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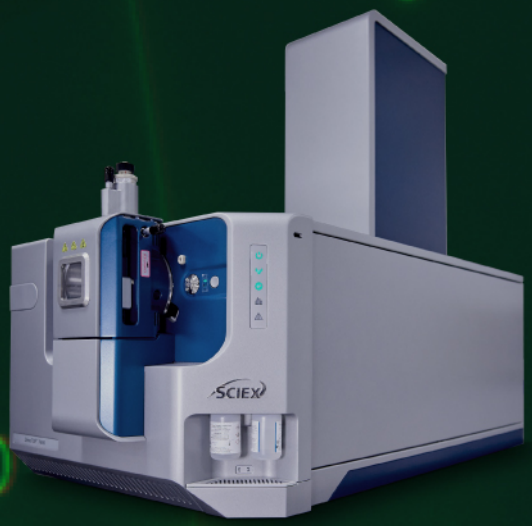

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REVIEW ARTICLE

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Sample preparation and culture condition effects on MALDI-TOF MS identification of bacteria: A review

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an excellent tool for bacterial identification. It allows high throughput, sensitive and specific applications in clinical diagnostics and environmental research. Currently, there is no optimal standardized protocol for sample preparation and culture conditions to profile bacteria. The performance of MALDI-TOF MS is affected by several variables, such as sample preparation, culture media and culture conditions, incubation time/growth stage, incubation temperature, high salt content, blood in the culture media, and others. This review thus aims to clarify why a uniformed protocol is not plausible, to assess the effects these factors have on MALDI-TOF MS identification score, and discuss possible optimizations for its methodology, in relation to specific bacterial representatives and strain requirements.

KEYWORDS

bacteria, culture conditions, diagnostics, MALDI-TOF MS, sample preparation

1 | INTRODUCTION

Accurate and rapid identification of microorganisms is critical in veterinary and human clinical diagnostics, as well as in environmental pollution management. Although many diagnostic laboratories still favor standard morphological and biochemical techniques for bacterial identification, they are giving way to molecular identification procedures. Many of these procedures possess strong differentiation power, but they also suffer from a number of shortcomings, such as being time-consuming, laborious, costly, and complex, requiring high technical skill and support, or having limited availability of primers (Buller, 2004; Okafor, 2011; Topić Popović et al., 2017). A number of challenges such as incorrect identifications, the need for urgent identification of pathogens and their resistance to antimicrobial drugs, as well as the need to identify rare bacteria, led to mass spectrometry (MS)-based microorganism identification (Campos Braga et al., 2013).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has proven to be an excellent tool for bacterial identification. It allows high throughput, sensitive and specific applications in clinical diagnostics and environmental research (Sauer &

Abbreviations: ACN, acetonitrile; BSL-3, biological safety level 3; CHCA, alpha-4-cyano-4-hydroxycinnamic acid (alpha matrix); CMBT, 5-chloro-2-benzothiazolethiol; FA, formic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MCA, MacConkey agar; MHB, Mueller–Hinton broth; MS, mass spectrometry; S/N, signal-to-noise ratio; TFA, trifluoroacetic acid; TSA, tryptone soy agar.

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Kliem, 2010). It enables profiling of bacteria to the genus, species, and even strain-level, although identification of individual strains often requires greater resolution than strain categorization or strain differentiation (Sandrin et al., 2013). Although MALDI-TOF MS is a reliable and rapid technique for phenotypic identification, some barriers remain unresolved. Variability was noted in reproducibility and accuracy related to various bacterial culture media and culture conditions of certain strains (Goldstein et al., 2013; Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019; Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019; Sandrin et al., 2013). At this time, there is no optimal standardized protocol for sample preparation to profile bacteria, but attempts have been made (Cuénod et al., 2021; Drevinek et al., 2012; Liu et al., 2007; Wang et al., 2021). Therefore, this review will focus on the assessment of effects of culture media and conditions on identification score at the strain level, as well as on effects of different sample preparation protocols on overall identification success, as they influence the overall performance of the MALDI-TOF MS method.

2 | MALDI-TOF MS METHOD AND BACTERIAL ANALYSIS

MALDI-TOF MS (Figure 1) is a vital tool in bacterial diagnostics, generating protein mass spectra which are used to group and identify bacteria. Such protein mass spectra mainly contain m/z peaks relating to ribosomal proteins relative to their high content in bacterial cells (Ghyselinck et al., 2011; Ryzhov & Fenselau, 2001). Ribosomal proteins are ancient molecules; over one-third of ribosomal protein families are conserved over Bacteria, Eucarya, and Archaea (Lecompte et al., 2002). They are, thus, used for the classification of bacteria, as identification is based on the detection of mass signals specific at genus, species, or subspecies taxonomic levels (Benagli et al., 2012). For ionization of protein samples, the matrix solution is mixed with the sample to be analyzed, enabling the formation of protein mass spectra with specific molecular weight ranges. Measured mass signals are compared with mass spectra from reference bacterial strains collected in a dedicated mass spectra library (library-based approach) or with publicly available proteomics/genomics data (bioinformatics enabled approach) (Sandrin et al., 2013; Topić Popović et al., 2017). There are several sample preparation options for bacterial profiling (Figure 2). The choice of using one over the others may significantly influence the identification score (Figure 1) and success of the method (Schumaker et al., 2012; Wang et al., 2021).

In majority of cases, pure cultures of bacteria grown on a solid medium are prepared for analysis. Typically, 10^4 cells is the minimum sample biomass needed for yielding a mass spectrum of sufficient quality for a reliable identification (Hsieh et al., 2007), but the accuracy and specificity of MALDI-TOF MS identification also depend on the bacterial species analyzed or on the mass spectrum of the

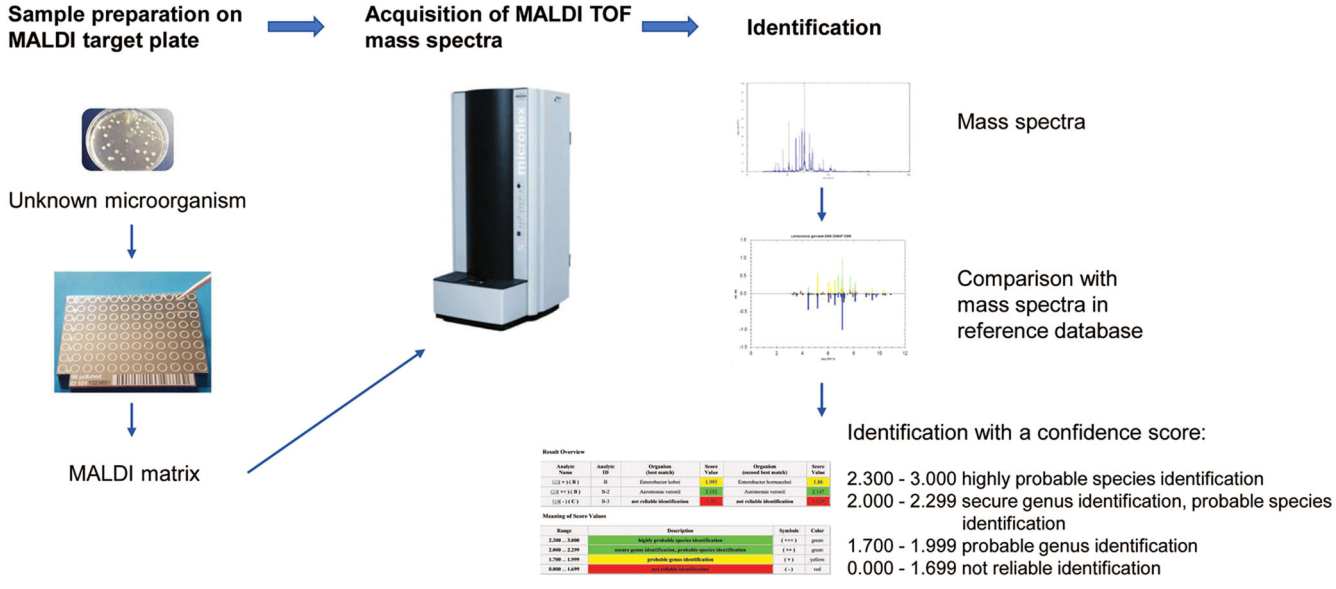

FIGURE 1 Schematic representation of the principles of a typical workflow of matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry bacterial identification. For ionization of protein samples, the matrix solution is mixed with the sample to be analyzed, enabling formation of protein mass spectra with specific molecular weight ranges. Measured mass signals are compared with mass spectra from reference bacterial strains collected in a dedicated mass spectra library or with publicly available proteomics/genomics data [Color figure can be viewed at wileyonlinelibrary.com]

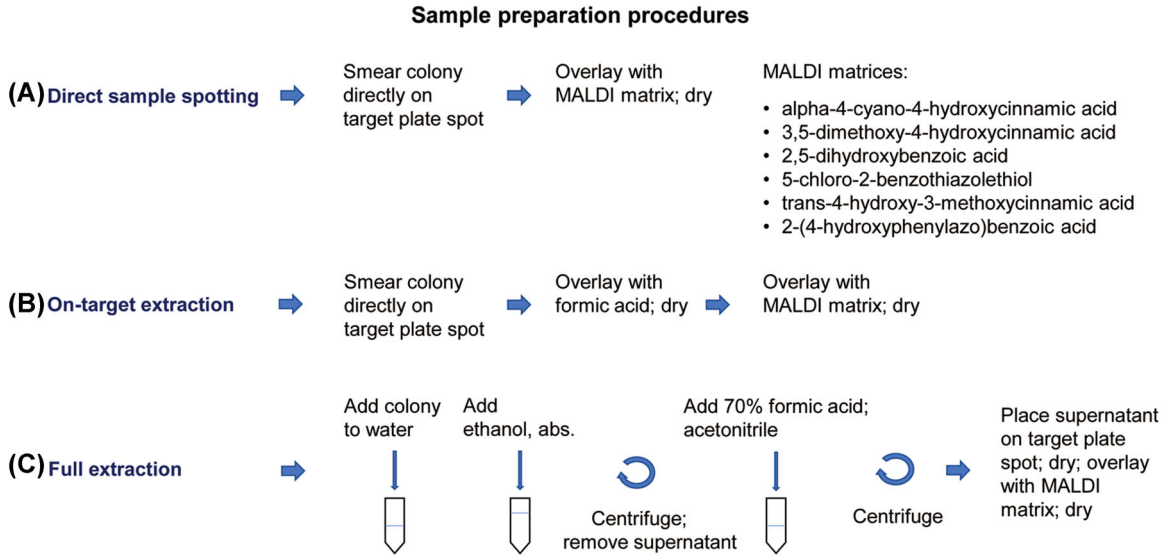

FIGURE 2 Schematic summary of sample preparation procedures in matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry bacterial identification: (A) Direct sample spotting, (B) on-target extraction, and (C) full extraction. The most commonly used matrices are listed [Color figure can be viewed at wileyonlinelibrary.com]

analyzed sample (van Belkum et al., 2017). However, in clinical and particularly in environmental samples, bacteria mostly occur in mixtures and biofilms, presenting a challenge for their accurate identification. One of the many impediments is the isolation and cultivation of pure cultures from such polybacterial samples (Sandrin & Demirev, 2018). In addition, the patterns of protein expression vary with the culture conditions of such mixtures (Reeve & Bachmann, 2019), which might also contain unculturable bacteria for which reference mass spectra do not exist (Stewart, 2012). For these reasons, there has been a considerable interest in the development of MALDI-TOF MS procedures for identification of bacterial mixtures without pure culture isolations (Mandrell et al., 2005; Mörtelmaier et al., 2019; Reeve & Bachmann, 2019; Wahl et al., 2002; Yang et al., 2018; Zhang et al., 2015). Most of these studies used model mixture systems by mixing several bacteria and comparing them to the reference mass spectra of pure cultures. To maximize the performance of the method and before demonstrating the practical application of MALDI-TOF MS in direct characterization, non-model bacterial mixtures with inherently higher variability in mixing ratios need to be investigated.

2.1 | Treatment of Gram-positive versus -negative bacteria

Gram-negative and -positive bacteria sometimes need different treatment due to differences in their cell wall structure. Mycobacteria, for example, have cell walls with low permeability, rich in lipids and peptidoglycans esterified with mycolic acids. These properties call for a treatment of cell walls to release the cellular compounds of interest for MALDI-TOF MS analysis (Balážova et al., 2014). Gram-positive bacteria frequently yield mass spectra with fewer m/z peaks, with a narrower mass range and lower intensities (Smole et al., 2002). Therefore, Gram-positives might require disruption of cell walls by lysis, whether mechanical, enzymatic, chemical, or heat treatment (Vargha et al., 2006). Mass spectra of Gram-positive bacteria have more m/z peaks after lysozyme treatment, with various signal-to-noise (S/N) ratios (R.A. Giebel et al., 2008; Vargha et al., 2006). Indeed, lysozyme treatment gives more effective cell wall disruption and MALDI-TOF MS profile enrichment for both Gram-positive and -negative bacteria, but the signal tends to decrease with the addition of other components to the mixture (Šedo et al., 2011). Trypsin treatment aiming to release more cellular proteins has unsatisfactory results (Liu et al., 2007). Overall, whole or intact bacteria comprise cells suspended in a solution, deposited directly on the target plate spot, while exposure to solvents, acids, or water in the matrix tends to lyse them (Fenselau & Demirev, 2001).

2.2 | Inactivation of potentially pathogenic bacteria

The destruction of bacterial viability without disrupting its protein structure can be attained by numerous

physical methods such as micro-beads, thermolysis, sonication, and corona-plasma discharge treatment (Šedo et al., 2011). Bacterial cells of lower pathogenicity can be inactivated by heating at 95°C for 30 min (Balážova et al., 2014), although it was shown that after contact with the organic compounds of the matrix solvent, such bacteria do not present risk for contamination (Pennanec et al., 2010).

However, potentially pathogenic or highly pathogenic bacteria require different, specific protocols. The sample preparation methods for bacteria requiring biological safety level 3 (BSL-3) calls for their inactivation due to the high biosafety risk (Drevinek et al., 2012). As it is often not possible to operate the MALDI-TOF MS within a BSL-3 laboratory, highly pathogenic bacteria need to be completely inactivated before being handled at a lower safety level (van Belkum et al., 2017; Lasch et al., 2016).

Gamma-irradiation is one alternative for processing highly virulent strains before subjecting them to MS (Moura et al., 2008; Shaw et al., 2004). In some cases, it can reduce the identification score values and thus affect the identification accuracy (Tracz et al., 2013), but generally, it does not induce notable changes of bacterial mass spectra patterns in the 2000–20,000 m/z range (Lasch et al., 2008, 2016). Bacterial spores, however, are more resistant than vegetative bacterial cells. Majority of bacteria are radiation-sensitive and inactivated by 0.5 kGy or less, while spores (of *Bacillus anthracis*, for example) need a minimum dose of 20 kGy (Lasch et al., 2008; Horne et al., 1959).

Chemical extraction methods are also relatively reliable for inactivation of highly pathogenic bacteria, and they comprise ethanol/formic acid (FA) and trifluoroacetic acid (TFA) inactivation (Lasch et al., 2008). The TFA inactivation is by far the best for bacterial endospores, including bactericidal capacity and information content of the mass spectra (Lasch et al., 2016; Liu et al., 2007; Ruelle et al., 2004; Welker et al., 2019). However, when preparing extracts with *B. anthracis* spores, Drevinek et al. (2012) suggest an additional centrifugal filtration step to overcome the biosafety risk of highly virulent strains. Nevertheless, since some highly pathogenic bacteria show unexpected resistance to chemical and physical inactivation treatments (Lasch et al., 2016; Wang et al., 2021), it is essential to develop robust and reliable test methods to upgrade databases of their mass spectra.

2.3 | Bacterial cell concentration

Bacterial cell concentration also influences the number and intensity of m/z peaks (Liu et al., 2007). Depending on the procedure for the protein extraction, for optimal MALDI-TOF MS detection, 10^4–10^5 (Hou et al., 2019) or 10^5–10^7 cells are needed (Freiwald & Sauer, 2009). However, cell concentrations are bacterium-specific, as optimal cell concentrations for *Arthrobacter* spp. with all matrices, based on S/N ratios are $1–5 \times 10^8$ cells μl^{-1} (Vargha et al., 2006). The ideal mass spectra for *Yersinia, Escherichia, Proteus, Morganella*, and *Salmonella* were obtained when samples contained 10^5–10^6 cells, whereas other cell concentrations, higher or lower, could not produce good quality mass spectra (Mazzeo et al., 2006). Overall, only one colony could be used (Freiwald & Sauer, 2009; Tsuchida et al., 2020) or up to five colonies in the case of *Pseudomonas* spp. (Anderson et al., 2012). As the optimal amount for successful identification varies between species, re-tests of the same sample are common. Dilution series of the sample in an assay might increase the success rate but add more workload and reduce the number of samples that can be processed. Therefore, being the most common procedure, re-testing of samples with low initial identification scores is likely more efficient overall. Contamination of cultivation medium should be brought to a minimum by aseptic selection of the bacterial colonies for analysis.

3 | SAMPLE PREPARATION: DIRECT SAMPLE SPOTTING OR EXTRACTION?

To allow the extraction of proteins from bacterial cells, general sample preparation approaches involve three possibilities: (A) direct sample spotting, (B) on-target extraction, and (C) the full extraction procedure (Figure 2) (Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019).

3.1 | Direct sample spotting

Direct sample spotting is the simplest of three approaches. For most bacteria, it involves smearing of a single colony from agar plate directly onto the target plate spot using a sterile toothpick or a swab, allowing it to dry, and adding the matrix (Anderson et al., 2012; Hou et al., 2019; Topić Popović et al., 2017). The choice of the matrix might have a dramatic effect on the m/z peak detection (Nilsson, 1999; Šedo et al., 2011) because it is well known that a matrix can promote ionization of specific families of compounds such as phospholipids, peptides, or proteins (Ruelle et al., 2004). For most bacteria, reliable readings can be attained by the addition of the alpha-matrix (alpha-4-cyano-4-hydroxycinnamic

acid, CHCA), after deposition of bacteria (whether intact cells, lysates, or extracts) on the target plate (Hou et al., 2019). It is commonly used for peptides in the lower mass range (<2500 Da), and results in small homogenous crystals which give good resolution for analysis (Jang & Kim, 2018). Besides CHCA, the most frequently used matrices for bacterial identification are sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 2,5-dihydroxybenzoic acid, 5-chloro-2-benzothiazolethiol (CMBT), ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid, FA), 2-(4-hydroxyphenylazo)benzoic acid (Beavis et al., 1989, 1992; Pennanec et al., 2010; Strupat et al., 1991). The CMBT is a favorable matrix for Gram-positive bacteria (R.A. Giebel et al., 2008; Walker et al., 2002).

For many environmental strains (e.g. *Legionella* spp., which may present biosafety risk and thus needs to be inactivated), the direct sample spotting in comparison with the extraction procedures has no significant differences in terms of identification and quality of the mass spectra (Pascale et al., 2020). However, for anaerobic bacteria, it is inferior to the on-target and full extraction procedures at all time points (Veloo et al., 2014). It is best suited for the high-precision identification of Gram-negative rod-shaped bacteria (Tsuchida et al., 2020). Overall, direct spotting usually gives lower identification rates, and in such cases, additional manual analysis is required (Anderson et al., 2012). It can reliably identify bacteria commonly isolated in clinical laboratories, excluding Gram-positive bacteria and some mycobacteria (Wang et al., 2021). Nevertheless, it is a good first step for identification to reduce the workload of subsequent manual sample extraction (Jussiaux et al., 2021; Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019; Wolk & Clark, 2018).

3.2 | On-target extraction

The on-target extraction also requires a bacterial colony smeared onto a target plate spot. For bacteria with sturdy cell walls or producing exopolysaccharide matrix, such as mucoid bacteria (*Pseudomonas* spp.), further processing is needed (Anderson et al., 2012). Each colony is overlaid by (mostly) 70% FA and after drying, by MALDI matrix (Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019; Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019). The direct deposition of bacteria often involves the treatment of cells with 40% ethanol to eliminate the formation of cell clusters and enhance sample homogeneity and shot-to-shot reproducibility (Madonna et al., 2000; Šedo et al., 2011). It is also possible to treat cells with absolute ethanol before the addition of the matrix solution (Ruelle et al., 2004). Some operators place intact cells in a mixture of solvent and matrix before administering them onto the target plate spot, resulting in good resolution to the strain level (Moura et al., 2008). It was observed that measurements performed in a linear positive-ion-mode result in higher sensitivity than in the reflector mode, producing mass spectra with sufficient precision and resolution (Freiwald & Sauer, 2009).

The environmental *Photobacterium damselae* subsp. *piscicida* samples prepared by the on-target extraction method were better identified than by the direct sample spotting and the full extraction method, yielding highly probable species-level identification, in all tested culture media, for all incubation times and in all replicates (Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019). Similarly, for clinical *Streptococcus salivarius* and *Lactobacillus* spp., all isolates were identified after the on-target extraction. Surprisingly, some misidentifications occurred at the genus level, while all misidentifications with the direct sample spotting occurred at the species level (Jussiaux et al., 2021). Tsuchida et al. (2021) found that the on-target extraction procedure under controlled humidity conditions might result in high identification scores, particularly for samples prepared at 30%–40% humidity. However, although humidity control effectively increases the identification accuracy for both Gram-positive and -negative bacteria, it does not enable conclusive bacterial identification in all cases (Tsuchida et al., 2021).

3.3 | Full extraction

The full extraction approach is a more complex, but the suitable procedure for inactivation of pathogenic bacteria without spore formation. Sample preparation methods have substantial variability in procedures (Sandrin et al., 2013). One of the most used variations is to suspend a loopful of a bacterial colony is in liquid chromatography-MS-grade water, vortex, add ethanol to the suspension and briefly centrifuge. The supernatant is, afterward, discarded and the pellet recentrifuged. After discarding the supernatant, the pellet is dried at room temperature and resuspended in FA. The suspension is mixed by pipetting and acetonitrile (ACN) added, mixed, and briefly centrifuged. Before overlaying with MALDI matrix, supernatant is added to each plate spot and allowed to dry (Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019; Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019). Approximately 10^6–10^7 bacterial cells are needed for this procedure (Freiwald & Sauer, 2009).

The choice of the extraction solvent, such as TFA or ACN, can affect which strain-specific biomarkers will be detected (Nilsson, 1999), although both of these solvents enhance the profile quality of all types of bacteria tested (Šedo et al., 2011). Although the full extraction method is reported to lead to more efficient ionization and higher quality identifications (Anderson et al., 2012), some environmental strains prepared by the on-target extraction were better identified than by the full extraction (Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019). Evermore optimized protocols are published for rapid identification by the adapted full extraction approach, for example for cereulide detection from *Bacillus cereus* cultures, or for identification of *Mycobacterium* spp., with recommended analyses in duplicate or triplicate (Bryson et al., 2019; Doellinger et al., 2020).

To summarize, the choice of sample preparation with direct sample spotting or extraction relies on several crucial facts: Gram-negative bacteria are typically identified with high probability using the direct sample spotting; The identification scores are lower for Gram-positive bacteria, such as *Staphylococcus* and *Enterococcus* (Goldstein et al., 2013; Tsuchida et al., 2020). This difference in confident identification is likely caused by insufficient extraction of proteins due to differences in the cell wall structure between Gram-positive and -negative bacteria (Tsuchida et al., 2020); The on-target extraction is used to promote cell wall disruption, where ethanol/FA is applied over the bacterial smear (McElvania TeKippe et al., 2013); The full extraction procedure is suitable for identification of pathogenic bacteria without spore formation (Sandrin et al., 2013).

4 | IMPACT OF CULTURE MEDIA ON IDENTIFICATION ACCURACY

The fingerprints of a specific bacterial strain could have differences depending on the medium, probably in relation to nutrients from the medium, which can induce or repress the synthesis of specific proteins (Al-Kass et al., 2020; Ruelle et al., 2004; Sandrin et al., 2013; Topić Popović et al., 2017). Accordingly, various nutrients affect the protease production pattern in bacteria (Longo et al., 1999). For example, several strains of *B. subtillis* have large variations in levels of galactose and galactosamine relative to culture medium type, demonstrating the variability in cellular composition (N. Valentine et al., 2005). Also, many components from the rich media are not well defined and consequently, there could be many variables associated with spectral differences (Wunschel et al., 2005).

4.1 | Solid and liquid media

Bacteria grown on solid media are more heterogeneous than in liquid media, but solid media reduce the sample complexity and improve control over the samples (Freiwald & Sauer, 2009). The heterogeneity is related to the fact that bacterial colonies consist of cells varying in age, having the older ones in the center and the newer ones at the perimeter of an agar medium. Conversely, liquid media have more homogenous cell populations, synchronized in growth (Madigan et al., 2009). In liquid media, nutrients are consumed and bacteria reject their waste, thus modifying their environment that stimulates adaptation of bacteria (Pennanec et al., 2010). Therefore, the same bacterium may yield different mass spectra if changing experimental factors such as medium type (Goldstein et al., 2013).

It was shown that bacterial cultures, grown in liquid media, enhance data richness in terms of increasing the number of m/z peaks, as well as spectrum quality when using pure cultures and protein extraction (Goldstein et al., 2013). Cells from liquid media may be more informative for growth phase-dependent physiological studies (Vargha et al., 2006). For direct identification of bacteria from liquid cultures or liquid clinical specimens, purification with short culture on rich media is required, followed by filtration, or combined centrifugation and washing steps (Welker et al., 2019). When Ruelle et al. (2004) compared Plate count and Luria–Bertani agars with Mueller–Hinton broth (MHB), they found that MHB allowed for good-quality mass spectra of both signal intensity and number of m/z peaks for all the tested strains. In contrast, not all strains from Plate count and Luria–Bertani agars yielded good-quality mass spectra, and thus MHB was selected as a preferred medium for identification of *E. coli*, *S. enteritidis*, *S. thyphimurium*, and *Acinetobacter* sp. (Ruelle et al., 2004). Various mycobacterial isolates grown in liquid and solid media were also tested (Balada-Llasat et al., 2013), and MALDI-TOF MS identified 93.8% accurately to the species level and 98.3% to the genus level, irrespective of the medium type. Regardless, protocols exist for washing bacteria from broth cultures to remove the elements of the broth and transferring the rinsed bacterial solution on a target plate spot (N. B. Valentine et al., 2002). However, in daily clinical practice, to attain rapid differentiation, bacteria are first developed on solid media and isolated as colonies (Tsuchida et al., 2020).

4.2 | Blood media

The impact of blood in the media on MALDI-TOF MS profiles was noted (Anderson et al., 2012; Dieckmann et al., 2008; Ferroni et al., 2010; Moura et al., 2008). Hemoglobin present in blood media adds to a variety of proteins affecting the identification (Tsuchida et al., 2020). To the end of limiting interference from blood cells, blood culture media (broths in particular) can be preprocessed, and the steps that follow are the same as for colonies from agar (Wolk & Clark, 2018). As several bacterial strains (*Bacillus* spp. and *Yersinia* spp.) are routinely grown on blood agar as an indicator medium, blood agar is important for developing signatures for these species (N. Valentine et al., 2005). Indeed, it was observed that *Staphylococcus aureus* from Columbia blood agar had extra m/z peaks than cells from Mannitol salt agar, which may be attributed to the blood components (Walker et al., 2002). A similar result was observed with *P. damselae* subsp. *piscicida* as it gave higher signals and S/N ratio if grown on blood agar (Figure 3). These ion signals did not alter the identification of the strain but confirmed that blood in the agar could affect protein mass spectra (Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019). There are a number of such examples; for instance, the profiles of *S. enterica* are specific to particular solid and liquid media and blood-enriched agars. However, its strain-specific m/z peaks are consistent within all the media (Dieckmann et al., 2008). Similarly, *S. aureus* grown on Mueller–Hinton and Columbia blood agars did not have significantly different identification profiles (Bernardo et al., 2002). In addition, higher level of background noise was detected for *Streptococcus pyogenes* grown on a blood agar, although a key set of m/z peaks representing potential biomarkers was consistently observed (Moura et al., 2008). N. Valentine et al. (2005) noted a difference in mass spectra of bacteria cultured on blood agar and regular growth media, with a series of the common reproducible ions for all tested conditions. There were 9 m/z peaks for *B. subtilis*, 11 m/z peaks for *E. coli*, and 8 m/z peaks for *Y. enterocolitica* in common to all culture conditions. The authors established that m/z peaks unique to each culture medium could be used for the identification of unknown samples. Therefore, it can be summarized that the presence of proteins prevailing in all culture conditions (blood agars and other agars) is related to housekeeping genes as many of such genes are constitutively expressed in cells (Savli et al., 2003; Tobisch et al., 1999; N. Valentine et al., 2005). One of the functions of housekeeping genes is protein synthesis, which implies that ribosomal proteins should represent stable protein markers.

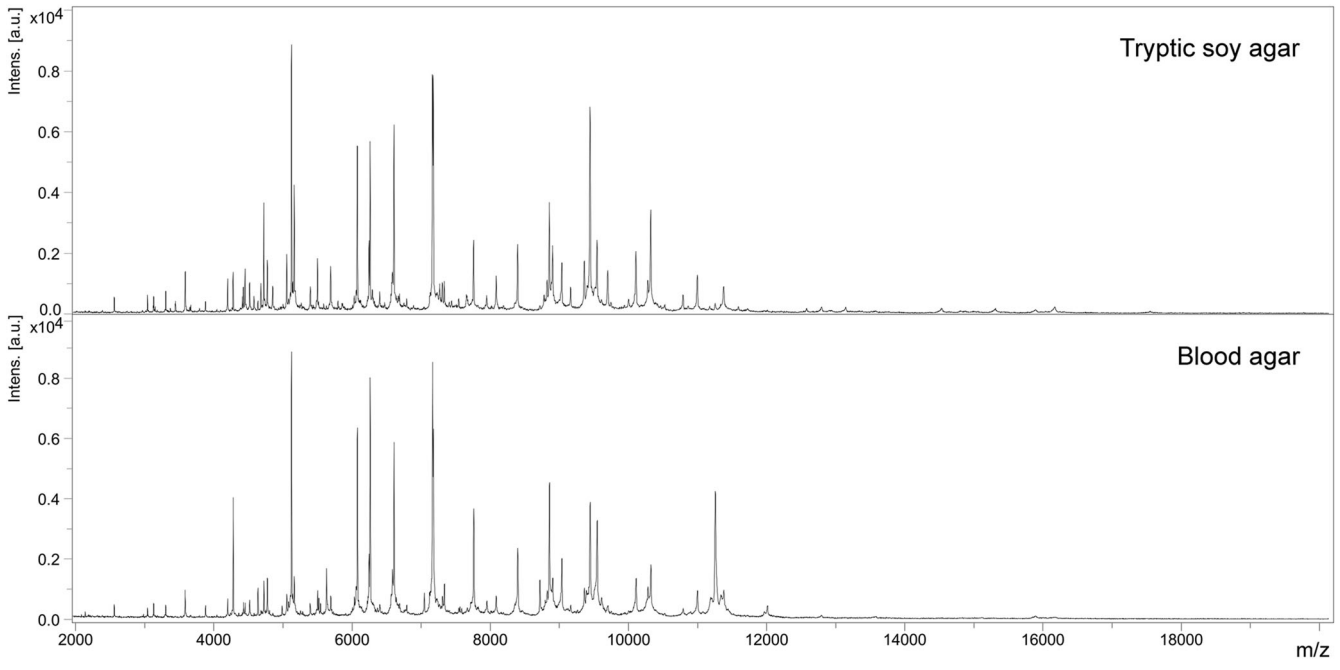

FIGURE 3 Representative mass spectra (m/z 2.000–14.000) for *Photobacterium damselae* subsp. *piscicida* from Blood agar and Tryptic soy agar enriched with 1.5% NaCl. A difference was observed regarding the culture media and successful acquisition of mass spectra. Identification of bacteria grown on Blood agar slightly outperformed identification on the other agar for the direct sample spotting and the on-target extraction methods (Adapted with permission from Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019)

4.3 | Manual and automatic acquisition of mass spectra

Mass spectra are generally considered to be of high quality if mass peaks have significant intensity and S/N ratio, with relatively high resolution (R. Giebel et al., 2010). Mass spectra can be acquired manually and automatically. Manual acquisition of MALDI-TOF mass spectra involves the operator analyzing several locations on a spot of sample. Most software that facilitates operation of MALDI-TOF MS perform automated data acquisition in which the operator provides criteria (e.g., mass peak S/N ratio, minimum mass peak intensity) by which the software determines whether the automatically acquired spectra are of adequate quality (R. Giebel et al., 2010; Schumaker et al., 2012). Schumaker et al. (2012) reported that automated data acquisition yielded less reproducible mass spectra than manual acquisition, while Zhang et al. (2014) sought to optimize automated data acquisition. They found that the base m/z peak resolution may have more limited applicability to microbial characterization with MALDI-TOF MS than to protein identification (Zhang et al., 2014). Moreover, culture conditions and sample preparation may affect the identification results in relation to the mass spectra acquisition procedures. In addition, the limits of taxonomic resolution of MALDI TOF MS profiling might vary across different bacteria, and be bacterium-specific (Sandrin et al., 2013). For example, up to 35% of the *Staphylococcus* spp., *Enterobacteriaceae*, and *Pseudomonas* spp. cultured on Columbia blood agar required manual analysis if using the direct sample spotting, while 10% needed manual analysis if using the extraction method (Anderson et al., 2012). Nevertheless, Balážova et al. (2014) call for the manual acquisition of mass spectra to avoid deterioration of method performance, as they found that automatic acquisition diminishes S/N ratio, data richness, and reproducibility. For *Staphylococcus* spp., the extraction method from blood agar increased identification accuracy to 100% for genus and 90% for species (Anderson et al., 2012). In the latter case, *Staphylococcus* spp. exhibited a lower identification rate if grown on colistin-nalidixic acid agar but was excellently identified from blood agar. Protein extraction enhanced its identification rate from blood agar, although somewhat prolonged the analysis (Lavigne et al., 2013).

5 | IMPACT OF PRESERVATION AND INCUBATION TEMPERATURE

Mass spectra obtained from samples subjected to freezing and lyophilization are generally of low quality and reproducibility. Lyophilized samples had a lower number of consistent m/z peaks (19 m/z peaks), as well as samples frozen in glycerol or dimethyl sulfoxide (20 m/z peaks) compared with 34 m/z peaks obtained with fresh cultures in the work of Mazzeo et al. (2006). Poorer mass spectra could arise from disruption of bacterial cells by freezing and lyophilization, which generally results in poor S/N, making mass spectra less useful.

Different growth temperatures affect the phenotypic properties of bacterial samples grown at various culture conditions. Indeed, the immediate response of bacteria to changing environmental conditions induced by temperature stress is the readjustment of cell-membrane fluidity by the alteration of their phospholipid composition (Ramos et al., 2001). The lipids provide a suitable environment for the functioning of the membrane proteins, which sense changes in the lipid bilayer triggered by a temperature change. Thus, temperature-induced membrane thickness variations affect the activity of membrane-embedded proteins (Mendoza, 2014). Such protein variations are reflected in mass spectra of tested bacteria. For example, the MALDI-TOF MS fingerprinting of *Y. enterocolitica* gave similar profiles from cultures grown at 32°C and 37°C, and less similar from 26°C grown on various media (Wunschel et al., 2005). The authors, thus, concluded that temperature was a greater factor for differences between fingerprints than the media used for culturing *Y. enterocolitica*. The similarity of fingerprints of *Y. enterocolitica* on higher temperatures might be related with the slower growth of the organism at higher temperatures. The incubation temperature of *Vibrio (Listonella) anguillarum* significantly impacted its MALDI TOF MS identification, and 37°C incubation resulted in the most unreliable results in the work of Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., et al. (2019). In that study, temperature was a more detrimental factor for the bacterial growth than salinity of the medium (Figure 4).

Subculturing might also have an effect on misidentifications. Frequent subcultures increased the number of unidentified Gram-positive bacteria, although various incubation temperatures did not alter their identification rate (McElvania TeKippe et al., 2013). Contrarily, there were no misidentifications of clinically relevant enteric Gram-negative bacteria grown at various incubation temperatures and repeatedly subcultured (Ford & Burnham, 2013). The effects of bacterial preservation and temperature stress on MALDI TOF MS identification accuracy thus also seem to be bacterium-specific.

6 | IMPACT OF HIGH SALT CONTENT ON MALDI-TOF MS SIGNATURES

Ion suppression, resulting from the presence of less volatile compounds which can change the test matrix, might decrease the efficiency of the ionization process.

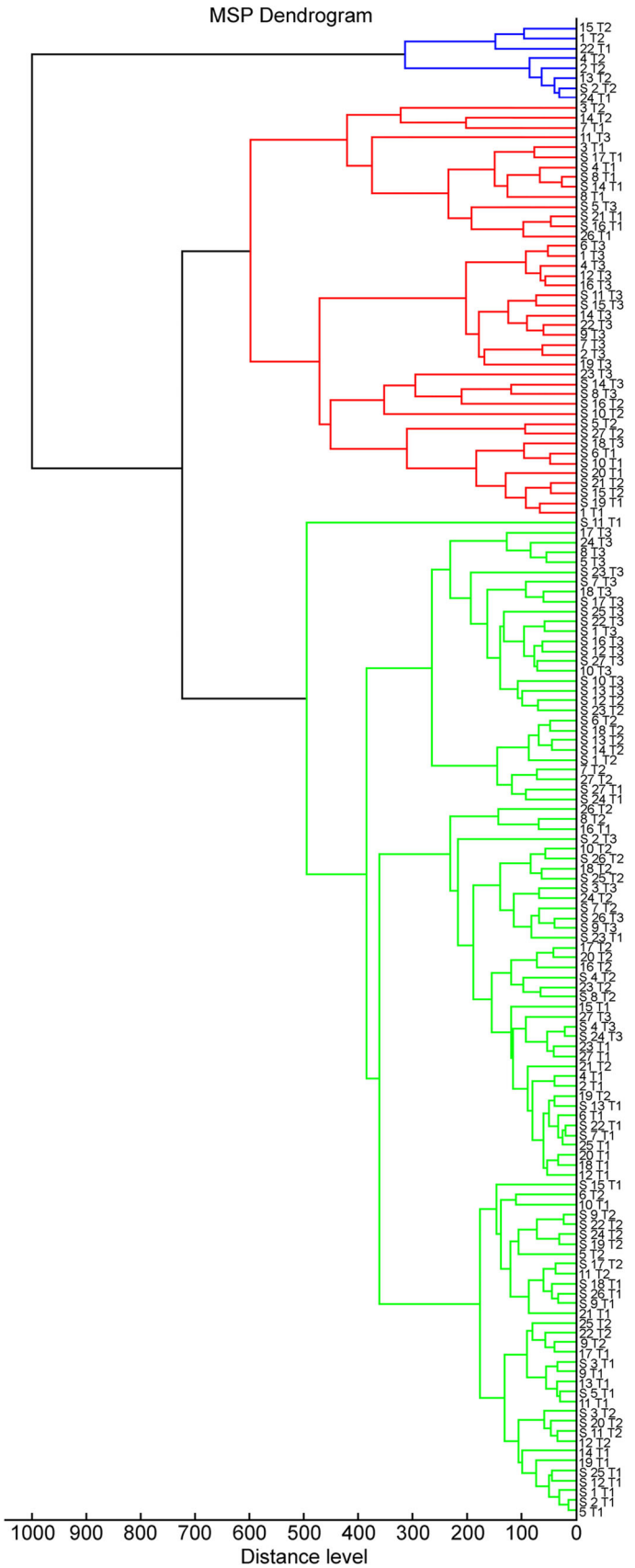

FIGURE 4 Dendrogram calculated on the basis of cluster analysis of mass spectra of identified *Vibrio* (*Listonella*) *anguillarum* strains, analyzed after three time-points when cultured at 15°C, 22°C, and 37°C on Tryptone soy agar (TSA) supplemented with 1.5% NaCl and nonsupplemented TSA. T1 = 15°C, T2 = 22°C, T2 = 37°C; S = NaCl-supplemented TSA medium (Adapted with permission from Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019) [Color figure can be viewed at wileyonlinelibrary.com]

High salt content may thus result in suppression of signal (Annesley, 2003). By desalting the bacterial sample, more species-specific ions are detectable. Ion suppression might be responsible for poorer identification scores of *Pseudomonas* spp. cultured on MacConkey agar (MCA) than on the blood agar (Anderson et al., 2012). MCA inherently contains bile salts as primary ingredients. *Pseudomonas* spp. grown on agars which contain higher levels of salts than MCA (Hektoen enteric and Salmonella-Shigella agar), have even lower species identification scores than bacteria grown on MCA (Anderson et al., 2012). Ruelle et al. (2004) showed that all the ions of identified bacteria in their study were present in mass spectra of both desalted and non-desalted bacteria, but many ions from salt-containing samples were just above the identification baseline.

Interestingly, identification of marine fish pathogen *V. anguillarum* grown on media supplemented with NaCl outperformed identification of cultures from non-supplemented agar (Figure 2) (Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019). That could be connected with the optimum growth conditions and metabolic properties of *V. anguillarum*, being a halophilic bacterium (Naka & Crosa, 2011). The best scores were obtained for strains grown on NaCl-supplemented tryptone soy agar (TSA) at 22°C and incubated for 48 h, followed by incubation at 37°C for 48 h. However, the majority of these isolates were correctly identified under most culture conditions, but only a fraction with highly probable species-level identification. Yet, for most environmental bacteria, a less restrictive cut-off score (≥2.000) (Figure 1) is tolerated for a secure identification (Mougin et al., 2020; Pérez-Sancho et al., 2016).

7 | IMPACT OF INCUBATION TIME/GROWTH STAGE

During their growth stages, bacteria exhibit morphological changes and cell wall composition changes, even if they do not exhibit obvious morphological differences (Arnold et al., 1999). To compare bacterial identifications, samples need to be grown under similar conditions, and be in the similar growth phase, either log or stationary phase (Veloo et al., 2014; Wunschel et al., 2005). Heterogeneous bacteria (bacteria from solid media) should not be screened in the lag phase or in the death phase (Freiwald & Sauer, 2009). Sample preparation after longer cultivation of bacteria could lead to a collection of cells with residues of the medium, as cells tend to attach to the solid medium more over time (Balážova et al., 2014).

The longer cultivation could result in a decrease in spectral quality and the number of consistently reproducible ions. Several studies have reported the impact of incubation time on identification accuracy. It was established that the prolongation of the incubation time affects the correct identification of *V. anguillarum*. The 7-day incubation enabled correct identifications of less than 30% of overall specimens in various culture conditions (Kazazić, Topić Popović, Strunjak-Perović, Babić, et al., 2019). Similarly, for *E. coli*, of all tested cultivation settings, the growth rate had the largest impact (Wunschel et al., 2005). Strains of *E. coli*, *S. enteritidis*, *S. typhimurium*, and *Acinetobacter* sp. grown for 24, 48, and 72 h had diverse mass spectra over time (Ruelle et al., 2004). The 48-h incubation had the highest signal intensity; the 24-h incubation gave the same m/z peaks but lower intensities, while the 72-h led to the disappearance of some m/z peaks for all the strains tested. The same could be speculated for *Arthrobacter* strains as each of the growth stages has comparable mass spectra in various cultivation media. The intensity of m/z peaks increased from the early phase to the mid-log phase, while the intensity of periodic m/z peaks decreased as the cells entered the stationary phase, and disappeared in the stationary phase, giving a unique strain-specific profile (Vargha et al., 2006).

For *Mycobacteria*, however, the identification of species to the genus level was not significantly influenced by cultivation time, and the longer cultivation time (72 h) was optimal for its identification to the species level (Balážova et al., 2014). Comparably, the age of the culture did result in variations in m/z peak intensities of *Legionella* species, but did not influence their identification, nor restrict their recognition by the database (Pennanec et al., 2010). A subset of bacterial proteins change with culture age and at different growth stages and thus influence MALDI-TOF MS spectral variability (Arnold et al., 1999). To overcome this variability for the profiling of *E. faecalis* in various growth/life stages, Kuehl et al. (2011) obtained comparable profiles of *E. faecalis* in an exponential growth phase, viable but not culturable phase, and after 4 and 24 h of reactivation. Changes in the mass spectra during these growth-/life-stage transitions were a consequence of shifts in the molecular composition of the bacterial cell wall during cultivation. The impact of longer cultivation could also be reduced by the method of sample preparation. In particular, the identification of *P. damselae* subsp. *piscicida* is better on younger cultures by direct spotting, but identification of cultures grown for 48 and 72 h is just as reliable if the on-target extraction approach is applied (Kazazić, Topić Popović, Strunjak-Perović, Florio, et al., 2019).

The anaerobic bacteria can be identified as soon as sufficient growth is recorded, after 24 h of incubation. The species-level identification of most anaerobes does not change even after 96 h, while identification of *Actinomyces israelii* is possible only after 96 h of incubation (Veloo et al., 2014). That is probably due to its colony morphology (Saeed et al., 2015) as it is difficult to spot on the target plate or obtain a homogenous suspension for full extraction in its early growth stages.

Currently, there is no clear consensus with regard to whether shorter or longer incubation time is needed for optimum identification. Further research is needed to determine the extent to which incubation time/growth stage affects MALDI-TOF MS identification accuracy of particular bacteria. Markedly, possible interactions between sample preparation procedures, culture conditions, and culture media should be closely investigated, especially for clinically and environmentally relevant bacteria.

8 | CONCLUSIONS

A large proportion of cellular proteins in bacteria are ribosomal proteins, and it is expected that at least some subset of these remains as stable markers for MALDI-TOF MS identification, independent of cultivation conditions (Wunschel et al., 2005). However, sample preparation methods, matrix solutions, and cultivation conditions definitely play a role in MALDI-TOF MS identification to the species level. These methods for characterization of bacteria should thus be optimized to set the key parameters for their discrimination. However, in many clinical cases, both human and veterinary, the choice of media to be used for the growth of unknown bacteria often depend on the clinical signs of disease and are not easy to standardize. We can conclude that bacterial mass spectra cultivated on solid or liquid media can generally be compared with each other and that the impact of the medium type on identification score might be species-specific, and not related to all bacteria under examination. These interactions need further investigation and quantification before protocols for (a) sample preparation, (b) type of media, and (c) culture conditions are optimized and set for environmental and clinical bacterial representatives to obtain good quality mass spectra and probable to highly probable species-level identification.

Apparently, the performance of MALDI-TOF MS depends on the species we want to identify and is limited for the identification of mixed bacterial populations. However, when applying the membrane glycolipid-based approach instead of the protein-based approach, bacterial cultures can be circumvented and polymicrobial infections detected directly (Fondrie et al., 2018; Leung et al., 2017). To that end, bacterial glycolipid mass spectra represent chemical barcodes that identify bacteria, potentially providing a useful alternative to existing diagnostics. Besides non-protein-based approaches, immunological and lectin-based approaches have shown promise to enhance the sensitivity and specificity of MALDI-TOF MS approaches to identify and characterize bacteria (Du et al., 2021; Sandrin et al., 2013).

The success of MALDI-TOF MS identification largely depends on reference databases. For the majority of routine medical and veterinary clinical bacteria, they are fairly adequate. On the contrary, environmental bacteria and new clinical species warrant continuous updating and amending of reference databases. It would be favorable that such databases be constructed with the mass spectra acquired under standardized culture conditions and sample preparation protocols, in relation to specific bacterial representatives and strain requirements.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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