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DETECTION METHODS OF FOODBORNE PATHOGENS

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ABSTRACT

Food borne pathogens are present in a variety of foods giving rise to foodborne illnesses that have become a major threat to human health globally. Detection of these pathogens is critical to ensure safe food supply and prevention of foodborne illnesses. There are varied ways of detection and the methods are categorized under two broad techniques which are culture dependent and culture independent. This review highlights different types of detection methods classified under culture-based and culture-independent methods namely, immunological assays, nucleic acid-based methods, biosensor-based, microarray based as well as the next generation sequencing; their strengths, limitations and challenges. Next-generation sequencing (NGS) technology has advanced our understanding of food microbiome by allowing the discovery and characterization of unculturable microbes with prediction of their function over other diagnostic assays. The need for improved, low cost, rapid and reliable detection techniques cannot be overemphasized and are highly recommended.

1.Introduction

Food safety can be defined as the assurance that food will not cause harm to the consumer when it is prepared and/or eaten according to its intended use. Food is often contaminated by bacteria, viruses, fungi and parasites, which induces several enteric diseases (Saravanan et al., 2021). The ability to rapidly detect viable pathogens in food is important for public health and food safety reasons (Foddai and Grant, 2020). Research in the food safety and security field continuously focuses on the search for improved methods of foodborne pathogen detection that are sensitive, accurate, rapid, and cost-effective (Vanegas et al., 2017). Detection methods of bacterial foodborne pathogens can broadly be divided into culture-based and culture-independent methods (Fig.1) (Park et al., 2023). This paper aimed to review different types of detection methods classified under the broad categories of culture-based and culture-independent methods.

2.Culture-based methods

Culture- based conventional methods also known as traditional techniques have been the oldest methods in detecting microorganisms, even the pathogenic strains (Priyanka et al., 2016). They are considered to be the "gold-standard" and are well known for their cost-effectiveness, sensitivity, ability to confirm cell viability, and ease of standardization (Senturk et al., 2018). Culture is a term used to describe the biological amplification of viable and cultivatable bacteria with manufactured growth media (Amani et al., 2015). It is the microbial growth on or in a nutritional solid or liquid medium; the increased number of the organisms simplifies detection as well as enhances the identification by the

morphological and biochemical studies of the microbial cells (Vazquez-Pertejo, 2023).

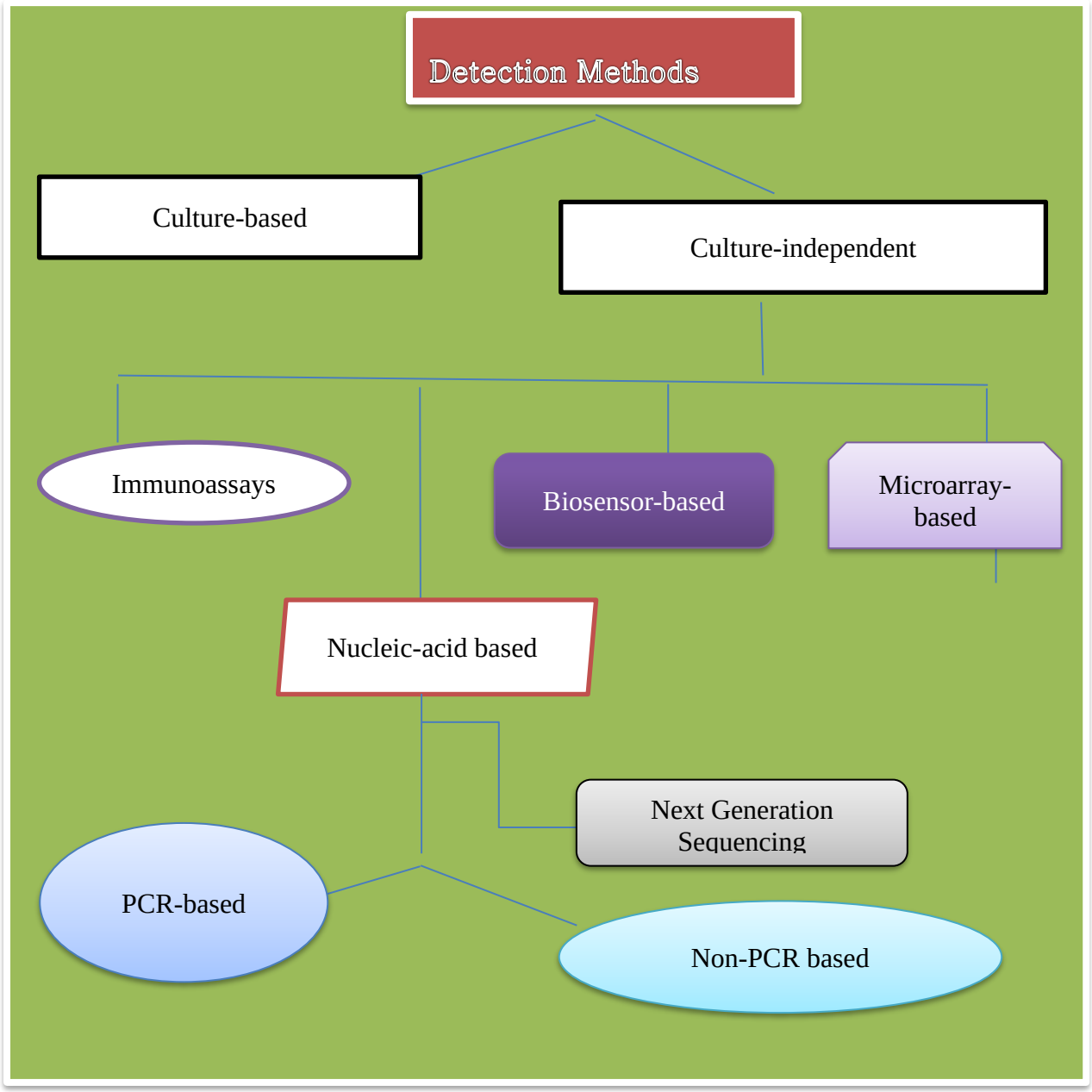

Figure 1. Detection methods of Foodborne Pathogens

Culture-based methods mainly rely on growing the pathogens on agar plates which are exhausting processes and take more than three days to a week, from preliminary observations to confirmation of the pathogens (Reddy et al., 2022). Routine detection of bacterial pathogens in most Nigerian laboratories is based on enrichment culture, microscopic observation and biochemical assays (Nasrabadi et al., 2017). Culture based methods have the advantage of delivering a bacterial isolate that can be subjected to further analyses such as antimicrobial susceptibility testing, test for virulence determinants (e.g. toxins) and also typing to support investigation in an epidemiologic study (Kaprou et al., 2021). Despite the merits stated and the benefits of being widely used and reproducible,

these methods are time-consuming, relatively expensive, prone to contamination due to extended time-lag, and the processes are cumbersome. Furthermore, the difficulty of quantitative analysis; that is the culture method is not appropriate for a number of important bacterial pathogens because they are difficult or impossible to grow under laboratory conditions (Nasrabadi et al., 2017). This has given rise to the phenomenon described as 'the great plate count anomaly'. A term coined by Staley and Konopka in 1985 to describe the difference between the numbers of cells from natural environments that form viable colonies on agar medium and the numbers obtained by microscopy (Harwani, 2013). This is largely explained by the development of cells into a dormant state regarded as viable but not culturable (VBNC) state. Cells in the VBNC state exhibit very low, but detectable, metabolic activity compared to actively growing cells. Such dormant cells maintain the integrity of their cell membranes and continue to express genes at low levels. When they are transferred to solid nutrient media, they fail to form colonies, as opposed to active cells (Ayrapetyan and Oliver, 2016). The presence of VBNC cells in food is widely documented. This was resulted due to stress and harsh environmental variations during the stages of food processing from farm to fork. Given that pathogenicity is maintained by some species during the VBNC state and the inability of culture method to detect the cells that are in a dormant state, poses a substantial food safety and public health risk (Ramamurthy et al., 2014). The use of such standard culture methods for the detection of pathogenic organisms is to be given urgent attention for improvement. It is vital that we be aware of their existence and understands how these dormant bacteria affect our experiments, and when their impact warrants the use of alternate methodologies (Ayrapetyan and Oliver, 2016). Other drawback of the culture-based method is the inability to identify the pathogen to species level.

2.1.Use of chromogenic media

Over the last few decades, a range of chromogenic media has been developed that are designed to target pathogens with high specificity. They serve as alternatives for rapid microbiological identification, as they make it possible to presumptively differentiate bacterial species and/or groups according to colony colour, reducing the need for biochemical tests (Garcia et al., 2021). These media exploit enzyme substrates that release coloured dyes upon hydrolysis, thus resulting in pathogens forming coloured colonies that can easily be differentiated from commensal flora. Ideally, commensal bacteria should either be inhibited completely by selective agents or form colourless colonies thus allowing pathogens to 'stand out' against background flora. This allows clear differentiation of microbes producing the target enzyme from those that do not. This is especially important when attempting to detect specific pathogens within polymicrobial cultures (Manal et al., 2015). The combination of agar media operating on different biochemical principles and characterized by different sensitivities and selectivities could allow for a more rapid and accurate detection of a broad spectrum of group members in food samples. Chromogenic reactions of enzymatic cleavages of substrates and the release of chromogens leading to higher specificities and improvement over the use of the conventional microbiological growth media (Fuchs et al., 2022). These culture media have therefore been found to outperform other conventional microbiological rapid methods in terms of specificity, sensitivity, and accuracy, in addition to identifying contamination in the samples more efficiently (Garcia et al., 2021). Chromogenic media are therefore regarded at least as convenient and sensitive as well as being more cost effective (Perry, 2017).

2.2. Microscopic techniques

Simple light microscopic observation and staining are the basis on which the oldest detection methods are formed after culture on

selectivity growth media, but often do not provide a clear answer (Mobed et al., 2019). It has the advantage of being inexpensive, rapid, and simple to perform. Microscopy however, is labour intensive and requires highly skilled scientists for optimal diagnostic performance (Anderson et al., 2014). Microscopic techniques over the years have been used for bacterial observation as a complement to scientific research and conventional diagnostic tests. Nowadays, they are applied in the areas of food and water quality amongst others, where biological detection and quantification are significantly important (Nasiłowska et al., 2021). The conventional light microscope suffers from limited throughput, relatively high cost, bulky size, lack of portability, and requirement for focus adjustment. These drawbacks partially limit the use (Zhang et al., 2015). Therefore, it is best used as an adjunct to traditional culture or molecular methods (Mobed et al., 2019). Several advancements in microscopy have evolved over the years and illustrated to achieve inspiring outcomes (Liu et al., 2021). Electron microscopy surpasses the use of light microscopy due to high resolution power and hence transmission electron microscopy becomes important to find number of bacterial cells and their biomass (Mishra et al., 2016). The scanning electron microscope (SEM) is quite useful to reveal morphological features of isolated organisms as well as for diagnosis, but difficulty with specimen preparation methods have in the past limited the use of SEM for routine Microbiology (Golding et al., 2016). One of the innovative techniques to improve microscopy is stimulated emission depletion (STED) microscopy (Ghithan et al., 2021). STED microscopy is a typical laser-scanning super-resolution imaging technology for studying live biological samples on a nanometer scale. The ability of the high laser power to induce severe phototoxicity and photobleaching, limits the application for live cell imaging and this has led to the development of low powered STED (Zhang et al., 2021). STED microscopy has many practical benefits including the production of details of objects smaller than 50nm in a direct optical implementation (Alonso, 2013).

The several drawbacks of the culture-based detection methods have led to the development of novel "rapid" detection methods. These methods decreased detection time dramatically.as they do not require cultural enrichment (Wang and Salazar, 2016). They are advanced techniques that have been developed and optimized as alternatives to or for use in combination with these traditional techniques (Alegbeleye et al., 2018). The methods have been found to be user-friendly, easy, precise, portable, cheap, rapid, and provide simultaneous results in the detection of pathogens. Broadly, these methods can be categorise into four major groups: immunological, molecular, biosensor- and microarray-based techniques amongst others (Senturk et al., 2018). It should be noted that no method has 100% superiority over another but rather the newly developed methods are improvements over the older methods. Each method has its benefits and limitations as stated in Table 1.

Table 1. Some Foodborne pathogen detection methods

S/N	Pathogen detection methods	Principle	Advantages	Limitations
1.	Culture-based methods	Microbial growth in a medium under controlled laboratory conditions involving steps like pre-enrichment, selective enrichment or/ and	Relatively inexpensive, easy quantification of cells. High sensitivity with appropriate media. Allows study of colonial morphology,	Labour intensive, time consuming, less accurate, overlooks microbes that are viable but not culturable (VBNC).

		selective plating, followed by biochemical identification and serological confirmation of the results.	phenotypic characterization of organisms e.g. antibiotic susceptibility testing.	
2.	Immunological methods	Based on highly specific binding between an antigen and an antibody.	High specificity, sensitivity and efficiency. Reagents are safe, eco-friendly; cost-effective and do not require complicated procedures.	Assay preparation is laborious and time consuming. Risk of false-positives, some methods are expensive (e.g. conventional ELISA)
3.	Nucleic acid-based methods	Amplification of genetic material of the organism or directly target specific sequences of the organism's genome.	Very much accurate, having high specificity and sensitivity, and very fast. Provides more information at relatively faster speed.	Highly skilled personnel needed. High cost of the complex operations and machines. Some types have risk of false positives.
4.	Biosensor-based methods	Generation of signals and transduces in response to a specified quantity of a physical phenomenon.	Quite sensitive, easy to design, specific, and accurate.	Susceptible to interference or food matrix effects caused by components present in food. Poses challenges with detection limit, detection time, and specificity.
5.	Microarray-based methods	The ability of DNA to bind to itself and to RNA, i.e. complementary sequences will bind to each other	Provides new insights into gene function, disease pathophysiology, disease classification and drug development. Allows for full sequencing of the whole transcriptome	High cost, low-specificity, lack of control over the pool of analyzed transcripts. Relatively low accuracy, precision and specificity. The purity and degradation rate of genetic material, amplification process may impact the estimates of gene expression.
6.	Next Generation Sequencing	Relies on capillary electrophoresis. It involves fragmenting DNA/RNA into multiple pieces, adding adapters, sequencing the libraries,	Highly efficient, fast and accurate identification of microbial taxa, including uncultivable	Huge datasets produced. Computational resources required for analysis of sequencing data.

		and reassembling them to form a genomic sequence	organisms and low frequency variants. Provides superior characterization of food-chain microbiomes.	

3. Immunological methods

The recent development of immunological-based methods in several studies has made it easier and faster to detect pathogens in foods (Hormsombut et al., 2022). Immunological assay is the gold standard for highly sensitive detection of biochemical targets, providing a good platform for food contaminants detection. Immunological detection relies on the reaction between an antigen and an antibody (Ahmed et al., 2020). In immunoassay, the antibody or antigen conjugated labels are the most important units because they play roles to convert the information of target analytes to easily detected signal (Pan et al., 2021). They have the advantage that only viable cells lyse and therefore are potentially quantitative assays or detection of antigens as opposed to the characterization of the antigens. They are usually one day rapid test techniques and also provide information of previous infections and therefore are crucial in vaccine development (Yu et al., 2015; Muralidharan et al., 2020). They also have the ability to detect both contaminating organisms and their toxins that may not be expressed in the organism's genome (Amani et al., 2015). However, immunological methods are limited by a number of shortcomings. These include the lengthy development time required to prepare the immunoreagents for new analytes; the unsuitability for multiresidue analysis as immunoassays are usually only capable of detecting about two to five related compounds. Also, very few immunoassays have official status as well as having very limited amount of information delivered in the assays (Ahmed et al., 2020). Two broad categories of culture-independent immunological methods for food pathogen detection are ELISA and Lateral flow immunoassay (Wang et al., 2016).

3.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is one of the immunoassay methods commonly used for the detection of surface markers on pathogens and pathogen-produced toxins based on the specificity of antibody–antigen interactions (Nehra et al., 2022). It is commonly used to measure antibodies, antigens, proteins, and glycoproteins in biological samples (Gouzalez et al., 2018). ELISA technique combined with the effective catalytic properties of enzymes; provide highly specific and sensitive detection of some special analytes. ELISA-based detection has been employed in a variety of formats, mostly immunofluorescence and lateral flow immunochromatography as well as in the development of immunosensors (Paramithiotis, 2023). ELISA has been used as a diagnostic tool in biotechnology, as well as a quality control check in various foodborne pathogen detection tests that relies on the specificity of the antibody–antigen interaction. Other immunological assays include enzyme-linked fluorescent assay (ELFA), and enzyme immunoassay (EIA). Double antibody sandwich technique is the standard immunoassay performed by ELISA. In this case, an antigen (pathogen or toxin) on the food pathogen binds to a specific antibody coated onto a solid support such as a microtiter plate. It is then washed and the pathogen is detected by the addition of an enzyme linked second specific antibody (Nehra et al., 2022; Iha et al., 2019). Enzymes usually used include alkaline phosphatase and horseradish peroxidase.

Detection of the conjugated enzyme linked antigen-antibody 'sandwich' can be performed using different systems including colorimetric substrates, chemiluminescence or fluorescence, and impedance (Iha et al., 2019; Alamer et al., 2018). The use of different substrates in ELISA has a major advantage as the substrates will bind to the respective conjugates specifically and will develop colouration which can be read in an ELISA reader in terms of wavelength. The colour change is visible to the naked eye. However, one of the disadvantages is that the binding of the chemical and conjugate is very specific, and contamination in the intermediate stages can lead to false positive result (Priyanka et al., 2016). The conventional ELISA method however is time-consuming and a costly plate reader to read the result. It is typically conducted in 96 well plates suitable for high throughput assays, thereby, allowing for many samples to be measured in a single experiment. It can take several hours to complete due to long incubation and blocking times needed. Furthermore, large volumes of expensive reagents, and the need for highly complicated and specialized instruments, limits their application in some point of care applications and in low-resource settings and developing countries (Iha et al., 2019). To circumvent these limitations, paper-based immuno-chromatographic strips have emerged for the rapid, reliable, easy-to-use, and on-site detection of pathogens (Gouzalez et al., 2018; Zhao et al., 2020). A novel paper-based enzyme-linked immunosorbent assay (p-ELISA) with shorter operation duration, lower cost, relatively higher sensitivity and wider application has been developed (Pang et al., 2018). This method provides result in less than an hour and requires 5μL of sample to complete the detection. The advantages of p-ELISA over the conventional ELISA includes the capacity to directly measure biomarker concentrations with significant increased sensitivity, use of inexpensive materials within a short duration made this platform handy for detection of pathogens especially in areas such as developing countries, lacking advanced analytical equipment (Tsao et al., 2018). The latest development, in 2012, was an ultrasensitive enzyme-based ELISA that manipulates nanoparticles as chromogenic reporters. This technique can generate a colour signal visible by naked-eye, with blue colour for positive results and red colour for negative results. Its advantage is in its ease of use, portability and speed of analysis. However, this method is qualitative and can determine only the presence or absence of an analyte and not its concentration (Alhajj et al., 2023). The technique has the challenge of being compromised by the food matrix and its accompanying microbiota. As its removal demands sample preparation and selective enrichment steps which are time consuming (Paramithiotis, 2023). Inadequate sensitivity often necessitates an enrichment step to increase bacteria count in the food sample, thus increasing the time required to deliver the result (Zhang, 2013).

3.2 Lateral flow immunoassay

A lateral flow immunoassay (LFIA) is a simple, paper-based diagnostic device operating on the chromatographic-like migration of a labelled analyte through multiple membrane endings in the visible result of an immobilized captured reagent (Tsai et al., 2018). Lateral flow immunoassays (LFIAs) are one of the urgent and prevalently applied quick recognition methods that have been settled for, recognizing diverse types of analytes (Sohrabi et al., 2022). It is a widely accepted technique owing to its on-site results, low-cost analysis, and ease of use with minimum user inputs, even though sensitivity is not quite equivalent to that of standard laboratory equipment (Jung et al., 2019).

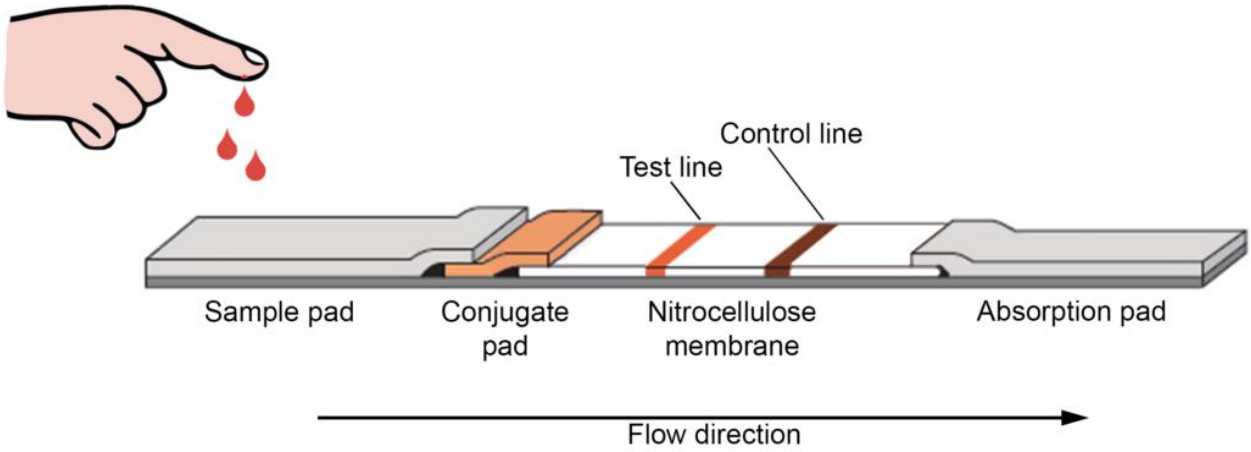

Figure 2. Schematic of a typical lateral flow assay device (Lateral flow assays, 2023)

As alternative to ELISA, LFIAs, which are designed in form of dipsticks and immuno-chromatographic strips, have emerged for the simple yet reliable detection of pathogens (Nehra et al., 2022). They have relatively long shelf-life and do not require refrigeration for storage and therefore suitable for use in resource-limited developing countries, small ambulatory care settings, remote regions and battlefields as well as refugee or internally disposed persons' camps (Contreras, 2019; Koczula and Gallotta, 2016). These tests are mostly used for qualitative assays by a visual interpretation of results. For the interpretation of the results, the colour intensity of the test zone is therefore very significant (Manta et al., 2020). Lateral flow immunoassay device is made up of four sections which are arranged orderly on a plastic backing, with sample pad starting at the bottom, followed by conjugate pad, nitrocellulose membrane which is further divided into test and control lines and then absorbent pad (Fig.2) (Nardo et al., 2021; Sajid et al., 2015).

The sample pad is the support where the sample is placed to perform the test. It absorbs the sample and allows the sample to migrate laterally to the conjugate pad. The dry conjugate is kept stable in the conjugate pad overtime (Contreras, 2019). On flowing to the conjugate pad, the analyte (sample) bind to the label biomolecules embedded in the conjugate pad and then flow together (analyte-conjugate complex) to the membrane where testing and control area are bound with immobilized protein for capturing the analytes. This part is significant as it determines the sensitivity of the assay (Kasetsirikul et al., 2020). The capillary flow-time is the duration required for the liquid to flow to fill the strip of the membrane (Koczula and Gallotta, 2016). The analyte-conjugate complex laterally flows through the third element, the nitrocellulose membrane, where specific biological compounds (typically antibodies, protein, or nucleic acids) are immobilized at pre-defined lines. The analyte, analyte-conjugate complex, and conjugates should react specifically to the compounds dispensed on the membrane. Lastly, the fourth element, the absorbent pad, should absorb any remaining sample of interest and conjugate complex (Lei et al., 2022). The captured antibodies immobilized on the test and control line on the nitrocellulose membrane form the basis of detection (Borse and Srivastava, 2019). Lateral flow immunoassays however, are not without limitations. They are of low sensitivity, they give qualitative and not quantitative results, and variability of the paper-based assay is also a concern as well as the fact that they are designed for single use and not for multiple sample analysis (Hristov et al., 2019).

The use of LFIAs in food safety procedures can help greatly in the management of foodborne risks by increasing the number of analyses, making them accessible, fast, and inexpensive. It can be recommended for used during food

safety monitoring alongside production chain, from raw materials to ready-to-eat products. It is the ideal device for testing in the Hazard Analysis and Critical Control Points (HACCP) procedures (Nardo et al., 2021).

4.Nucleic acid-based methods

Nucleic acid-based methods are by far the most sensitive and effective for the detection of a low number of target pathogens whose performance is greatly improved by combining with the sample preparation methods. They were previously considered unsuitable for routine testing of food products for pathogens because the techniques were only comprehended in research laboratories with skilled technicians. However, in the recent years, nucleic acid-based methods have gradually been mastered for use to replace or complement culture-based methods and immunochemical assays in routine laboratory analysis for food control (Lim and Kim, 2017; Souii et al., 2016). Polymerase chain reaction (PCR) is the foundational method of most DNA- and RNA-based rapid detection methods (Pleitner et al., 2014). Therefore nucleic acid-based detection methods can be broadly categorise into PCR-based and non-PCR based methods of pathogen detection (Jamdagni et al., 2016). Nucleic acid-based detection generally offers high sensitivity, but can be time-consuming, costly, and require trained staff (Cassedy et al., 2021). Also the separate detection of viable and dead bacteria is a major issue in nucleic acid-based diagnostics (Rudi et al., 2002).

4.1. Simple or conventional PCR

PCR is the oldest method of nucleic acid-based analysis; it has a dramatically reduced detection time compared with culture-based methods and provides increased sensitivity, thereby improving the possibility of detecting bacterial pathogens (Kim and Oh, 2021). PCR is a laboratory technique for DNA replication that allows a "target" DNA sequence to be selectively amplified (Wang, 2021). It is currently a widely used and incredibly potent technology that allows for rapid exponential amplification of a specific target sequence, reducing the need for culture enrichment (Akkina et al., 2022). It is one of the most commonly used molecular based methods for detection of foodborne pathogens (Wang, 2021*)*. The requirements for a PCR process include thermo cyclers, DNA template, two primers, Taq polymerase, nucleotides, buffers etc (Rajalakshmi, 2021). PCR is based on three simple steps required for any DNA synthesis reaction: first is the denaturation of the template into single strands; second is the annealing of primers to each original strand for new strand synthesis; and the third is the extension of the new DNA strands from the primers. These reactions may be carried out with any DNA polymerase and result in the synthesis of defined portions of the original DNA sequence (Delidow et al., 1993). PCR provides various benefits over culture-based and other traditional procedures; in terms of sensitivity, specificity, accuracy, speed and the ability to detect minute amounts of target nucleic acid in samples (Akkina et al., 2022). However, it cannot effectively distinguish the bacteria with different physiological states (Chen et al., 2022). Also, methods derived from PCR require sophisticated instruments and lifting temperature, and they are not suitable for the point-of-care testing (POCT) (Liu et al., 2019).

4.2. Real-time PCR/quantitative PCR

Real-time PCR or quantitative PCR (qPCR); it should be noted that the usage of RT-
PCR is inappropriate as this abbreviation is dedicated to reverse transcription PCR (Kralik and Ricchi, 2017). Real-time PCR is a variation of the PCR assay to allow monitoring of the PCR progress in actual time. Real-time PCR is currently one of the most powerful molecular approaches and is widely used in biological sciences and medicine because it is quantitative, accurate, sensitive, and rapid (Artika et al., 2022). However, there are limitations with assays based on qPCR. They

include its inherent incapability of distinguishing between live and dead cells. Its usage is limited to the typing of bacterial strains, detection, and possibly quantification in foods. To overcome this problem, a pre-enrichment of sample in culture media could be placed prior to the qPCR procedure. This step may include enrichment step or the use of specific selective media for the respective bacterium. This procedure is primarily intended to allow resuscitation or recovery and subsequent multiplication of the bacteria for the downstream qPCR detection (Kralik and Ricchi, 2017). This brings back the ill-experience of conventional culture-based technique. Also, qPCR is machine-dependent which is often expensive and requires regular maintenance (Foo et al., 2020).

4.3. Multiplex PCR

The basic principle of mPCR is similar to conventional PCR (Law et al., 2015). Multiplex PCR has the advantage of detection of many specific DNA markers in the same reaction and under specific experimental conditions (Al-Hindi et al., 2022). This has the benefit of saving time, workload, and efficiency is improved (Nguyen et al., 2016). Primer design is very important for the development of mPCR, as the primer sets are multiple and should have similar annealing temperatures in order for the mPCR assay to be successful and not produce primer dimers (Law et al., 2015). Despite the numerous advantages, mPCR has several pitfalls that have restricted its further development and broad application in food safety researches. These include the self-inhibition among different sets of primers; low amplification efficiency; and the no identical efficiency on different templates (Xu and Shang, 2016). Standardization of mPCR assays that enables the simultaneous detection of multiple pathogens has been carried out by several Researchers (Babu et al., 2013; Boukharouba et al., 2022).

4.4. Loop-mediated isothermal amplification (LAMP)

The Loop-Mediated Isothermal Amplification (LAMP) is a unique nucleic acid amplification technique for diagnosis of various pathogens which is simple, easy, rapid and cost effective when compared to PCR due to its high specificity, sensitivity, and rapidity (Akram,. 2017). LAMP results can also be easily read with the naked eye through colour-based reporters that can be added to the reaction mixture (Al-Hindi et al., 2022). Over several isothermal-based techniques, the loop-mediated isothermal amplification technique (LAMP) has many applications in the field of point-of-care (POC) testing as well as valuable means for food testing (Garg et al., 2021). LAMP assays can be carried out in regular laboratories, with minimal incubation sources like water bath or heating block and therefore suitable for application in resource-limited regions of the world (Xu et al., 2013; Al-Hindi et al., 2022). LAMP is highly specific and increases the amount of amplified DNA even up to a billion copies over less than an hour, compared to a million copies yielded by the PCR with the use of several primers (from four to six), which can distinguish up to eight specific locations on the DNA template, compared to only two in typical PCR (Soroka et al., 2021). LAMP is considered a promising alternative method to PCR technique (Bashar et al., 2022).

4.5. Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence based amplification (NASBA), also known as self-sustained sequence replication (3SR) or transcription mediated amplification (Fakruddin et al., 2013). It is an mRNA based technology. NASBA is a continuous, isothermal, enzymatic RNA-based nucleic acid amplification technique developed in 1991 by J. Compton. NABSA mimics the retroviral RNA replication (Gilbridle, 2013; Anjorin et al., 2019; Oliveira et al., 2021). The technique is isothermal (41 °C), and the RNA is amplified

to a billion-fold within a duration of about 2 h (Zhai et al., 2019). The reaction typically consists of three enzymes, including T7 RNA polymerase, RNase H, and avian myeloblastosis virus (AMV) reverse transcriptase (RT), all of which act together to amplify sequences from an original single-stranded RNA template. The reaction also includes buffering agents and two specific primers and takes place at approximately 41°C (Zhao et al., 2014). Nucleic acid sequence-based amplification methods rely on the detection of certain gene sequences (signature sequences) in the target organism's genotype. Sequences can be chosen to detect a certain group, genes, species, or strain of the microbe (Lopez-Campos et al., 2012). NASBA operates by detecting specific DNA or RNA sequences of the target pathogenic organism (Manta et al., 2020). NASBA mechanism is in two phases: first is the non-cycling phase, where the target RNA is converted to dsDNA by reverse transcription; and second is the cycling phase, where the dsDNA molecules are actively transcribed into RNA products, leading to a yield of 10–100 copies of RNA from each template molecule (Oliveira et al., 2021). NASBA has the advantages of simple operation, strong specificity, high sensitivity and being less prone to genomic DNA contamination and therefore more suitable for applications where the testing of microbial viability is important (Jaksik et al., 2015). It eliminates the need for a thermal cycler and may therefore facilitate potential clinical testing in resource-poor settings (Zhai et al., 2014). Continuous research is on-going in different parts of the world to make these methods available technically and economically as alternative for polymerase chain reaction (Fakruddin et al., 2013).

Although NASBA is more commonly used for detection of RNA viruses, it has also being used to detect pathogenic bacteria in food and environmental samples, such as, *Campylobacter* spp., *Listeria monocytogenes*, *Vibrio cholera*, *Escherichia coli*, *Salmonella enterica* and even *Cryptosporidum parvum* in water (Everett et al., 2010; Zhai et al., 2014; Adetunji et al., 2023; Srinivasa et al., 2023). NASBA does have some disadvantages. First, to make it isothermal reaction thermolabile enzymes must be used. This means that the reaction must be effected at a lower temperature than the PCR process using thermocycling, sometimes resulting in an increase in nonspecific primer interactions. NASBA has some pittfalls nevertheless, dead or inactivated cells do not always have compromised cell membranes, so false positives may result. It is relatively expensive and requires highly specialized skills. It also requires highly specialized skills. Besides, the technology is mostly in the research stage, and a true constant temperature for the amplification is not realized due to the requirement of preheating treatment prior to the testing (Jaksik et al., 2015; Gao et al., 2022).

5.Biosensor- based methods

Biosensors are sensing devices that can be used to analyze and diagnose substances by transforming a biological response into a signal (Yasmin et al., 2016). A biosensor is a combination of two elements: the biological sensing element and a transduction unit (sensor) to produce an electrochemical, optical, mass, or other type of signal in proportion to the quantitative information on the analyte of interest in a given sample (Fig. 3) (Chepyala, 2020). The sensor or a transducer is a device that can convert energy from one form to another (Naresh and Lee, 2021). The bio-element (receptor) receives the physical or chemical stimulus and transmutes this information in the form of electrical energy while transducer performs the function of transducing this energy into valuable analytical signal which can further be analyzed and presented in an electronic form (Ali et al., 2017).

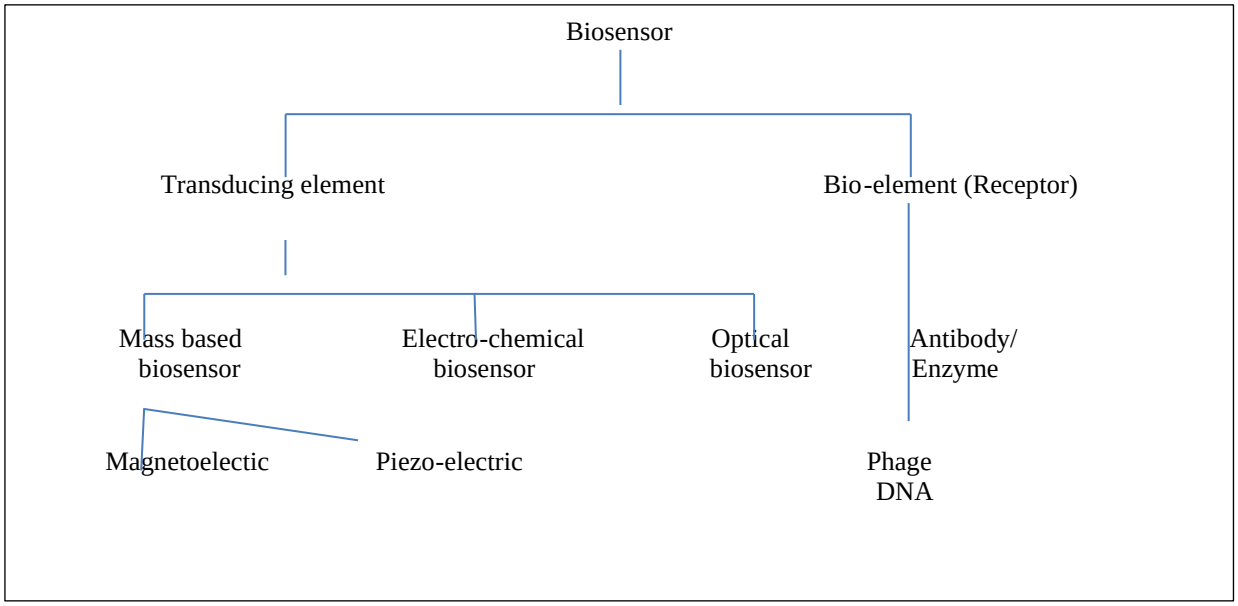

Figure 3. Working principle of biosensors. (Annonymous, 2023)

Biosensors function by coupling a biological sensing element with a detector system using a transducer (Punasiya et al.,. 2019). Biosensors are of significance use in a variety of disciplines and are employed in biochemical, electrochemical, agricultural, and biomedical fields. They are integrated in various point-of-care applications, such as disease monitoring, drug discovery, and detection of pollutants, disease-causing micro-organisms and markers that are indicators of a disease in bodily fluids (blood, urine, saliva, sweat), as well as in the food, healthcare, environmental monitoring, water quality, forensics, drug development, and other biological domains (Bhalla et al., 2016; Kulkarni et al., 2022). In Food processing, biosensors are used for the detection of pathogens such as *Escherichia coli* in vegetables, which is a bio-indicator of faecal contamination in foods (Mehrotra, 2016). A general working principle of biosensors is shown in Fig. 3.

The important components of a biosensor can be divided into three: first is a bioreceptor (e.g., enzymes, antibody, microorganism, or cells); the second is a transducer of the physicochemical signal, and lastly is a signal processor to interpret the information that has been converted. (Fatoyinbo et al., 2012). Biosensors can be classified according to transducers employed. The transducers are of different types such as electrochemical, optical, calorimetric, piezoelectric, microbial biosensor or enzyme biosensor (Dar et al., 2018).

5.1. Bacteriophage-based detection methods

Bacteriophages (phages) are viruses that can infect bacteria alone; they are predominant in nature, with more researchers paying attention toward bacteriophages as a promising tool to treat bacterial pathogens (Wei et al., 2019). With the advances made in genetic engineering and synthetic biology, phage-based methods for bacterial detection have become more iterative and elegant. Leveraging the unique characteristics of phage provides a wide variety of potential applications in the food and health industries (Wang et al., 2021). The high specificity and natural affinity of bacteriophages for their host cells make-phage based methods an attractive proposition (Foddai and Grant, 2020). Bacteriophages are reported as only been able to replicate inside living cells, hence phage-based methods can be used to demonstrate cell viability (Richter et al., 2018). Phages therefore could also assist in the discrimination between dead and living or viable but non-culturable bacteria (Paramithiotis, 2023). Bacteriophages

(i.e., viruses with bacterial hosts) pose advantages such as great specificity, robustness, toughness and cheap preparation, making them popular biorecognition elements in biosensors and other assays for bacteria detection. There are several possible designs of bacteriophage-based biosensors. The development of phage-based biosensors as a tool for the direct detection of live pathogens in food is an important and attractive approach (Richter et al., 2018).

6.Microarray-based techniques

Microarray is the advanced form of the southern blotting technique (Al-Hindi et al., 2022). Although originally developed for whole genome gene expression analysis, microarrays have become applicable in the detection of foodborne pathogens and in the investigation of the evolutionary relatedness between different bacterial species. This is due to the advantageous combination of powerful nucleic acid amplification strategies with an immense screening capability of the technique, to produce a high level of sensitivity, specificity, and throughput capacity (Sharma et al., 2022). Presently, different platforms are being used for microbial diagnostic microarrays. Microarrays not only allow characterization of microbes by information provided for specific identification of isolates, but also enhance the understanding of microbial pathogenesis based on the presence of virulence genes and an indication of the evolutionary trend of new pathogenic strains (Parolin et al**.** 2017). There are different types of microarrays, such as DNA microarrays, cellular microarrays, protein microarrays, antibody microarrays, etc (Hormsombut et al., 2022). They can be differentiated according to characteristics such as the nature of the probe, the solid surface support used, and the specific method used for probe identification and/or target detection (Sarengaowa et al., 2020).

6.1. Oligonucleotide DNA microarray

A DNA microarray is a nucleic-acid sequence based microarray technique composed of a collection of microscopic dots in which DNA is arranged and attached to a solid surface or membrane (Sarengaowa et al., 2020). Information on the differential expression of genes between food samples can be ascertained by DNA microarray. This technique uses small DNA probes capable of hybridizing complementary DNA (cDNA) of an mRNA, extracted from samples. The cDNA from food samples are tagged with fluorescent tags to facilitate the study of the differential expression of genes. Microarray can estimate the copy number of genes which in turn would aid in the study of the relative gene expression (Nehra et al., 2022).

The traditional gene chip arrays comprise of some probes that mark out the coding sequence of the virulence gene of pathogens of interest. Arraying many specific probes with molecular diagnostic markers and setting a significantly high threshold for the positive identification of pathogens that may be present, would considerably circumvent the false-positive results due to cross-contamination between foodborne pathogens. However, targeting only one area of the genome is no longer considered reliable for the identification of foodborne pathogens (Parolin et al**.** 2017). Rapid developments in the field of DNA arrays have led to a number of methods for their preparations (Sharma et al., 2022).

The strategy of tiling arrays on the gene chip can target the adjacent genome regions of the foodborne pathogen of interest, and detect the base sequences of the target gene (Parolin et al**.** 2017). However no such chip is commercially available in microarray detection technology therefore it is necessary to design and synthesize the tiling arrays for specific purposes. Microarrays have been criticized to be expensive for routine use and it is also said to be a method which fails to identify relevant information that can be transferred directly into clinical application (Miller and Tang, 2009; Everett et al., 2010).

6.2. In situ-synthesized array

In situ-synthesized arrays are extremely high-density microarrays where oligonucleotide probes are synthesized directly on the surface of

the microarray. Because in situ-synthesized probes are typically short (20–25 bp), multiple probes per target are included to improve sensitivity, specificity, and statistical accuracy (Jaksik et al., 2015; Lopez-Campos et al., 2012). For the rapid detection and identification of 10 food and waterborne bacterial pathogens including *Escherichia coli*, *Shigella* spp. *Vibrio cholera, Salmonella* spp., *Brucella* sp., and *Legionella pneumophila*, specific long oligo-microarray probes were designed (Dasari and Alex, 2014). The DNA microarray chip was able to identify all 10 bacterial agents tested simultaneously; however a professional bioinformatician would be needed for the design of the appropriate multifunctional microarray probes in order to increase the accuracy of the outcomes; developed an in situ-synthesized gene chip with 141 specific probes for the detection of *L. monocytogenes, S. aureus, E. coli* O157:H7, *Salmonella typhimurium*, and *V. parahemolyticus* on fresh-cut fruits and vegetables. They recorded a detection limit of approximately 3 log cfu/g without culturing and with a detection time of 24 h for the five target pathogens. They concluded that their detection technology can rapidly detect and monitor the foodborne pathogens on fresh-cut fruits and vegetables throughout the logistical distribution chain, from processing to sale (Parolin et al**.** 2017; Dasari and Alex, 2014). Despite some interesting features, most microarrays have integrated detection systems that require to be further developed and improvements in sensitivity and stability (Hormsombut et al., 2022). Furthermore, microarray analysis of food samples requires the application of specific conditions, and a major point of consideration is that the target microbes have to be detected in a background microflora with varying composition and abundance, depending on the type of food sample. Apart from been expensive for laboratory routine use, microarrays have also been criticized for their relatively low accuracy, precision and specificity. Also, the lack of control over the pool of analyzed transcripts is a concern since most of the commonly used microarray platforms utilize only one set of probes designed by the manufacturer (Everett et al., 2010; Ranjbar et al., 2017).

7.Next generation sequencing

Next-generation sequencing (NGS) provides new ways of detecting microorganisms beyond the microbial culture-based methods (Adenaike et al. 2023). It can allow detection in scenarios where traditional methods have generated negative or inconclusive results (Parker et al., 2023). This method with powerful bioinformatic approaches are revolutionizing Food Microbiology and serves as a powerful tool for rapidly and cost-effectively identifying and characterizing microbial species present in mixed food samples. The technology is developing at a rapid pace, with continuous improvement in quality and cost reduction and is having a major influence on Food Microbiology (Kostić and Sessitsch, 2012; News Story, 2021). Application of NGS in food safety issues does not only predict the existence of microorganisms in food samples but also to elucidate the molecular basis of their response to both intrinsic and extrinsic factors associated with the food.

This offers tremendous opportunities to predict and control the growth and survival of desirable as well as undesirable microorganisms in food (Jagadeesan et al. 2019). It also enables both culturable and non-culturable taxa to be characterized (Solieri et al., 2013).

One of the most impressive advantages of NGS over several other diagnostic assays is that it requires little or no prior knowledge of the pathogen. Therefore for pathogen discovery and detection, NGS is very valuable and reliable. NGS in Food Microbiology is applied in two major approaches: whole genome sequencing (WGS) and metagenomics. Whole-genome sequencing (WGS) is a comprehensive method for analyzing entire genomes. It identifies the entire gene content and when coupled to transcriptomics or proteomics, allows the identification of functional capacity and biochemical activity of microbial populations.

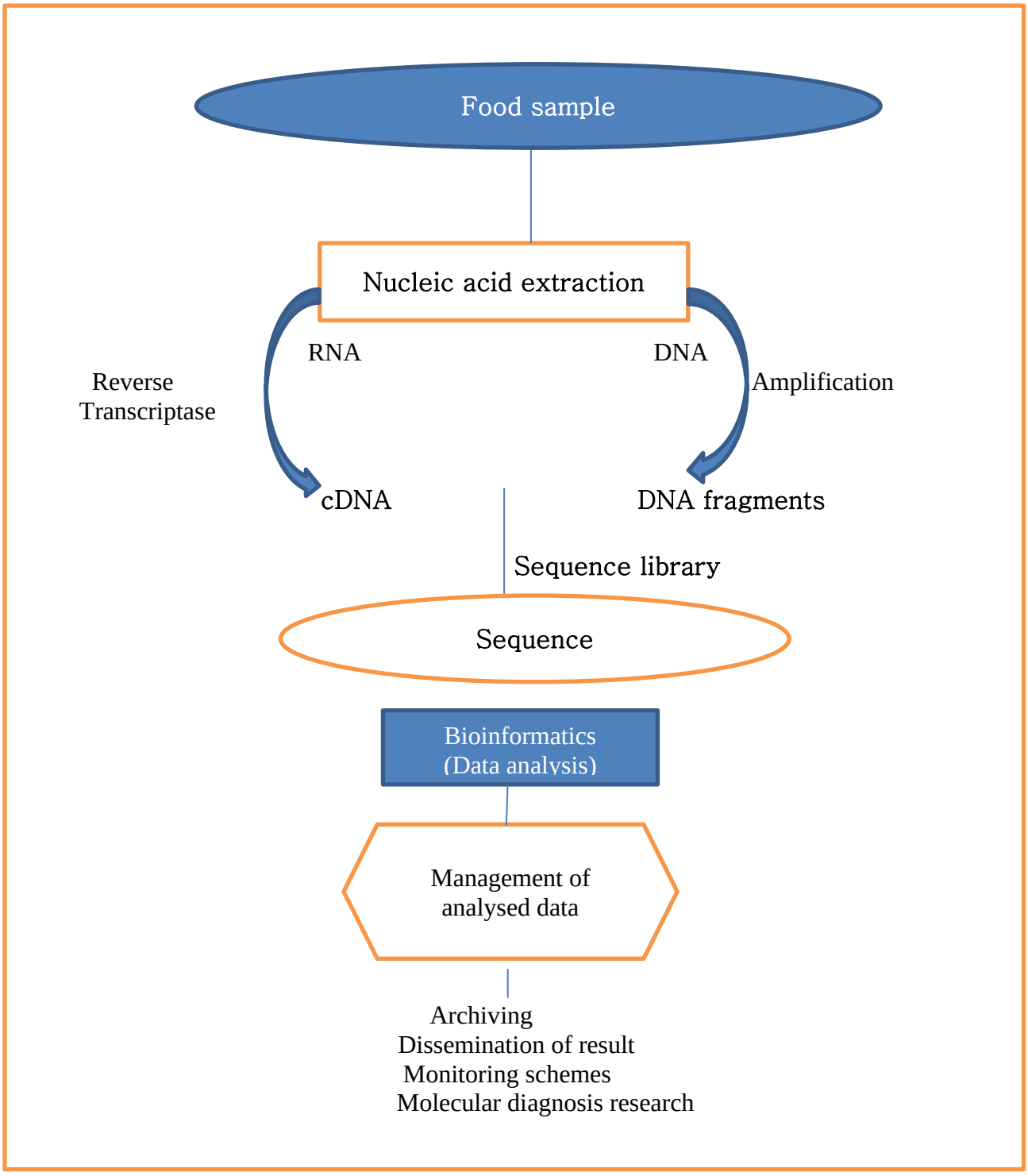

Figure 4. Stages of Next Generation Sequencing in Food sample analysis (Zhou et al., 2022)

Whole genome sequencing (WGS) of isolates consists of quality control, read trimming and assembly, bacterial characterization, strain typing, antimicrobial resistance characterization, variant calling, phylogenetic analysis and visualization tasks. WGS results can be obtained within few days rather than weeks, also provides accurate type and sub-type identification (Truchado and Randazzo, 2022; Frey and Bishop-Lilly, 2015; Jagadeesan et al. 2019; Qiagen Digital Insights, 2021). Metagenomics ensures the genetic analysis of the genomes contained in a food sample. It provides access to the functional gene composition of microbial communities and thus gives a much broader description (Zhou et al., 2022).

Next generation sequencing provides a more direct approach that does not rely on PCR and hence avoiding many of the PCR-associated potential biases (Thomas et al., 2012).

8.Conclusion

Foodborne illnesses pose important challenge to public health and cause significant economic problems in many countries of the world (Kozińska et al., 2019). Pathogens exist along the food chain and impact the quality and safety of foods in several negative ways. Identifying and understanding the behaviour of these microbes enable the implementation of preventative or corrective measures in public health and food industry settings; thus, emphasis on methods of pathogen detection as a means of prevention and control of food-borne diseases cannot be overemphasized (Adenaike et al., 2022). Classical culture-based methods can be applied to a broad range of pathogens but have long turnaround times and are not sufficient to detect and prevent all outbreaks of food-borne illnesses (Priyanka et al., 2016). Advances in technology like next-generation sequencing (NGS) have led to an explosion in the discovery and characterization of microbes, because NGS methods do not rely on traditional culture techniques and can thus detect the unculturable microbes. In foods, it is of great importance to distinguish between viable, active and inactive cells. (Yap et al., 2022; Wensel et al., 2022). Therefore, Next-generation sequencing-based methods have rapidly evolved and more or less to replace existing detection methods and platforms (Mayo et al., 2014). The continuous research for rapid, sensitive and low cost detection of pathogens in food is recommended (Ji et al., 2022).

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

Author OA-Conceptualization. Formal analysis and writing original draft. ENF-Writing original draft, proofread final draft. GOA-Writing original draft. OAO-Proofread first draft, made extensive contributions for improvement.