Recent trends for detection, quantification and identification of microorganisms in food industry

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ABSTRACT

Phenotypic characterization of microorganisms was originally performed using culture and physiological/biochemical methods. Throughout the 90s, traditional PCR was being replaced by the second-generation PCR, the real-time PCR (qPCR). This method is very effective for negative screening and also to confirm an isolate. However, limitations of qPCR are the inability to distinguish viable from dead cells and the sensitivity of amplification reactions to inhibitors. Sensitivity, specificity, robustness, time of response and the cost per analysis are often the criteria used for choosing a bacteria identification protocol. In this context, bacterial identification based on MALDI-TOF MS is becoming a method of choice for determining the genus, species and even subspecies of bacterial and fungal isolates in food analysis. MALDI-TOF MS is a promising method to identify bacteria and yeast in a few minutes directly from colonies grown in culture dishes. However, the identification of new isolates is possible only if the spectral database contains fingerprints of peptide mass from the strain type of specific microorganism. All current methods have their own guarantees, and so far, no method meets all attributes desired by the laboratory; therefore, recent research studies are associating two or more methods with different technologies.

Keywords: Microorganisms, food, alternative methods, qPCR, MALDI-TOF MS, technology.

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FINDINGS

In food microbiology, culture-dependent methods are still the most direct way to study the presence of microorganisms but are considered laborious and inconvenient caused by the isolation conditions and culture media applied, as well as the potential stress of target microorganisms. In addition, the time to obtain a result is high in comparison with some molecular methods (Mirotto et al., 2019).

The diversity of microorganisms and discoveries in various fields of science (physics, biochemistry, (micro) biology, medicine, (bio) informatic) were the main drivers of the development and progress of microorganism identification methods. Phenotypic characterization of the microorganisms was originally performed using macroscopic/microscopic methods, those based on culture and physiological/biochemical methods. Although some of these methods are still used today for economic/regulatory/gold standard reasons. Recent methods of identification are now based on cellular, immunological, and molecular techniques. Among the latter, tools dedicated to nucleic acid analysis are the most developed, for example, the use of enzymatic restriction, PCR-based techniques and their variations, based on hybridization, isothermal amplification and others (Nacef et al., 2017).

These molecular approaches have been applied to the
systems of microbial species, including restricted or amplified fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), multilocus sequence typing (MLST), and use of microsatellite markers. However, it is necessary to investigate useful methods for differentiation and taxonomic classification of known and new (or enigmatic) species with efficacy determination of certain species. According to a recent study done by Rico-Munoz et al. (2019), a number of qPCR tests, either single or multiplex, have been developed during the last two decades to detect and quantify these species in different food products.

Some methods that are currently being used by the scientific community or the industry for the detection, quantification or identification of microorganisms are given in Table 1.

Among the independent culture methods, qPCR is widely used in routine food laboratories. It is well adapted to specific and precise bacterial quantification, provided that a strong standardization and evaluation of the method performance. However, two major limitations of qPCR are particularly troubling: the inability to distinguish viable from dead cells and the sensitivity of amplification reactions to inhibitors. Reverse-transcription-qPCR (RT-qPCR) has been used to quantify different microbial states but remains delicate for reliable quantification due to high degradation of bacterial RNA and difficulties in extracting high quality RNA from complex matrices. More recently, the "viability-PCR" approach has been proposed as an alternative method to RT-qPCR, based on the treatment of the sample with DNA intercalating dye, ethidium monoazide (EMA) or propidium monoazide (PMA) or thiazole orange monoazide (TOMA) which penetrates only into cells with damaged membranes or binds to free DNA, inhibiting subsequent amplification. These results related to the amplification show that living cells with intact membranes are viable (Cao et al., 2018; Gobert et al., 2018; Scariot et al., 2018).

Multiplex assays, specific probes (MGB, Taq Man) and intercalating dyes applied to qPCR have made it more flexible in various applications in microbiology, including quantification of pathogens, as qPCR is based on the calculation of increased fluorescence, yielding results over a period of three days or less when compared to agar gel analysis (Wang et al., 2018). Although qPCR has been so common in the detection of foodborne pathogens, there are also limitations in respect to absolute quantification. Compared with qPCR, digital PCR (dPCR) has recently emerged as an interesting variation of qPCR based on the amplification of single target DNA molecules, which provides absolute quantification without the need for standard curves. Digital PCR allows the detection of rare target sequences of nucleic acids in the presence of a large abundance of standard sequences and is less sensitive to inhibitions than qPCR. This property seems particularly interesting for the specific quantification of food samples containing PCR inhibitors and many different bacteria (Gobert et al., 2018; Wang et al., 2018).

As already discussed, independent culture methods have become the main trend for microbial research. These techniques are based on the combination of genome and bioinformatics analysis and provide more detailed microbial information without the requirement of bacteria isolation. Some studies have applied 16S rRNA sequencing of the fragment to examine microbial diversity in isolation plaques from environmental samples, but still less frequent in food microbiology (Yu et al., 2019).

The main criteria for choosing the method of bacterial identification are often the sensitivity and specificity of the identification tests used; their robustness, time of response and the cost per analysis are also some useful aspects to have into account. In this context, bacterial identification based on MALDI-TOF MS is becoming a method of choice for determining the genus, species and even subspecies of bacterial and fungal isolates. Initially developed for academic research, the use of MALDI-TOF MS to obtain the molecular fingerprint of a given microorganism is now widely diffused in different activity areas: human and animal health, environment and hospital. However, this methodology is underused in the food industries, mainly due the high cost of the equipment. MS specialists have developed various types of ion sources, mass analyzers, and detectors to measure the molecular masses of different molecules.

Although several types of mass spectrometers are capable of analyzing complex mixtures of microorganism proteins, the MALDI-TOF mass spectrometer has emerged as the tool of choice for the adequacy between the solid nature of bacterial samples and the source; the absence of complex and critical sample preparation steps (even if several standard operating protocols are available); a fast acquisition time of directly usable MS data and a low cost per analysis. The MALDI-TOF mass spectrometer is composed of a source of solid sample ions coupled to a mass analyzer that classifies the ions according to their time-of-flight to go through a certain distance in a free field environment (Hsieh et al., 2018; Huber et al., 2018; Nacef et al., 2017; Quéro et al., 2019; Sauget et al., 2017; Thouvenot et al., 2018; Yu et al., 2019).

Recent increase in the identification of bacteria based on the MALDI-TOF MS profile depends on several advantages ranging from high productivity, robustness, recognition-based method, ultra-fast trend test, ease of use and low cost per test. MALDI-TOF MS profile can be easily implemented as routine analysis and complements the phenotypic methods for rapid identification in the first stage of characterization of the microbial community cultivable in dairy products, for example. This rapid, inexpensive, robust and reliable method of identification of bacteria presents itself as an attractive alternative to biology-based, biochemical and molecular identification approaches that do not require extraction of nucleic acids.
and PCR or sequencing steps (Yu et al., 2019).

However, bacterial identification based on MALDI-TOF MS is not devoid of disadvantages: fields of restricted application without prior and fresh culture (small quantity of culture that grew less than 24 h) of isolated microorganisms are required; a method of low analytical sensitivity because the insufficiency of biomass; an essential sample preparation method; a difficult but not impossible discrimination of phylogenetically close related microorganisms, and finally, a restricted content of MS profile database. In addition, the need for a comprehensive quality control program of the method and that the identification principle is based on distinct databases from one manufacturer to another, act both as strengths and limitations. The establishment of a single worldwide database of MALDI-TOF MS bacterial reference spectra of a larger set of microbial strains and/or a dedicated database for the food industry correlated with genomic data would be critical to the dissemination of this method of bacterial identification (Nacef et al., 2017).

There are still few available studies related to the performance of this technique in food-related strains. The results obtained with MALDI-TOF MS in the identification of clinical strains can not be transmitted directly to several bacterial species. It is known that *Campylobacter* strains, for example, related to food are much more stressed than clinical isolates. Changes in certain pathways, expression profiles, virulence and resistance patterns were reported in strains; attributed this variability to cell structures that interfered with whole-cell extraction techniques used in MALDI-TOF MS analysis (Ziino et al., 2019).

Although there are different molecular typing methods for comparing strains in foodborne outbreak investigations, the PFGE technique remains the gold standard for typing. One of the great advantages of this technique is that it is less expensive when compared to the Next Generation Sequencing (NGS); being this a promising tool as it allows rapid detection of genes and toxins, but requires knowledge of bioinformatics and software for data analysis. Therefore, NGS and software tools developed to analyze complete genomic sequence data can enable rapid detection of toxin genes such as staph (Denayer et al., 2017).

To ensure the safe production of food, it is necessary to monitor the occurrence of pathogenic organisms, including fungi, in different stages, such as the cultivation, harvesting, storage and processing of raw materials in the final product. Fungi are eukaryotic organisms with cell walls rich in glycans and chitin, producing enzymes that facilitate the absorption of organic materials as a source of energy and carbon. They are ubiquitous microorganisms, ranging in size from massive mycelium to a single microscopic yeast cell; known for producing a wide variety of metabolites, including mycotoxins, which play an important ecological role in the diversification and adaptation of these microorganisms. The major problem of fungal food contamination is that some species have the potential to produce mycotoxins that may have significant adverse effects on human health, mainly associated to the presence of toxigenic fungi including

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**Table 1.** Recent detection methods used in food microbiology studies for different targets and matrices.

| Method          | Target                      | Matrix                        | Gene                  | Reference             |
|-----------------|-----------------------------|-------------------------------|-----------------------|-----------------------|
| MALDI-TOF-MS    | Yeast, bacteria             | Cherry, flours                | n.a.                  | Bellili et al., 2019  |
| MALDI-TOF-MS    | *Escherichia coli* O157:H7 | Lettuce                       | *rfbE, stfA, sx2*     | Weiss et al., 2019    |
| MALDI-TOF-MS / PCR | Lactic acid bacteria       | Cheese                        | 16S rDNA              | Nacef et al., 2016    |
| MALDI-TOF-MS / sequencing | Total aerobic bacteria     | Poultry carcasses             | 16S rRNA              | Yu et al., 2019       |
| TOMA-qPCR       | *Escherichia coli* O157:H7 | Potable water                 | n.a.                  | Cao et al., 2018      |
| PMA-qPCR        | *Lactobacillus paracasei*   | Probiotic yogurt              | *tuf*                 | Scariot et al., 2018  |
| dPCR / qPCR     | *Salmonella Typhimurium*    | Milk                          | *timY*                | Wang et al., 2018     |
| qPCR            | *Escherichia coli*          | Oysters                       | *uidA*                | Miotto et al., 2019   |
| qPCR            | Acetic acid bacteria        | Vinegar, fermented milk       | 16S rRNA (K-AAB)      | Kim et al., 2019      |
| Tradicional ISO 6579 / MDS | *Salmonella* spp. | Herbs and spices               | n.a.                  | Lins (2018)           |
| MPN/ qPCR (BAX Vibrio) | *Vibrio parahaemolyticus* | Oysters                       | n.a.                  | Lindemann et al., 2019 |
| PFGE            | *Staphylococci enterotoxin* | Food associated with outbreaks | n.a.                  | Denayer et al., 2017  |
| HRM-PCR         | *Aspergillus*               | Grapes                        | ITS2 ribosomal region | Xanthopoulou et al., 2019 |

Legend: MALDI-TOF MS [Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass Spectrometry]; qPCR [real-time PCR]; MPN [Most Probable Number]; PFGE [Pulsed Field Gel Electrophoresis]; MDS [Molecular Detection System]; HRM-PCR [High Resolution Melt-Polymerase Chain Reaction]; n.a. [not applicable].
the genera *Aspergillus*, *Penicillium* and *Fusarium*. Representatives of *Aspergillus* can produce specific mycotoxins, such as aflatoxins, while *Penicillium* is able to produce citrinin and ochratoxin. *Fusarium* is capable of producing various mycotoxins, of which fumonisins, deoxynivalenol and zearalenone are the most important because of their negative impact on human and animal health.

Since molds and yeasts usually have slower growth, conventional culture-based quantification methods are time-consuming, taking around 5 to 7 days until the results are finished. Alternative solutions for the quantification of molds and yeasts such as SimPlate Yeast and Mold and 3M Rapid Yeast and Mold can shorten the time to 2-3 days. Similar to bacteria, the use of alternative techniques used to quantify and identify fungi in food grows toward faster, more automated testing. Molecular identification of mycotoxin producers can increase the efficiency and reduce the cost of quantification of individual mycotoxins by methods such as ELISA (enzyme-linked immunoassay) or HPLC (high performance liquid chromatography). Until now, several molecular approaches have been applied to the design of *Aspergillus* species, including restriction fragment length (RFLP) or amplified polymorphism (AFLP) (Vyhnánek et al., 2018). In addition, the high-resolution melting (HRM) PCR has been a useful tool in the characterization and differentiation of *Aspergillus* isolates (Xanthopoulou et al., 2019).

Genomic methods, such as Polimerase Chain Reaction (PCR) based techniques and loop-mediated isothermal amplification, have been reported as fast and accurate alternatives. Limitations of the genomic methods due to false results should be monitored using the internal amplification control and avoiding experimental conditions that favor the formation of mutations. The MALDI-TOF MS method is also already available for the early and accurate detection of filamentous spoilage fungi in food (Lima and Santos, 2017; Quéro et al., 2019; Rodríguez et al., 2015).

Most microorganism detection techniques are based on the combination of assays or techniques. While much work has been done to develop methods of automation, combination and miniaturization, the practical applications of these new devices remain challenging, as for example the PCR method is still an expensive technology for most service labs. This is due to the fact that it includes steps of extraction and purification of genetic material, without considering the development, validation of primers, probes, and others performance test inherent to the technology.

As pointed out in this short review, few developments in microbiological diagnostics have had such a rapid impact on the identification of species-level microorganisms like MALDI-TOF MS. Conventional methods of differentiation are based on biochemical criteria and require additional pre-testing procedures and long incubation periods. In comparison, MALDI-TOF MS can identify bacteria and yeasts in a few minutes directly from small colonies grown from culture dishes until 24 h. This relatively new approach to food and methodically simple reduces the cost of consumables and the time spent on diagnostics. The reliability and precision of the method have been demonstrated in numerous studies, reviews and different systems are already commercially available, with a database that is constantly improving.

This technology was readily absorbed by microbiologists who reported using MALDI-TOF MS for various purposes such as microbial identification of *Salmonella* spp. (Demirci et al., 2019); *Listeria* spp. (Thouvenot et al., 2018); *Escherichia coli* STEC (Mclean et al., 2018); *Campylobacter* (Lawton et al., 2018); *Staphylococcus* enterotoxin (Tonacini et al., 2019); *Bacillus cereus* cereulide (Ulrich et al., 2019); epidemiological studies (Camarasa and Cobo, 2018; Welker et al., 2019), detection of *Legionella pneumophila* in water system (Trnková et al., 2018), resistance to antibiotics (Carvalho et al., 2017; Cordovana et al., 2019) and pathogens in blood and urinary tract (Opota, 2018; Sánchez-Juanes et al., 2018), among other research studies. The limitation of the technology is that the identification of new isolates is possible only if the spectral database contains fingerprints of peptide mass of the strain type of specific genera, species or subspecies.

It is expected that soon detection methods in food microbiology will be fast, inexpensive, reliable and will not require further confirmation. If a fast detection method can save time and cost, it will be more acceptable and become popular in the analytical routine. However, all current methods have their own advantages or limitations, and so far, none detection, quantification or identification method meets all the desired facilities.

Rapid methods and automation in microbiology will continue to grow in number and kind of tests in the future, due to the increased concern over food safety and public health globally. Today, food analysis laboratories usually use various technologies in combination to detect and quantify microorganisms. This is necessary because there is a need for quick release of results and simplifies the analyst's routine, reducing steps. Furthermore, new and differential developments should be required in quantitative detection of microorganisms.

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