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Species Identification of Food Spoilage and Pathogenic Bacteria by MALDI-TOF Mass Fingerprinting

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1. Introduction

Food quality and safety is an increasingly important public health issue. Nowadays, the topics "food quality" and "food safety" are very close and two important issues in the food sector, due to the globalization of the food supply and the increased complexity of the food chain. The consumers need to purchase safe products that do not involve any kind of risk for health. On one hand, the aim of the "food safety" is to avoid health hazards for the consumer: microbiological hazards, pesticide residues, misuse of food additives and contaminants, such as chemicals, biological toxins and adulteration. On the other hand, "food quality" includes all attributes that influence the value of a product for the consumer; this includes negative attributes such as spoilage, contamination with filth, discoloration, off-odors and positive attributes such as the origin, color, flavor, texture and processing method of the food (FAO, 2003).

The contamination of food products with microorganisms presents a problem of global concern, since the growth and metabolism of microorganisms can cause serious foodborne intoxications and a rapid spoilage of the food products. Thus, the acceptance and safety of a food product for the consumers depends in great part on the presence and nature of microorganisms. Besides molds and yeasts, bacteria are the principle responsible for various types of food spoilage and foodborne intoxications (Blackburn, 2006). It has to be mentioned that a food product naturally contains an indigenous microbiota that can include spoilage and/or pathogenic bacterial species. Depending on the preservation method these species

can proliferate and adulterate the product. However, most bacterial contamination occurs during processing and manipulation of the food products.

1.1 Spoilage bacteria

Spoilage bacteria are microorganisms that cause the deterioration of food and develop unpleasant odors, tastes, and textures. A spoiled food has lost the original nutritional value, texture or flavor and can become harmful to people and unsuitable to eat. The microbial spoilage of food products constitutes an important economic problem, as it results in high economic losses for the food industry, especially under incorrect refrigeration conditions. Thus, spoilage bacteria are able to grow in large number in food, decompose the food and cause changes in the taste/smell, which affect the quality of the products. Spoilage bacteria normally do not cause illness; however, when consumed in high concentration, they can cause gastrointestinal disturbance (Blackburn, 2006). There are different bacterial species that can cause spoilage in food products and the spoilage microbiota depends in great part on the processing and preservation method. Storage temperature also plays a key role in the growth of undesirable microbiota in food. Thus, fresh foodstuffs such as fish and meat, stored at refrigeration temperatures can result in the growth of *Pseudomonas* spp., including spoilage species, such as *P. fragi* and *P. putida*. A light preservation and change in the atmosphere, e.g. by vacuum-packaging, may inhibit these bacterial species and favour the growth of other species, such as lactic acid bacteria (LAB), *Enterobacteriaceae*, *Bacillus* spp. and *Clostridium* spp. These last two genera are able to produce spores that can survive heat treatments and germinate after a pasteurization process, being an important issue in food safety. Spoilage species may be more food-specific and, thus, *Erwinia* spp. has been reported in products of vegetal origin. Otherwise, seafood products are commonly spoiled by species such as *Shewanella* spp. or *Photobacterium* spp. In general, bacteria can spoil different foods depending on the physical-chemical preservation profile (Gram et al., 2002).

1.2 Pathogenic bacteria

Foodborne diseases are caused by agents that enter the body through the ingestion of food. Food can transmit disease from person to person, as well as serve as a growth medium for bacteria that can cause food poisoning. The global incidence of foodborne diseases is difficult to estimate, but it has been reported that in 2005 alone 1.8 million people died from diarrheal diseases. A great proportion of these cases can be attributed to the consumption of contaminated food and water. In industrialized countries, the percentage of the population suffering from foodborne diseases each year has been reported to be up to 30% (WHO, 2007). Pathogenic bacteria often do not change the color, odor, taste or texture of a food product, being hard to recognize if the product is contaminated. Food-borne infection is caused by bacteria in food. If bacteria become numerous and the food is eaten, bacteria may continue to grow in intestines and cause illness. Food intoxication results from consumption of toxins (or poisons) produced in food as a by-product of bacterial growth and multiplication in food. In this case the toxins and not bacteria cause illness. Toxins may not alter the appearance, odor or flavor of food. Common bacteria that produce toxins include *Staphylococcus aureus* and *Clostridium botulinum*. In some cases, such as *Clostridium perfringens*, illness is caused by toxins released in the gut, when large numbers of vegetative cells are eaten. Bacterial food poisoning is commonly caused by bacterial pathogenic species

such as *Escherichia coli, Salmonella* spp., *Listeria monocytogenes, S. aureus, Bacillus cereus, C. perfringens, Campylobacter* spp., *Shigella* spp., *Streptococcus* spp., *Vibrio cholerae*, including O1 and non-O1, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Emerging foodborne pathogens may refer to new pathogens, pathogens that emerge due to changing ecology or changing technology that connects a potential pathogen with the food chain or emerge de novo by transfer of mobile virulence factors (Tauxe, 2002). Emerging foodborne pathogens include *E. coli* O157:H7, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas sobria*, *Mycobacterium* spp., vancomycin-resistant enterococci, non-gastric *Helicobacter* spp., *Enterobacter sakazakii*, non-jejuni/coli species of *Campylobacter*, and non-O157 Shiga toxin-producing *E. coli*.

2. Bacterial identification methods

In order to control and minimize the microbiological hazard of foodborne pathogens, as well as to predict and enhance shelf-life of food products, pathogenic and spoilage bacteria need to be identified in a rapid and unequivocal way. Several methods have been designed to achieve bacterial identification.

2.1 Bacterial identification by classic methods

Traditionally, bacterial species have been identified by classic tools relying on culturing processes coupled to morphological, physiological, and biochemical characterization. Phenotypic identification is based on direct comparison of phenotypic characteristics of unknown bacteria with those of type cultures. The reliability of this kind of identification is in direct proportion to the number of similar phenotypic characteristics.

When classifying microorganisms, all known characteristics are taken into account. However, certain characteristics are selected and used for the purpose of identification. Primary identification usually involves a few simple assays such as colony morphology, Gram staining, growth conditions, catalase and oxidase tests (Duerden et al., 1998). Testing the requirements for growth includes the presence or absence of oxygen and the growth ability on different culture media. For a better approximation of bacterial identification, other laborious techniques are employed, such as microscopic observation, type of hemolysis, and biochemical arrays like tests for aminopeptidase, urease, indol, oxido-fermentation, coagulase test, analysis of resistance to different substances, etc. To make these analyses faster and less laborious, biochemical assays with multitest galleries are applied. With these arrays inoculation, incubation and lecture can be carried out on a minimal space and the whole process can be automated. Using these tests it is usually possible to characterize the bacterial genus and even species to that an unknown strain more likely belongs to. Conventional identification methods are widely used despite some disadvantages. Apart from being slow and laborious, they can be used only for organisms that can be cultivated in vitro and furthermore, some strains exhibit unique biochemical characteristics that do not fit into patterns that have been used as a characteristic of any known genus and species.

In the last decades, the progress of microbiological identification turned to more rapid and sensitive methods, including antibody-based assays and DNA-based methods, together with important advances in bioinformatic tools. Thus, some methodologies such as ELISA

or PCR already became classic. PCR coupled to sequencing tools has provided a big amount of information that has been deposited in public databases and is freely available. Recently, the development of rapid and high sensitive techniques, such as real-time PCR (RTi-PCR), DNA microarrays and biosensors, provoked the replacement of traditional culturing methods in the field of bacterial identification in clinical diagnostics, as well as in the food sector (Feng, 2007; Mohania et al., 2008). Furthermore, Fourier transform infrared spectroscopy (FT-IR) has been described as a new method for rapid and reliable bacterial identification (Sandt et al., 2006). At the same time, proteomic tools, such as mass spectrometry were introduced for the identification of microorganisms (Klaenhammer et al., 2007).

2.2 Bacterial identification by DNA-based methods

Nowadays, in the field of bacterial diagnostics, traditional culturing methods have been replaced by molecular techniques based on the analysis of DNA, being much faster, more sensitive and accurate. However, they usually have a superior cost due to that they require specific industrial equipment and more qualified personal than conventional techniques.

The sequencing of the 16S rRNA gene is a common tool for bacterial identification. Universal primers are designed and bind to conserved regions to amplify variable regions. The amplification is carried out by means of PCR (Polymerase Chain Reaction). Sequences of the 16S rRNA gene have been determined for an extremely large number of species and are accessible on huge DNA databases, such as the GenBank of the National Center for Biotechnology Information (NCBI). For bacterial identification the 16S rRNA sequence of an unknown strain is compared to the database of published sequences and the most similar bacterial strains are determined using the common bioinformatic tool BLAST.

Other DNA genes have also been used for the study of phylogenetic relationships, such as the 23S rRNA, the intergenic spacer region of 16S-23S (ITS), rpoB (subunit β of RNA polymerase) and gyrB (subunit β of the DNA girasa). However, for bacterial identification these genes are not yet applied, due to the lack of amply databases as reference.

More recently, RTi-PCR has been introduced for the detection and quantification of major foodborne spoilage and/or pathogenic bacteria. As a consequence, a large number of RTi-PCR procedures are currently available for the specific detection and quantification of foodborne bacteria, such as *Salmonella spp.*, *L. monocytogenes*, *S. aures* or *Leuconostoc mesenteroides* (Hein et al., 2001; Malorny et al., 2004; Elizaquível et al., 2008).

The state-of-the-art DNA-based technique is DNA microarray that consists of gene arrays that can hybridize multiple DNA targets simultaneously, and thus, have enormous potential for detection and identification of pathogens. Thanks to the increase in the complete microbial genome sequences, DNA microarrays are becoming a common tool in many areas of microbial research in the field of bacterial identification in clinical diagnostics, as well as in the food sector (Severgnini et al., 2011). Microarray is a powerful, sensitive and specific technology that allows an accurate identification based on single target detection and can determine subtle differences in the genome.

2.3 Mass spectrometry for bacterial identification

In the past few years, proteomic tools, such as mass spectrometry, were introduced for microbial identification (Klaenhammer et al., 2007). According to the ionization source, mass spectrometry can be divided in two techniques electrospray ionization (ESI) and matrix-assisted laser desorption/ ionization (MALDI). The mass analyzer uses an electric or magnetic field in order to accelerate the ions and produce their separation, by means of the ratio mass/charge (m/z). In the field of bacterial identification, mass spectrometric methods have a high potential, due to the ability to detect and identify bacterial proteins (van Baar, 2000).

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) proved to be a technique with high potential for microorganism identification due to its rapidness, reduced cost and minimal sample preparation compared to conventional biochemical and molecular techniques (Seng et al., 2009; Bizzini et al., 2010; Giebel et al., 2010). With MALDI-TOF MS intact bacterial cells can be analyzed in a rapid way, obtaining high specific spectral profiles in the low-mass range of 1500 – 20000 Da. The soft ionization technique allows the analysis of intact high mass molecules, such as proteins. Studies, in that bacterial cells were treated with trypsin and lysozyme before analysis by MALDI-TOF MS showed that the spectral patterns detected by this method are generally attributed to intracellular proteins (Conway et al., 2001). In further studies the proteins detected by MALDI-TOF MS were identified and resulted that most peaks in the spectral profiles are derived from ribosomal proteins (Ryzhov and Fenselau, 2001).

Bacterial identification can be carried out by either identifying ion biomarker masses that could be correlated with theoretically determined protein masses in databases or by comparing the whole spectral profile to a reference database (van Baar, 2000). In the first approach, bacterial strains are identified by determining the masses of biomarker proteins by MALDI-TOF MS and searching against a protein database by matching the masses against sequence-derived masses (Demirev et al., 2004). A number of studies have been carried out, determining protein biomarkers by MALDI-TOF MS for bacterial species identification (Demirev et al., 2004; Fagerquist et al., 2005). A critical challenge of protein database searches is the necessary high mass accuracy, since some proteins have very similar masses. Furthermore, identification is limited to well-characterized microorganisms with known protein sequences available in proteome databases (Dare, 2006).

The second approach, also named "fingerprint approach" is the most applied for bacterial identification. It relies on spectral differences of bacterial species and identification is carried out by comparison of the spectral profile of an unknown strain to a reference database of spectral profiles (Giebel et al., 2010; Mazzeo et al. 2006). This approach allows the differentiation of bacterial strains, due to the high specific spectral profiles, named "fingerprints", obtained. For this purpose, it is not necessary to identify the proteins but just to determine a number of characteristic peaks that are representative for the corresponding species and/or genus.

3. MALDI-TOF MS fingerprinting, a rapid and reliable method for bacterial identification in food

MALDI-TOF MS fingerprinting proved to be applicable for bacterial identification at the genus-, species- and even strain level. In routine bacterial identification in the clinical sector

it demonstrated to be a rapid, cost-effective and accurate technique that achieved correct species identification of more than 92%. This is a significantly better result than conventional biochemical systems or even 16S rRNA sequencing for bacterial identification (Seng et al., 2009; Bizzini et al., 2010). Several authors agree that the costs of bacterial identification by MALDI-TOF MS fingerprinting is around two-thirds less than conventional methods, when taking into account the cost of materials and staff (Hsieh et al., 2008; Nassif, 2009). Furthermore, it has several advantages over other fast methods relying on genomics, such as DNA-microarrays, because fewer steps are necessary to achieve bacterial identification and thus, fewer errors are introduced along the analyzing process. Another advantage of MALDI-TOF mass fingerprinting is the effortless analysis of results, since no extensive data processing and statistical analysis is required, as it is the case in other rapid methods for bacterial identification, such as FT-IR and DNA-microarrays.

Until recently, MALDI-TOF MS techniques for the identification and typing of microorganisms remained confined to research laboratories and to certain species and the comparison with other species is limited by the accessibility of spectra. In the last years, the availability of MALDI-TOF MS devices and the reduction of costs, which enabled their use in either clinical, food or environmental microbiology laboratories, helped to increase the number of studies for the identification of different food pathogens. Thus, regarding to the application to routine bacterial identification, MALDI-TOF MS has shown to be a fast, reliable and cost-effective technique that has the potential to replace and/or complement conventional phenotypic identification for most bacterial strains isolated in clinical, food or environmental microbiology laboratories. Some authors conclude that the identification of microbial isolates by whole-cell mass spectrometry is one of the latest tools, forging a revolution in microbial diagnostics, with the potential of bringing to an end many of the time-consuming and man-power-intensive identification procedures that have been used for decades. However, to increase the reliability of the method, it should be taken into account that for routine identification of bacterial isolates, correct identification by MALDI-TOF MS at the species or strain level should be achieved (Bizzini et al., 2010).

In this sense, bacterial differentiation at the species level is not always possible with the commonly applied methods for bacterial identification. Thus, the analysis of the 16S rRNA gene resulted to be complicated in some cases due to the high similarity of sequences of species of the same genus, such as *Bacillus* spp. and *Pseudomonas* spp. However, the correct identification of the corresponding species is of great importance for food safety and quality, since the pathogenic and spoilage character can vary significantly. With MALDI-TOF MS fingerprinting a higher discrimination potential has been described, allowing the differentiation and correct identification of much close bacterial species and even strains of the same species (Keys et al., 2004; Donohue et al., 2006; Vargha et al., 2006).

In recent years, several reports have shown the feasibility of using MALDI-TOF MS for identifying microorganisms (Seng et al., 2009; Giebel et al., 2010). The detection and comparison of protein mass patterns has become a convenient tool for the rapid identification of bacteria, due to the high specific mass profiles obtained. It has to be mentioned that most studies of bacterial identification by MALDI-TOF MS fingerprinting are targeted at clinical diagnostics of bacterial strains associated with human infectious diseases.

In contrast, only few works have been done in the field of microbial food analysis by MALDI-TOF MS for the identification of foodborne pathogens and/or spoilers. These works

included the classification and identification of several widespread pathogens causing foodborne diseases such as *Aeromonas A. hydrophila, Arcobacter spp., Campylobacter spp., Clostridium spp., Listeria spp., Salmonella spp., Staphylococcus spp., V. parahaemolyticus, Yersinia* spp., *Bacillus* spp. and species of the *Enterobacteriaceae* family (Bernardo et al., 2002; Bright et al., 2002; Keys et al., 2004; Mandrell et al., 2005; Donohue et al., 2006; Carbonnelle et al., 2007; Barbuddhe et al., 2008; Grosse-Herrenthey et al., 2008; Hazen et al., 2009; Alispahic et al., 2010; Ayyadurai et al., 2010; Dubois et al., 2010; Stephan et al., 2010; Stephan et al., 2011). Furthermore, some studies were aimed at the detection of foodborne pathogens and food spoilage bacteria, including genera such as *Escherichia*, *Yersinia*, *Proteus*, *Morganella*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Lactococcus*, *Pseudomonas*, *Leuconostoc* and *Listeria* (Mazzeo et al., 2006). In further studies an ample spectral library was created, including the main pathogenic and spoilage bacterial species potentially present in seafood (Böhme et al., 2010a; Fernández-No et al., 2010; Böhme et al., 2011b). These works included genera, such as *Acinetobacter*, *Aeromonas*, *Bacillus*, *Carnobacterium*, *Listeria*, *Pseudomonas*, *Shewanella*, *Staphylococcus*, *Stenotrophomonas*, *Vibrio* and genera of the *Enterobacteriaceae* family.

In Figure 1 spectral profiles of some important foodborne pathogens and spoilers are shown, demonstrating the high specificity. Böhme et al. (2010a, 2011b) also determined characteristic biomarker peaks for every studied genus and species. Such unique or characteristic peak masses can serve for the rapid identification of a bacterial genus and/or species. However, unequivocal identification can not be carried out based on a single biomarker protein, but under consideration of a number of characteristic mass patterns, representing the spectral fingerprint.

Furthermore, when working with microbial mixtures, such biomarkers become more important, since the presence or absence of unique peak patterns could lead to a conclusion of the present bacterial species. The detection of biomarker proteins by MALDI-TOF MS has been successfully applied for the identification of two bacterial species isolated from contaminated water, lettuce and cotton cloth (Holland et al., 2000). However, until now, the application of MALDI-TOF MS fingerprinting for microbial mixtures has not yet been demonstrated.

Another critical challenge of MALDI-TOF MS fingerprinting is that the classification of a bacterial genus or species, as well as the determination of unique biomarker patterns, is only possible in the frame of the content of the spectral reference library. However, the number of studies on bacterial species identification by MALDI-TOF MS in foodstuffs is continually increasing and so does the reliability of the identification.

Figure 2 shows the scheme of the whole process of bacterial identification by MALDI-TOF MS in food products. Bacteria are isolated from food samples and cultivated to obtain single colonies. Afterwards, the bacterial cells are lysed by an organic solvent and a strong acid, being the most applied ones Acetonitrile (ACN) and Trifluoracetic acid (TFA). Once obtained the spectral profiles for each bacterial strain, data analysis is carried out, including the extraction of representative peak mass lists and the comparison of spectral data, with the aim of bacterial discrimination. Furthermore, cluster analysis of the peak mass lists reveals phyloproteomic relationships between bacterial species, allowing the identification of unknown strains, as well as the typing of closely related strains. For the sample preparation and data analysis a number of different protocols and techniques have been described and are discussed in the following sections.

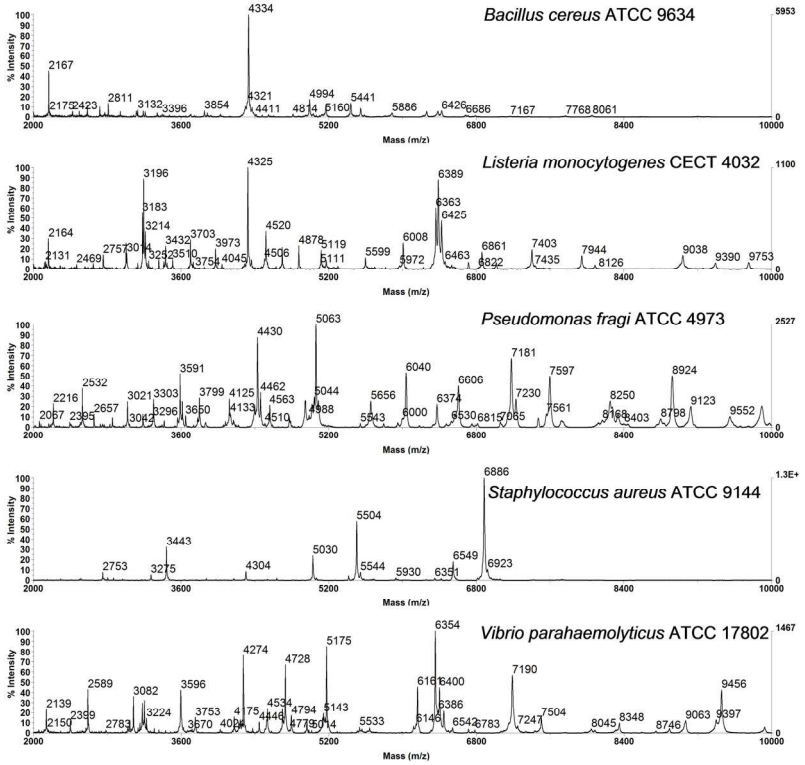

Fig. 1. MALDI-TOF MS profiles of some foodborne and spoilage bacteria.

For bacterial identification by MALDI-TOF MS fingerprinting, the spectral profile of the strain of interest is compared to a spectral library of reference strains. Several private databases have been created, including spectral profiles of more than 500 bacterial strains, such as The Spectral Archive And Microbial Identification System (Saramis™; *AnagnosTec* GmbH, Potsdam, Germany) (Erhard et al., 2008) and the Microbelynx bacterial identification system (*Waters* Corporation, Manchester, UK) (Keys et al., 2004; Dare, 2006). The MALDI Biotyper 2.0 (Bruker Daltonics) search against an ample database of more than 1800 bacterial species and new spectral profiles are being added on a daily basis. The database demonstrated to be applicable for the routine bacterial identification in the clinical sector, being a rapid, cost-effective and accurate technique that achieved correct species identification of 92% (Seng et al., 2009; Bizzini et al., 2010). As already mentioned, most studies, as well as these databases, are targeted at human pathogens causing infectious diseases. Nevertheless, the databases also include bacterial species that play an important role in food safety and quality and could represent important reference data for the identification of food pathogens and food spoilage bacterial species. However, the critical challenge of these databases is the limited availability. In this sense, it would be desirable a public database for the submission of the spectral information for each species that would allow the comparison with results from different researches and favoring a more precise method for identification of intact bacteria based on a huge amount of data.

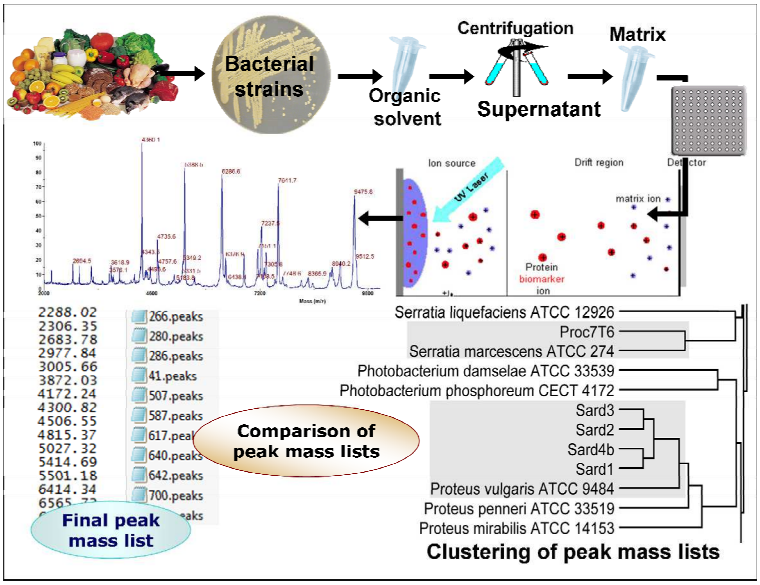

Fig. 2. Scheme of the protocol for the identification of foodborne and spoilage bacteria by MALDI-TOF MS fingerprinting.

Thus, a few attempts to start a public database have been achieved. Mazzeo et al. (2006) constructed a library containing spectra of 24 food-borne bacterial species, including *Escherichia spp., Yersinia spp., Proteus spp., Morganella spp., Salmonella spp., Staphylococcus spp., Micrococcus spp., Lactococcus spp., Pseudomonas spp., Leuconostoc spp.* and *Listeria spp.* Although, the spectral profiles and peak mass lists are freely available on the Web (http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm), the library only includes a few bacterial species important in food-borne diseases and/or food spoilage.

A reference library of mass spectral fingerprints of the main pathogenic and spoilage bacterial species, potentially present in seafood products has been created, including more than 50 bacterial species with interest in the food sector (Böhme et al., 2010a; Böhme et al., 2011b). In further studies, the library showed to be applicable for the correct identification of unknown bacterial strains isolated from commercial seafood products (Böhme et al., 2011a). It should be emphasized that the compiled reference library of seafood borne and spoilage bacterial species can be applied to any other foodstuff. The constituted spectral library may easily be enlarged by further bacterial species and strains that are of interest in the corresponding food product.

3.1 Sample preparation protocol

To allow the comparison of bacterial spectral fingerprints, the obtained spectra have to be representative and reproducible. For that, a standardized protocol has to be followed, beginning from sample preparation to instrumental parameters. It has been shown that spectral profiles were less sensitive to culture conditions but can show significant variability depending on the sample preparation protocol (Bernardo et al., 2002; Wunschel et al., 2005).

In the first studies of bacteria by MALDI-TOF MS, protein fractions were isolated from bacterial cells but shortly turned to the analysis of whole cells directly without any sample pre-treatment, called intact cell mass spectrometry (ICMS). Many authors focused on the optimization of the sample preparation protocol, with the aim to establish a standardized protocol to obtain specific and reproducible spectral profiles in a rapid and labor-saving way (Williams et al., 2003; Mazzeo et al., 2006; Liu et al., 2007). Nowadays, three different sample preparation protocols are commonly applied. Table 1 resumes these different methods, highlighting their advantages and disadvantages. The most rapid and labor-saving method is based on the direct application of bacterial biomass taken from culture plates to the MALDI-TOF MS sample plate. Afterwards, the bacterial cells are overlaid with the matrix solution (Bright et al., 2002). Apart from being the most rapid and labor-saving method, the direct spotting of biomass to the sample plate had several disadvantages. The difficulty in taking the correct amount of biomass complicates to get a homogenous distribution of the sample and matrix. Although, this technique was successfully applied for bacterial species identification (Keys et al., 2004; Erhard et al., 2008), it has been shown that spectra showed more noise and less peak resolution with this fast method, making difficult to obtain reproducible spectral profiles (Böhme et al., 2010b). Another sample preparation technique analyzed cell suspensions that were obtained after harvesting bacterial biomass in a solvent, including one or two washing steps and resuspension of the pellet in the matrix solution (Mazzeo et al., 2006; Vargha et al., 2006). The disadvantages of this method are the time-consuming washing steps and the loss of small soluble proteins. Some authors also described a similar sample preparation method, but where no washing step was applied and the bacterial colonies were harvested in a solvent to obtain cell suspensions (Carbonnelle et al., 2007).

Whole Cells	Whole Cell Suspension	Cell extract
Sample preparation		
Direct application of biomass to target well	Harvest biomass in organic solvent 1-2 cycles of Washing/ Centrifugation steps	Harvest biomass in organic solvent 1 Centrifugation step Analysis of supernatant
Advantages and disadvantages		
Very fast method	Time consuming washing steps	Very fast method
Less homogenous crystallization	Homogenous crystallization	Homogenous crystallization
Less reproducibility	Good reproducibility	Best reproducibility
More noise	More noise	Low noise
Less resolution of peaks	Good resolution of peaks	Best resolution of peaks

Table 1. Comparison of different sample preparation protocols.

In a third approach, based on the latter one, cell suspensions were centrifuged and spectra obtained by the analysis of the supernatant. When comparing to the spectra resulting from cell suspensions without centrifugation step, the spectral profiles obtained of the supernatant showed a better reproducibility, a higher resolution and less noise. The decreasing of noise, lessening the background, and the increase in resolution leads to more representative and characteristic peaks for each bacterial species improving the reproducibility (Böhme et al., 2010b). It should be mentioned that, in general terms, a more

homogenous distribution of sample and matrix is expected with cell extracts, than with cell suspensions. In addition, this sample preparation method is rapid and effortless, since the extracts were obtained directly from cell cultures in just one dilution/centrifugation step. Although, when working with the extracts it should be expected to find small, soluble proteins, spectral profiles showed a high number of peaks, similar or even higher than those obtained by the analysis of whole cell suspensions. Nowadays, most applications of MALDI-TOF MS for bacterial identification analyzes bacterial cell extracts, obtained by just one dilution/ centrifugation step.

As mentioned before, for the direct comparison of spectral profiles, with the aim of bacterial differentiation, a strict standardized protocol has to be followed. It has been shown that, when applying the same culture conditions, sample preparation, matrix, organic solvents and MALDI-TOF MS analyzer, reproducible spectral profiles are obtained (Keys et al., 2004; Barbuddhe et al., 2008). Although, different protocols can lead to a high variability in the resulting spectral profiles, it has to be mentioned that some peaks were detected, even if different protocols were applied. Such characteristic and conserved peaks could serve as biomarker proteins for the corresponding genus and/or species.

3.2 Data analysis and phyloproteomics

For the identification of an unknown bacterial strain by MALDI-TOF MS fingerprinting, the spectral profile is compared to a spectral library of reference strains. The existing microbial identification databases MALDI Biotyper 2.0 (Bruker Daltonics), SaramisTM (AnagnosTec GmbH) and Microbelynx (Waters Corporation) include an amply library of spectral data. At the same time, the software for analyzing spectral data and carry out bacterial identification are incorporated to these databases. Identification of unknown microorganisms is performed by comparing their individual peak lists to the database. A matching score based on identified masses and their intensity correlation is generated and used for ranking of the results. Furthermore, based on the similarity scores dendrograms can be constructed and principle component analysis can be carried out. The critical challenges of these databases and software are that both are conditioned to the corresponding instrument and are not available for other investigators without paying high charges.

Thus, in a number of studies, smaller, "in house" libraries have been constructed. The difficulty of developing an "in house" database lays in the need for particular algorithms to analyze or compare obtained spectra and to carry out searches against the constructed reference library. Peak matching techniques eliminate the subjectivity of visual comparison. Jarman et al. (2003) developed an automated peak detection algorithm to extract representative mass ions from a fingerprint and to compare spectra to fingerprints in a reference library. This algorithm carries out the identification of an unknown spectrum by calculating a degree of matching and is robust to the variability in the ion intensity (Jarman et al., 2003). Other authors developed a software (BGP-database, available on http://sourceforge.net/projects/bgp) to analyze and compare spectral profiles, allowing the rapid identification. This software determines the best match between the tested strain and the reference strains of the database, taking into account a possible error of the *m/z* value (Carbonnelle et al., 2007).

In further studies, the freely available web-based application SPECLUST (http: //bioinfo.thep.lu.se/speclust.html) was used to extract representative peak masses and to

obtain final peak mass lists for each bacterial strain. Later on, required mass lists can be compared and common peak masses defined. The web interface calculates the mass difference between two peaks taken from different peak lists and determines if the two peaks are identical after taking into account a certain measurement uncertainty (σ) and peak match score (s) (Alm et al., 2006). The web program resulted very fast, easy to handle, and could be extended by new spectral mass lists in a simple manner. Although, it was not possible to search an unknown spectrum directly against the library, comparison of peak mass lists could be carried out and common peaks determined with the aim of identifying a spectral profile of an unknown strain. The web-application was successfully applied to identify pathogenic and spoilage bacterial strains, isolated from commercial seafood products (Böhme et al., 2011a). In addition, the program allows the rapid determination of specific biomarker peaks and includes a clustering option.

Further bioinformatics programs, such as Statgraphics Plus 5.1 (Statpoint Technologies, inc., Warrenton, USA), offer a variety of functionalities. First, spectral data have to be transformed to a binary table, indicating the presence (1) and absence (0) of a peak mass. Afterwards, various algorithms for cluster analysis can be applied, as well as Principal Component Analysis (Böhme et al., 2011b). In a different study, the BioNumerics 6.0 software (Applied-Maths, Sint-Martens-Latem, Belgium) was used for data analysis and machine learning for bacterial identification by MALDI-TOF MS (De Bruyne et al., 2011).

Clustering of the spectral data obtained by MALDI-TOF MS represents another approach for bacterial identification and classification. Conway et al. (2001) introduced the term "Phyloproteomics" and the clustering of peak mass lists allowed a better visualization of similarities and differences of spectral comparison. The construction of a dendrogram based on mass spectral data is a rapid technique to analyze spectral profiles and to visualize spectral relations by grouping the obtained peak mass lists of bacterial strains. Thus, on one hand, clustering has been successfully applied for the differentiation and identification of bacterial strains at the genus and species level (Conway et al., 2001; Wunschel et al., 2005; Vargha et al., 2006; Carbonnelle et al., 2007; Böhme et al., 2010a; Dubois et al., 2010; Böhme et al., 2011b). On the other hand, clustering of mass spectral data has been applied as a typing method for the phyloproteomic study of different strains of the same species, with the aim to classify the strains (Siegrist et al., 2007; Teramoto et al., 2007). When comparing the dendrograms representing phyloproteomic relations to the phylogenetic trees, a high concordance were found by these authors. Since the peak patterns observed by MALDI-TOF MS are generally attributed to ribosomal proteins (Ryzhov and Fenselau, 2001), the similarity of the MALDI-TOF MS cluster to phylogenetic trees obtained by the analysis of ribosomal genes is not surprising (Dubois et al., 2010). However, in comparison to the sequence analysis of the 16S rRNA gene that is commonly used for phylogenetic studies, the classification of bacterial strains by MALDI-TOF MS fingerprinting resulted to be more discriminating. This is important for some genera, such as *Bacillus* and *Pseudomonas*, for that the differentiation at the species level is difficult with 16S rRNA analysis.

4. Conclusion

In order to ensure food safety and quality, the objective of this chapter is to review and update MALDI-TOF MS-based molecular methods that allow for the early detection and identification of the main pathogenic and spoilage bacteria. In recent years, molecular

diagnosis has increased in importance, representing an attractive alternative to the traditional techniques of cultivation and characterization for the rapid detection and identification of microorganisms in food products that may produce foodborne illnesses.

MALDI-TOF MS fingerprinting proved to be a feasible, rapid and cost-effective technique for the classification and discrimination of bacterial strains. Besides the high potential in clinical diagnostics, it demonstrated to be applicable for the identification of unknown bacterial strains isolated from food samples. In addition, the methodology of MALDI-TOF MS fingerprinting can be applied to different foodstuff, since bacterial strains are isolated from the corresponding matrix before analysis and the constituted spectral library may easily be enlarged by adding bacterial species and strains that are of interest in the corresponding food product. The vast spectral data can be effectively examined by searching common peaks masses and cluster analysis. Characteristic peak patterns can be determined and serve as biomarker proteins for the rapid identification of an unknown spectrum. The creation of a dendrogram based on phyloproteomic relations, allow for the typing of closely related strains, extending conventional typing methods. When comparing phyloproteomic clustering with the classical taxonomy approach based on 16S rRNA gene analysis, high similarities were observed in the grouping of bacterial strains. However, genetic analysis does not always permit differentiation between species of the same genus due to the high similarity of DNA sequences, whereas spectral profiles obtained by MALDI-TOF analysis can reveal specificities for individual bacterial species. Thus, proteomic tools and phyloproteomic clustering resulted in more discrimination capability of different species.

The critical challenge of the MALDI-TOF MS fingerprinting approach for bacterial identification in food is the limited availability of reference data. A huge public database, such as the GenBank database of the NCBI, which facilitate the comparison of an unknown DNA sequence to millions of reference strains, is not available for mass spectral data obtained by MALDI-TOF MS. In future the main objective should be the publication of the created spectral libraries, including strains obtained from culture collections, as well as strains isolated from food samples, to allow other researchers the comparison of their spectra to the library and thus, a more accurate and rapid identification of bacteria in food.

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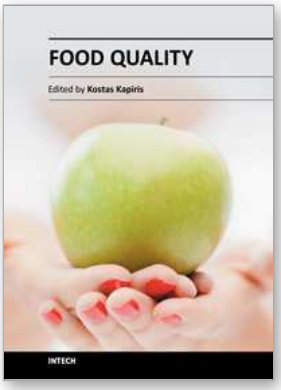

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The book discusses the novel scientific approaches for the improvement of the food quality and offers food scientists valuable assistance for the future. The detailed methodologies and their practical applications could serve as a fundamental reference work for the industry and a requisite guide for the research worker, food scientist and food analyst. It will serve as a valuable tool for the analysts improving their knowledge with new scientific data for quality evaluation. Two case study chapters provide data on the improvement of food quality in marine and land organisms in the natural environment.

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