Chapter 2

Fingerprinting for Detecting Contaminants in Food

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2.1 INTRODUCTION

Food quality and safety are increasingly important public health issues. Every year, about 600 million persons fall ill after consuming contaminated food or water and 420,000 of them die. Diseases caused by unsafe food can cause severe diarrhea, life-threatening intoxications, and cancer (WHO, 2015). Consumers need to purchase safe products that do not involve any kind of health risk. The aim of "food safety" is to avoid health hazards for the consumer, such as microbiological and chemical contaminants, as well as adulteration.

Chemical food contaminants are veterinary drugs, feed additives, growth promoters, dioxins, heavy metals, and pesticides that are known or suspected to be carcinogenic, causing cardiovascular disease, kidney and liver dysfunction, etc., when humans are exposed by ingestion of the contaminated food or water. Thus, food products are under stringent laboratory control to assure they comply with the regulatory limits for residues and contaminants. The progress made in analytical chemistry in the last decades toward significantly higher sensitivity and specificity now allows the detection of chemical contaminants in complex food matrices, down to parts per billion. Routine analytical methods for the detection of single or multiple chemical contaminants include rapid screening tests, such as enzyme-linked immunosorbent assays and microbial inhibition tests, as well as complex multitarget instrumental analysis based on chromatography, mass spectrometry, and vibrational and atomic spectroscopy.

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. Thus, the safety of a food product depends in great part on the presence and nature of the microorganisms.

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Besides molds and yeasts, bacteria are the principal microorganisms responsible for various types of food spoilage and foodborne intoxications. It should be mentioned that a food product naturally contains an indigenous microbiota that can include pathogenic bacterial species; however, most bacterial contamination occurs during processing and manipulation of the food products. The global incidence of foodborne disease is difficult to estimate, but it has been reported that every year 230,000 people die due to diarrheal diseases after consuming contaminated food or water. In industrialized countries, the percentage of the population suffering from foodborne diseases each year has been reported to be up to 30% (WHO, 2015). In order to control and minimize the microbial hazard, pathogenic bacteria need to be identified in a rapid and unequivocal way. Traditionally, bacterial species have been identified by classic tools relying on culturing processes coupled to morphological, physiological, and biochemical characterization. In the last few decades, the field of microbiological identification has turned to more rapid and sensitive methods, including antibody-based assays and DNA-based methods, together with important advances in bioinformatics tools. Thus, some methodologies such as ELISA or PCR have already become classics. Recently, the development of rapid and highly sensitive techniques, such as real-time PCR, DNA microarrays, and biosensors, has provoked the replacement of traditional culturing methods in the field of bacterial identification in clinical diagnostics, as well as in the food sector. 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 have been introduced for the identification of microorganisms (Klaenhammer et al., 2007).

This chapter aims to review the detection of food contaminants by fingerprinting techniques. The term fingerprinting has its origin in forensics, where specific DNA profiles are used to differentiate individuals. DNA fingerprinting is the most common approach for species differentiation and identification, with many applications in the food sector, as much for food authentication purposes as for microbial pathogen identification. The different techniques, such as amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), repetitive sequence-based PCR (rep-PCR), multiple-locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE), create sets of characteristic DNA profiles, specific for every individual. All these techniques have been extensively studied with the aim of determining plant varieties, animal species, and the source and/or geographic origin of a food or food ingredient. Besides food authenticity, DNA fingerprinting is a common tool for bacterial species differentiation at the genus, species, and strain levels (Mandal et al., 2011). On the one hand, identification of the microbiota of a food product allows shelf life to be determined and any necessary measures to be taken to assure quality. On the other hand, the detection of certain

microbial contaminants with a foodborne pathogenic character is crucial to avoid a microbial health risk for consumers. Furthermore, DNA typing of bacterial strains at the subspecies level is carried out to classify the strains in relation to their origin, antibiotic resistance and virulence, being crucial for microbial source tracking (MST), and epidemiological studies.

Nowadays, molecular fingerprinting is not restricted to DNA-based methods, but describes a variety of analytical methods that can measure the composition of a sample in a nonselective way, such as by collecting a spectrum. In following sections, molecular fingerprinting techniques, different from DNA fingerprinting, and their applications for detecting food contaminants are described. Analytical methods, such as spectroscopy and spectrometry generate spectral profiles that represent fingerprints of the analyzed target. As with DNA fingerprints, the information obtained may be used to differentiate and identify individuals for food authenticity and microbial identification purposes. This chapter focuses especially on (1) the detection of bacterial contaminants and (2) bacterial discrimination at the species and subspecies levels. Closely related species are sometimes difficult to distinguish by traditional and DNA-based methods, but the pathogenic character may differ significantly. This is even more important in those cases where different strains of the same species exhibit different virulent potentials. That is why identification at the species level is not always sufficient and bacterial typing methods that give information about virulence factors and antibiotic resistance are key aspects of ongoing research. Table 2.1 gives an overview of the main foodborne pathogenic bacterial species studied by molecular fingerprinting techniques and the corresponding references are listed. Spectral approaches are also being applied to the detection of mycotoxins and food contaminants different from those of microbial origin. In this chapter, the use of spectral fingerprinting techniques to detect chemical contaminants is summarized, including detection of antibiotics, drugs, hormones, melamine, pesticides, and some further banned food ingredients.

2.2 MALDI-TOF MASS SPECTROMETRY

Matrix-assisted laser desorption/ionization (MALDI) is a very soft ionization technique in that large molecules, such as proteins, can be analyzed without fragmentation of the molecules. For this the sample is mixed and crystallized with a matrix solution that protects the target molecules when submitted to shots from a laser beam, absorbing the applied energy. Following this, the matrix ions transfer the energy to the sample molecules, resulting in ions of low charge (+1/+2). Subsequently, the ions produced are separated in an electric field with relation to their mass/charge (m/z) ratio by a time of flight (TOF) analyzer.

TABLE 2.1 Bacterial Food Contaminants Studied by Fingerprinting Techniques With the Corresponding References

Species	Method	Reference
Acinetobacter baumanii		
	MALDI-TOF MS	Alvarez-Buylla et al. (2012), Sousa et al. (2014)
***Bacillus* spp.**		
	MALDI-TOF MS	Branquinho et al. (2014), Fernández-No et al. (2013)
	SERS	Patel et al. (2008)
***Campylobacter* spp.**		
	MALDI-TOF MS	Bessède et al. (2011), Kolínská et al. (2008), Mandrell et al. (2005), Zautner et al. (2013)
***Clostridium* spp.**		
	MALDI-TOF MS	Grosse-Herrenthey et al. (2008), Reil et al. (2011)
	FT-IR	Kirkwood et al. (2006)
Escherichia coli		
	MALDI-TOF MS	Christner et al. (2014), Clark et al. (2013), Khot and Fisher (2013), Matsumura et al. (2014), Novais et al. (2014), Siegrist et al. (2007)
	FT-IR	Al-Qadiri et al. (2006)
	Raman/SERS	Cho et al. (2015), Yang and Irudayaraj (2003)
	LIBS	Barnett et al. (2011), Diedrich et al. (2007), Marcos-Martinez et al. (2011), Mohaidat et al. (2011), Multari et al. (2013)
***Legionella* spp.**		
	MALDI-TOF MS	Gaia et al. (2011), Moliner et al. (2010), Pennanec et al. (2010)
***Listeria* spp.**		
	MALDI-TOF MS	Barbuddhe et al. (2008), Jadhav et al. (2014)
	FT-IR	Al-Holy et al. (2006), Janbu et al. (2008), Rebuffo et al. (2006)
	SERS	Green et al. (2009)
Salmonella enterica		
	MALDI-TOF MS	Dieckmann et al. (2008), Dieckmann and Malorny (2011), Kuhns et al. (2012), Sparbier et al. (2012)
	FT-IR	Al-Qadiri et al. (2008), Baldauf et al. (2006), Männig et al. (2008)
	SERS	Duan et al. (2015)
	LIBS	Barnett et al. (2011), Marcos-Martinez et al. (2011), Multari et al. (2013)

(*Continued*)

TABLE 2.1 (Continued)

Species	Method	Reference
***Shigella* spp.**		
	MALDI-TOF MS	Khot and Fisher (2013)
Staphylococcus aureus		
	MALDI-TOF MS	Böhme et al. (2012), Carbonnelle et al. (2007), Du et al. (2002), Dubois et al. (2010), Jackson et al. (2005), Josten et al. (2013), Szabados et al. (2010), Wolters et al. (2010)
	FT-IR	Amiali et al. (2011)
	Raman	Harz et al. (2005)
	LIBS	Barnett et al. (2011)
***Vibrio* spp.**		
	MALDI-TOF MS	Dieckmann et al. (2010), Erler et al. (2015), Hazen et al. (2009)
Yersinia enterocolitica		
	MALDI-TOF MS	Ayyadurai et al. (2010), Lasch et al. (2010), Stephan et al. (2011)
	FT-IR	Kuhm et al. (2009)

MALDI-TOF MS emerged as a new tool for bacterial differentiation due to its speed, simplicity, and cost effectiveness. Whole bacterial cells can be analyzed directly without any sample pretreatment and the resulting spectral profiles are highly specific, representing a fingerprint for the corresponding organism (Clark et al., 2013). Furthermore, culturing conditions and growth media have been shown to influence the fingerprints to a lower extent, allowing the comparison and identification of bacterial strains even if different protocols have been applied. With MALDI-TOF MS soluble and low-weight proteins (1,500−20,000 Da) are detected and the majority have been associated with ribosomal proteins and to a lower degree with structural proteins, such as cold-shock proteins and DNA-binding proteins (Ryzhov and Fenselau, 2001). The number of studies aimed at the application of MALDI-TOF MS fingerprinting to microbial identification has increased significantly in the last decades and several detailed reviews give an overview of the methodology, sample preparation and application to bacteria, yeasts, and fungi (Clark et al., 2013; Welker and Moore, 2011). The applicability of MALDI-TOF MS fingerprinting to clinical routine analysis has been demonstrated in a number of studies, achieving 92−98% of correct species identification. This is a significantly better result than that obtained with commonly applied

microbial identification tools. MALDI-TOF MS has been compared to conventional phenotypic and molecular methods, highlighting the higher discrimination potential, in addition to the speed and cost effectiveness of the proteomic approach. More recently, MALDI-TOF MS fingerprinting has also been applied to the detection of foodborne pathogens isolated from food products (Böhme et al., 2013).

Bacterial identification by MALDI-TOF MS is carried out by comparing the spectral profile to a previously created library of reference spectra. The whole spectral profile is representative for the corresponding strain and the determination of a number of characteristic peaks allows the classification of the strain at the genus, species, and even subspecies or strain level.

2.2.1 Spectral Databases

Whereas the first work in this area was realized at an intralaboratory level with few reference spectra, some manufacturers of mass spectrometers created commercial spectral databases. The MALDI Biotyper system from Bruker Daltonics (Bremen, Germany) includes an ample database of bacterial strains, mycobacteria, and fungi. A robust, standardized procedure for automated bacterial analysis, including sample preparation and data analysis, has been described (Sauer et al., 2008). The entries in the database consist of representative main spectra that have been created by replicative measurements of the corresponding reference strain. The Biotyper system has been validated in several studies, such as the identification of 1371 clinical isolates, achieving 93.2% of correct identification (Bizzini et al., 2010) and the study of 980 clinical isolates, resulting in 92.2% correct species identification (van Veen et al., 2010). Another microbial identification system based on MALDI-TOF MS, including a spectral archive, is the VITEK MS platform from bioMérieux (Marcy-l'Étoile, France). This database consists of SuperSpectra that are created by the determination of a set of biomarker peaks, representative for the corresponding genera, species, and strain. The VITEK MS platform has been validated for the identification of 1129 clinical isolates, achieving 93% of correct identification (Martiny et al., 2012). When compared to the Biotyper database, similar results were obtained. The Biotyper platform has been approved by the Food and Drug Administration (FDA) for the official identification of 40 bacterial species and the VITEK MS database for 194 species (Deak et al., 2015). Further databases are the Andromas database from Andromas SAS (Paris, France) and the MicrobeLynx bacterial identification system from Waters Corporation (Manchester, UK). Today, the Andromas database is frequently used for clinical routine analysis in Europe and includes reference data for bacteria, mycobacteria, yeasts, and fungi (Dupont et al., 2010). It has been implemented into the clinical microbiology laboratory of the Necker-Enfants Malades Hospital to identify all microorganisms isolated routinely, achieving 93% and 99% of correct species identification after single and

two acquisitions, respectively (Bille et al., 2012). Likewise, the MicrobeLynx database has been successfully applied to identify clinical isolates (Rajakaruna et al., 2009).

The spectral libraries mentioned are only available commercially and require high charges for access. In this sense, the main drawback of the MALDI-TOF MS fingerprinting approach is the lack of public spectral libraries. In addition, most studies carried out are aimed at the identification of clinically relevant strains. Although many human diseases are caused by the consumption of contaminated food and the previously described commercial databases also include bacterial species that are of interest in the field of food safety, the application of these libraries to food control is not always indicated due to the lack of bacterial strains isolated from food products. For this reason, the laboratory intern database SpectraBank has been created and made publicly available (www.spectrabank.org) (Böhme, Fernández-No et al., 2012a). The whole process for the identification of an unknown bacterial strain, including sample preparation, MALDI-TOF MS measurement and data analysis by the web tool Speclust (Alm et al., 2006) and comparison to the SpectraBank library, are explained in detail. Fig. 2.1 shows the schematic procedure of the identification process. At present, SpectraBank includes open access spectral information obtained by MALDI-TOF MS for more than 200 bacterial strains of 56 different bacterial species with interest in food safety and quality. Continuous extension of the data is intended, by adding new strains and improving the data processing, analysis', and sharing. Recently, a further in-house spectral database has been created for *Vibrio* spp. (VibrioBase), since the entries in the Biotyper database were not sufficient for correct species identification of *Vibrio* spp. strains (Erler et al., 2015).

2.2.2 MALDI-TOF MS Fingerprinting for the Detection of Bacterial Food Contaminants

Most reviews and studies that have been published about MALDI-TOF MS fingerprinting for bacterial identification have been aimed at application to the clinical sector and the routine identification of human pathogens in clinical isolates. Nevertheless, as mentioned before, many bacterial foodborne pathogens cause human infectious diseases and are therefore included in these studies, such as *Bacillus* spp., *Escherichia coli*, *Listeria* spp., *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus*, and *Streptococcus* spp. (Farfour et al., 2012; Hsieh et al., 2008; Smole et al., 2002). In Table 2.1 studies based on MALDI-TOF MS analysis of bacterial species with foodborne pathogenic character are listed. Recently, the fast, accurate, and easy-to-handle MALDI-TOF MS fingerprinting technology has also been applied to bacterial identification in veterinary diagnostics, environmental isolates, and food samples. Mazzeo et al. (2006) and Böhme et al. (2013)

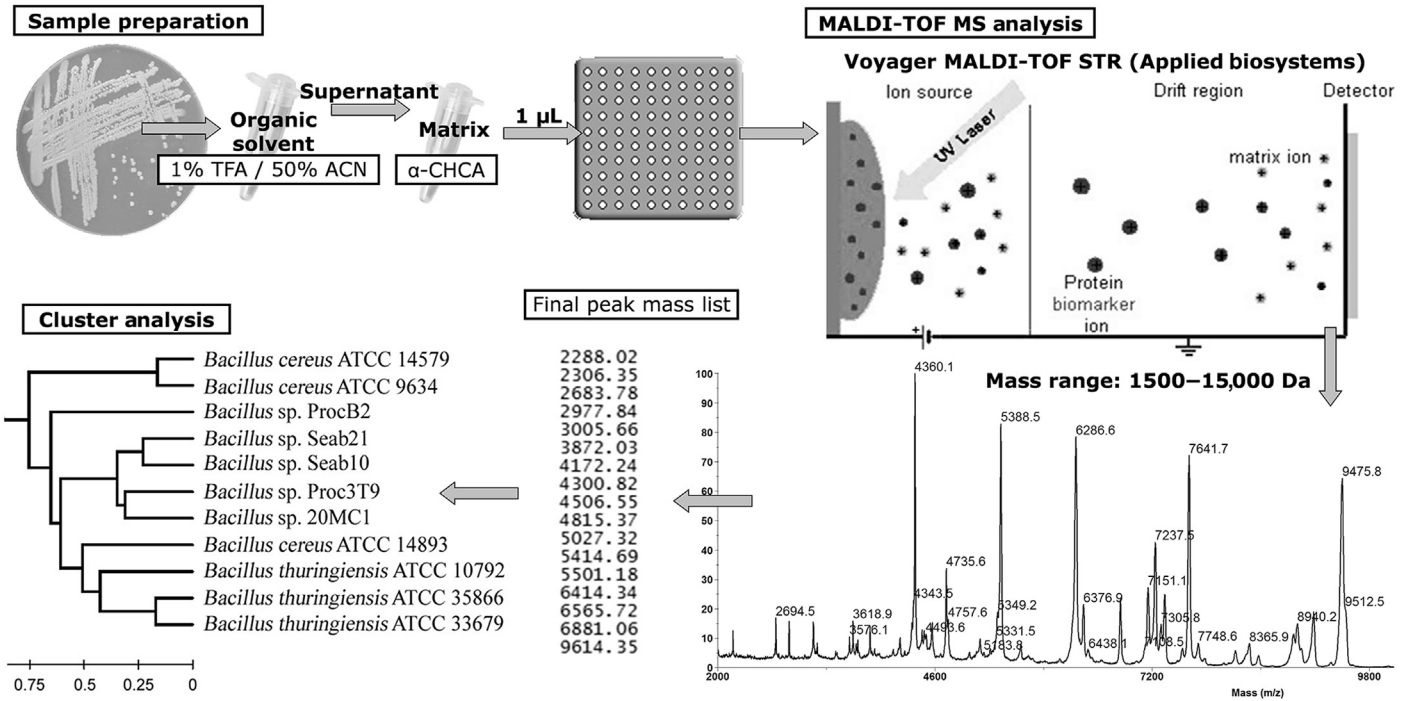

FIGURE 2.1 Scheme of the bacterial identification process by MALDI-TOF MS fingerprinting.

constructed spectral libraries containing spectra of 24 and 58 foodborne bacterial species, respectively. Mazzeo et al. (2006) made the spectral profiles and peak mass lists freely available on the Web (http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm). The library includes the genera *Escherichia*, *Yersinia*, *Proteus*, *Morganella*, *Salmonella*, *Staphylococcus*, *Micrococcus*, *Lactococcus*, *Pseudomonas*, *Leuconostoc*, and *Listeria*. Böhme et al. (2013) created the spectral library SpectraBank (www.spectrabank.org), where other researchers can download spectral information of 58 bacterial species that are of interest for food safety and quality, including the genera *Acinetobacter*, *Aeromonas*, *Bacillus*, *Carnobacterium*, *Clostridium*, *Listeria*, *Photobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Shewanella*, *Staphylococcus*, *Vibrio*, and a number of genera of the Enterobacteriaceae family.

In a further study, histamine-producing bacterial species have successfully been differentiated by MALDI-TOF MS fingerprinting (Fernández-No et al., 2010).

In most studies, MALDI-TOF MS analysis has been applied to bacterial reference strains. Although these strains correspond to bacterial species for which the pathogenic potential is known, it has been shown that real isolates can differ significantly from type strains in their phenotypic and proteotypic properties, due to modifications caused by environmental changes or the food matrices. To perform efficient bacterial species identification of foodborne pathogens that are isolated from food products, it is important to include spectral information of strains isolated from different food matrices into the databases. Therefore, for subtyping of *Yersinia enterocolitica* (Stephan et al., 2011) and *E. coli* (Novais et al., 2014) a few strains isolated from food products have been included. Likewise, four strains isolated from food have been studied for species level differentiation of *Bacillus pumilus* and *Bacillus safensis* (Branquinho et al., 2014). Dubois et al. (2010) studied 92 *Staphylococcus* spp. strains isolated from food and plants in relation to species identification inside the genus *Staphylococcus* (Dubois et al., 2010). In further studies, Dieckmann et al. (2011) analyzed *S. enterica* subsp. *enterica*, *Aeromonas* spp., and *Vibrio* spp. strains, including isolates from food, environment, animals, and humans. *Cronobacter sakazakii* is getting attention as an emerging foodborne pathogen and due to its presence in infant formulas. MALDI-TOF MS has been successfully applied to identify *Cronobacter* spp. strains at the species level with high accuracy. In these studies, besides reference strains, strains have been isolated from infant formulas, milk powder producing plants, and further food samples (Stephan et al., 2010; Zhu et al., 2011). *Vibrio parahaemolyticus* is a main causative agent of pandemic outbreaks of seafood-borne gastroenteritis. MALDI-TOF MS has been applied to distinguish *V. parahaemolyticus* from other *Vibrio* spp. For this, *V. parahaemolyticus* strains have been isolated from illness-related human and food samples of diverse outbreaks and analyzed by

MALDI-TOF MS (Hazen et al., 2009). A new approach for the rapid identification of *Listeria monocytogenes* has been described by Jadhav et al. (2014). Isolates from different food products (UHT milk, cheese, chicken paté, and cantaloupe), spiked with bacterial pathogens, have been submitted directly to analysis in selective enrichment broth, after incubation of 30 h (24 h first enrichment and additional enrichment of 6 h). This methodology represents a fast and simple way to identify *L. monocytogenes* at very low concentrations in food products. MALDI-TOF MS has been successfully applied to the differentiation of the closely related species *Streptococcus uberis* and *Streptococcus parauberis* and to the correct identification of two *S. parauberis* strains isolated from vacuum-packaging refrigerated seafood products (Fernández-No et al., 2012). Some species of the genus *Streptococcus* are of special interest for the dairy industrial sector, since they are important mastitis-causing agents. In this sense, MALDI-TOF MS has been successfully applied to differentiate mastitis-causing *Streptococcus* spp. strains, isolated from mastitis causes, blood and food at the species and subspecies level (Raemy et al., 2013; Schabauer et al., 2014). Finally, in a number of studies of Böhme et al. the ability of MALDI-TOF MS fingerprinting in identification of bacterial strains isolated from seafood products has been tested. For that, a total of 50 bacterial strains have been isolated from different fish and processed seafood products.

In general, MALDI-TOF mass spectral profiles exhibit a high interspecific variability and at the same time a high intraspecific similarity for most bacterial species, allowing identification at the genus and species levels. Exceptions have been reported for some very closely related species, such as *Escherichia coli* and *Shigella* spp., *Streptococcus* spp., and *Listeria* spp. that could not be differentiated by the commonly applied databases at the species level (Farfour et al., 2012; Risch et al., 2010). Nevertheless, MALDI-TOF MS demonstrated a high discriminatory potential at the intraspecies level. The differentiation of subspecies and serotypes is of crucial importance for risk assessment in the food sector, due to the varying pathogenic character. Likewise, determination of clonal lineages is fundamental for epidemiological studies of foodborne disease outbreaks. Clustering of mass spectral data has been successfully applied for chemotaxonomic studies of bacterial strains and compared to phylogenetic trees based on DNA analysis. The high similarity observed is not surprising, since the molecules detected by MALDI-TOF MS are generally attributed to ribosomal proteins that serve as taxonomic markers for the corresponding genus, species, or strain (Welker and Moore, 2011). In addition, in many cases a higher discriminatory potential has been observed for the proteomic approach when compared to conventional bacterial classification tools (Böhme et al., 2013; Risch et al., 2010). Recent reviews pay special attention to MALDI-TOF MS applications as a bacterial typing tool, with the aim of detecting antimicrobial resistance

and carrying out epidemiological studies (Clark et al., 2013; Sandrin et al., 2013). In some cases, the available spectral databases do not exhibit sufficient resolution at the species, subspecies or strain levels to perform bacterial typing of serotypes, pathotypes, or clonal lineages. Thus, the use of bioinformatics tools and the determination of subtype-specific biomarker peaks is required (Suarez et al., 2013). In this sense, subtyping of *Y. enterocolitica* (Stephan et al., 2011) and *Yersinia pestis* (Ayyadurai et al., 2010) strains into different serotypes could be achieved. Likewise, strains of the highly infective *Campylobacter jejuni* have been classified by MALDI-TOF MS, resulting in the separation of hyperinvasive strains and strains with an extended amino acid metabolism from the other strains (Mandrell et al., 2005). A critical aspect for *E. coli* identification is the high similarity to *Shigella* spp. The differentiation of *E. coli* and *Shigella* spp. by MALDI-TOF MS has been demonstrated recently by determining 15 biomarker peaks with the ClinProTool from Bruker (Khot and Fisher, 2013). A further challenge is the bacterial typing of *E. coli* isolates and differentiation of clonal groups to realize epidemiological studies. In a number of studies, MALDI-TOF MS has been successfully applied to the differentiation of *E. coli* clones (Christner et al., 2014; Matsumura et al., 2014; Novais et al., 2014). In these studies, clonal groups related to extended-spectrum-β-lactamase (ESBL) producers have been identified at high percentages. Likewise, in the spectral profiles of the Shiga-Toxigenic *E. coli* O104:H4 two peaks have been determined that were not present in the spectra of preoutbreak strains. MALDI-TOF MS has also been successfully applied for discrimination of the five most important serovars of *S. enterica* subsp. *enterica* based on a decision tree and specific biomarkers (Dieckmann and Malorny, 2011). In another study, the differentiation of *S. enterica* typhy from nontyphy was not possible with the Biotyper database, but clear identification could be achieved after identifying serovar-specific biomarkers (Kuhns et al., 2012).

MST aims at the detection of foodborne pathogens in the food chain and determination of the source of contamination and consequent corrective actions to be taken, as well as prevention of foodborne outbreaks. MALDI-TOF MS fingerprinting has been successfully applied to analyze *Enterococcus faecium* and *Enterococcus faecalis* and differentiate the strains in relation to their isolation sources (meat or dairy products) (Quintela-Baluja et al., 2013). Similarly, spectral variability could be observed at the strain level in relation to the geographical origin and moment of isolation of *V. paraheamolyticus* strains (Hazen et al., 2009).

High interest exists in the ability to distinguish methicillin-resistant *S. aureus* strains (MRSA) from methicillin-sensitive *S. aureus* strains (MSSA). MALDI-TOF MS fingerprinting has been successfully applied to this matter and was also able to distinguish clonal types of *S. aureus* (Du et al., 2002; Jackson et al., 2005; Wolters et al., 2010). The analysis of MRSA strains isolated during an outbreak allowed differentiation from

MSSA strains, as well as rapid typing of the outbreak strains and detection of epidemic lineages (Josten et al., 2013). In Fig. 2.2, spectral differences observed for *S. aureus* at the strain level are highlighted. The corresponding study demonstrated the discriminatory potential of MALDI-TOF MS, since the studied strains exhibited 100% identical 16S rRNA gene sequences, but could be classified into subgroups by the spectral profiles (Böhme, Morandi, et al., 2012b). Nevertheless, the subtypes could not be related to the production of toxins, as also confirmed by further studies (Szabados et al., 2010).

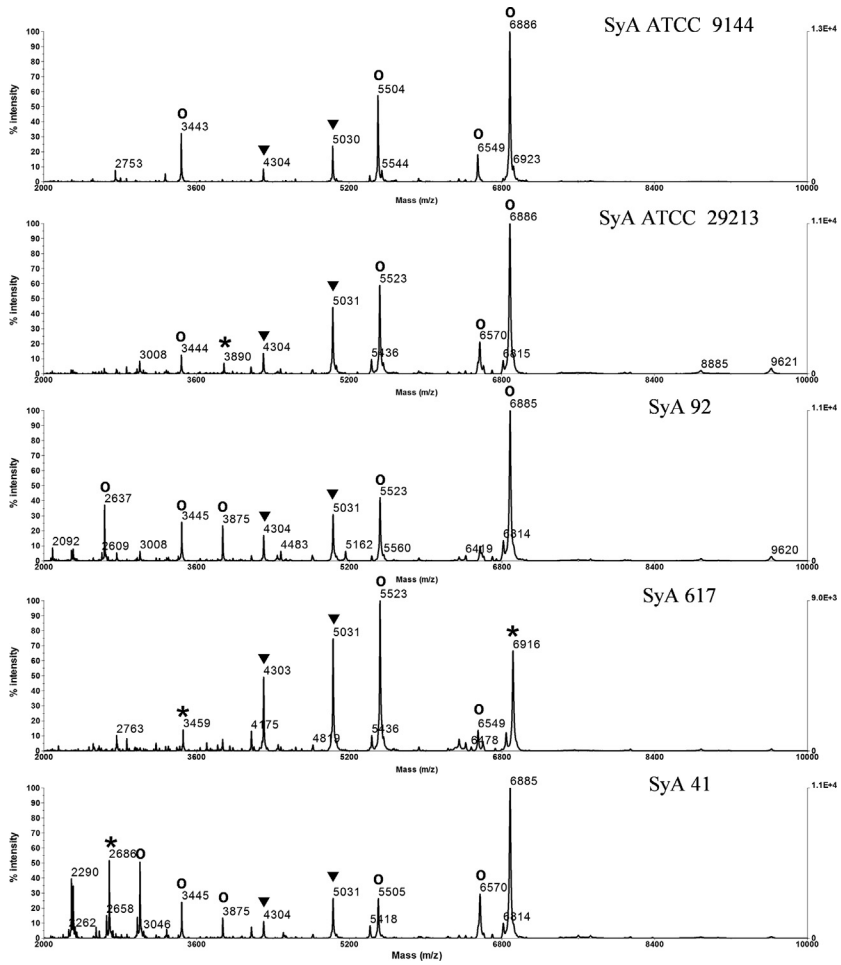

FIGURE 2.2 Spectral profiles of different *S. aureus* strains. Symbols correspond to species-specific (▼), subgroup-specific (*), and further characteristic peaks (○).

The genus *Bacillus* is known to comprise closely related species, with differentiation by DNA-based approaches not always possible. However, the different species corresponding to the *Bacillus cereus/thuringiensis* and the *Bacillus subtilis/amyloliquefaciens* complexes exhibit very different pathogenic and spoilage potential, thus requiring their correct identification. With MALDI-TOF MS and cluster analysis, a clear grouping of the *B. subtilis* strains was achieved (Böhme et al., 2013). In the case of *B. cereus* and *B. thuringiensis*, the 16S rRNA sequences are nearly 100% identical, inhibiting any differentiation at the species level. By phyloproteomic clustering, the strains could not be identified; however, a differentiation of the strains into subgroups was obtained. These findings were confirmed in a further study, where a number of *Bacillus* spp. strains, isolated from fresh and processed food products, were analyzed by MALDI-TOF MS (Fernández-No et al., 2013).

2.3 VIBRATIONAL SPECTROSCOPY

Vibrational spectroscopy consists of two approaches, infrared (IR) absorption and Raman scattering, and provides structural and chemical information about molecules based on their vibrational transitions. Both approaches are fast, low-cost, highly specific for target molecules, robust, and easy to use. Furthermore, no or a minimum sample preparation is required and chemical constituents can be determined qualitatively and quantitatively down to very low concentrations, with these characteristics making vibrational spectroscopy very suitable for food contamination detection. Furthermore, the advantage over other analytical techniques is the fact that the measurements are nondestructive, reagentless, and may be applied directly to food surfaces. In addition, the instruments can be transferred to portable devices, allowing immediate analysis of the food products and onsite evaluations throughout the whole food-processing chain.

In the last few years, the number of studies aimed at the use of vibrational spectroscopy for food safety applications increased markedly and different applications have been reviewed recently for FT-IR (Huang et al., 2008; Jimaré Benito et al., 2008; Woodcock et al., 2008) and Raman spectroscopy (Craig et al., 2013; Yang and Ying, 2011), as well as for different food classes, such as milk (Cattaneo and Holroyd, 2013), fish (Cheng et al., 2013), and water (Lopez-Roldan et al., 2013).

IR spectroscopy has widely been studied for bacterial identification purposes and in lesser extents for the detection of chemical contaminants, such as melamine and pesticides. Raman spectroscopy gives complementary information to IR spectroscopy and has gained increasing attention in the last decades for both foodborne pathogen and chemical contaminants detection. Although IR spectroscopy is cheaper and easier in instrumentation and handling, Raman spectroscopy has several advantages over conventional IR

spectroscopy, such as less interference of water and more detectable features over the same wave-number range (Lu et al., 2011). Nevertheless, Raman signals are weak and the spectra are highly interfered with by noise signals and fluorescence background, making it difficult to obtain good results for low concentrations of contaminants. New advances in nanotechnology have made it possible to enhance Raman signals by surface-enhanced Raman spectroscopy (SERS). Raman spectroscopy is combined with nanomaterials, such as gold or silver nanosphere colloids, solid surface gold-coated nanosubstrates, bimetallic nanosubstrates, or spherical magnetic-core gold-shell nanoparticles (Fan et al., 2014).

Fingerprints obtained by vibrational spectroscopy are affected by various factors and other nontargeted food components may significantly interfere with the obtained signals. To overcome these challenges and permit its use in routine analysis of food control laboratories, advanced data preprocessing and statistical analyses are required. The use of chemometrics for the interpretation of high-dimensional spectral fingerprints have made complex analyses possible.

2.3.1 Vibrational Spectroscopy for Bacterial Identification

Vibrational spectroscopy has been extensively applied to the study of bacteria in food stuffs (Lu et al., 2011; Pahlow et al., 2015). The characteristic fingerprints generated contain information about the biochemical constitution of bacterial cells and enable the differentiation of the organisms at the species and even strain level.

Many studies have analyzed bacterial foodborne pathogens and were aimed at either the identification of bacterial species present in food products or the detection and in some cases quantification of a concrete species with pathogenic character. In further studies, various foodborne pathogenic bacterial species have been studied together and could be successfully differentiated and identified by SERS approaches (Fan et al., 2011; Sundaram et al., 2013; Xie et al., 2013). A spectral library of 19 bacterial species of the most important harmful and nonpathogenic bacteria associated with meat and poultry has been created and tested for the identification of spiked meat samples. The test samples were correctly assigned to their genus and in most cases down to the species level by Raman fingerprinting and a three-level classification model by means of support vector machines (Meisel et al., 2014).

Bacterial identification by FT-IR achieved 100% of correct species identification for Gram-positive bacteria and 80% for Gram-negative bacteria (Janbu et al., 2008; Sandt et al., 2006). IR and Raman spectroscopy have also been applied to differentiate strains at the subspecies level with the aim of typing different biotypes and serotypes. In this sense, an FT-Raman

procedure was successful in discriminating different *E. coli* strains on whole apples and accurately differentiated the nonpathogens from pathogens, including *E. coli* O157:H7 (Yang and Irudayaraj, 2003). Likewise, strains of *Y. enterocolitica* have been distinguished into the main biotypes and serotypes (Kuhm et al., 2009). In this study, species of the genus *Yersinia* that cannot be differentiated by conventional biochemical methods exhibit specific IR fingerprints, allowing the clear discrimination at the species level. In addition, the presence of the ail gene, one of the main pathogenicity markers, was demonstrated using FT-IR and correct identification of isolates concerning the ail gene was achieved in 98.5%. Fan et al. (2011) successfully applied SERS to identify *E. coli* O157:H7 and *Staphylococcus epidermidis* in a mixed bacterial sample. Nevertheless, analysis of bacterial mixtures of different species remains challenging. Furthermore, bacterial identification generally includes cultivation procedures previous to analysis. This implies that only cultivable bacterial cells can be detected and the risk of the presence of injured but viable cells, spores, and already produced toxins remains. Al-Qadiri et al. (2008) applied FT-IR spectroscopy to detect sublethally heat-injured *S. enterica* var. *typhimurium* and *L. monocytogenes* cells. The studies of injured bacterial cells by vibrational spectroscopy have been reviewed and discussed by Lu et al. (2011). The detection of thermo-resistant spores of *Bacillus* spp. has also been successfully carried out by SERS (Alexander and Le, 2007; He et al., 2013). Further works have reported the application of Raman and FT-IR fingerprinting to detect the contamination of food products with mycotoxins, such as aflatoxins in maize kernels (Lee et al., 2014) and deoxynivalenol in ground wheat and barley (Liu et al., 2009).

To avoid time-consuming culturing steps, different approaches have been described to isolate bacterial strains from food matrices previous to IR or Raman analysis. The most promising practice is the implementation of nanoparticles that are targeted against the bacterial species of interest, concentrated, and then submitted to spectral analysis. In this sense, magnetic nanoparticles have been applied, functionalized with anti-*E. coli* O157:H7 or anti-*Salmonella typhimurium* antibodies (Ravindranath et al., 2009). The pathogens could be detected using a portable mid-IR spectrometer in complex food matrixes with a detection limit of 10(4)−10(5) CFU/mL. In further studies, bacterial cells have been captured by using antibody−antigen interaction with nanoparticles immobilized on SERS active surfaces and detected by direct analysis with spectral fingerprinting (Chae et al., 2013).

Besides bacterial species identification and detection of single pathogens, the monitoring of microbial spoilage of food products by FT-IR spectroscopy has been described for fish (Tito et al., 2012), meat (Ellis et al., 2002), and milk (Nicolaou and Goodacre, 2008), allowing the quantification of the microbial load by direct analysis on the food surface without any culturing or isolation processes.

2.3.2 Vibrational Spectroscopy for Detection of Chemical Contaminants

The capability of vibrational spectroscopy to detect all the biochemical compounds of a cell has led to the emergence of FT-IR and SERS as competent tools for the analysis of trace amounts of chemical hazards in various food products. The applications of vibrational spectroscopy fingerprinting to detect chemical contaminants in various food stuffs, such as dairy products, fish, fruits, vegetables, and condiments, have been reviewed in detail (Fan et al., 2014; Zheng and He, 2014).

Most studies reported are targeted on detecting melamine in milk, milk powder, infant formula, and egg white (Betz et al., 2012; Hu et al., 2015; Wang et al., 2015). Besides detection of melamine at very low concentrations (63 ppb to 2 ppm), quantification was also possible.

The characteristic of vibrational spectroscopy to be applicable directly on food surfaces without destroying the food gives it an enormous potential to detect pesticides on fruit and vegetable surfaces. In this sense, pesticide residues could be detected on apple surfaces and in apple juice samples at concentrations of 0.125 $\mu g/cm^2$ and 3 $\mu g/mL$ (3 ppm), respectively (Wijaya et al., 2014). Pesticides could also be detected in mixtures (Zhai et al., 2015) and on the surfaces of further fruits, such as bananas and citrus fruits (Müller et al., 2014).

Further chemical contaminants that have been investigated by FT-IR and Raman spectroscopy are banned food additives, such as sudan I, rhodamine B, and malachite green (He et al., 2015). Likewise, the contamination of fish with heavy metals could be detected by vibrational spectroscopy (Chen et al., 2013).

2.4 LASER-INDUCED BREAKDOWN SPECTROSCOPY

Laser-induced breakdown spectroscopy (LIBS) is another spectral fingerprinting technology that has emerged in the last few years. It uses a laser pulse as excitation source and emits an atomic spectrum of the analyzed target, representing the whole elemental composition. Since a very small amount of material is required, normally a few picograms, LIBS is also considered a nondestructive technique. Measurements can be carried out directly on surfaces of food or surfaces used for food manipulating and processing, with minimal or no sample preparation. Thus, real-time analysis can be carried out, exhibiting high sensitivity and the ability to detect all elements, including biological and chemical hazards. In addition, LIBS is a cost-effective, fast and robust technique that can be implanted into portable systems for nonspecialist users. All these attributes make LIBS fingerprinting a competent tool for food analysis purposes. Nevertheless, since it is a very recent methodology, only a few reports exist describing the use of LIBS for the detection of food

contaminants. More work has been done and reviewed recently for biomedical and military applications (Pathak et al., 2012; Rehse et al., 2012).

A challenge of LIBS fingerprinting is the complex data analysis, since the obtained spectral profiles contain mostly all the same elements. The use of chemometrics for computerized data analysis is necessary and allows a search for similarities and specificities between two spectral fingerprints. The so-called discriminant function analysis (DFA) is a computational discrimination technique that analyzes the entire observed elemental composition of a sample target and reduces the entire information of a spectrum to a quantity known as the discriminant function score. The scores from each spectrum are plotted against each other and the grouping obtained represents the differences and similarities that are immediately visible (Rehse et al., 2012).

2.4.1 LIBS for Bacterial Identification

Most of the reported studies are targeted at the identification of bacterial pathogens, responsible for human diseases. The differences detected by LIBS analysis are based on the chemical compositions of the outer membranes, which vary between different species of bacteria. These membranes contain divalent cations such as Mg^{2+} and Ca^{2+} that are the dominating spectral features in the LIBS profiles. Emission lines from other trace inorganics such as iron, potassium, sodium, manganese, and phosphorus, as well as molecules such as CN, OH, and NH, are also present and create all together a spectral fingerprint of the analyzed target organism.

LIBS fingerprinting has been successfully applied to the discrimination of bacterial foodborne pathogens, including specimens from 13 distinct taxonomic bacterial classes representative of 5 bacterial genera (Putnam et al., 2013). In further studies, the foodborne pathogens *E. coli*, *S. enterica*, and *S. aureus* have been analyzed by LIBS and could be clearly identified from each other due to the obtained specific spectral profiles (Barnett et al., 2011; Multari et al., 2013a; Singh, 2014). The authors also showed the applicability of LIBS to the detection of *S. enterica* and *E. coli* in different food matrices, such as milk, chicken, eggshell, ground beef, bologna, and lettuce. For bacterial identification the spectral fingerprints of an unknown bacterial strain are compared against a previously compiled spectral library. The development of an application of LIBS together with neural networks (NNs) for analysis and comparison of the spectra achieved over 95% correct species identification for bacterial strains of the species *P. aeroginosa*, *E. coli*, and *S. typhimurium* (Marcos-Martinez et al., 2011). LIBS also exhibits great potential to discriminate bacterial strains at the intraspecies level. In this sense, three clonal methicillin-resistant *S. aureus* (MRSA) strains could be distinguished and differentiated from one methicillin-sensitive *S. aureus* (MSSA) strain (Multari et al., 2010). Likewise, an *E. coli* O157:H7

strain could be clearly discriminated from nonpathogenic commonly occurring environmental *E. coli* strains (Diedrich et al., 2007).

When studying different culture conditions, the fingerprints obtained by LIBS apparently have proven to be independent to growth conditions and culture media. Those spectra of the same strain cultured under different conditions have demonstrated that they are more similar than those spectral profiles obtained for different strains grown under the same conditions (Diedrich et al., 2007; Marcos-Martinez et al., 2011; Rehse et al., 2010). In addition, LIBS profiles did not change between viable and killed bacterial cells, nor with time when aging on abiotic surfaces, this being of special interest for the analysis of processed food products and processing surfaces (Mohaidat et al., 2011; Multari, Cremers, Dupre, et al., 2013a).

Quantification of bacterial pathogens and detection of bacterial mixtures by LIBS fingerprinting remains challenging, but has been reported by Rehse et al. (2010). The authors showed that the intensity of the LIBS spectrum is linearly dependent on the cell number, but does not influence the specificity. When analyzing bacteria in mixed samples, the dominant bacterial component could be reliably identified if it comprised 70% or more of the mixture.

2.4.2 LIBS for the Detection of Chemical Contaminants

Advances in LIBS spectral data analysis and chemometrics techniques for data analysis have led to the ability to detect chemical contaminants in complex matrices such as foods. Pesticides and dioxins could be successfully detected in tissue fats and rendering oils. The pesticide concentrations in the samples ranged from 0.005 to 0.1 μg/g (Multari et al., 2013b).

Similarly, spinach and rice have been analyzed by LIBS with the aim of detecting contamination with pesticides. The results demonstrated that the LIBS technique together with a chemometric method (PLS-DA) could be a great tool to distinguish pesticide-contaminated samples from pesticide-free samples in a rapid manner (Kim et al., 2012).

In a further study, heavy metals have been analyzed in different tissues of contaminated fish samples. External calibration has been applied to quantify the contamination in the food products (Wan et al., 2013). Similarly, harmful metals have been detected by LIBS in rice (Hemati Farsani et al., 2014).

2.5 FUTURE TRENDS

The spectral fingerprinting techniques described here all represent accurate and fast methods in comparison to other bacterial identification techniques. However, the challenge of food security and foodborne pathogen detection is that very low doses of a critical strain may cause serious infectious diseases after multiplying rapidly in a few hours or days. Unfortunately, recent

identification methods also require multiplication of bacterial cells to reach a detectable concentration. In addition, one disadvantage of most fingerprinting techniques is that isolated bacterial strains are required. That means that culturing steps are still necessary to isolate and concentrate the bacterial strains from the food matrices. In this sense, approaches that apply spectral fingerprinting directly to a sample without or with minimal sample pretreatment are challenging. In clinical routine analysis, direct bacterial identification by MALDI-TOF MS has been achieved for urine and blood samples (Clark et al., 2013). Similar approaches could be applied to liquid food samples, as already demonstrated for contaminated water or the rinse water after washing lettuce and cotton cloth (Holland et al., 2000). Nevertheless, a critical point of food matrices is the presence of a mixture of different bacteria and, until now, the application of MALDI-TOF MS fingerprinting for microbial mixtures has not yet been demonstrated. For that to occur, specific biomarker proteins have to be determined that allow an unequivocal identification of the corresponding strain on the basis of a few peaks. The identification of biomarker proteins is also of interest for bacterial typing and the correlation to virulence factors, toxin production, and/or antibiotic resistance that could significantly improve the risk assessment in the food chain.

Shortening the culturing process has also been the focus of several studies based on vibrational spectroscopy. A real-time detection and identification approach to food pathogens has been described and was based on SERS measurement of labeled immunoassay reagents in cultural enrichment vessels while culturing is ongoing. The approach allowed sensitive detection of the pathogens *E. coli*, *Salmonella*, and *Listeria* in complex food matrices (Weidemaier et al., 2015).

Promising future prospects are those applications where the high specificity of molecular fingerprints is combined with the high selectivity of nanomaterials, such as aptamers, nanoparticles, or further recognition molecules. The use of magnetic nanoparticles allows the separation of target strains from the food matrix and nontarget strains and the posterior analysis by spectral fingerprinting.

Future trends will probably focus strongly on the analysis of a food product without any culturing step. An interesting study has been carried out aimed at the direct detection and quantification of the microbial load in meat and milk, using MALDI-TOF analysis (Nicolaou et al., 2012). Such rapid screening tests are essential for an effective risk assessment in the food control sector and are required for the fast and accurate detection of food contaminants, such as bacterial pathogens, as well as mycotoxins, drug residues, heavy metals, etc. To realize onsite analysis and field tests, portable and automated instruments are the objectives of ongoing research. A portable and automated optofluidic SERS system has been successfully applied to detect food and water contaminants (Yazdi and White, 2012).

Finally, the spectral fingerprinting techniques described can be combined with further analytical techniques, since this can give much more complete information as a single technique, especially in the field of bacterial strain characterization. As an example, vibrational spectroscopy is commonly implemented into microscope systems to recover cells after enrichment on agar and following spectral analysis. More interestingly, the combination of LIBS, vibrational spectroscopy, and mass spectrometry has great potential to unite all the information and create a "whole-organism spectral fingerprint."

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