012 [152]. Analyses of the infectious risk, therapeutic effectiveness, or inactivation process can be aided by the viability of live/dead analyses with additional data on the intact cell count (ICC) [153-155]. Here, a combination of cell-permeant and cell-impermeant nucleic acid stains are used to target the membrane integrity. Propidium iodide (PI; a cellimpermeant dye) and SYTO and SYBR stain families (cell-permeant dyes), such

as SYBR® Green I (SG1) or SYTO 9TM, are the fluorochromes that are most frequently utilized for this purpose [156, 157]. During the wastewater treatment process, Ma et al. [158] used a fast staining approach with SG1-PI (green vs. red fluorescence) to measure the TCC of bacteria and viruses as well as the quantity of live/dead bacterial cells. It was necessary to ultrasonically treat the cells in order to get free single cells for FCM analysis. Even more sophisticated datasets may be obtained using TCC measurement as fluorescence signals and scattered light are said to create a bacterial community's "fingerprint" [159]. Based on SSC gating, Liang, Soupir, Rigby, Jarboe, and Zhang [160] separated environmental E. coli cells connected to clay from those devoid of clay particles. It is possible to distinguish between bacteria with high and low nucleic acid levels using SCC gating and green fluorescence [161,162,155,156]. It is frequently used for marine environments to define water communities by identifying and separating high- and low-nucleic-acid content clusters [81]. Low-nucleic-acid-content bacterial cells were linked to inactive or dead cells, whereas high-nucleic-acid-content cells were said to be more sensitive and dynamic to changes [82,93]. Recent research, however, revealed that bacteria with modest nucleic acid concentrations have active metabolisms. Drinking water contamination was connected to an increase in high-nucleic acid-content cells, according to a study by Prest et al. [163]. It showed a connection between a rise in TCC and a concentration of elevated nucleic acids. According to a recent study by Farhat et al. [162], bacteria with high nucleic acid concentration were more responsive to chlorine dioxide treatment than bacteria with low nucleic acid content. Cytometric fingerprints enable more comprehensive data analysis in addition to high and low nucleic acid content. The Bray-Curtis dissimilarity was used as an online tool in a study by Favere et al. [167] to distinguish various microbial communities in drinking water. This parameter was created for the first time by Bray and Curtis [168] and is a simple, unambiguous tool. When comparing two fingerprints, their dissimilarity is calculated and assigned a number between zero (identical samples) and one (completely distinct samples) [169]. FCM fingerprinting offers real-time monitoring and detection within 10 minutes as a completely automated online tool, enabling quick monitoring and a sensitive early warning of changes or contamination in aquatic milieus [170,161,159]. Only a few publications, in contrast to water analysis, have concentrated on the flow cytometrical detection of air-related microorganisms [171,172,173]. The first study to use FCM with FISH as a quantification and identification approach for airborne microorganisms from agricultural environments and to produce results that were comparable to those of fluorescence microscopy was Lange et al.'s [172] study. An all-glass impinger-30 and a May multistage liquid impinger, which included a collection liquid including a surfactant (Tween 80) and an antifoaming agent (Antifoam A), were utilized for air sampling. By using FSC, SSC, autofluorescent measures, and numerous gatings, Day et al. [171] were able to distinguish between pollen and other fungal spores and airborne Phytophtora infestans spores. Additionally, their research indicated that the Calcofluor white M2R dye, which was deemed non-toxic and displayed a brighter fluorescence than other stains,

allowed for more precise distinction. To measure Aspergillus versicolor in indoor air, one recent work combined FCM and qPCR [173]. The particles from the air were collected in sterile water using a liquid cyclone air sampler. Prior to qPCR quantification, Flow cytometry (FCM) was employed to quickly count and calibrate *A. versicolor* particle concentrations.

Flow cytometry (FCM) thus improves comprehension of the mechanism of action of antibiotics, antimetabolites, antimalarial medicines, inhibitors and other medications. It is also used to track medication resistance to antibiotics. In different natural or artificial ecosystems, Flow cytometry (FCM) may also be utilized to identify the agents of microbial competition. For instance, we are currently researching the aggressive behavior of antagonistic bacteria in Limoges and Poitiers [174]. *Listeria monocytogenes*, a food-borne pathogen is inhibited from growing by a peptide produced by the lactic acid bacterium *Leuconostoc mesenteroides*. This proteinaceous toxin is a member of the same family of bacteriocins as the well-known colicins made by some strains of E coli. These bacteriocins kill sensitive cells by various ways but they are often only effective against a small number of bacterial strains that are present in the same environment as the producer. Flow cytometry (FCM) investigated the behavior of each population in a Listeria/Leuconostoc co-culture. Specific fluorescent probes and light scattering measurements were used to track the impact on morphology, cellular components and physiology. In addition to allowing kinetics investigations, Flow cytometry (FCM) revealed the bactericidal effect of one strain on another more quickly [174]. According to flow cytometry (FCM) investigations using rhodamine 123, the action of the Leuconostoc bacteriocin causes the Listeria membrane to depolarize, most likely by pore creation [160].

Chapter 5

New and Rapid Microbial Determination Methods

5.1 Miniaturized System

In order to reduce the volume of chemicals and medium used in microbiological testing, miniature systems are primarily created. In its simplest form, a miniaturized system consists of clean Micro titer plates, a multiple inoculation tool, and containers for both solid and liquid media. Pathogens are quickly and precisely identified when diagnostic kits are used, thereby saving countless lives. An organism is identified (keyed out) using a manual identification code after each well's color reaction in a diagnostic kit has been read. In recent years, automatic readers have also been created to quickly and accurately identify new cultures [82]. Comparison analyses of diagnostic kits and selection standards for small-scale systems were examined by Cox et al. and Fung et al. Their analysis shows that tiny systems are affordable, precise, and effective systems. In addition to being more efficient than traditional methods, these methods also save space and labor. There are a few commercially available miniaturized systems, such as yeasts and molds as well as enterics (Salmonella, Shigella, Proteus, and Enterobacter) and non-fermentors, anaerobes, and gram positive bacteria [73,83,82].

5.2 Biosensor and its Recent Advances for Potential Applications

A biosensor is a tool that monitors, transmits, and records data on a biochemical or physiological change. According to technical definitions, it is a probe that combines a biological element with an electronic transducer to transform a biochemical signal into a quantifiable electrical response. Various transducers, including electrochemical, optical, acoustic, and electronic ones, are used in biosensors [91, 92, and 93]. The biochemical specificity of the biologically active substance affects how well a biosensor works. The specificity, storage, operational, and environmental stability of the biological material will all be important considerations. The analyte to be detected, such as chemical substances, antigens, microorganisms, hormones, nucleic acids, or any subjective criteria like smell and taste, will also influence the selection process. As biological sensing components, enzymes, antibodies, DNA, receptors, organelles, microbes, and animal and plant cells or tissues have all been employed. Specificity, sensitivity, dependability, portability, the capacity to operate in optically opaque liquids (in most circumstances), real-time analysis, and ease of use are some of the key characteristics of a successful bio sensing device.

The need for quick, precise analytical equipment for food and fermentation analysis has grown and is constantly growing in recent years. In order to ensure that food products meet quality standards, both the food business and governmental health organizations demand a wide

variety of various analytical techniques. In order to monitor nutritional parameters, food additives, food pollutants, microbiological counts, shelf life assessment, and other sensory qualities including smell and odor, analysis is required. Electronic noses [94, 95] and a variety of sensors based on enzymes and antibodies have all been reported (96-99). According to Mulchandani and Rogers (1998), microbial biosensors have also demonstrated promise for food analysis. Because current approaches include bulk collection and extended storage, gauging milk quality is a crucial metric. The release of short-chain fatty acids (C4-C12), which are what, causes rancidity or off-flavor in milk and milk products. It has been discovered that Arthrobacter nicotianae differs from other bacteria in that it has enzymes that are highly selective for short-chain fatty acids in the -oxidation pathway. These cells have been used to make a microbial sensor for flow injection measurement of short-chain free fatty acids in milk [100, 101]. This sensor was made in conjunction with an oxygen electrode. Using *A. nicotianae* immobilized in Ca-alginate directly on the oxygen electrode surface, Schmidt et al. (1996) presented a microbial biosensor based on thick film technology. For the purpose of determining the amount of free fatty acids in milk, a batch system was utilized with the sensor. No sample preparation was required, and the sensor's response time was only three minutes. Thiobacillus thiooxidans is used as a microbial biosensor in the food business to measure sulphite levels in a variety of foods. The total sulphite content of 40 different food types, including dried vegetables, has been determined using this microbial sensor [102, 103]. In the field of pharmaceuticals and medicine, microbial-based sensors have been investigated for the estimation of steroids such as cholesterol (Nocardia erythropolis), androstendione (N. erythropolis), and testosterone (N. erythropolis); antibiotics such as nystatin (S. cereisiae); and other compounds such as gonadotropin-releasing hormone (B. subtilis), urea (nitrifying bacteria), uric acid (Alten Angiotensin converting enzyme (acetylcholine esterase) inhibitors, which include enalapril maleate (EMa), are a new class of antihypertensive medications. Induced B. subtilis cells have recently been used to produce a microbial biosensor for EMa [104]. This biosensor, which has been successfully used to identify the active ingredient in pharmaceutical tablet formulations, detects the acceleration of respiration during particular metabolic pathways of this medicine.

5.2.1 Technical Strategies

Biosensors employ technical solutions based on label-free and label-based detection [175]. The primary determinant of target detection in label-based detection is the unique characteristics of label molecules. These biosensors are trustworthy; however they frequently need a particular detecting element made with an immobilized target protein in conjunction. However, the label-free strategy [176, 177] makes it possible to identify target molecules that are hard to tag or lack labels. Label-free biosensors for a variety of detection techniques with a broad range of applications in the disciplines of medical and environmental science have been made

possible by recent interdisciplinary approaches combining biotechnology with bioengineering, electrical, and electronics engineering.

5.2.2 Electrochemical Biosensors

The first in the line of discovery of electrochemical biosensors is the classical discovery of the glucometer employing glucose oxidase-based biosensors [178]. Since glucose biosensors are necessary for diabetic patients to periodically monitor their blood glucose, hospitals and diagnostic clinics frequently use them.

However, unstable enzyme activity or in homogeneity are frequently the cause of glucose biosensor shortcomings [179], necessitating additional calibration. Actually, because of these possible limitations, a variety of biomolecules with varying electrochemical characteristics were developed [175, 181], opening the door to the development of more practical glucose biosensors. Nowadays, biomaterials like enzymes, antibodies, or DNA are used to change the surface of metal and carbon electrodes in order to create electrochemical biosensors [181]. The output signal from a biosensor is often produced by the particular binding or catalytic reactions of biomaterials on the electrode surface [181]. The development of electrochemical sensors became essential for the clinical diagnosis of illnesses [183]. Early monitoring or detection appears to be crucial. The creation of non-enzymatic biosensors is frequently taken into consideration in this area by substituting synthetic materials for proteins. It's interesting to note that different kinds of biomolecules exhibit differing electrode stability and selectivity, which eventually aids in the creation of novel electrochemical biosensors with a range of applications. On the basis of their intended use, several varieties of electrochemical biosensors were created. Glucose biosensors have evolved quickly since their creation, as previously mentioned [179]. Given that ferrocene (Fc)-modified boronic acids have an electrochemically active portion (an Fc residue) and a binding site (a boronic acid moiety), Wang et al. (2014) examined the development of ferroceneboronic acid (FcBA) and Fc-modified boronic acids for biosensor development. One special ability of FcBA and its derivatives is their ability to bind to sugars' 1, 2- or 1, 3-diol residues, forming cyclic boronate ester linkages in the process. Different from free FcBA, the redox characteristics of the FcBA-sugar adduct provide the foundation for electrochemical detection. Furthermore, boronic acids exhibit high propensity for binding to Fe− ions, providing an added benefit for the development of unconventional ion-selective electrodes that utilise F− ions. The hydrocarbon chains included in the HbA1c polypeptide chain can be quantified using electrochemical detection based on FcBA. The immobilization of FcBA derivatives [182] on the electrode surface is a major drawback of this method because these derivatives are introduced to sample solutions as reagents. The FcBA electrochemical sensor may be improved by using polymers and/or silver electrodes with appropriate modification of FcBA derivatives for use in biological domains, such as diabetes diagnosis, where glucose

measurement will be supplementary. An interesting contemporary innovation is the electrochemical biosensor, which measures the concentrations of reactive oxygen species and antioxidants in physiological systems [184]. The principal end product of bodily fluid purine metabolism, uric acid, is detected in this line of work [185], and it can be used as a diagnostic tool for a number of clinical abnormalities and disorders. Yet, creating an economical and delicate approach is crucial. Just as glucose quantification looks best achieved using an electrochemical-based method, so too does uric acid oxidation. The development of an electrochemical biosensor with high sensitivity is hampered by the similarity between uric acid and ascorbic acid in terms of oxidation. In order to get around this, researchers have created a biosensor based on amperometric detection that can monitor both oxidation and reduction potentials [185]. It is crucial to immobilize or screen print the enzyme on electrodes, or alternatively on electrodes based on nanomaterial, given the expense and reproducibility of this process. These electrodes are perfect for developing disposable, sensitive, economical, and selective uric acid biosensors for routine analysis. In this context, recent developments in 3D bioprinting [175] have aimed to produce biosensors that contain living cells contained in three-dimensional microenvironments. A novel wireless mouth guard biosensor was created to continuously and real-time measure salivary uric acid levels [186]. The technique can also be applied to wearable monitoring devices, instruments for a range of fitness and wellness applications. Although hormone measurements have been accomplished with effectiveness using electrochemical biosensors [187], its perspective needs to be thoroughly examined. Targeting nucleic acids is a promising avenue for biosensor technology development. Cellular miRNA expression is a well-established biomarker for illness onset diagnosis, and targeting it enhances the effectiveness of gene therapy for hereditary disorders. Typically, polymerase chain reaction, microarray, and northern blotting are used to identify miRNAs. Utilizing guanine oxidation as a label-free detection method following the hybrid synthesis of the miRNA and its inosine replacement capture probe, modern technology offers the best electrochemical biosensors for miRNA detection [188]. The development of electrochemical-based biosensor technology in biomedicine has led to the creation of all these innovations through contemporary biofabrification techniques. In order to quickly identify pesticidal residues and avert health risks, environmental monitoring is another crucial area where biosensor technology is needed [189, 190]. Although there are drawbacks, such as complexity, labor-intensive procedures, the need for expensive equipment, and operational capabilities, traditional methods like high-performance liquid chromatography, capillary electrophoresis, and mass spectrometry are useful for analyzing pesticides in the environment [190]. Therefore, while basic biosensors appear to offer many benefits, creating a single biosensor that can analyze many pesticide classes is difficult. Consequently, a number of enzyme-based biosensors were created to comprehend the physiological effects of pesticides in the environment, food safety, and quality control [190, 191]. Biosensors based on acetylcholinesterase (AChE)

inhibition were created for this reason [191]. In the past ten or so years, advancements in AChE inhibition-based biosensors, such as immobilization techniques and alternative fabrication methodologies, have further refined this methodology for quick analysis. Similar to this, piezoelectric biosensors have been created to identify the environmental impact of carbamate and organophosphate pesticides [192]. It is well known that organochlorine pesticides have an impact on the ecosystem (Senthilkumaran, 2015), with endosulfan being one of the most harmful. These pesticides do in fact affect male and female fishes' reproductive systems differently [193]. In light of this, the development of biosensors to monitor the aquatic ecosystem will be more important in the context of biomagnification. The electrochemical biosensor saw a revolution in response to the demand, as seen by the swift advancements in the manufacture and application of nanomaterials, quartz, and silica [175, 190]. Particular attention should be paid to the selection of receptors for the construction of biosensors, the use of various transduction processes and quick screening methods for biosensor applications in food, and environmental safety and monitoring. Biosensor fabrication appears to be crucial for enabling this, and the developments in this area were succinctly outlined below.

5.2.3 Optical/Visual Biosensors

As previously said, environmental or biological applications necessitate the creation of quick, easy, and extremely sensitive biosensors. This might be achievable with immobilizers made of glass, silica, quartz, carbon-based compounds, gold, or other materials [194, 195, 175, 196, 197]. Actually, utilizing microfabrication to incorporate gold nanoparticles or quantum dots offers novel technologies [198] for the creation of extremely sensitive and portable cytochrome P450 enzyme biosensors for specific applications. Additionally, fiber-optic chemical sensors are particularly relevant in a number of domains, including biosensing, biomedicine, and drug development. Hydrogels are a new class of materials for fiber-optic chemistry immobilization that have emerged more lately. They are employed as DNA-based sensors [199]. Hydrogels immobilize in three dimensions as opposed to other materials, allowing for a large loading capacity of sensing molecules. Hydrogels, or polyacrylamide, are hydrophilic cross-linked polymers that can be immobilized in a variety of ways, including thin films and nanoparticles [200]. Hydrogels are thought to be an easy substrate for DNA immobilization that also has benefits such trapping, controlled release, improved analyte quality, and DNA protection. When considering alternative materials that provide biomolecular immobilization, hydrogels are the only ones with these characteristics [200]. Moreover, hydrogels' high optical transparency offers a practical method for visual detection. In the field of biosensor technology, detailed techniques for immobilizing DNA biosensors in monolithic polyacrylamide gels and gel microparticles are frequently regarded as technological advancements [200].

5.2.4 Silica, Quartz/Crystal and Glass Biosensors

Glass, quartz, or crystal, and silica materials are used in biosensor development processes nowadays because of their special qualities. Because of its biocompatibility, abundance, electrical, optical, and mechanical qualities, silicon nanoparticles provide the most promise for technical advancements in biosensor applications. Furthermore, a crucial requirement for biological and biomedical applications is the non-toxicity of silicon nanoparticles. Applications for silicon nanoparticles are numerous and include cancer therapy, biosensing, and bioimaging [196, 197]. Additionally, there are long-term uses for luminous silicon nanoparticles in bioimaging. Remarkably, hybrids made of silicon nanowires and gold nanoparticles are employed as innovative silicon-based nano-reagents for successful cancer treatments [197]. Thiol-modified DNA oligomers covalently bond to silica or glass to produce DNA films that are more suited for UV spectroscopy and hybridization techniques [200]. Even though using silicon nanoparticles has many benefits, there are still a number of issues that need to be considered, including the creation of low-cost, large-scale production techniques and biocompatibility following biomolecular contact. When these problems are fixed, silicon nanostructures will be able to function as contemporary biosensor components. High-sensitivity analysis of biomolecule interactions can also be done on another platform–the wire- and electrode-free quartz crystal microbalance biosensors. Without the need of wire connections, antennas used electromagnetic waves to stimulate and detect the pulses of quartz oscillators. A crucial component of creating ultrahigh-sensitive protein detection in liquids with quartz-crystal biosensor-based instrumentation is this exact non-contacting measurement [195]. Many new biosensors were created using cutting-edge technology to improve bioinstrumentation to biomedicine technology, taking into account the special qualities of silica, quartz, and glass materials; however, cost-effectiveness and biosafety require attention [195, 196, 197, 175]

5.2.5 Nanomaterial based Biosensors

For the development of biosensor immobilization, a wide variety of nanomaterials are used, including graphite, carbon nanotubes, graphite and silver nanoparticles, and carbon-based materials [201, 202, 203, 204, 205, 206, 207, 177, and 197]. Furthermore, materials based on nanoparticles offer excellent sensitivity and specificity for creating electrochemical and other kinds of biosensors. Of the metallic nanoparticles, gold nanoparticles show promise due to their resistance to oxidation and near-zero toxicity [208]. In contrast, silver nanoparticles oxidize and manifest toxicity when used internally in medical applications, such as drug delivery. Generally speaking, there may be issues with using nanomaterials for biosensors, which should be resolved if they are to be used in biomedicine [209]. Additionally, there may be benefits and drawbacks to nanoparticle-based signal amplification techniques [210]. However, just by raising the sensitivity and detection limits for single molecule detection, nanomaterials are regarded as

essential parts of bio-analytical apparatuses [175]. It is important to note in this regard that platinum-based nanoparticles for single-label response electrochemical amplification have been developed for the purpose of detecting low DNA concentrations [211]. Similar to this, tumor-targeting ligands like monoclonal antibodies, peptides, or small molecules can be efficiently coupled with semiconductor quantum dots and iron oxide nanocrystals possessing both optical and magnetic properties to target tumor antigens with high affinity and specificity. Both the administration of nanomedicine and the understanding of the tumor microenvironment for therapeutic purposes can be accomplished with the use of quantum dots technology [212]. Because of its potential for use in a variety of disciplines, the use of cantilever size (milli, micro and nano-cantilevers) biosensors is even critically evaluated.

5.2.6 Genetically Encoded or Synthetic Fluorescent Biosensors

Understanding biological processes, including different molecular pathways inside the cell was made possible by the development of tagged biosensors employing synthetic or genetically encoded fluorescence [213, 214, and 15]. Actually, it was to image fixed cells that fluorescent-tagged antibodies were first produced [214]. In fact, this tactic offered fresh approaches to the development of such sensors that make use of biological proteins, tiny molecules that bind to analytes and second messengers. More recently, single molecule detection with precise analyte concentration was used to analyze motor proteins using fluorescent biosensors [213]. Despite these benefits, the approach for detecting and analyzing probes appears to be challenging. Up to the last ten years, mitochondrial physiology was better understood thanks to genetically encoded biosensors that targeted chemicals linked to energy production, reactive oxygen species, and cAMP [216]. Similarly, cGMP is a crucial signaling molecule and a target for medications that affect the cardiovascular system. Considering this biosensors based on FRET (Förster resonance energy transfer) have been created [217] to visualize Ca2+, cGMP, and cAMP in cells, Numerous of these sensors function well in live-cell in vivo imaging and primary culture [214, 215]. Many important factors have now been worked out for the development of sensors for imaging living animals. Among the greatest biosensor techniques in contemporary physiology are small-angle X-ray scattering for the development of calcium sensors and fluorescence resonance energy transfer probes for kinase sensing [214]. Thus, a number of biosensors based on microbes and cell organelles were created, each with a particular target [218]. As was previously mentioned, biosensors that are electrochemical, electromechanical, and optical have been designed to detect miRNA far more effectively than other molecular approaches [219]. A deeper comprehension of cellular activity and numerous more molecules, including DNA, RNA, and miRNA, have been identified with the introduction of in vivo imaging using small molecule biosensors [200, 175, and 219]. With the development of small molecule biosensors for in vivo imaging, cellular activity has been better understood, and numerous more molecules, including DNA, RNA and miRNA, has been found. The current revolution in this

sector calls for a whole genome approach with improved optical genetic biosensors. Furthermore, it is now generally acknowledged that optically based biosensors that combine fluorescence with small molecules or nanomaterials have had more success in terms of sensitivity and applicability.

5.2.7 Microbial Biosensors

Utilizing cutting-edge, novel technologies based on genetic/protein engineering and synthetic biology to program microorganisms with particular signal outputs, sensitivity, and selectivity is a more recent trend in environmental monitoring and bioremediation. For instance, a living cell that is active with enzymes to break down xenobiotic substances will be used in bioremediation more extensively [220]. Likewise, biosensors based on microbial fuel have been created with the intention of tracking Environmental toxicity and the need for biochemical oxygen. It is possible for bacteria to break down the organic substrate and produce energy for fermentation. The method basically uses a bio-electrochemical apparatus to regulate the power of microbial respiration, which turns organic substrates into electrical energy directly. Notwithstanding these opportunities, the low power density of microbial biosensors poses production and operating cost constraints. With the use of new systemic techniques, efforts are being made to drastically improve performance and reduce costs. Technologies have made it possible to create self-powered designed microbial biosensors [221, 222]. Another field in which eukaryotic microorganisms have an advantage over prokaryotic cells is the detection of heavy metals and pesticides using microbial biosensors [223]. The primary reason for this is the benefit of creating whole cell biosensors, which have sensitive and selective applications in the detection of pesticidal toxicity and heavy metals [223]. Moreover, higher eukaryotic microorganisms may be more sensitive to many harmful substances and may be relevant to higher animals. It's interesting to note that microbial biosensors have a wide range of uses, from energy production to environmental monitoring. Novel biosensors with high sensitivity and selectivity from microbial origin, ranging from eukaryotes to modified prokaryotes, will be made possible by inventive methodologies. These microbial biosensors will be used more widely in the future to monitor environmental metal contamination and the production of sustainable energy [221, 222].

5.3 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

For routine identification and typing of Gram-positive and Gram-negative bacteria, mycobacteria, actinomycetes, yeasts, and mold fungi for more than ten years [105-108], MALDI-TOF has been employed. Furthermore, this method was used to examine phyloproteomic interactions [109]. MALDI-TOF is also appropriate for protein quantification

[110, 111]. Additionally, MALDI-TOF mass spectrometry (MS) can be utilized to find additional biomolecules such sugars, nucleic acids, and tiny compounds (105). Using the chemotaxonomic method MALDI-TOF MS, ribosomal proteins that are specific to a particular family, genus, species, or even microorganism strain are analyzed. A modest amount of test material (105–106 cells; whole cells or cell extracts) is initially added to a plate in order to execute this examination. The sample is then treated with a matrix solution consisting of -cyano-4-hydroxycinnamic acid, 2,5-dihydroxy-benzoic acid, or 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) in a combination of solvents including water, 2.5% trifluoroacetic acid (TFA), and 50% acetonitrile (ACN), ethanol, or methanol. By entering the microbial cells and extracting the internal proteins, these matrix components make them accessible for examination. Co-crystallization is the process by which protein molecules and other biological components crystallize in matrix crystals after the solvents from the cell suspension have evaporated. The dried sample is then put into the analyzer chamber, where under the influence of a laser, matrix molecules and proteins are desorption (released) and ionized (without being degraded), preserving the bacteria. The matrix is crucial in the desorption and ionization process that results in the release of analyte molecules because of its capacity to absorb a significant amount of the energy of the laser light. The molecules that are subsequently ionized are accelerated in a vacuum analyzer column, and the time of ion flight (TOF) is gauged. First, low-mass ions and then highly ionized charged molecules enter the detector. Then, based on the distribution of peptides according to their molecular weight, charge, and ion flight time, the MALDI-TOF MS system automatically generates a peak spectrum (mass spectrum), in which the peaks correspond to ions with various mass to-charge ratios (m/z). The mass spectra that were collected are like a fingerprint for a certain species of microbe. The MALDI-TOF MS system also enables peak intensity, quantity, and peak correlation analysis, as well as comparison of the mass spectra of a given living organism with the spectra in the mass spectral database [111, 105, 112, 113, and 114]. It is possible to identify microorganisms at the level of genus, species, and even strain by comparing the ion biomarker masses of an unknown microorganism with the protein masses in the database or by comparing the whole-protein profile with a library made up of the mass spectra of the reference strains (fingerprint approach; a designation of the number of peaks that are characteristic of the genus and/or species of microorganisms) [115, 116]. Figure 5 displayed the MALDI-TOF MS technique's fundamental idea.

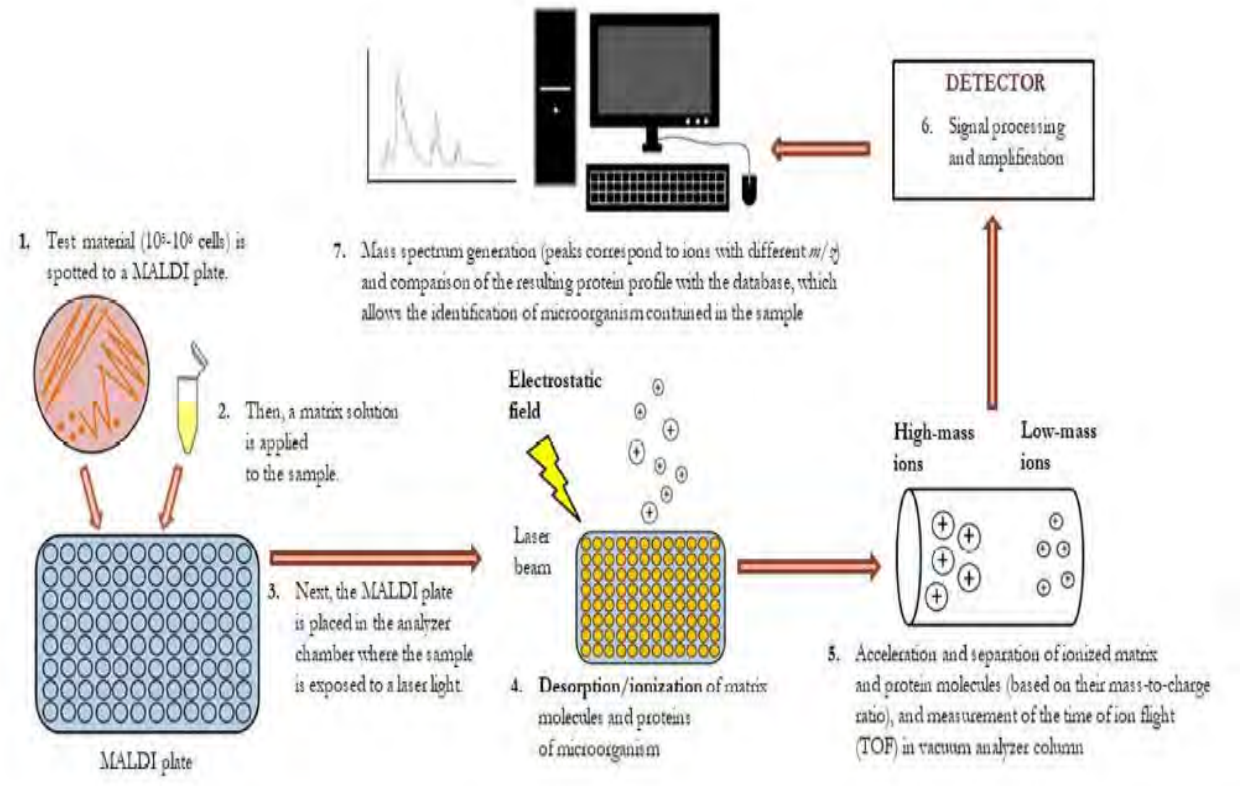

Figure 5: According to Clark et al. (2013), Ouedraogo et al. (2013), Singhal et al. (2015), Lo et al. (2017), and Mazari et al. (2017), a sample's MALDI-TOF MS analysis is depicted in a scheme that outlines the general principles involved.

Although filamentous fungus, such as those found in Penicillium, Fusarium, and Aspergillus species, offers numerous advantageous traits like the capacity to generate organic compounds, In addition to being used to generate fermented foods, acids, enzymes, and antibiotics also produce mycotoxins and contaminate a variety of food items. Fungal deterioration is thought to be responsible for 5–10% of the global food loss. Quéro and colleagues employed the MALDI-TOF MS method to quickly identify the food-spoilage molds [117]. The mass spectra of 618 fungal strains from 136 different species that were acquired by MS were used to establish a database. No matter the incubation time or culture media used (assessment based on cross-validation), the database enabled roughly 95% accurate species-level identification of fungi [117]. In addition to being used in brewing, winemaking, and baking, *Saccharomyces cerevisiae* strains are also crucial to the pharmaceutical and nutraceutical sectors.

These microbes also generate L-lactic acid and a few other secondary metabolites. In comparison to molecular techniques like PCR amplification employing delta elements and contour-clamped homogeneous electric field gel electrophoresis (CHEF), Moothoo-Padayachie et al. demonstrated that the MALDI-TOF MS approach is a far better bio typing tool for the

identification of industrial yeast strains [118]. The MS approach successfully identified *S. cerevisiae* to the level of the genus and species with a 100% accuracy rate and 18 out of the 20 tested strains with a 90% accuracy rate. Two samples that included incorrectly classified yeasts at the strain level, according to the investigators, were contaminated. All of the investigated *S. cerevisiae* strains could not be reliably differentiated using molecular techniques. But the MALDI-TOF MS technique, used in conjunction with multivariate statistics and principal component analysis, enabled a definite separation between the brewing yeast strains SAB MC CO and SAB MC UF. The authors came to the conclusion that the MALDI-TOF MS method can be applied in the industry for starting culture quality control and contamination detection [118]. "Wild yeast" can contaminate fermented drinks like beer, wine, or cider as well as starting cultures like the ones indicated above, spoiling the finished product. Such deteriorated goods appear turbid, have less ethanol, and have an unpleasant aftertaste (phenolic, for example). Usbeck and colleagues demonstrated that the MALDI-TOF MS approach is a quick and accurate way for detecting yeast species that can ruin fermented beverages, including *S. cerevisiae* var. diastaticus, Wickerhamomyces anomalus and Debaryomyces hansenii [119]. The authors did note that the method is constrained by the database's lack of reference spectra, though. Additionally, they recommended using ethanol for extraction since this solvent enables getting spectra with the highest and most repeatable number of peaks. Beer can also be spoiled by certain germs. Therefore, in the brewing sector, the distinction and classification of these microorganisms is crucial. The capacity of Lactobacillus brevis strains isolated from wheat, lager, and pilsner beer to ruin beer as well as their tolerance to iso-alpha acids, which have antibacterial activities, were assessed by Kern and colleagues [120]. Additionally, they used the MALDI-TOF MS method to identify these strains. The examined bacteria had varying degrees of beer-spoiling potential (low, moderate, and high). The potential of germs to taint beer increases with iso-alpha acid resistance. The scientists also pointed out that a number of variables, including the presence or absence of ethanol or nutrition, affected how resistant bacterial isolates were to iso-alpha acids. The MALDI-TOF approach provided 90% accurate strain-level identification of beer spoiling bacteria [120]. In an adverse environment, several types of lactic acid bacteria (LAB) can enter a state known as VBNC, or viable but non-culturable, where they can still be a danger to the safety of food. Bacteria in this stage cannot be detected using conventional microbiology techniques, although identification based on peptide markers may be helpful in this situation [121]. Mycotoxins like zearalenone (ZEA), which are present in food products, pose a major hazard to human health. Król and colleagues examined the MALDI-TOF method to determine how well LAB neutralized ZEA produced by the Fusarium genus of fungi, such as Fusarium graminearum, Fusarium cerealis, or Fusarium culmorum [122]. The researchers verified the uptake of mycotoxin by *Lactococcus lactis* and Bifidobacterium sp. bacteria based on modifications in the mass spectra (appearance or disappearance of signals as a function of time) following the incubation of bacteria with ZEA for 0, 10, and 20 min. The

amount of technic acids, polysaccharides, proteins, and peptidoglycan in Gram-positive bacteria's cell wall correlated with the degree of ZEA neutralization and binding [122]. The heat-stable cyclic depsipeptide cereulide generated by some strains of the *B. cereus* species causes emetic food poisoning in humans when it contaminates food (such as rice, pasta, herbs, milk, and dairy products). To distinguish between emetic and nonemetic bacterial strains, Urlich et al. used the MALDI-TOF technique, the PCR method, and bioassays (HEp-2 cytotoxicity test). 120 out of 121 examined strains/isolates could be accurately distinguished by the MS technique as having the ability to produce emetic toxin in 99.1% of the cases. Only one emetic strain that produced a minuscule quantity of poison was incorrectly identified. All emetic strains were shown to have two distinct biomarkers (m/z 171 and 1187 Da) in the mass spectrum [123]. The bacteria contaminating pasteurized milk and pork were found and measured by Nicolaou et al. using the MALDI-TOF technology in conjunction with chemo metric analysis. According to their research, there were minor differences between the mass spectra of fresh and rotten milk and pork. Proteins or peptides found in the food products were digested by microorganisms that cause food to degrade, creating new proteins or peptides. As a result, the mass spectrum's peaks underwent changes in their size, intensity, and location, and some even vanished altogether. The researchers also carried out linear and nonlinear regression analysis to calculate the bacterial count. They suggested that the meat and dairy industries may employ the MALDI-TOF MS approach in conjunction with chemometry to detect and quantify bacteria [110]. It is relatively common to extract Enterococcus bacteria from food products, and these germs are frequently utilized as starter cultures. However, due to their ability to create biogenic amines and virulence factors, two species of this genus, *Enterococcus faecalis* and *Enterococcus faecium* can infect humans and pose a risk to their health and lives. The identification of these microorganisms that have been isolated from food is crucial for this reason. Using MALDI-TOF MS, Quintela-Baluja et al. classified 36 strains of various Enterococcus species. They were able to identify between distinct species and even some strains of the same species based on the presence or absence of specific peaks in the mass spectrum and differences in those peaks. According to their research, the biomarker for the Enterococcus genus is a peak at m/z 426±1. The origin of the strain (dairy or meat products) was also discovered to alter the occurrence of mass spectra (biomarkers) with various m/z values, as well as the frequency of particular peaks in the mass spectrum [124]. The MALDITOF method is suitable for the quick identification of enteric and marine bacteria that produce histamine, according to Fernández-No et al. Histamine poisoning can result from eating fish or other seafood, cheese, wine, or other foods that contain bacteria like Staphylococcus xylosus, Klebsiella pneumoniae, Proteus vulgaris, Lactobacillus sp. 30A, or Photobacterium phosphoreum, which can perform histidine decarboxylase activity.

The study's bacterial strains were clearly distinguishable thanks to their mass spectra. All Enterobacteriaceae family members that produce biogenic amines had similar protein profiles

(peak intensities and their number). The authors were able to identify the bacteria at the species level based on variations in peak masses [125]. The Cronobacter genus of bacteria are opportunistic food-borne diseases that can seriously affect newborns. The nonpathogenic bacterial species *Enterobacter helveticus, Enterobacter turicensis, and Enterobacter pulveris* are phenotypically similar to these germs. However, using the MALDI-TOF MS method, Stephan et al. successfully identified every Cronobacter strain at the genus and species levels [126].

Chapter 6

Conclusion

The ongoing evolution of microbial determination methods within pharmaceutical and food quality control stands as a testament to the relentless pursuit of precision, safety, and efficacy in these industries. Throughout this review, a spectrum of innovative techniques including Biburden test, ELISA, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), PCR-based assays, microfluidics, biosensors and omics technologies has been showcased, reflecting the diverse approaches aimed at ensuring product integrity and consumer safety. The multifaceted nature of these methodologies has significantly enhanced our ability to rapidly and accurately identify, quantify, and characterize microorganisms. From comprehensive genomic analyses to real-time detection of specific microbial components, these methods offer unprecedented insights into microbial communities and their functionalities. However, the integration of these advanced techniques into routine quality control protocols necessitates addressing persistent challenges. Standardization across platforms, cost considerations, and the need for continuous adaptation to emerging microbial threats remain focal points demanding concerted efforts from stakeholders and researchers. The future trajectory of microbial determination methods hinges on collaborative endeavors aimed at refining existing methodologies, fostering interoperability, and enhancing accessibility. Embracing technological advancements and establishing robust frameworks for method validation and regulatory compliance will be instrumental in harnessing the full potential of these techniques. As these methodologies continue to evolve, their seamless incorporation into industry practices will not only fortify product safety but also reaffirm consumer trust in pharmaceutical and food products. Moreover, sustained research initiatives and strategic collaborations hold the promise of overcoming current limitations, thereby solidifying the pivotal role of microbial determination methods in safeguarding public health and maintaining the highest standards of quality. Advantages and some shortcomings of the discussed methods are gathered in Table 2.

Finally, the dynamic landscape of microbial determination methods in pharmaceutical and food quality control epitomizes a relentless quest for excellence. The fusion of cutting-edge technologies with collaborative efforts is pivotal in ensuring the continual advancement and integration of these methodologies, ultimately guaranteeing the safety and reliability of products consumed by global populations.

Table 2. Advantages and shortcomings of microbial determination methods discussed in this paper

Method	Sample	Advantage/Disadvantage
Bioburden Test	Pharmaceutical and food sample	Advantage: It helps ensure the safety and quality of products by assessing the microbial contamination levels, aiding in compliance with regulatory standards. Disadvantages: There can be instances of false positive or negative results due to contamination during sampling or testing procedures, leading to incorrect assessments.
RT-PCR method	Pharmaceutical product	Advantages: A precise and accurate method with ability to provide accurate detection nucleic acids with a low concentration of starting amount. Disadvantages: It is an indirect method for detection of amikacin. It is also a complex method that involves expensive equipments, as well as very labor-intensive procedures.
Multiplex PCR	Pharmaceutical products	Advantages: Ability to determine low levels of microbial contamination less than 10 cfu per milliliter or gram of product. Disadvantages: The microbial quality control of nonsterile pharmaceutical products can be performed in a cost-effective and timely manner in pharmaceutical industry.
Multiplex PCR	Clinical, dairy and poultry samples	Advantages: Rapidly identify the entrococci in clinical, dairy and poultry samples

Method	Sample	Advantage/Disadvantage
Enzyme-linked immunosorbent assay (ELISA)	Pharmaceutical and food sample	Advantages: ELISA often provides relatively quick results compared to some other analytical methods, allowing for efficient monitoring of quality and safety in pharmaceuticals and food products. Disadvantages: Accurate interpretation and execution of ELISA assays require skilled personnel, and the technique may be less accessible in settings lacking trained professionals.
ATP bioluminescence and impedance method	Pharmaceuticals and cosmetic products (like dextrose, potassium dihydrogen phosphate, magnesium phosphate heptohydrate, ammonium sulphate)	Neither ATP bioluminescence nor DEFT methods gave a satisfactory dose/response curve while impedance method presented a satisfactory dose/response results.
Fuorescent staining	Herbal medicines	Advantages: Simplicity and short time for staining and enumeration.
Mechanized and automated MPN method	Pharmaceutical and food samples	Precise and automation in test, lowering human interventions and contaminations
Direct Epifluorescent Filter Techniques (DFET)	Pharmaceutical and food samples	Advantages: DEFT provides rapid and real-time enumeration of viable microbial cells in pharmaceutical and food samples, allowing for quick assessment of microbial contamination levels. Disadvantages: DEFT does not differentiate between different microbial species or strains. It provides a total count of viable cells without specifying the types of

		microorganisms present, limiting its specificity compared to some other methods.
Method	Sample	Advantage/Disadvantage
Biosensor techniques	Pharmaceutical and food samples	Advantages: Biosensors provide fast and real-time detection of target analytes, allowing for quick assessment of contaminants, pathogens, or quality parameters in pharmaceuticals and food samples. They offer high sensitivity and specificity, enabling the detection of target molecules or substances at very low concentrations, ensuring accurate and reliable results. Disadvantages: Designing and developing biosensors tailored for specific targets or applications can be complex and time-consuming requiring expertise in various disciplines such as biology, chemistry, and engineering.
Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)	Pharmaceutical and food samples	Advantages: It allows for the rapid identification of microorganisms including bacteria, fungi and viruses aiding in microbial control and ensuring the safety and quality of pharmaceutical products and food items. MALDI-TOF MS allows for high-throughput analysis enabling the screening of numerous samples in a relatively short time enhancing efficiency in quality control processes.

		Disadvantages: The initial investment in MALDI-TOF MS instruments and their maintenance costs might be substantial as well as limiting accessibility for some laboratories or facilities. While MALDI-TOF MS provides qualitative information, it might have limitations in quantitative analysis especially for certain molecules or compounds.
Flow Cytometry	Pharmaceutical and food samples	Advantages: It is a rapid analysis technique. Flow cytometry enables quantitative measurements of cell counts, viability and specific biomarkers aiding in precise quantification and assessment of sample characteristics. It offers high sensitivity in detecting rare cell populations, low-abundance biomarkers or contaminants present in pharmaceuticals or food samples. Disadvantages: The sensitivity of flow cytometry instruments might be affected by variations in sample quality, matrices or the presence of interfering substances, potentially impacting the accuracy of results. Interpreting flow cytometry data requires expertise and the complexity of data analysis might be a barrier in some settings without access to skilled personnel or specialized software.

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