Chapter 9
MALDI-TOF MS as a Novel Tool for Dereplication and Characterization of Microbiota in Bacterial Diversity Studies

Freek Spitaels, Anneleen D. Wieme and Peter Vandamme

An Introduction to Dereplication in Bacterial Diversity Studies

Determining the number and identity of all cultivable species of an ecosystem and, if possible, distinguishing among different strains of each of these species is a major challenge in bacterial diversity studies. A main reason for this is that there are no comprehensive databases that allow rapid, affordable, and accurate species-level identification of large numbers of isolates, irrespective of their origin. This is even more true for the recognition of different strains within a species. Publicly available 16S rRNA gene sequence databases cover (virtually) the entire known bacterial species diversity, but this gene lacks resolution to allow differentiation between closely related species, let alone strains belonging to the same species (Vandamme et al. 1996). Accurate species-level identification of numerous isolates obtained in the frame of biodiversity studies is therefore either not attempted, or it is based on polyphasic approaches in which methods with different taxonomic resolution, cost, and workload are applied sequentially to examine different subsets of isolates in a stepwise manner. Very often such a procedure involves first a "dereplication" step in which all isolates are examined to group those that represent the same taxon in a rapid and cost-effective manner. Dereplication is thus the assessment of novelty and aims to reduce a (large) number of isolates to a smaller, nonredundant set for further, more labor-intensive identification. It involves a rapid and affordable screening of all isolates to recognize and eliminate those that represent the same taxon.

P. Vandamme (✉) · F. Spitaels · A. D. Wieme
Laboratory of Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium
e-mail: Peter.Vandamme@UGent.be

A. D. Wieme
Laboratory of Biochemistry and Brewing, Faculty of Bioscience Engineering, Ghent University, Valentin Vaerwyckweg 1, 9000 Ghent, Belgium

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In a second step, representative isolates of these unidentified taxa are selected for further identification. The latter very often consists of determining the 16S rRNA gene sequence of these representative isolates to generate a tentative identification or to reveal the genera or species clusters the unknowns belong to. Especially the universal polymerase chain reaction (PCR) primers and the near-complete public database render this "16S rRNA approach" very appealing and very commonly more accurate identification is not required. However, 16S rRNA gene sequence analysis may be complemented by the application of validated methods that can be used for accurate species-level identification of the bacteria concerned (Prakash et al. 2007; Russo et al. 2010; Vandamme et al. 1996; Vandamme and Peeters 2014). For instance, until recently, many studies of fermented food ecosystems such as cocoa, sourdough, and others applied repetitive element (rep) primed PCR to group all isolates in a first step after which representative strains were selected and analyzed by partial 16S rRNA gene sequence analysis to obtain a tentative identification; members of the lactic acid bacteria (LAB) were subsequently accurately identified to the species level through sequence analysis of protein-encoding genes such as *pheS, rpoA,* and *atpA* using LAB-specific PCR primers. The latter was feasible and scientifically correct, because earlier taxonomic studies had generated PCR primers and *pheS, rpoA,* and *atpA* sequences of taxonomic reference strains of all LAB species and demonstrated that comparative sequencing of these housekeeping genes indeed allowed accurate species-level identification of LAB isolates, whereas comparative 16S rRNA gene sequence analysis did not (De Bruyne et al. 2007, 2008; Naser et al. 2005, 2007). The identification results of these representative strains are then extrapolated to all members of the same taxon (Doan et al. 2012; Papalexandratou et al. 2011; Scheirlinck et al. 2008).

The differentiation of genetically different strains belonging to the same species is mostly referred to as "typing" and is commonly done in medical microbiology, for instance, to reveal the epidemiology of outbreak strains, or in food microbiology in the frame of source tracking. Ideally, the dereplication method applied in diversity studies is universally applicable so that prior knowledge of the microorganisms present in a sample is not needed, and it has a resolution that allows to distinguish species and strains simultaneously. This ideal method has not yet been discovered. Several dereplication techniques have been described and include cellular fatty acid methyl ester analysis and $(GTG)_5$-PCR fingerprinting (Coorevits et al. 2008; De Clerck and De Vos 2002; De Vuyst et al. 2008; Faimali et al. 2010; Gevers et al. 2001; Ishii and Sadowsky 2009; Vandecandelaere et al. 2010; Versalovic et al. 1994). After identification of representative strains of the clusters delineated by dereplication, a cumulative database can be constructed which will allow to compare profiles generated in subsequent dereplication studies with those delineated and identified earlier. As a result, a growing dereplication database will increasingly allow identification as well, and further polyphasic identification efforts can be limited to those representative strains with patterns not observed earlier.

The Use of MALDI-TOF MS for Dereplication in Environmental Microbiology

To understand the taxonomic resolution of a dereplication method used is key to knowing how useful it is in diversity studies. For more than a decade, $(GTG)_5$-PCR fingerprinting has been used as a dereplication standard for many bacteria. This method can be used for both species- and strain-level differentiation, but it does not consistently differentiate all strains in a species (De Vuyst et al. 2008; Švec et al. 2005). It also suffers from limited long-term reproducibility of PCR fingerprints and has a limited throughput capacity (Gevers et al. 2001; Ghyselinck et al. 2011). The introduction of matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) in diagnostic microbiology provided a technically appealing alternative. Indeed, sample preparation can be as simple as the deposition of (inactivated) biological material on a ground steel plate and the addition of a suitable matrix. Furthermore, universal sample preparation methods preclude the need for prior knowledge of the identity of the isolates studied and allow analysis of both bacteria and yeasts. Moreover, provided sample preparation is properly standardized, the spectra are highly reproducible (Freiwald and Sauer 2009) and enable the creation of a mass spectral database that can be shared between users. The consumable cost is low, the generation of the mass spectra is fast, and the preparation and deposition of the samples can be automated (Cherkaoui et al. 2010; Seng et al. 2009)—all of these contribute to an impressive and affordable throughput capacity.

Today, MALDI-TOF MS has primarily been used as a dereplication tool aiming at grouping isolates at the species level. Similarity values used for delineating groups of spectra are mostly generated using algorithms based on the presence or absence of peaks (e.g., the Jaccard or Dice coefficient), or taking peak intensity into account (e.g., Pearson product-moment correlation coefficient). Alternatively, spectra can be analyzed using a curve-based algorithm without prior peak picking, using the Pearson product-moment correlation coefficient. Finally, more advanced similarity coefficients that yield normalized similarity values can be used, such as correlation coefficient index analysis (Koubek et al. 2012). The delineation of individual clusters can be based on visual inspection of the tree topology, preset cluster cutoff values, or calculated cluster cutoff values. These cluster cutoff values are similarity levels that are used as thresholds below which the spectra are regarded as belonging to different clusters, and therefore they are expected to represent different species. Preset cluster cutoff values are mostly derived from preliminary experiments and are used as a rule of thumb for the cluster delineation. Follow-up experiments performed on representative isolates of each cluster must yield the species-level identification of all isolates (see above) and will intrinsically confirm or contradict the validity of the cutoff values used. Calculated cluster cutoff values are mostly obtained through the analysis of a limited set of technical and biological replicates, and through the analysis of taxonomically well-characterized reference strains of some species. The latter will allow to quantitate the variance of similarity between spectra of different strains of those species. The degree of variance is however often species dependent and the delineation of clusters is very often based on results of preceding experiments, trial and error, and personal expertise (see below).

MALDI-TOF MS was first used as a dereplication tool in a study of bacterial isolates from marine sponges, in which mass spectra that shared five of the most intense peaks were grouped together (Dieckmann et al. 2005). Subsequent analyses of the isolates by partial 16S rRNA gene sequence analysis revealed a congruence between the obtained MALDI-TOF MS groups and the tentative identifications based on partial 16S rRNA gene sequences (Dieckmann et al. 2005). Ichiki et al. (2008) used MALDI-TOF MS to delineate groups of bacteria that were able to degrade alkylphenol polyethoxylate. The isolates were studied using PCR-restriction fragment length polymorphism (RFLP), MALDI-TOF MS, and *gyrB* sequence analysis. The latter two methods yielded the same results and grouped isolates on the basis of their ability to degrade this compound (Ichiki et al. 2008). MALDI-TOF MS was also used for the dereplication of halophilic prokaryotes from solar saltern sediments (Munoz et al. 2011). In this study, the mass spectra were clustered and divided into groups delineated using the peak-based Jaccard algorithm (Munoz et al. 2011). Some representative strains were selected for 16S rRNA gene sequence analysis and DNA–DNA hybridization experiments which demonstrated that the groups delineated consisted of isolates representing a single species (Munoz et al. 2011). Yet, random amplified polymorphic DNA (RAPD) typing revealed that such isolates could represent genetically distinct strains (Munoz et al. 2011). Similarly, MALDI-TOF MS was also used as a species-level dereplication tool in a study aiming to isolate soil and endophytic *Burkholderia caledonica* isolates (Verstraete et al. 2014). Field isolates were dereplicated by means of MALDI-TOF MS, after which clusters representing *B. caledonica* were identified by sequence analysis of the *recA* gene of representative isolates (Verstraete et al. 2014).

The use of a preset cutoff value was also applied to nine isolates from polychlorinated biphenyl (PCB)-contaminated sediment (Koubek et al. 2012). The MALDI-TOF mass spectra were subjected to a correlation coefficient index analysis and the cluster cutoff was adopted from previous tests and set at 0.6 (Koubek et al. 2012). Isolates were investigated using a commercial biochemical test kit, MALDI-TOF MS, and 16S rRNA gene sequence analysis, of which the latter two resulted in the same number of taxonomical units delineated in the sample analyzed (Koubek et al. 2012). Similarly, bacterial isolates were screened using MALDI-TOF MS for novel carotenoids with UVA-blue light absorbing properties (Stafsnes et al. 2013). The mass spectral clusters were delineated using a preset distance cutoff value of 500, although the authors noticed that not all species were delineated appropriately (Stafsnes et al. 2013). Cluster delineation was evaluated by comparison with the results of the pigment profiles and the inclusion of previously identified strains (Stafsnes et al. 2013). A preset distance cutoff value of 500 was used as a rule of thumb in other studies as well (Kopcakova et al. 2014). This cutoff value was, however, determined for the delineation of MALDI-TOF mass spectra of *Erwinia* species (Sauer et al. 2008), and it seems not appropriate to use the same value without reevaluation for other groups of bacteria as species-level cutoff values can vary, depending on the identity of the isolates studied (Christensen et al. 2012; Stafsnes et al. 2013; Wang et al. 2012).

In contrast, a carefully determined cluster cutoff value was used by Ghyselinck et al. (2011), who were the first to report a detailed comparison of the taxonomic resolution of MALDI-TOF MS and $(GTG)_5$-PCR fingerprinting in a study of potato rhizosphere isolates. They aimed to distinguish between strains rather than species and based the cluster delineation upon the reproducibility of both techniques by analyzing some of the isolates in triplicate (Ghyselinck et al. 2011). The technical variance of the triplicate measurements was then used for the calculation of the most appropriate cutoff level (Ghyselinck et al. 2011).

The Use of MALDI-TOF MS for Dereplication in Food Microbiology

In food microbiology, dereplication of both bacterial and yeast isolates has been reported but the taxonomic resolution aimed at was primarily species-level differentiation. Doan et al. (2012) compared $(GTG)_5$-PCR and MALDI-TOF MS for the dereplication of LAB isolates from Vietnamese fermented meat and used *pheS* sequence analysis for accurate species-level identification of representative strains. Although the dendrograms based on the $(GTG)_5$-PCR and MALDI-TOF MS fingerprints were not identical, both methods yielded the same species-level identification for all 119 isolates studied. These authors further confirmed the potential of MALDI-TOF MS as a dereplication tool by analyzing the LAB diversity of a fermented mustard sample and by again using *pheS* sequence analysis as an identification method (Doan et al. 2012). They also analyzed the LAB diversity of Vietnamese fermented mustard, beet, and eggplant using the same approach (Nguyen et al. 2013). Similarly, MALDI-TOF MS was applied for the dereplication of 348 beer spoilage isolates obtained from multiple types of beer cultivated onto different growth media and in different growth conditions (Wieme et al. 2014b). All isolates were subsequently identified at the species level using sequence analysis of various protein-encoding genes.

Spitaels and coworkers used MALDI-TOF MS as a dereplication tool for yeast and bacterial isolates in their studies of traditional lambic beer, an acidic beverage which is the result of a spontaneous fermentation that lasts for up to 3 years (Spitaels et al. 2014c). A total of over 2000 bacterial and yeast isolates, taken from consecutive samples during the first 2 years of fermentation, were dereplicated using a cumulative, purpose-built database. MALDI-TOF MS clusters were delineated visually, and representative isolates of each cluster were identified using sequence analysis of 16S rRNA (bacteria), 26S rRNA (yeasts), and/or housekeeping genes (both groups). The combination of MALDI-TOF MS-based dereplication coupled to sequence analysis-based identification was paramount for the thorough characterization of the microbial community during this long fermentation process. The same approach was applied on industrially produced lambic beer fermentations of which over 1300 bacterial and yeast isolates were dereplicated and/or identified using MALDI-TOF MS (Spitaels et al. 2015). The resulting mass spectra were used for the construction of a mass spectral database that is now used for the identifica-

tion of numerous bacterial and yeast isolates from other traditional acidic Belgian beers (unpublished data). The long-term application of this method demonstrated that isolates with highly similar mass spectra and therefore grouping in one cluster consistently represented the same species, but it also revealed that very distinct clusters may also represent the same species. The latter was mostly due to genuine differences between mass spectra and therefore revealed differences between strains of the same species, but it was occasionally caused by varying quality of the spectra.

MALDI-TOF MS Can Combine Dereplication and Identification

We mentioned earlier that the use of MALDI-TOF MS as a dereplication tool in biodiversity studies coupled to accurate species-level identification by means of validated taxonomic methods increasingly facilitates direct identification through MALDI-TOF MS and therefore increasingly precludes the need for further polyphasic identification. This is especially true when using commercial MALDI-TOF MS instruments and databases to which the mass spectra of unknowns are matched (Clark et al. 2013; Croxatto et al. 2012). Most of the recent biodiversity studies have been performed using instruments with commercial identification databases that can be extended in-house with mass spectra of organisms that are not in the database because they lack clinical or pharmaceutical interest (Calderaro et al. 2013, 2014; Christensen et al. 2012; Edouard et al. 2012; Ferreira et al. 2011; Kopcakova et al. 2014; Vavrova et al. 2014). MALDI-TOF MS therefore has the technical capacity to simplify dereplication and identification dramatically (Bille et al. 2012; Seng et al. 2009), and recent biodiversity studies that use MALDI-TOF MS are increasingly skipping the dereplication step (Dec et al. 2014; Egert et al. 2014; Ferreira et al. 2011; Uhlik et al. 2011; Vavrova et al. 2014). In such studies, mass spectra of isolates that cannot be identified directly can be clustered and representative isolates can be chosen for further polyphasic identification (Dubourg et al. 2014; Kopcakova et al. 2014; Plenz et al. 2014). With this approach, the cost of additional sequencing is minimized, and unreliable identifications caused by poor-quality mass spectra can be detected. This way, the mass spectral database can be further completed and application-specific databases can be constructed (Campos et al. 2010; Plenz et al. 2014; Wieme et al. 2014b).

To facilitate direct and accurate species-level identification, it is important to have an exhaustive reference database containing high-quality mass spectra generated from a sufficient number of reference strains of the same species, grown on multiple media and in different growth conditions (Bille et al. 2012; Seng et al. 2009). The number of shared peaks appeared to decrease with an increasing number of strains per species examined, and a growth medium-dependent species-specific core set of peaks and therefore peptides has been reported (Wieme et al. 2014a). For instance, the core set of peaks of two *Lactobacillus malefermentans* strains reported earlier (Wieme 2014) was reduced considerably through the acquisition and analysis of three additional *L. malefermentans* strains (Fig. 9.1). This confirmed that the intraspecies diversity

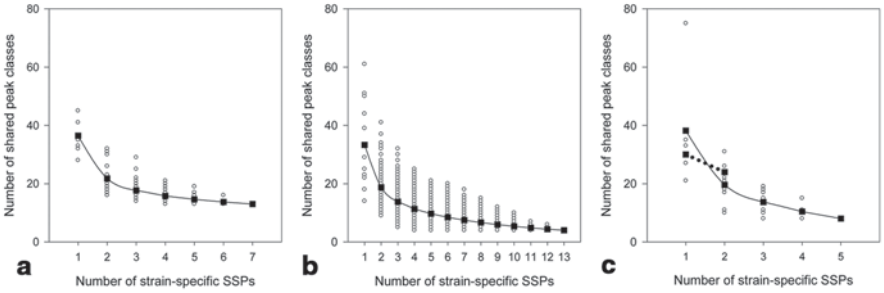

Fig. 9.1 The decrease in shared peak classes among summary spectra (SSPs; theoretical spectra that are generated by peak matching of multiple spectra per strain and that include only peaks that are present in all individual spectra, binned in peak classes) generated from 7 *L. brevis* strains grown on MRS (**a**), 13 *L. paracasei* strains grown on MRS (**b**), and 2 *(dotted curve)* and 5 *(full curve) L. malefermentans* strains grown on MRS (**c**). The number of shared peak classes is plotted as a function of the number of strain-specific SSPs sequentially added. *Squares* represent the average of shared peak classes per sequential addition of a strain-specific SSP. *MRS* de Man, Rogosa, and Sharpe medium

should be properly represented in an identification database before a reasonably accurate representation of the core peptides can be obtained (Lartigue et al. 2009). An inadequate representation of the intraspecies diversity in a MALDI-TOF MS identification database may be the cause of misidentification or lack of discriminatory power in some groups of microorganisms (Seng et al. 2009; Wieme et al. 2014a).

Detection of Taxonomic Novelty

To date, only a fraction of the bacterial diversity has been taxonomically characterized and only about 11,000 species are given formal binomial names; hence, the isolation of novel bacteria in the frame of bacterial diversity studies is fairly common (Tamames and Rosselló-Móra 2012; Vandamme and Peeters 2014; Yarza et al. 2014). The absence of matching mass spectra in a MALDI-TOF MS identification database might point to a missing organism in the database as discussed above, but might also reveal that an isolate represents a novel species. MALDI-TOF MS has aided in the detection and description of several new species in a range of genera (Kadri et al. 2014; Korczak et al. 2014; Li et al. 2014; Snauwaert et al. 2013; Spitaels et al. 2014a, b), and mass spectral data are increasingly added to novel species descriptions (Li et al. 2014; Spitaels et al. 2014a, b). Alternatively, spectra can be made available in public online repositories, such as Spectra, an initiative of the public health agency of Sweden (http://spectra.folkhalsomyndigheten.se), SpectraBank (Böhme et al. 2012), or via a private laboratory database shared online (Mishra et al. 2012; Djelouadji et al. 2012). When using commercial MALDI-TOF MS identification systems such novel species cannot be detected when solely looking at the identification scores that are produced; the raw spectral data as well as the clustering of these mass spectra should be carefully analyzed (Srinivas et al. 2014).

Furthermore, MALDI-TOF MS not only facilitates the detection of novel species but also can reveal taxonomic anomalies (Wieme et al. 2014b). The clustering of the *Pediococcus lolii* type strain among *Pediococcus acidilactici* strains suggested that it might have been misclassified (Wieme et al. 2012). The latter was indeed confirmed by using a polyphasic taxonomic analysis which demonstrated that both organisms represented the same species (Wieme et al. 2012). Similarly, Buddruhs et al. (2013) used MALDI-TOF MS in a polyphasic study to prove that strain DSM 17395 did not correspond with the type strain of *Phaeobacter gallaeciensis*.

MALDI-TOF MS As a Dereplication Tool in Culturomics

The potential of MALDI-TOF MS as a dereplication tool is maximally exploited in so-called culturomics studies. Culturomics is a term used for the exhaustive application of many culture media and growth conditions for a maximal recovery of cultivable microorganisms from a biological sample (Lagier et al. 2012). Although metagenomics (performed by either shotgun sequencing or target enrichment strategy) is a well-established cultivation-independent technique for characterizing the microbial diversity in samples (Gilbert and Dupont 2011), it is increasingly recognized that it has pitfalls too, as rare community members may remain undetected and sequence data generated prove insufficient for identification (Dubourg et al. 2013b; Lagier et al. 2012). There is a renewed interest in cultivation-dependent analyses of microbial diversity to complement other "omics" studies (Lagier et al. 2012). Culturomics approaches aimed at isolating the whole microbial community used more than 200 isolation conditions (Dubourg et al. 2013a, b, 2014; Lagier et al. 2012) and yielded several thousands of isolates. This huge number of isolates necessitated the application of a fast and cost-effective dereplication and identification technique to rapidly process these isolates in order to reduce the risk of losing part of the isolates and therefore valuable resources and information. The dereplication and subsequent identification of all isolates is a limiting factor in these studies, rather than the number of bacterial species that can be cultivated (Lagier et al. 2012). Not surprisingly, MALDI-TOF MS is currently used in such culturomics studies (Bittar et al. 2014; Dubourg et al. 2013a, b, 2014; Lagier et al. 2012). Since these studies addressed the microbial diversity of the human gut microbiome, the databases of commercial MALDI-TOF MS systems were fairly complete and many isolates were identified during dereplication (Samb-Ba et al. 2014). Unidentified isolates have been subjected to 16S rRNA gene sequence analysis, which revealed the presence of many novel species (Dubourg et al. 2013b).

The Influence of Experimental Factors on MALDI-TOF Mass Spectra Generated

Reproducibility is a major factor to consider when characterizing microorganisms using MALDI-TOF MS (Carbonnelle et al. 2011; Hinse et al. 2011; Welker 2011). Therefore, standardized growth conditions (e.g., culture medium, incubation time)

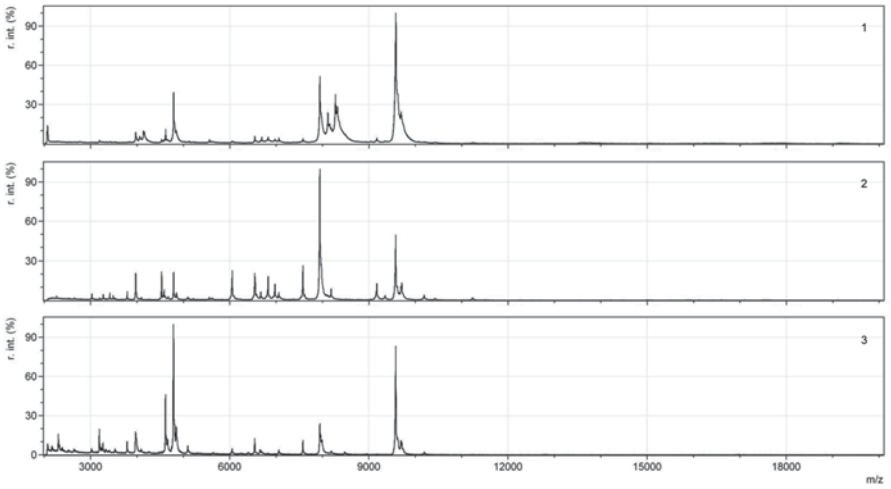

Fig. 9.2 *Pediococcus acidilactici* strain LMG 25667 grown on a commonly used growth medium for the detection of beer spoilage bacteria, known as "Nachweismedium für bierschädliche Bakterien" *(panel 1),* on de Man, Rogosa, and Sharpe medium *(panel 2),* and Raka-Ray medium *(panel 3)*. Spectra were visualized using the free mMass software. (Strohalm et al. 2010)

and optimized data acquisition for the generation of mass spectra have been recommended (Hazen et al. 2009; Sedo et al. 2011; Seibold et al. 2010; Siegrist et al. 2007; Williams et al. 2003). Yet, in biodiversity studies, multiple selective and non-selective isolation media are commonly used to maximize the isolation of different microbial populations. In order to use MALDI-TOF MS as an efficient and effective dereplication tool, the effects of the culture conditions used on the mass spectra generated should be minimized. The mass spectra of bacteria consist of signals derived mostly from ribosomal and other abundant proteins (Alispahic et al. 2010; Arnold et al. 1999; Barbuddhe et al. 2008; Dieckmann et al. 2008; Guo et al. 2009; Ryzhov and Fenselau 2001; Teramoto et al. 2007; Welker 2011). For ribosomal proteins, the effect of growth conditions on the mass spectra and therefore the identification result is expected to be minimal (Cherkaoui et al. 2010; Welker 2011). However, the growth medium used can influence the expression pattern of other proteins and hence alter the mass spectrum (Carbonnelle et al. 2011; Dieckmann et al. 2008; Giebel et al. 2010; Valentine et al. 2005; Walker et al. 2002; Welker 2011). Medium compounds can interfere with the ionization of the bacterial biomolecules, especially if the bacterial cells have the tendency to adhere onto the culture medium surface (Alispahic et al. 2010; Dieckmann et al. 2008; Giebel et al. 2010; Hettick et al. 2004; Walker et al. 2002) and culture media that do not sustain optimal growth can strongly affect the mass spectra generated (Wieme et al. 2014a). For instance, a commonly used growth medium for the detection of beer spoilage bacteria, known as the "Nachweismedium für bierschädliche Bakterien," did not support the growth of *P. acidilactici* strain LMG 25667 very well. Hence, the mass spectra generated from cells grown on this medium differed considerably from those obtained when the strain was grown on de Man, Rogosa, and Sharpe medium or Raka-Ray medium (Fig. 9.2). Besides growth conditions, other experimental factors such as the age of

the cell culture at the time of analysis, cell concentration, sample treatment, spotting method, and data acquisition all can contribute to variation in mass spectra and alter their reproducibility (Arnold et al. 1999; Balážová et al. 2014; Chean et al. 2014; Chen et al. 2008; Giebel et al. 2010; Goldstein et al. 2013; Hettick et al. 2004; Holland et al. 1996; Horneffer et al. 2004; Hsu and Burnham 2014; Karger et al. 2013; Lotz et al. 2010; Sedo et al. 2011; Toh-Boyo et al. 2012; Veloo et al. 2014; Williams et al. 2003; Usbeck et al. 2013). Also, as described earlier, mass spectra may be compared using either peak- or curve-based algorithm (Croxatto et al. 2012; Welker 2011). With a peak-based algorithm, the presence, and possibly also the intensity, of specific biomarker peaks in the unknown isolate's mass spectrum is verified, or peak lists of mass spectra of different organisms are compared (Barbuddhe et al. 2008; Böhme et al. 2011). A curve-based method considers the complete spectrum, that is, not only the presence of certain peaks but also the variation in peak signal intensity, taking into account all data points of the mass spectrum and not only the data points that describe peaks. The latter approach requires less data processing and thus allows a higher throughput, but is more prone to experimental variation (Dieckmann et al. 2005). In dereplication studies, curve-based analysis therefore introduces more variation into the clustering of isolates.

In general, however, the experimental factors used for the generation of the mass spectra do not modify the species-level identification of unknown microorganisms, irrespective of the identification algorithm used (Bille et al. 2012; Conway et al. 2001; De Bruyne et al. 2011; Dieckmann et al. 2008; Grosse-Herrenthey et al. 2008; Lartigue et al. 2009; McElvania TeKippe et al. 2013; Rezzonico et al. 2010; Seibold et al. 2010; Valentine et al. 2005; Wieme et al. 2014a). The inclusion of biological and technical replicates of strains grown at specific culture conditions reduces the biological or technically induced variations of the mass spectra generated. Commercial MALDI-TOF MS systems use robust algorithms and adequately build the proprietary database to enable a robust identification under different experimental conditions. Nonetheless, a drawback of the wide distribution and user-friendly interfaces of commercial databases is that most users report only the output identification scores of the mass spectral software, without publishing the mass spectral data or the parameters applied during mass spectral quality control, thus preventing to evaluate the quality of the spectra generated.

The culture medium used, in particular, can have a profound effect on the mass spectra generated as revealed by the presence and varying signal intensities of the peaks observed (Wieme et al. 2014a). Therefore, strain-level differentiation can be strongly affected by the growth medium selected as it may be based on minor discernible mass spectral differences (Sandrin et al. 2013; van Baar 2000; Wieme et al. 2014a). Consequently, the growth medium used is likely far more important if strain-level differentiation is aimed at, compared to species-level differentiation (Sandrin et al. 2013).

Dereplication at an Infraspecific Level?

The use of MALDI-TOF MS for the dereplication and identification of bacteria and yeasts is now well established. The resolution of MALDI-TOF MS for the identification of bacteria and yeasts is comparable or superior to the taxonomic resolution that can be obtained using rRNA gene sequence analysis (Benagli et al. 2011; Mellmann et al. 2008). Very commonly, MALDI-TOF MS will allow accurate species-level differentiation in groups of bacteria where comparative 16S rRNA gene sequence analysis lacks taxonomic resolution (Böhme et al. 2013). However, for some genera, the taxonomic resolution of MALDI-TOF MS is insufficient when mass spectra are analyzed using conventional identification algorithms only (He et al. 2010; Tan et al. 2012; Werno et al. 2012). The mass spectra of such species show too few discriminating peaks to be sufficiently differentiated when using conventional identification algorithms; yet, more rigorous peak analysis can facilitate species-level identification of some of these bacteria, as was illustrated for *Listeria* species (Barbuddhe et al. 2008).

In an ideal scenario, dereplication is not limited to the differentiation and/or identification of different species in a sample, it also allows to distinguish among strains of the same species. At present, only a limited number of studies have addressed strain-level resolution. Some of these studies aimed to distinguish among subspecies of, for example, *Bifidobacterium* or *Leuconostoc* species, but did not consistently succeed in doing so (Ruiz-Moyano et al. 2012; Pennanec et al. 2010; Zeller-Péronnet et al. 2013; De Bruyne et al. 2011), a result which may also be influenced by the data analysis algorithm used. Other studies aimed to distinguish among strains with or without certain characteristics such as antimicrobial resistance determinants (Wolters et al. 2011) or beer spoilage potential (i.e., their tolerance towards iso-alpha acids and capacity to grow in beer; Kern et al. 2014). These studies also did not prove consistently successful.

The ultimate goal however is genuine strain-level differentiation or typing, as is aimed at, for instance, when characterizing starter cultures, when source-tracking contaminants in food microbiology, or when performing epidemiological studies of outbreak strains. A growing number of studies are addressing this application of MALDI-TOF MS but commonly report an insufficient resolution. For instance, Lasch et al. (2014) could not differentiate among several clinical isolates of *Enterococcus faecium* and *Staphylococcus aureus,* whereas Schirmeister et al. (2014) could not identify reliable biomarker peaks to differentiate among isolates of *Vibrio cholerae.* Similarly, MALDI-TOF MS allowed to differentiate only a few β-lactam-resistant *Klebsiella pneumoniae* strains (Sachse et al. 2014). However, some other applications proved feasible. *Gluconobacter cerevisiae* strains isolated from different lambic breweries and from a spoiled yeast starter from a third brewery had different RAPD fingerprints and their MALDI-TOF MS spectra comprised differentiating peaks at 7491, 8102, and 10483 Da (Fig. 9.3; Spitaels et al. 2014b). Similarly, MALDI-TOF MS was recently used for quality control monitoring of a brewer's yeast: the mass spectra allowed to distinguish the brewing yeast from a nonbrewing

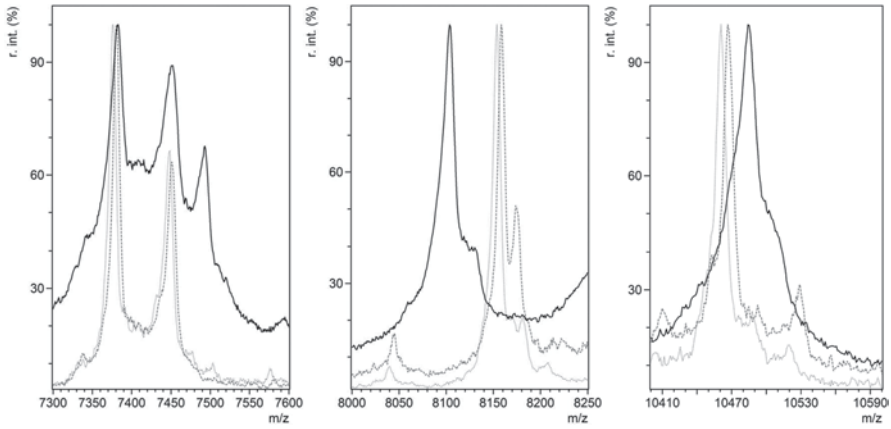

Fig. 9.3 Peaks differentiating *G. cerevisiae* LMG 27749 *(full black line)* from *G. cerevisiae* LMG 27748T *(dotted gray line)* and *G. cerevisiae* LMG 27882 *(dashed gray line):* 7491, 8102, and 10483 Da. Spectra were visualized using the free mMass software. (Strohalm et al. 2010)

wild-type yeast, although both yeasts were identified as *Saccharomyces cerevisiae* (Wieme et al. 2014c). Also, Moothoo-Padayachie et al. (2013) optimized and applied a MALDI-TOF MS protocol for the rapid biotyping of *S. cerevisiae* strains applied in different industrial fermentation backgrounds, and Usbeck et al. (2014) used MALDI-TOF MS for typing commercial yeast starters used in wine fermentation. The approaches for using MALDI-TOF MS for typing have been reviewed (Sandrin et al. 2013; Spinali et al. 2014), but strain typing using MALDI-TOF MS as the sole technique remains difficult, not the least because current algorithms are designed for the identification of microorganisms, while strain typing requires more, in depth peak-based analysis of the spectra in which the common peaks within a species can be filtered out (Sandrin et al. 2013; Spinali et al. 2014). The latter allows to focus on often subtle but reproducible differences in mass spectra of different strains (Giebel et al. 2008; Schumaker et al. 2012; Siegrist et al. 2007). Such subtle differences should represent genuine and reproducible characteristics of the mass spectra rather than experimental variation. Hence, it is plausible to assume that the use of MALDI-TOF MS for the dereplication of both species and strains in biodiversity studies will not be achieved in a single step. Strain-level identification may become feasible when isolates picked up from different cultivation media are reanalyzed after growth in standardized cultivation conditions using biological and technical replicates and the exclusion of common peaks. Indeed, the mass spectral reproducibility and quality are equally important to enable accurate and reliable infraspecific-level discrimination (Goldstein et al. 2013).

Future Perspectives

MALDI-TOF MS has become a standard method for microbial identification in clinical laboratories (Croxatto et al. 2012), while in other fields of microbiology, it is often used as a dereplication tool. Commercial systems with accompanying databases allow the direct identification of isolates during dereplication, but fail to identify many or most species in diversity studies of food or environmental samples. Commercial databases will therefore have to be expanded with appropriate numbers of reference strains to facilitate direct identification of isolates during dereplication studies of such samples as well. Although the advantages of using MALDI-TOF MS in other microbiology disciplines are obvious, the initial investment cost for both instrument and database represents a serious hurdle towards its wider application. To maximize the exploitation of the throughput capacity of MALDI-TOF MS in biodiversity studies, especially those using a culturomics approach, automation of colony picking, direct smearing or extract preparation, and subsequent MALDI-TOF MS analysis will be needed. The increased use of MALDI-TOF MS in biodiversity studies will lead to more comprehensive databases, which could also benefit from the inclusion of MALDI-TOF MS data and/or description of specific biomarkers in the taxonomic descriptions of novel species (Tindall et al. 2010).

Dereplication of microbial communities to the strain level requires further studies but may become a two-step procedure that involves the recultivation of all isolates that must be compared in identical cultivation conditions to be able to evaluate the value of individual peaks as strain markers. There is a need for flexible algorithms that allow to filter out common peaks and focus on differential peaks in the mass spectra.

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