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Recent Advances in the Identification and Characterization of Fermentative Microorganisms: An Exploratory Review

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Abstract

Fermentative microorganisms bring about transformative processes in food, thereby enhancing flavor and appearance indices, inhibiting undesirable components, improving shelf and storage life, as well as improving health and nutritional quality parameters. Traditional methods for identification of fermentative microorganisms involve cultivating and analyzing phenotypic characteristics, which are labor-intensive and time-consuming. Advanced molecular techniques such as polymerase chain reaction, whole gene sequencing, next generation sequencing, fluorescent in situ hybridization (FISH), Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), fourier transform infrared spectroscopy and hyper-spectral imaging offer rapid, cost-effective, and reliable identification of bacteria and fungi cultured on agar plates or in liquid media. Techniques, such as genotyping, examine the DNA sequence to determine the genetic makeup of microorganisms, alongside the grouping of isolates via application of phylogenetic tree similarity indices. Overall, modern molecular techniques have revolutionized the identification of fermentative microorganisms, providing efficient and more elaborate alternatives to traditional methods. In this review work, an attempt is made to examine the historical and analytical trends from the conventional methods to the most recent modern tools for effective identification of industrially-important fermentative microorganisms.

Keywords: Fermentative microorganisms; identification; characterization; analytical methods

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1. Introduction

Fermentations are a set of procedures that help break down large organic molecules, using microorganisms, into simpler molecules; for example, the conversion of sugars and starch into alcohol with the use of yeast enzymes and the breakdown of protein into amino acids/peptides. Fermentation could be spontaneous or controlled with the introduction of microbial starters (Oyedokun et al., 2016); or it could be via seed culture or back-slopping for product reproducibility (Enujiugha, 2020). It aids in improving vitamin production, essential amino acids, food appearance and aroma enhancement all in the most natural form (Nkhata *et al*., 2018; Xiang *et al*., 2019). Typically, fermentation involves microorganisms, which can take the form of individual cells or clusters of cells (Adisa and Enujiugha, 2020); they are commonly bacteria, and can also occur as fungi, algae and other cells derived from plants and animals (Sudhanshu *et al*., 2019). Chemical and physical changes occur in food, which shows the significant role microorganisms play during fermentation (Enujiugha et al., 2002; Isaac-Bamgboye et al., 2020; Sharma et al., 2020). The presence of microorganisms in foods brings about transformative processes to the substrate, imparting the flavor and appearance, inhibiting undesirable constituents, improving the shelf life and enhancing nutritional quality (Merlyn and Subathra, 2021).

Every individual organism shows diversity in its phenotypic traits (morphological, biochemical) due to its genetic variations despite a high degree of phenotypic similarity (Adisa et al., 2024). To achieve the most accurate identification, classification and systematics of microorganisms, it is vital to select suitable techniques and possess a comprehensive scientific understanding of the technological operations (Ricardo *et al*., 2019). Conventional methods presently employed for microorganism identification rely on cultivating the organism and analyzing their

phenotypic characteristics. Nevertheless, these approaches are labor-intensive, time consuming (requiring up to three days) and frequently insufficient for distinguishing between microorganisms with similar phenotypes (Buszewski *et al*., 2017).

In recent years, numerous sophisticated molecular culture-dependent techniques (such as library cloning, TGGE/DGGE, LH-PCR, RISA, RT-Q-PCR, FISH, RAPD, and RFLD) have been developed and are regarded as useful tools for isolating and identifying new bacterial strains with degrading capabilities (Al-Dhabaan, 2018). Currently, molecular techniques are utilized to analyze the nucleic acids of microorganisms extracted from samples. Genotyping, which involves examining the DNA sequence to determine the organism's genetic makeup has evolved with several genetic methods being developed for bacterial genotyping (Omogbai and Aghahowa, 2017). More recently, MALDI-TOF MS has been adopted by microbiological diagnostic laboratories worldwide as a rapid, cost effective and dependable method for identifying bacteria and fungi that have been cultured on agar plates or in liquid media (Schubert and Kostrzewa, 2017). The present investigation provides a comprehensive overview of the recent advances in the identification of fermentative microorganism and future perspectives in the early adoption of these techniques in the food industry.

1. Fermentative Microorganisms

The microbial or enzymatic process of fermentation impacts upon food and contributes to the breakdown of the food macromolecules, inducing favourable biochemical transformations that bring about significant modifications to the food (Enujiugha et al., 2008; Nkhata *et al*., 2018). Fermented foods offer several benefits which include (Melini *et al*., 2019; Sanlier *et al*., 2019):

i. Extended shelf life compared to their original state

ii. The elimination of detrimental/unwanted components from raw materials which is evident in garri processes, where the poisonous cyanide content of cassava is reduced and the flatulence factors in soybean are removed during fermentation

iii. The improvement of nutritional properties is due to the presence of fermenting microorganisms such as yeast in bread, and yeast as well as lactic acid bacteria in garri contributes to the nutritional quality

iv. Fermented food exhibits an elevated antioxidant capacity. For instance, fermented milk and yoghurt consist of higher antioxidant properties in comparison to regular milk which is attributed to the release of biopeptides due to the proteolysis of milk proteins (α-casein, α-lactalbumin and β-lactoglobulin). The composition of the utilized substrate and the fermentating microorganisms are pivotal factors influencing the characteristics of fermented foods (Sharma *et al*., 2020). Fermentative microorganisms play a crucial role in enhancing food quality by influencing its physicochemical and sensory attributes as well as contributing to food safety through the suppression of pathogenic and spoilage microorganisms (Chinma et al., 2023; El Sheikha, 2018). Consequently, characterizing these fermenting microbes is essential to enhance the food quality and safety measures for the final food products. To achieve this a variety of methods, both traditional and molecular are employed for this purpose (El Sheikha and Hu, 2018). Main microorganisms found in the ecosystem of Fermented foods include;

1.1 Bacteria

Bacteria play a predominant role not only in naturally occurring (or spontaneously-fermented) food products, but also in those fermented using starter cultures. Lactic acid bacteria (LAB), in particular are frequently encountered in the production of acidic fermented products from cereals (Adejobi et al., 2024; Adisa et al., 2024). Although there is a wide array of bacteria directly or indirectly contributing to the production of fermented foods, they are categorized into three main Phyla: Firmicutes, Proteobacteria, and Actinobacteria. Lactic acid bacteria (LAB), which is a compound of gram positive bacteria and a key microbial component in fermented food products are found within Firmicutes group (Voidarou *et al*., 2021). They include *L. plantarum/paraplantarum, L. pentosus, L. salivarius, L. rhamnosus, L. curvatus, L. brevis, L. sakei, L. acidophilus, L. acidipiscis, L. reuteri, L. johnsonii, L. kefiri, L. parakefiri, P. acidilactici, P. damnosus, P. parvulus, P. pentosaceus subsp. pentosaceus, P. pentosaceus* (Enujiugha and Badejo, 2017; Voidarou *et al*., 2021). Most of these LAB species are involved in different cereal-based fermentations, such as in sourdough fermentation (Adepehin et al., 2018; Babatuyi et al., 2023), breakfast gruels and complementary meals (Adejobi et al., 2024), and as probiotics in commonly consumed beverages (Enujiugha and Badejo, 2017; Ukeyima et al., 2010). Some other non-LAB microorganisms, such as *Bacillus subtilis* and *Bacillus licheniformis*, have been implicated in tropical legume and pulse fermentations that produce various condiments (Chinma et al., 2023; Enujiugha, 2009; Oguntimehin *et al*., 2023). In most of the non-LAB fermentations, there are evidences of mucilage and poly gamma glutamic acid (gamma-PGA) production (Oyedokun et al., 2016). Some selected fermentative microorganisms and their respective products are presented in Table 1.

1.2 Moulds

Although the presence of mould in food typically indicates contamination and the need for disposal (Fagbemi et al., 2023), there are certain foods where visible fungal mycelium is an integral part of the microbiota and food dynamics, such as in cheese production where *Penicillium* species (e.g., *Penicillium camemberti* and *Penicilliun roqueforti*) appear as main fermentative organisms,or in fermentated rice where *Rhizopus oryzae* plays a significant role. Filamentous fungi encompassing various species are frequently employed as key agents in the production of essential microbiota for producing dry starters used in alcohol production, fermented milk and cheese (Tamang et al., 2016). Notably filamentous fungi found in traditional Asian starters serve multiple functions including saccharification, liquefaction, and ethanol production contributing to the production of diverse low-alcoholic beverages and highly alcoholic distilled liquors. Different fungal genera exist, such as *Actinomucor, Amylomyces, Aspergillus, Bjerkandera, Brettanomyces, Candida, Cryptococcus, Cyberlindnera, Cystofilobasidium,*

Debaryomyces, Dekkera, Fusarium, Galactomyces, Rhizopus, Saccharomyces, Saccharomycodes, Saccharomycopsis, Schizosaccharomyces (Voidarou *et al*., 2021).

1.3 Yeasts

Traditionally, yeasts and their metabolic byproducts have found widespread use in various food processing and preservation methods with a primary focus on baking and brewing. Presently, yeast biotechnology has become integral to commercially significant sectors, such as food, beverages, pharmaceuticals, industrial enzymes and more (Nandy and Srivastava, 2018). A diverse array of products ranging from ethnic fermented milks to alcoholic beverages like wines and beer is achieved through yeast fermentation (Dimitrios and Dimitrios, 2019). Species such as *Saccharomycopsis, Pichia, Candida and Zygosaccharomyces* play a role in most of these fermentation processes (Voidarou *et al*., 2021). Yeasts have been severally implicated in sourdough fermentations (Adisa et al., 2020; Adepehin et al., 2018), with excellent and organoleptically well-accepted final products such as sour bread (Adepehin et al., 2023).

Table 1: Commonly prepared fermented foods/beverages along with their associated fermenting microorganisms

Fermented foods/Beverages	Substrates	Fermentative Microorganisms
Cereals		
Kunu-zaki, Dosa, Ambali, Bahtura,	Sorghum, wheat, millet, rice	*L. pantheris, L. plantarum, Penicillium sp., S. cerevisiae, L. mesenteroides, E. faecalis, Trichosporon pullulans, Pediococcus acidilactici, P.cerevisiae*
Beverages		
Kombucha, wine, beer,	Grapes, rice, cereals	*Aspergillus oryzae, Zygosaccharomyces bailii, S. cerevisiae, Acetobacter pasteurianus, Gluconacetobacter, Acetobacter xylinus, Komagataeibacter xylinus*
Vegetables		
Kimchi, Tempeh, Natto, Sauerkraut, Miso	Cabbage, soybean, ginger, cucumber, radish, brocolli	*L. plantarum, L. rhamnosus, Rhodotorula rubra, Leuconostoc carnosum, Bifidobacterium dentium, Leuconostoc mesenteroides, Aspergillus spp., Rhizopus oligosporus, R. oryzae, L. sakei*
Dairy products		
Yoghurt, Cheese, Kefir,	Milk and milk casein	*Lactobacillus bulgaricus, Lactococcus lactis, L. acidophilus, L. cremoris, L. casei, L. paracasei, L. thermophilus, L. kefiri, L. caucasicus, Penicillium camemberti*
Meat products		
Salami, Sucuk, Sausage, Salchichon	Meat	*L. sakei, L. curvatus, L. plantarum, Leuconostoc carnosum, Leuconostoc gelidium, B. licheniformis, E. faecalis, E. hirae, E. durans, Bacillus subtilis, L. divergens, L. carnis*

Sources: Marco et al. (2017); Anagnostopoulos and Tsaltas (2019); Sharma et al. (2020)

3. Methods of Identification of Fermentative Organisms

3.1. Conventional Methods

Conventional methods for detecting microorganisms such as culturing, colony counting, polymerase chain reaction (PCR) and immunoassay can identify initially low cell numbers and do not demand specialized equipment. However, these methods are typically time consuming, labor intensive, destructive and requires well trained operators and significant time to yield results (Wang *et al*., 2018). Biochemical tests and microscopic identification of bacteria has been routinely carried out in laboratories in which its execution ranges from a couple of hours to several days (Rave *et al*., 2017; Enujiugha et al., 2008). Conventional methods such as traditional culturing methods which include sample preparation, enrichment, serial dilution, plating, counting and further characterization after isolation of species or colonies (Ferone *et al*., 2020) are commonly executed in developing countries where advanced technological equipment are not easily obtainable (see Table 2 and Figure 1).

3.1.1. Morphological Identification

It involves the examination of colour, shape, transparency, margin and the microscopic features of the isolated microbes by gram staining (Al-Dhabaan, 2018). Bacterial isolates identification, gram staining is carried out and the characteristics including colour (purple or pink), shape (cocci or rods) and arrangement (singles, pairs, chains or clusters) are observed, while for yeast/mould isolates, it is stained with lactophenol cotton blue which is carried out for microscopic examination of cell shape, size and sporulation (Omemu *et al*., 2018).

3.1.1.1. Microscopic identification

The microscope serves as a crucial tool for identifying microorganism within a natural sample. Microscopy images facilitates the analysis of shape, observe motion, and categorizing biological objects in which these processes are commonly used to define morphological differences (Franco-Duarte *et al*., 2019). Nevertheless, relying on microscopy for

microorganism identification has several limitations such as: small cells which are often present, pose challenges in identification. When observing natural samples these small cells can be easily overlooked especially in the presence of abundant particulate matter or larger cells (Madigan *et al*., 2012).

3.1.1.2. Gram staining

The gram staining technique elucidates the morphological characteristics of bacteria such as cocci, rods, or spiral-shaped forms and it differentiates between gram-positive (stained violet) and gram negative (stained red) bacteria based on the variations in their cell wall structure, particularly differences in the thickness of the peptidoglycan layer and permeability. This method is widely employed in laboratories due to its simplicity and quick results achieved under 5minutes (Boyanova, 2017).

3.1.2. Biochemical tests

Chemical traits also hold significance in the identification of fermentative organism, encompassing factors like sugar fermentation, antibiotic resistance and enzyme production, all of which can be readily examined. A range of biochemical tests can be advantageous in the process of identification, they include; catalase activity, cellulose activity, starch hydrolysis, hydrogen sulfide production and profiling the utilization of carbon sources (Hameed *et al*., 2018). Phenotypic identification techniques usually admit reactions to different chemicals (Franco-Duarte *et al*., 2019).

3.1.2.1. Catalase test

This test is employed to detect microorganisms that generate the catalase enzyme, which serves to neutralize hydrogen peroxide (H_2O_2) by decomposing it into water and oxygen gas. The primary producers of the catalase enzymes are obligate aerobes and facultative anaerobic bacteria. This examination is conducted on a slide or within a test tube by combining a bacteria colony with a few drops of 3% H_2O_2 and observing the formation of bubbles within a span of 10 seconds. If there are bubbles, it indicates positive catalase bacteria if there are no bubbles, it also indicates negative catalase bacteria (Al-Joda and Aziz, 2021; Dawodu and Akanbi, 2021).

3.1.2.2. Oxidase test

This test is used in the identification of microbes that produces the enzyme cytochrome oxidase which is based on electron transfer from the donor to the final acceptor (oxygen). It is majorly used in differentiating between oxidase-negative Enteriobacteriaceae and oxidase-positive Pseudomaceae. A filter paper is soaked with 1% Tetramethyl-p-phenylenediaminedihydrochloride (oxidase reagent), the colonies are then smeared on the filter paper strip. If the organism produces oxidase, it oxidizes the phenylenedimine in the reagent to deep purple colour. The colour change is checked after 10seconds to determine if it's a positive result (Al-Joda and Aziz, 2021; Dawodu and Akanbi, 2021).

3.1.2.3. Indole test

This test is employed in the identification of organisms that have the ability to produce tryptophanase enzyme which aids in the conversion of amino acid tryptophan into indole gas. Several reagents are used in checking the gas, in which, in isoamyl alcohol and conc. HCl, Kovac indicator contains para-dimethyl amino benzaldehyde but Ehrlich indicator contains ethanol. The reagent reacts with the indole gas forming the red rosindole dye (positive result) (Al-Joda and Aziz, 2021).

3.1.2.4. Sugar fermentation test

Carbohydrate fermentation test is used in the identification of bacteria that can ferment a specific carbohydrate. It analyzes the presence of gas/ acid produced from carbohydrate fermentation and makes use of a basal medium containing a single carbohydrate source (glucose, lactose and sucrose). Due to acid production which causes lowering of the pH medium, a pH indicator bromothymol blue is added for its detection, while for the production of gas (hydrogen/carbondioxide), it is tested by immersing inverted Durham tubes into the medium (Dawodu and Akanbi, 2021).

3.1.2.5. Methyl red test

Certain bacteria aids in the conversion of glucose into pyruvic acid which is then converted into other acids such as lactic acid, acetic acid and formic acid depending on the bacteria species. The production of acid lowers the pH of the medium and changes the colour of the methyl red from yellow to red, which shows the ability of the bacteria to utilize glucose in the culture medium (Al-Joda and Aziz, 2021).

3.1.2.6. Voges-Proskaur Test

This test is used in the detection of microorganisms that can produce butylene as a byproduct. Acetoin is oxidized to diacetyl in the presence of 40% KOH, in which in the presence of alpha-naphtol, diacetyl will react with peptone (guanidine component) which results in the formation of red colour (positive test) which can be further analyzed using magnetic resonance imaging (MRI) test (Al-Joda and Aziz, 2021).

3.1.2.7. Urease test

This is used for the identification of microbes that has the ability to hydrolyse urea (organisms that produces urease) to produce ammonia and carbondioxide. It undergoes incubation for 1or 6 hours, the organism is urease negative if the culture medium gives a yellowish colour (retaining original color) while if the organism produces the enzyme urease, the colour changes from light orange to magenta (Dawodu and Akanbi, 2021).

3.1.2.8. Gas production (CO_2)

This test is carried out by inoculating isolates in Man Rogosa Sharpe liquid media in an analysis tube with a durham tube, after incubation for a period of five days at 30^{o}C, the air bubbles produced is observed. Air bubbles formed indicates the production of carbondioxide and can be used in identifying lactic acid bacteria species (Prastujati *et al*., 2022).

Table 2: Review of the conventional methods used in the identification of fermentative microorganisms

Fermentative organisms	Source of isolation	Method	Reference
Saccharomyces cerevisiae, Zymomonas mobilis	*Hibiscus sabdariffa* drink and *Citrus aurantium*	Gram staining, motility test Catalase test, Oxidase test, Indole test	(Dahiru *et al*., 2018)
Aspergillus niger	Maize grains	Urease test, Sugar fermentation, Methyl red, Voges-proskaeur (VP) test	(Dahiru *et al*., 2018)
Lactic Acid Bacteria	Bekamal (Banyuwangi traditional fermented meat)	Gram stain test, Motility test Catalase test, Gas production test (CO_2)	(Prastujati *et al*., 2022)
Lactococcus spp. and Lactobacillus spp.	Cocoa heaps	Catalase reaction and Gram reaction test	(Ayertey *et al*., 2017)

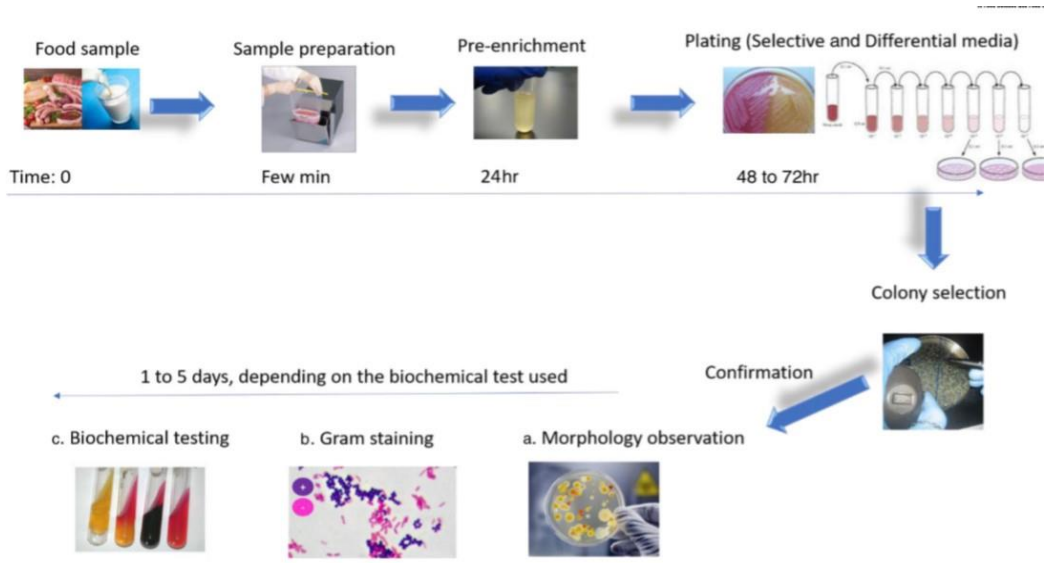

Figure 1: A standard procedure for microbial identification using conventional culture and plating methods (Ferone *et al*., 2020).

3.2. Molecular and Genomic Methods of Identification

Molecular methods of identification are used in the detection of specific microorganisms in a tested material that are difficult to be detected by phenotypic methods (Enujiugha, 2020). The molecular methods used include; amplified species of PCR products, PCR based methods and rRNA/rDNA gene sequencing (Buszewski *et al*., 2017). These methods range from simple DNA sequencing to intricate methods which are based on targeted genes, whole gene sequencing and restriction fragment analysis (Franco-Duarte *et al*., 2019). The molecular methods involve the analysis of established genetic markers which are non-culture dependable methods that corresponds to specific nucleic acid sequences. Amplification of gene, characterization, and direct sequencing of ribosomal genes permits the identification of microorganisms and provides information on the viability and antimicrobial susceptibility at genus level (Ferone *et al*., 2020).

3.2.1. PCR (Polymerase Chain Reaction) technique

The polymerase chain reaction originated in the 1980's. This technique is also similar to a molecular photocopier because of its ability to identify a specific sequence of DNA and rapidly synthesize a large number of copies, as demonstrated on Figure 2 (Jalali *et al*., 2017). Real time polymerase chain reaction (qPCR) and reverse transcriptase real time PCR can be used to accurately detect microorganisms on the basis of 16S rRNA gene or 26S rRNA (Ferone *et al*., 2020). PCR-based method involves the PCR amplification of the 16S rRNA, sequencing of the gene and comparing with known databases for identification, these methods are not only faster than the conventional culture based methods but are also beneficial in the identification of microorganisms that are difficult to grow in laboratory conditions (Franco-Duarte *et al*., 2019).

Polymerase chain reaction is used in the amplification of DNA chains by continuously subjecting the isolates to change in temperature (heating and cooling) in the presence of a polymerase and primers (short DNA fragment complementary to the analyzed gene). Some examples of PCR application are presented in Table 3. The DNA molecule is visualized to the expected size when the product of reaction is subjected to gel electrophoresis, in which the amplified products are then sequenced (Buszewski *et al*., 2017). The PCR cycle comprises of three stages which include;

i. **Denaturation:** This involves heating the reaction mixture to over 90^{o}C in order to disentangle the double helix of DNA by breaking the hydrogen bonds (Jalali *et al*., 2017).

ii. **Primer annealing:** The reaction mixture is then cooled to temperature 45ºC-65ºC for primer annealing, through complementary base pairing the forward and reverse primers hybridize to opposite strands of the DNA (Jalali *et al*., 2017).

iii. **Extension:** For the DNA polymerase enzyme activity, the reaction mixture is heated to optimum temperature (72ºC), the polymerase binds to the primer-template hybridized complex and then produces a new complementary strands from free nucleotides in the mixture (Jalali *et al*., 2017).

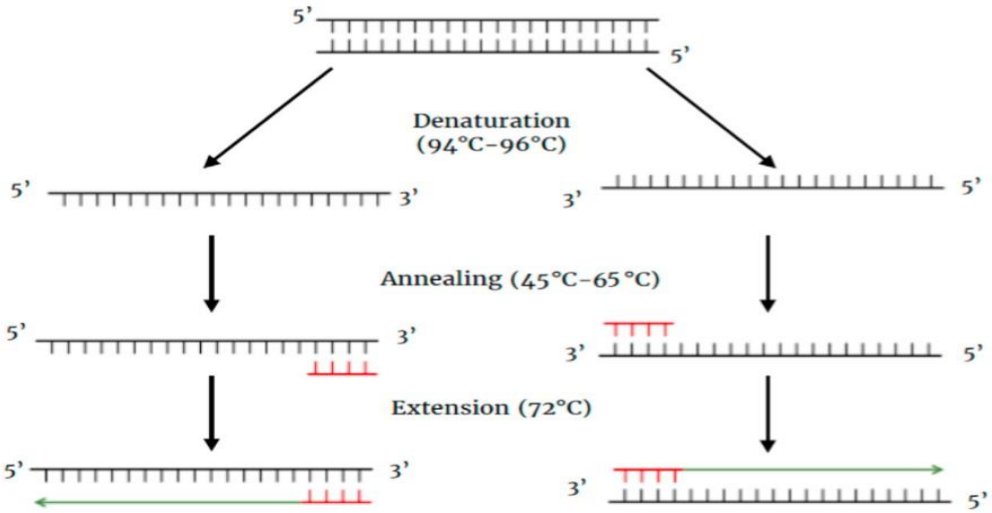

Figure 2: Synthesis of a new DNA strand complementary to the template strand (Jalali *et al*., 2017).

Real Time PCR (RT-PCR) provides many benefits than the conventional PCR; they are accurate, highly sensitive and have the ability to monitor the amplification of DNA in real time through fluorescence intensity and nullifies the need for any post-PCR detection technique (Franco-Duarte *et al*., 2019). The major advantages of amplification methods are that they can provide results in few hours, which is dependent on the number of samples to be analyzed, real-time PCR also provides quantitative data on specific sequences within a mixed population sample within a short period (Ferone et al., 2020). The sensitivity and specificity characteristics of the amplification methods are important in the identification of organisms that grows slowly due to its low detection limit (Ferone *et al*., 2020). The setback that occurs during amplification is that the risk of contamination increases as million-fold amplification of the sample takes place (Ferone *et al*., 2020).

Table 3: Review of the polymerase chain reaction technique for the identification of different groups of fermentative organism

Fermentative microorganism	Source of Isolation	Method	Reference
Lactobacillus delbrueckii ssp. bulgaricus and Lactobacillus plantarum.	Curd and pickle	16SrRNA multiplex PCR analysis	Thakur *et al*., 2017
Fermentative bacteria	Golden snail	Amplification PCR by using MyTaq Red Mix (Bioline).	Retnowati and Katili, 2021
Leuconostoc citreum	Fermented foods	Rep-PCR amplification	Kaur *et al*., 2017
Lactobacillus spp., Debaryomyces spp.	Qingcai paocai, (traditional Chinese fermented vegetable food)	Quantitative PCR (Lightcycler Nano System)	Liang *et al*., 2018
Lactobacillus, Pediococcus, Oenococcus and Leuconostoc	Cider (fermented apple juice)	MultiplexPCR (DreamTaq Green PCR Master Mix)	Cousin *et al*., 2019
Lactic Acid Bacteria	Nemchua	(GTG)5-PCR analysis	Doan *et al*., 2012

3.2.2. Pulsed-Field Gel Electrophoresis-PFGE

Pulsed-field gel electrophoresis is a technique employed to separate substantial DNA fragments and is especially valuable for characterizing and typing bacteria (Franco-Duarte *et al*., 2019). During this process, pure bacterial strain is encapsulated in agarose plug which undergoes treatment with enzymes and detergent (such as proteases and SDS) to liberate chromosomal DNA. Subsequently the agarose plug is then exposed to restriction enzymes which cleaves to specific sites and produces a restricted number of DNA fragments. These

plugs are then subjected to an electric current and alternate rotations within a magnetic field enhancing the mobility of larger DNA fragments, leading to the separation of DNA fragments by size and the formation of a distinctive banding pattern (Parizad *et al*., 2016).

3.2.3. Ribotyping

Ribotyping, unlike certain previously described molecular typing methods, utilizes rRNA-based phylogenetic analysis as a method for bacterial identification and characterization. The rRNA (16S rRNA) tends to be highly conserved within bacterial species, detecting variations in the 16S rRNA gene reflects the evolutionary lineage of the bacterial specie which provides insights into bacterial classification, taxonomy and population biology. Ribotyping encompasses a multi-step procedure that initiates with the utilization of restriction enzymes targeting the genomic sequence of interest, this is followed by Southern blot transfer and hybridization with probes concluding with the analysis of ribotype RFLP bands (Franco-Duarte *et al*., 2019).

3.2.4. Whole gene sequencing

Whole gene sequencing (WGS) has recently become widely accessible and cost effective method for bacterial genotyping, examining the complete bacterial genome not only offers novel insights into bacterial classification and evolutionary lineages but also improved strategies in antimicrobial resistance (Franco-Duarte *et al*., 2019). Another advantage of whole gene sequencing is its non-dependence on specific targeting unlike PCR, so there is no development of primers as bacteria mutations (Ferone *et al*., 2020).The main drawback is the substantial amount of bioinformatic work needed to process the data generated by this method, along with the shortage of personnel proficient in this domain (Low and Tammi, 2017).

3.2.5. Next Generation sequencing

Next Generation sequencing (NGS) technology allows for the efficient and comprehensive examination of intricate microbial populations by focusing on short amplicons, typically targeting the highly variable regions of prokaryotic 16S rRNA (Ohshima *et al*., 2019). It has the ability to sequence nucleic acids by multiplying the amount of sequence data that can be produced in a swift and cost-effective manner (Kumar *et al*., 2019). Next generation has suggested a progression in the evolution of DNA sequencing technology, hinting at further advancements and indicating the potential of future potentials to be labelled with a next-next generation nomenclature (Slatko *et al*., 2018). Despite the significant variations in NGS methods, the overall process generally adheres to three main stages: sample preparation, nucleic acid sequencing and data analysis (Kumar *et al*., 2019).

3.2.6. Fluorescent In situ Hybridization

Fluorescent in situ hybridization (FISH) allows the identification of specific microorganism such as bacteria, yeasts and protozoa at either genus or species level, which is achieved by employing short, fluorescence-labelled, target specific oligonucleotide probes that binds to ribosomal RNA, followed by an analysis using a fluorescence microscope (Frickmann *et al*., 2017). Following fixation onto standard microscope slides and appropriate slide preparation which involves target-specific permeabilization process steps in which the hybridization process takes place (Frickmann *et al*., 2017). FISH is well suited for use in resource constrained areas as it demands limited technical equipment. The material costs for FISH are significantly lower than those associated with all exisiting PCR-based techniques. (Frickmann *et al*., 2017). It also involves visualization, identification, counting and mapping of individual cells (El Sheikha and Hu, 2018).
The effective execution of FISH demands a skilled and extensively trained operator because it is a microscopic method that lacks standardization and necessitate expertise in the interpretation of results. Its sensitivity is notably lower than that of PCR when analyzing primary materials (Frickmann *et al*., 2017)

3.3. Immunological Methods

Immunological methods rely on the specific interaction between antibodies and antigens, where regions of the antigen bind to available epitopes on the antibody. Common immunological techniques employed in food analysis includes enzyme-linked immunosorbent assay (ELISA) which uses antibodies to detect substances through colour changes, enzyme-linked fluorescent assay (a highly sensitive biochemical test similar to ELISA) and immune-magnetic separation (a laboratory tool for efficient isolation from cultured cells) (Hameed *et al*., 2018).

3.3.1. Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay is a method that consists of sorbent substrate, immune-recognition and enzyme label. The principle of ELISA includes the specific immune reaction between antibody and antigen which acts as sorbent substrate and binds to the supporting material. A bioconjugation is formed in which the enzyme-based biomolecules bind to the sorbent, while the detection signal is achieved by utilizing a chromogenic reagent (Wu *et al*., 2019). Sample pretreatment is carried out in ensuring that the samples are free from salt, acid, metal ions, or other compounds that might strongly affects the results of the detection (Shen *et al*., 2014).

This antibody based detection systems are usually distinctive and sensitive with detection limit that can be low as small number of CFU/g (Ferone *et al*., 2020). ELISA offers significant benefits compared to other methods, with its exceptional specificity, sensitivity, simplicity and reproducibility, as demonstrated on Figure 3 (Hameed *et al*., 2018; Wu *et al*., 2019). It has an advantage due to its good selectivity of reagents, strong performance and easy accessibility in most laboratories (Wu *et al*., 2019). It requires specific antibodies and has a higher detection limit which is a major setback in its use (Ferone *et a*l., 2020).

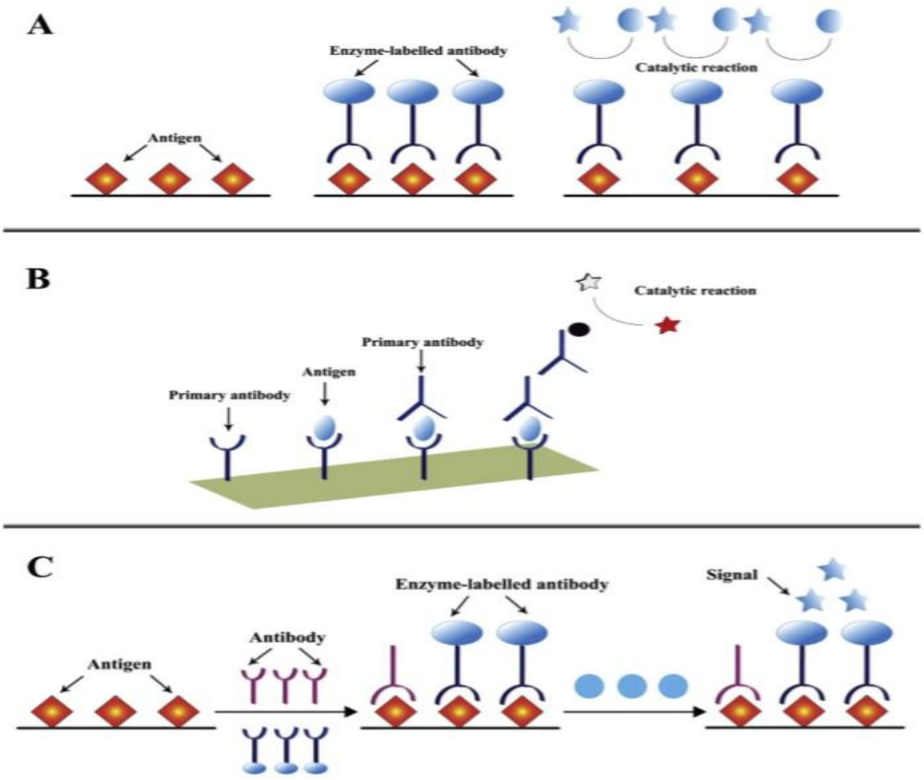

Figure 3: ELISA technique (A) The fundamental three stages of direct ELISA: coating, identification, colour production (B) Secondary antibody labelled with an enzyme in Sandwich ELISA (C) Utilizing competitive ELISA for the immunoassay of an antigen (Wu *et al*., 2019)

3.4. Spectroscopic Techniques

Spectroscopy involves examining matter through its interaction with electromagnetic radiation. The technique vary depending on the species under analysis (molecular or atomic spectroscopy), the type of interaction between radiation and matter to be observed (absorption, emission, or diffraction) and the specific region of the electromagnetic spectrum employed for the analysis. The unique macromolecular composition of bacterial cells including nucleic acids, proteins, carbohydrates, proteins and fatty acids can yield distinctive absorption spectra (Ferone *et al*., 2020).

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

MALDI-TOF MS is an analytical method that involves ionizing particles, separating them based on their mass- charge ratio and quantification is done by measuring the time it takes for the ions to reach a detector at the end of a time-of-flight tube. This technology has the capacity to identify a wide range of microorganisms including gram-positive, gram negative bacteria, aerobic and anaerobic species, yeasts and molds usually achieving species-level accuracy (Rychert, 2019).

Microorganisms are ionized, creating a distinct molecular fingerprint or spectra profile which is registered while identification is carried out by comparing this spectral profile to a database through an automated program. The primary MS technique used for microorganism identification is the analysis of protein (Buszewski *et al*., 2017). MALDI TOF analysis output is a characteristic spectrum of peptide mass fingerprint (PMF) in which the proteins used for identification are called ribosomal proteins represents (60-70%) of the microbial cell dry weight (Ferone *et al*., 2020).

In most instances, pure bacterial cultures cultivated on solid media are prepared for analysis in which a minimum sample biomass of 10^4 cells is required to generate a high- quality mass spectrum for dependable identification (Popovic *et al*., 2023). However, the precision and selectivity of MALDI-TOF MS identification also vary based on the bacterial species under examination and the mass spectrum of the sample being analyzed (van Belkum *et al*., 2019).

3.4.1.1. Identification of Microorganisms using MALDI-TOF MS

In MALDI-TOF MS analysis (Figure 4), the samples are prepared by either mixing or applying a solution of an energy-absorbing organic compound known as matrix (Popovic *et al*., 2023). As the matrix solution dries and crystallizes, the embedded sample also co-crystallizes within it (Han *et al*., 2021). The selection of the matrix can significantly impact the detection of microorganism peaks, as matrices are recognized to facilitate the ionization of specific compounds like phospholipids, peptides or proteins (Popovic *et al*., 2023).

The matrices frequently employed for biological samples like proteins or peptides include α-cyano-4-hydroxycinnamic acid (CHCA or HCCA), 5- chloro-2-mercapobenzothiazole (CMBT), sinapinic acid (SA) and dihydroxybenzoic acid (DHB) (Yoo *et al*., 2020; Kobylis *et al*., 2021). The application of laser leads to the desorption and ionization of the sample in the matrix, resulting in the creation of distinct protonated ions derived from the analytes present in the sample. Subsequently, these protonated ions are propelled through a constant electric potential and separated from one another based on their mass-to - charge ratio (Han *et al*., 2021). Table 4 shows some examples of MALDI-TOF applications in biological materials.

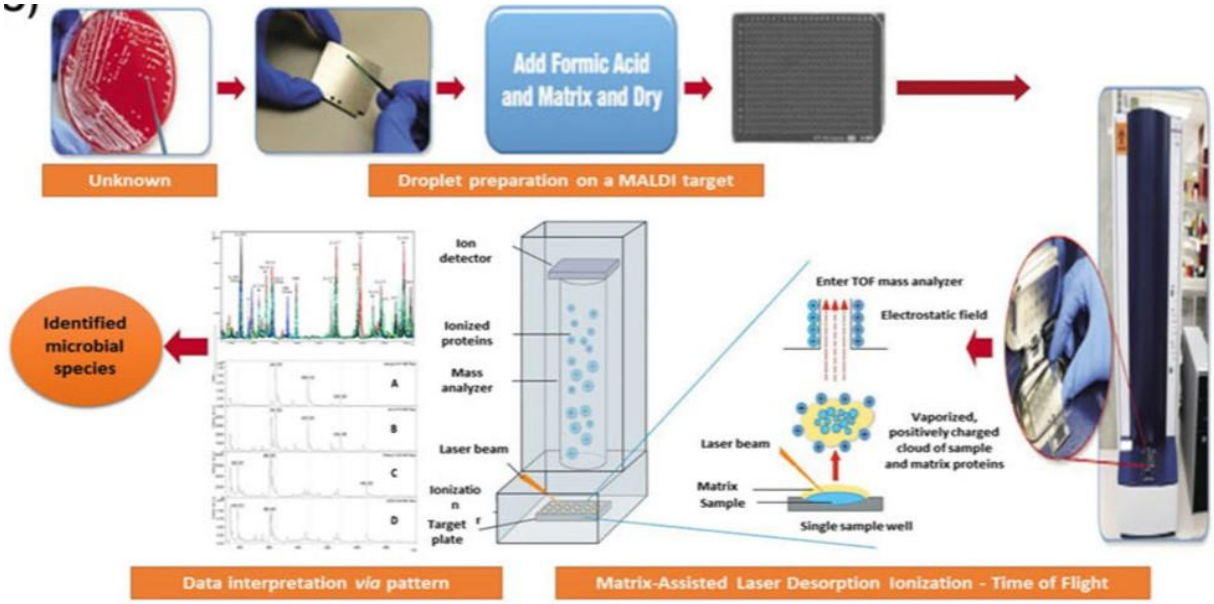

Figure 4: Workflow diagram of Matrix-Assisted Laser Desorption Ionization-Time of Flight (El Sheikha and Hu, 2018).

Advantages of matrix assisted laser desorption ionization time of flight

i. MALDI-TOF demonstrates the capability to differentiate closely related bacterial species with a substantial level of certainty (Rychert, 2019).
ii. Utilizing MALDI-TOF MS as the principal method of identification in the laboratory can result in notable cost reductions (Rychert, 2019).

Disadvantages of of matrix assisted laser desorption ionization time of flight

i. Insufficient spectra in the database can also contribute to the misidentification of similar species. In such situations, there is a possibility of receiving either an inaccurate species-level identification or no identification at all (Rychert, 2019).
ii. Another drawback of MALDI-TOF involves the requirement for intricate extraction procedures with organisms possessing thick cell walls such as Gram-positive bacteria, yeast or filamentous fungi. These extraction procedures necessitate the use of hazardous substances and elevates the costs associated with waste disposal (Buszewski *et al*., 2017).
iii. The initial cost of MALDI-TOF is expensive (El Sheikha and Hu, 2018).
iv. Only cultivable microorganisms can be identified (Ferone *et al*., 2020).

Table 4: Review of MALDI-TOF in the identification of different groups of fermentative organisms

Fermentative Microorganism	Source of isolation	MS-ID system	Reference
Lactobacillus brevis *Lactobacillus plantarum*	Fermented Kimchi	MALDI Biotyper	(Kim *et al*., 2021)
Weissella isolates	Kimchi and Jeotgal.	MALDI bioTyper	Kim *et al*., 2017
Lactobacillus Plantarum, Hanseniaspora opuntiae, Saccharomyces cerevisiae, Acetobacter pasteurianus	Fermented cocoa bean	MALDI Biotyper	Schwenninger *et al*., 2016
Lactic Acid Bacteria	Nemchau	MALDI TOF/TOF analyzer	Doan *et al*., 2012
Saccharomyces cerevisiae, Pichia kudriavzevii, Kluyveromyces marxianus	Sourdough fermentation	MALDI Biotyper	Sevgili *et al*. 2021
Candida crusei, Candida parapsilosis, Saccharomyces cerevisiae	Fermented milk	MALDI Biotyper	Corbu *et al*. 2021

3.4.2. Fourier transform infrared spectroscopy (FTIR) FTIR spectroscopy involves the detection of molecular vibrations when exposed to infrared light, and the resulting absorbance spectrum serves as a distinct fingerprint that characterizes any biochemical or chemical substance. Each bacterial species possesses a distinct cell wall or cell

membrane composition leading to unique IR fingerprint resulting from the molecular vibrations in bio-organic compounds. These molecular compositions differ from one cell to another, enabling the identification of individual cells through FTIR spectroscopy which are then cross referenced with the exisiting spectral library for verification (Hameed *et al*., 2018).

FTIR can serve as a quantitative quality control tool in the food industry, it offers a fast and non-invasive analytical approach that eliminates the need for time consuming sample preparation, special consumables or highly skilled labour (Papadopoulou *et al*., 2021).

This non-invasive method approach is more resilient to variations in bacterial species related to physiology or biochemistry compared to fluorescent dyes and PCR methods. It is particularly well suited for accessing the physiological state of cells, distinguishing between live, dead and injured states (Hameed *et al*., 2019). It is also a sensitive method that requires small quantity of the sample (El Sheikha and Hu, 2018).

The disadvantage is that the differences in the spectra can be attributed to environmental conditions surrounding the FT-IR instrument and a comprehensive spectrum library is recommended to facilitate detection (El Sheikha and Hu, 2018).

3.4.3. Hyperspectral Imaging

Emerging as a non-invasive and cutting edge optical technology, it involves image data technology with numerous narrow bands. It integrates machine vision and spectral technology to capture both the two-dimensional spatial and one-dimensional spectral information of the targets, resulting in the acquisition of high resolution image and spectral data (Hu *et al*., 2014). Hyperspectral technology simplifies the detection of objects that would be challenging to identify using broad-spectrum methods (Zhu *et a*l., 2020). The foundation of HIS for detecting microorganism relies on the assumption that metabolites offers unique patterns that signify the presence of microorganism (Wang *et al*., 2018). The benefit of HSI lies in its capability to offer both spectral and spatial data for the detection, identification and quantification of microorganism (Herrero-Langreo *et al*., 2018). Hyperspectral detection (Figure 5) minimizes numerous errors originating from external factors such as reagents, instruments and operators, ensuring accurate, swift and non-invasive detection (Zhu *et al*., 2020). It is also a non-destructive method of identification (Ferone *et al*., 2020).

One of the primary limitations when utilizing HSI systems for microbiological analyses is the challenge of comparing across different studies, owing to the distinct data structures obtained from each instrument settings (Ferone *et al*., 2020). The hyperspectral photography system captures data exclusively from the surface of the object and cannot penetrate the interior of the sample, making it unsuitable for detecting chemical components with low homogeneity (Zhu *et al*., 2020).

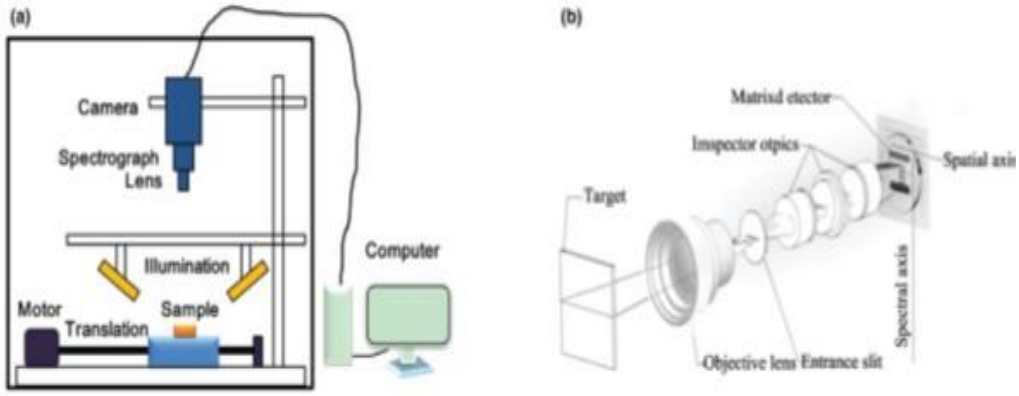

Figure 5: The fundamental concepts and procedure of hyperspectral imaging technology (a) Hyperspectral imaging setup (b) Schematic illustrating the principles of hyperspectral imaging (Zhu *et al*., 2020).

3.4.4. Raman Spectroscopy

Raman spectroscopy is a method relying on the inelastic scattering of monochromatic light, typically emitted from a laser source. Inelastic scattering describes the alteration in the frequency of photons in monochromatic light as they interact with a sample. The samples absorbs photons from the laser light and subsequently re-emits them (Ferone *et al*., 2020). This procedure uses vibrational, rotational, and other low-frequency modes in the system in order to produce a structural fingerprint by which molecules can be identified (Franco-Duarte *et al*., 2019). It is easy to use, high speed of analysis, it gives comprehensive information regarding the structure, chemical composition and interactions of biomolecules within the microorganism, measurements can be conducted at the single cell level, which avoids the pre-cultivation steps and the analysis of non-cultivable microorganism (Ferone *et al*., 2020). Raman is a more expensive technique which limits its usage (Ferone *et al*., 2020).

3.5. Future Directions and Emerging Technologies

Machine learning has the capability to recognize statistical dependencies in data, taking into account non-linearity and interaction effects among features (Han et al., 2021). Following current advances, machine learning technology can unravel novel information embedded in the MALDI TOF mass spectrum which is useful for the identification and differentiation of species, especially those that are phylogenetically closer at the subspecies level (Tran *et al*., 2021). Hence, they combined a single-cell MALDITOF MS with an ML algorithm to experimentally demonstrate that the

resulting spectra were useful in distinguishing between different bacterial species (Han *et al*., 2021). Machine learning can be employed for the direct analysis of fermentation parameters or to deduce insights from high-dimensional omics data. Machine learning techniques, including ensemble methods, have demonstrated enhancements in refining genome-scale metabolic reconstructions (Khaleghi *et al*., 2021).

4. Conclusion

The development of rapid, low-cost, sensitive, and reproducible methods for the screening identification of microorganisms is an important issue in modern science. Identification methods for determining the identity of a microbial sample have been emerging in the last decades. In general, spectroscopic techniques can achieve rapid, nondestructive, or label-free detection, rendering them attractive for routine microorganism detection in the food industry. Several challenges must be overcome in the development and application of such new and contemporary approaches in food science. There is an urgent need to fill in the gap that exists between conventional techniques and advanced identification methods.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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