Identifying Anaerobic Bacteria Using MALDI-TOF Mass Spectrometry: A Four-Year Experience

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Because of the special culture requirements of anaerobic bacteria, their low growth-rate and the difficulties to isolate them, MALDI-TOF MS has become a reliable identification tool for these microorganisms due to the little amount of bacteria required and the accuracy of MALDI-TOF MS identifications. In this study, the performance of MALDI-TOF MS for the identification of anaerobic isolates during a 4-year period is described. Biomass from colonies grown on Brucella agar was directly smeared onto the MALDI-TOF target plate and submitted to on-plate protein extraction with 1 μl of 100% formic acid. Sequencing analysis of the 16S rRNA gene was used as a reference method for the identification of isolates unreliably or not identified by MALDI-TOF MS. Overall, 95.7% of the isolates were identified to the species level using the updated V6 database vs 93.8% with previous databases lacking some anaerobic species; 68.5% of the total were reliably identified with high-confidence score values (≥2.0) and 95.0% with low-confidence values (score value ≥1.7). Besides, no differences between Gram-positive and Gram-negative isolates were detected beyond a slight decrease of correct species assignment for gram positive cocci (94.1% vs 95.7% globally). MALDI-TOF MS has demonstrated its usefulness for the identification of anaerobes, with high correlation with phenotypic and conventional methods. Over the study period, only 2.1% of the isolates could not be reliably identified and required molecular methods for a final identification. Therefore, MALDI-TOF MS provided reliable identification of anaerobic isolates, allowing clinicians to streamline the most appropriate antibiotic therapy and manage patients accordingly.

Keywords: MALDI-TOF, mass spectrometry, protein spectrum, anaerobic bacteria, routine identification

INTRODUCTION

Over the last decade, MALDI-TOF MS has demonstrated to be a rapid, accurate and inexpensive alternative for the identification of bacteria species encountered in the microbiology laboratory (Croxatto et al., 2012; Dingle and Butler-Wu, 2013; Rodríguez-Sánchez et al., 2014; Patel, 2015). This technology has proved to be highly useful for the identification of anaerobic bacteria since only
2-3 colonies from agar plates are enough to successfully identify the species they belong to, the identification can be obtained in 5-10 minutes and only a few reagents are needed in very small amounts (Nagy et al., 2012; Schmitt et al., 2013; Garner et al., 2014; Lee et al., 2015; Rodríguez-Sánchez et al., 2016; Xiao et al., 2016; Ferrand et al., 2018).

The level of expertise acquired on the implementation of MALDI-TOF for the identification of anaerobic isolates has also enabled their direct identification from blood cultures (Jeverica et al., 2018) and the determination of their antibiotic susceptibility patterns (Nagy et al., 2011; Treviño et al., 2012). The only drawback of MALDI-TOF MS so far has been the lack of identification of species either missing or underrepresented in the available databases. MALDI-TOF users have detected this limitation, especially in the case of Gram positive anaerobic cocci, underrepresented in the available databases (Veloo et al., 2016). A multicenter study has been performed in order to expand and validate new reference spectra (Main Spectral Profiles, MSPs) corresponding to less common anaerobic bacteria. The input from this study has allowed the V6 database from Bruker Daltonics (Bremen, Germany) - containing 6903 MSPs - to increase the number of MSPs from clinically important anaerobic bacteria and to comprise a higher number of anaerobe species (Veloo et al., 2018).

A previous study carried out in our laboratory demonstrated that the implementation of MALDI-TOF MS for the routine identification of anaerobes reduced the number of isolates that required DNA sequencing analysis for a conclusive species assignment to 3.1% (9/295). Besides, correct species-level identification was achieved in 85.8% of the cases and no misidentifications at the genus level were detected (Rodríguez-Sánchez et al., 2016). Since the database available at that time contained 5627 MSPs and was previous to the enrichment with anaerobic reference spectra we hypothesize that the current database could increase the rate of species-level identification of anaerobic species. For that purpose, we analyzed the anaerobic isolates routinely identified in the Hospital Gregorio Marañón (Madrid, Spain) between 2013 and 2016 using MALDI-TOF MS and the V6 database, enriched in anaerobic species. The reference method in our study was the analysis of the 16S rRNA gene sequence, performed on the isolates not reliably identified by MALDI-TOF MS and on those that belonged to species that had not been evaluated in our previous study.

**MATERIAL AND METHODS**

**Isolates**

During the study period - January 2013 to December 2016-, 4094 anaerobic strains were isolated from clinical samples and subsequently identified in the microbiology laboratory from the Hospital Gregorio Marañón (Madrid, Spain). The isolates belonged to 190 species and 50 genera (Supplementary Table 1). None of the isolates within this study had been included in previous articles focusing on the evaluation of MALDI-TOF for the identification of anaerobic bacteria. *Clostridiodes difficile* was considered in this study as *Clostridium difficile*, since this is how MALDI-TOF MS currently identifies this microorganism, even with the most upgraded library (9234 MSPs) –Bruker Daltonics-.

All clinical samples –sourced from abscesses (32.7%), wound exudates (12.5%), blood (8.4%), peritoneal fluids (8.2%) and others (15.0%)– were cultured on Brucella agar (Becton Dickinson, NJ, USA) and incubated at 35°C for 48 hours in anaerobic conditions. An aerotolerance test was performed on suspect colonies grown on the agar plates and those confirmed as anaerobic bacteria were submitted to identification by MALDI-TOF MS. Only those isolates unreliably identified by MALDI-TOF MS or belonging to a species not encountered previously in our laboratory (Rodríguez-Sánchez et al., 2016) were further identified by DNA sequencing analysis.

Conventional and Genomic Identification of the Anaerobic Isolates

Direct microscopic observation of the bacteria grown under anaerobic conditions was performed. Gram staining was also performed when more than one species from the same clinical sample was suspected and for confirmation purposes. Besides, all those isolates whose identification by MALDI-TOF MS was genus-level, not reliable or yielded a species that had not been previously evaluated in our laboratory were further identified by amplification of the 5’ end 16S rRNA gene with the universal primers 5’-AGAGTTTGATCCTGGCTCAG-3’ and 5’-TTACCG CGGCTGCTGGCA-3’ (Baker et al., 2003; Rodríguez-Sánchez et al., 2016). Further details about the amplification conditions, PCR product purification and sequencing have been provided before (Rodríguez-Sánchez et al., 2014). The identification obtained was interpreted following the CLSI guidelines (CLSI, 2008) and considered as the reference identification of the anaerobic isolates included in this study (Supplementary Table 2).

Identification Using MALDI-TOF MS

All anaerobic isolates were analyzed using a Microflex LT bench top mass spectrometer (Bruker Daltonics, Bremen, Germany). FlexControl 3.3 and Maldi Biotyper 3.1 (Bruker Daltonics) were used for the mass spectrometer control and comparison with the database, respectively. The MBT library (Bruker Daltonics) containing 9234 MSPs was used. All spectra acquired before the V6 database was released were re-identified with it for this study.

Sample preparation has been described elsewhere (Rodríguez-Sánchez et al., 2014). Briefly, it consisted on spotting a small amount of bacteria with a 1μl sterile loop or a toothpick onto a MALDI target plate. An on-target protein extraction step was performed by overlaying the sample with 1μl of 100% formic acid and allowing it to dry at room temperature. Once dried, the spots were covered with 1μl of matrix -α-HCCA, prepared according to the manufacturer’s instructions-. When the mixture was dried, spectra acquisition was performed using default settings and compared with the database.

A Bacterial Test Standard provided by the manufacturer was included in every run for calibration purposes. Default settings (acquisition of mass spectra in the linear positive mode within the 2-20kDa range) were applied. All isolates were analyzed by MALDI-TOF MS in duplicates and the higher score value was recorded as well as the identification provided by MALDI-TOF MS.
| LIST OF MICROORGANISMS | Number of isolates | MICROORGANISMS IDENTIFIED BY MALDI-TOF (%) |
|-------------------------|--------------------|------------------------------------------|
|                         | Species Level      | Genus Level | Not Reliable/No ID | Score ≥2.0 | Score 1.99-1.70 | Score 1.69-1.60 | Score <1.6 |
| **Gram-negative bacilli** |                    |             |                    |            |                |                  |            |
| *Alistipes finegoldii*    | 1                  | 1           | –                  | –            | 1              | –                | –           |
| *Alistipesnderdonkii*     | 5                  | 5           | –                  | –            | 5              | –                | –           |
| *Bacteroides caccae*      | 8                  | 8           | –                  | –            | 7              | 1                | –           |
| *Bacteroides fragilis*    | 359                | 356         | 3                  | –            | 332            | 20               | 2           |
| *Bacteroides ovatus*      | 73                 | 72          | 1                  | –            | 48             | 20               | 3           |
| *Bacteroides pyogenes*    | 11                 | 11          | –                  | –            | 6              | 4                | 1           |
| *Bacteroides thetaiotaomicron* | 152           | 151         | 1                  | –            | 127            | 23               | 2           |
| *Bacteroides uniformis*   | 33                 | 33          | –                  | –            | 32             | 1                | –           |
| *Bacteroides vulgatus*    | 92                 | 91          | 1                  | –            | 65             | 25               | 1           |
| *Bacteroides sp.*         | 32                 | 32          | –                  | –            | 14             | 18               | –           |
| *Bilophila wadsworthia*   | 3                  | 3           | –                  | –            | 1              | 2                | –           |
| *B. sp.*                  | 3                  | 3           | –                  | –            | 1              | 2                | –           |
| *Butyricimonas virosa*    | 1                  | 1           | –                  | –            | 1              | –                | –           |
| *Campylobacter rectus*    | 2                  | 2           | –                  | –            | 1              | 1                | –           |
| *Campylobacter ureolyticus* | 2                | 2           | –                  | –            | 1              | 1                | –           |
| *Capnocytophaga gingivalis* | 3                     | 3           | –                  | –            | 1              | 2                | –           |
| *Capnocytophaga granulosa* | 2                   | 2           | –                  | –            | 2              | –                | –           |
| *Capnocytophaga ochracea* | 2                  | 2           | –                  | –            | 2              | –                | –           |
| *Capnocytophaga sp.*      | 4                  | 4           | –                  | –            | 3              | 1                | –           |
| *Capnocytophaga sp.*      | 3                  | 3           | –                  | –            | 3              | –                | –           |
| *Dialister micraerophilus* | 4                  | 4           | –                  | –            | 4              | –                | –           |
| *Dialister pneumosintes*  | 25                 | 25          | –                  | –            | 25             | –                | –           |
| *Fusobacterium naviforme* | 19                 | 17          | 2                  | –            | 6              | 10               | 2           |
| *Fusobacterium nucleatum* | 61                 | 60          | 1                  | –            | 50             | 10               | –           |
| *Fusobacterium periodonticum* | 135              | 128         | 2                  | 5            | 63             | 53               | 7           |
| *Fusobacterium necrophorum* | 6                   | 6           | –                  | –            | 4              | 2                | –           |
| *Fusobacterium sp.*       | 16                 | 14          | 2                  | –            | 8              | 6                | 2           |
| *Odontobacter splanchnicus* | 1               | 1           | –                  | –            | 1              | –                | –           |
| *Parabacteroides distasonis* | 41               | 41          | –                  | –            | 41             | –                | –           |
| *Parabacteroides goldsteinii* | 6                     | 6           | –                  | –            | 6              | –                | –           |
| *Parabacteroides johnsonii* | 11               | 11          | –                  | –            | 1              | 8                | 2           |
| *Porphyromonas endodontalis* | 2                 | 2           | –                  | –            | 2              | –                | –           |
| *Porphyromonas gingivalis* | 1                  | 1           | –                  | –            | 1              | –                | –           |
| *Porphyromonas somerae*   | 9                  | 9           | –                  | –            | 6              | 2                | 1           |
| *Porphyromonas uenonis*   | 2                  | 2           | –                  | –            | 1              | 1                | –           |
| *Prevotella baroniae*     | 26                 | 26          | –                  | –            | 20             | 6                | –           |
| *Prevotella bergensis*    | 10                 | 10          | –                  | –            | 5              | 5                | –           |
| *Prevotella bivia*        | 53                 | 53          | –                  | –            | 41             | 12               | –           |
| *Prevotella buccae*       | 57                 | 56          | 1                  | 45           | 11             | –                | 1           |
| *Prevotella denticola*    | 37                 | 36          | 1                  | 30           | 6              | –                | 1           |
| *Prevotella disiens*      | 20                 | 19          | 1                  | 11           | 7              | –                | 2           |
| *Prevotella intermedia*   | 55                 | 53          | 2                  | 36           | 17             | –                | 2           |
| *Prevotella melaninogenica* | 52               | 52          | –                  | 19           | 26             | 5                | 2           |
| *Prevotella nigrescens*   | 31                 | 31          | –                  | 24           | 6              | 1                | –           |
| *Prevotella oris*         | 20                 | 20          | –                  | 19           | 1              | –                | –           |
| *Prevotella sp.*          | 87                 | 54          | 17                 | 16           | 31             | 30               | 3           |
| 1578                     | 1514               | 95.9        | 36 (2.3)           | 28 (1.8)     | 1143           | 344 (21.8)      | 35 (2.2)   |
| (72.4)                   |                    |             |                    |              |                |                  |            |
| **Gram-positive cocci**   |                    |             |                    |              |                |                  |            |
| *Acidaminococcus intestini* | 8                  | 8           | –                  | –            | 7              | 1                | –           |
| *Megasphaera micronucleiformis* | 2                  | 2           | –                  | 2            | –              | –                | –           |
| *Veillonella atypica*     | 23                 | 23          | –                  | –            | 21             | 2                | –           |
| *Veillonella dispar*      | 15                 | 14          | 1                  | 10           | 4              | 1                | –           |
| *Veillonella parvula*     | 137                | 137         | –                  | –            | 124            | 12               | 1           |
| *Veillonella ratti*       | 2                  | 2           | –                  | –            | 1              | 1                | –           |
| 187                      | 186                | 99.5        | 0 (0.0)            | 1 (0.5)      | 165 (88.2)     | 20 (10.7)       | 1 (0.5)    |
| (Continued)              |                    |             |                    |              |                |                  |            |
| **Gram-positive bacilli** |                    |             |                    |              |                |                  |            |
| *Actinomyces europaeus*   | 17                 | 16          | –                  | 2            | 14             | –                | 1           |

(Continued)
### TABLE 1 | Continued

| LIST OF MICROORGANISMS | Number of isolates | MICROORGANISMS IDENTIFIED BY MALDI-TOF (%) |
|------------------------|--------------------|------------------------------------------|
| Lactobacillus sp. | 28 26 1 1 2 | Species Level 1 1 1 1 1 |
| Actinomyces meyeri/ | 82 77 1 1 | Genus Level 1 1 1 1 1 |
| odontolyticus | 24 23 1 1 1 | Not Reliable/No ID 1 1 1 1 1 |
| Actinomyces neuii | 17 17 1 1 | Score ≥2.0 1 1 1 1 1 |
| Actinomyces oris | 15 15 1 1 | Score 1.99-1.70 1 |
| Actinomyces radigae | 15 15 1 1 | Score 1.69-1.60 1 |
| Actinomyces tunicenis | 31 31 1 1 | Score <1.6 1 |
| Actinomyces urorogenitalis | 8 8 1 1 |  |
| Actinomyces sp. | 6 6 1 1 |  |
| Actinotignum schaali | 24 23 1 1 |  |
| Alloscardovia omnicolens | 1 1 1 |  |
| Atopobium minutum | 7 7 1 1 |  |
| Atopobium parvulum | 31 30 1 1 |  |
| Atopobium rima | 13 13 1 1 |  |
| Atopobium vaginae | 5 5 1 1 |  |
| Billobacterium longum | 12 12 1 1 |  |
| Billobacterium sp. | 11 11 1 1 |  |
| Blautia cocoides | 1 1 1 1 |  |
| Clostridium clostridioforme | 10 8 1 1 |  |
| Clostridium difficile | 29 25 1 1 |  |
| Clostridium innocuum | 37 36 1 1 |  |
| Clostridium perfringens | 76 73 1 1 |  |
| Clostridium ramosum | 13 13 1 1 |  |
| Clostridium sp. | 55 53 1 2 |  |
| Collinsella aerofaciens | 8 8 1 1 |  |
| Coprobacillus cateniformis | 1 1 1 1 |  |
| Eggertihella lenta | 71 66 1 1 |  |
| Eggertihella catenaformis | 6 6 1 1 |  |
| Eubacterium brachy | 6 6 1 1 |  |
| Eubacterium limosum | 3 3 1 1 |  |
| Eubacterium yunii | 1 1 1 1 |  |
| Flavonifractor plautii | 6 6 1 1 |  |
| Hungstella hathewayi | 13 13 1 1 |  |
| Lachnionaerobaculum 1 | 4 4 1 1 |  |
| Lachnionaerobaculum umeense | 9 9 1 1 |  |
| Lactobacillus fermentum | 10 8 1 1 |  |
| Lactobacillus gasseri | 29 29 1 1 |  |
| Lactobacillus jensenii | 12 11 1 1 |  |
| Lactobacillus paracasei | 28 26 1 1 |  |
| Lactobacillus rhamnosus | 51 50 1 1 |  |
| Lactobacillus sp. | 34 33 1 1 |  |
| Mobiluncus curtisii | 6 3 2 1 |  |
| Leucostoc 1isatis | 11 1 1 1 |  |
| Olsenella uli | 12 11 1 1 |  |
| Propionibacterium acidifaciens | 13 13 1 1 |  |
| Propionibacterium acnes | 409 400 1 1 |  |
| Propionibacterium avidum | 42 41 1 1 |  |
| Propionibacterium granulosum | 10 10 1 1 |  |
| Propionibacterium sp. | 11 1 1 1 |  |
| Propionimicrobiyum lymphophilum | 6 6 1 1 |  |
| Ruminococcus gravis | 3 3 1 1 |  |
| Slackia exigua | 43 43 1 1 |  |
| Treuerralia bernardiae | 7 5 1 1 |  |
| Slobacterium moroei | 35 34 1 1 |  |
| 1406 1347 (85.6) | 23 (1.6) | 36 (2.6) |
| Gram-positive cocci | 866 (63.0) | 448 (31.9) |
| Anaerococcus hydrogenalis | 19 13 4 1 |  |
| Anaerococcus murdocchii | 15 15 1 1 |  |
| Anaerococcus vaginalis | 68 66 2 1 |  |
| Anaerococcus sp. | 32 16 1 1 |  |
| Finegoldia magna | 299 290 1 1 |  |

(Continued)
Among the isolates analyzed, *Bacteroides* was the most commonly encountered genus with 763 isolates included in this study (18.5%); *Propionibacterium* spp. (now *Cutibacterium* spp. [Scholz and Kilian, 2016]) was the second most abundant genus (n=485, 11.8%) followed by *Prevotella* spp. (n=448, 10.9%), *Finegoldia* spp. (n=299, 7.3%) and *Parvimonas* spp. (n=255, 6.2%) (Table 1).

### Identification of Anaerobic Strains

The implementation of MALDI-TOF MS for the identification of anaerobic isolates yielded 95.7% (n=3916), 2.3% (n=94) and 2.1% (n=84) species-level, genus-level and unreliable identifications, respectively (Table 1). For the last two categories 16S rRNA gene sequencing was needed for species assignment (Supplementary Table 2). Besides, 237 isolates identified at the species level by MALDI-TOF MS yielded species that had never been found before in our laboratory and were identified for confirmatory purposes. These isolates belonged mainly to genera *Bacteroides*, *Fusobacterium*, *Prevotella*, *Actinomyces*, *Clostridium*, *Lactobacillus* and *Propionibacterium* (Supplementary Table 2).

From the Gram negative microorganisms, 1514/1578 bacilli (95.9%) and 186/187 cocci (99.5%) were identified at the species level. Most of the isolates not reliably identified belonged to the species *Fusobacterium nucleatum* (n=5) and to the genus *Prevotella* (n=15). Overall, 72.4% of the bacilli and 88.2% of the cocci were identified with high-confidence score values (score ≥ 2.0) and with low-confidence values (score ≥ 1.7, 21.8% of the bacilli and 10.7% of the cocci (Table 1). Besides, 90.0% of the bacilli and 98.4% of the cocci were reliably identified at the species level with score values ≥ 1.8, a cut-off proposed for high-confidence species-level assignment by different authors (Fedorko et al., 2012; Hsu and Burnham, 2014; Rodríguez-Sánchez et al., 2016) - (Supplementary Table 1).

From the Gram positive microorganisms, 1347/1406 bacilli (95.8%) and 869/923 cocci (94.1%) were identified at the species level.

### Ethics Statement

The Hospital Gregorio Marañón Ethics Committee approved and gave consent for the performance of this study (Code: MALDI-Anaerobios). The study has been carried out using microbiological samples, not human products. Therefore, all the conditions to waive the informed consent have been met.
level. Besides, 23 bacilli (1.6%) and 35 cocci (3.5%) were identified at the genus level. The bacilli belonged mainly to the genera *Clostridium* (n=4), *Lactobacillus* (n=5) and *Propionibacterium* (n=10) and the cocci to the genera *Anaerococcus* (n=21) and *Peptostreptococcus* (n=13) – Table 1. Finally, 36 bacilli (2.6%) and 19 cocci (2.1%) could not be reliably identified by MALDI-TOF MS. They belonged mostly to the genera *Actinomyces* (n=6), *Clostridium* (n=8), *Eggerthella* (n=5) and *Propionibacterium* (n=10) in the first case and to *Finegoldia magna* (n=9) in the second case. The lower score values registered lie within this group of unreliably identified isolates (Table 1).

According to the cut-off established by the manufacturer, 68.5% of the isolates (2806) were identified with score values ≥2.0 and 26.5% (1084) with score values ≥1.7, accounting for a total of 95.0% reliable identification. From the remaining 5.0%, isolates belonging to commonly encountered species and well represented in the databases such as *Bacteroides fragilis* or *Prevotella melaninogenica*, were reliably identified despite the low score values.

The enrichment of the available databases has made possible the identification of an increasing number of anaerobic isolates. In our study, 70 isolates that could not be previously identified using older databases obtained correct species-assignment when the Biotyper V6 library or a more upgraded database was applied (Table 2). The addition of reference spectra from anaerobic isolates to this library allowed the identification at the species level of 56/70 isolates (Figure 1). Only 8 isolates belonging to *Prevotella* spp. one *Propionibacterium* spp. and 5 to *Anaerococcus* spp. were identified only at the genus level. Besides, their identification was achieved with score values ≥1.6 in all but 8 cases, but the identification was reliable nonetheless due to the consistency within the top ten identifications provided by MALDI-TOF MS.

**DISCUSSION**

The implementation of MALDI-TOF for the routine identification of anaerobic isolates has allowed the rapid and reliable identification of a high number of anaerobic species. This statement has been demonstrated in the present study: from a large number of isolates analyzed (n=4094), 95.7% of them were correctly identified at the species level. Besides, correlation with phenotypic and conventional methods was shown and consistency with DNA sequencing was demonstrated for a limited number of isolates. Although this is one of the limitations of the study, a previous study carried out by our research team showed 85.8% correct species assignment between MALDI-TOF and DNA sequencing for 295 anaerobic isolates (Rodríguez-Sánchez et al., 2016). The increased percentage of species-level identifications can be explained by the enrichment of the available databases with further reference spectra from anaerobic species.

The ENRIA (European Network of Rapid Identification of Anaerobes) project has represented a significant improvement for the identification of anaerobic isolates using MALDI-TOF MS (Veloo et al., 2018). The addition of well-characterized anaerobic isolates from more than 60 different genera allowed the identification of 79.2% of the isolates included in the validation set. The impact of the enriched library on the identification of Gram positive anaerobic isolates at the species-
The level was also measured: 86.4% using the Biotyper V6 library including the isolates from the ENRIA project versus 69.2% using the previous library version (V5). In our case, the implementation of the Biotyper V6 library allowed the reliable identification of 94.1% of the Gram positive anaerobic cocci from 10 different genera, but failed to identify 19/923 isolates (2.1%). Although the rate of unidentified Gram positive cocci has been reduced to half by implementing the V6 database, these results still pinpoint the need to include further reference spectra from this group of bacteria to future versions of the commercial libraries, but they also render the number of unidentified Gram positive anaerobic cocci similar to other anaerobic groups (1.8% Gram negative bacilli and 2.6% Gram positive bacilli). Thus, this group of bacteria no longer represents a hindrance for MALDI-TOF thanks to the enrichment of the updated libraries with anaerobic isolates. Actually, these rates of unidentified anaerobes represent a realistic number of samples that a routine laboratory can identify by molecular methods without delaying the final identification results or causing unaffordable over-costs.

When anaerobic species are considered globally, correct species assignment of anaerobic species between 70.8% and 91.2% have been reported using different MALDI-TOF MS platforms (Nagy et al., 2012; Schmitt et al., 2013; Garner et al., 2014; Lee et al., 2015; Rodriguez-Sánchez et al., 2016; Xiao et al., 2016; Ferrand et al., 2018). As expected, the lowest rates corresponded to the identification of less common anaerobic species (Ferrand et al., 2018). This fact was also demonstrated in the present study, where infrequent species (e.g. Prevotella disiens, Clostridium subterminale, Mobiluncus curtisi, etc.) could not be identified by MALDI-TOF due to their absence or underrepresentation in the available database. However, other equally infrequent species in our setting were successfully identified (e.g. Murdochiella asaccharolytica or Peptoniphilus lacrimalis) thanks to the reference spectra included in the most recent databases.

Recent studies have also reported rapid and reliable identification of anaerobic isolates directly from blood cultures (Jeverica et al., 2018; Shannon et al., 2018). Jeverica et al. reported 84.9% correct identifications with score values ≥1.6 from blood cultures spiked with anaerobic isolates using 5% saponin while Shannon et al. demonstrated that short-incubation (4-6 hours) of a few drops of blood culture broths allowed at least genus-level identification in 33.0% of the cases in a small set of samples. All in all, MALDI-TOF MS provided a high rate of species-level identifications for anaerobic isolates from clinical samples. The rapid and reliable identification of these isolates has provided clinicians with valuable information about the involvement of these microorganisms in important pathologies such as

| IDENTIFICATION BY VISUAL INSPECTION | IDENTIFICATION WITH BIOTYPER V6 LIBRARY | SCORE |
|------------------------------------|----------------------------------------|-------|
| Peptoniphilus gorbachi            | 1.66                                   |       |
| Peptoniphilus gorbachi            | 1.71                                   |       |
| Peptoniphilus gorbachi            | 1.76                                   |       |
| Peptoniphilus harei              | 1.49                                   |       |
| Peptoniphilus lacoenceniæ         | 2.05                                   |       |
| Peptoniphilus lactimalis          | 2.26                                   |       |
| Peptoniphilus lactimalis          | 2.40                                   |       |
| Peptoniphilus tymalæ              | 2.00                                   |       |
| Peptostreptococcus anaerobius      | 1.58                                   |       |
endocarditis or meningitis (Kestler et al., 2017; Kalay et al., 2019). The results from the present study support these statements. In this scenario, the role of MALDI-TOF MS as a reliable tool for the identification of anaerobic bacteria is becoming critical for laboratory personnel and clinicians alike in order to identify these microorganisms in a rapid and reliable way and provide an optimal management of the affected patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The Hospital Gregorio Marañón Ethics Committee approved and gave consent for the performance of this study (Code: MALDI-Anaerobios). The study has been carried out using microbiological samples, not human products. Therefore, all the conditions to waive the informed consent have been met.

AUTHOR CONTRIBUTIONS

Study design (LA and BR-S). Morphological characterization of the isolates (LA, MZ-C, and MF-C). Identification of the isolates by DNA sequencing (MM). MALDI-TOF identification (AR, LQ, and BR-S) Manuscript writing (BR-S) Manuscript review (LA, MM, PM, and BR-S). All authors contributed to the article and approved the submitted version.

REFERENCES

Baker G. C., Smith J. J., Cowan D. A. (2003). Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55, 541–555. doi: 10.1016/j.mimet.2003.08.009
CLSI (2008). Interpretive criteria for identification of bacteria and fungi by DNA target sequencing; approved guideline. CLSI document MM18-A (Wayne, PA: Clinical and Laboratory Standards Institute).
Croxatto A., Prod’hom G., Greub G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol. Rev. 36 (2), 380–407. doi: 10.1111/j.1574-6976.2011.00298.x
Dingle T. C., Butler-Wu S. M. (2013). Maldi-tof mass spectrometry for microorganism identification. Clin. Lab. Med. 33, 589–609. doi: 10.1016/j.cll.2013.03.009
Fedorko D. P., Drake S. K., Stock F., Murray P. R. (2012). Identification of clinical isolates of anaerobic bacteria using matrix-assisted laser desorption ionization–time of flight mass spectrometry. Eur. J. Clin. Microbiol. Infect. Dis. 31, 2257–2262. doi: 10.1007/s10096-012-1563-4
Ferrand J., Bonnet I., Alauzet C., Lozniewski A. (2018). Evaluation of the Vitek MS and the MALDI Biotyper systems for the identification of less commonly isolated but clinically relevant anaerobes and facultative anaerobes. Anaerobe 54, 210–216. doi: 10.1016/j.ananae.2018.05.014
Garner O., Mochon A., Branda J., Burnham C. A., Bythrow M., Ferraro M., et al. (2014). Multi-centre evaluation of mass spectrometric identification of anaerobic bacteria using the VITEK® MS system. Clin. Microbiol. Infect. 20, 335–339. doi: 10.1111/1469-0691.12317
Hsu Y. M., Burnham C. A. (2014). MALDI-TOF MS identification of anaerobic bacteria: assessment of pre-analytical variables and specimen preparation techniques. Diagn. Microbiol. Infect. Dis. 79, 144–148. doi: 10.1016/j.diagmicrobio.2014.02.007
Jeverica S., Nagy E., Mueller-Premru M., Papst L. (2018). Sample preparation method influences direct identification of anaerobic bacteria from positive blood culture bottles using MALDI-TOF MS. Anaerobe 54, 231–235. doi: 10.1016/j.ananae.2018.05.003
Kalay G. N., Dalgic N., Bozan T., Ulger-Toprak N., Bayraktar B., Soyiletir G. (2019). Polymicrobial Anaerobic Meningitis Caused By Bacteroides Fragilis, Bacteroides Thetaiotaomicron, Fusobacterium Necrophorum And Slackia Exiguia In A Patient With Mastoiditis Following Otitis Media. Anaerobe 56, 95–97. doi: 10.1016/j.ananae.2019.02.003
Kestler M., Muñoz P., Marín M., Goenaga M. A., Idigoras Viedma P., de Alarcón A., et al. (2017). Endocarditis caused by anaerobic bacteria. Anaerobe 47, 33–38. doi: 10.1016/j.ananae.2017.04.002
Lee W., Kim M., Yong D., Jeong S. H., Lee K., Chong Y. (2015). Evaluation of VITEK mass spectrometry (MS), a matrix-assisted laser desorption ionization–time-of-flight MS system for identification of anaerobic bacteria. Ann. Lab. Med. 35, 69–75. doi: 10.3343/alm.2015.35.1.69
Nagy E., Becker S., Šokl J., Urbán E., Kostmárová M. (2011). Differentiation of division I (cfA-negative) and division II (cfA-positive) Bacteroides fragilis strains by matrix-assisted laser desorption/ionization–time–of–flight mass spectrometry. J. Med. Microbiol. 60, 1584–1590. doi: 10.1099/jmm.0.031336-0
Nagy E., Becker S., Kostmárová M., Barta N., Urbán E. (2012). The value of MALDI-TOF MS for the identification of clinically relevant anaerobic bacteria in routine laboratories. J. Med. Microbiol. 61, 1393–1400. doi: 10.1099/jmm.0.043927-0
Patel R. (2015). MALDI-TOF MS for the diagnosis of infectious diseases. Clin. Chem. 61, 100–111. doi: 10.1373/clinchem.2014.221770
Rodríguez-Sánchez B., Marín M., Sánchez-Carrillo C., Cercenado E., Ruiz A., Rodríguez-Créixems M., et al. (2014). Improvement of matrix-assisted laser desorption/ionization–time–of–flight mass spectrometry identification of difficult-to-identify bacteria and its impact in the workflow of a clinical microbiology laboratory. Diagn. Microbiol. Infect. Dis. 79, 1–6. doi: 10.1016/j.diagmicrobio.2014.01.021
Rodríguez-Sánchez B., Alcalá L., Marín M., Ruiz A., Alonso E., Bouza E. (2016). Evaluation of MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization...
Time-of-Flight Mass Spectrometry) for routine identification of anaerobic bacteria. *Anaerobe* 42, 101–107. doi: 10.1016/j.anaerobe.2016.09.009

Schmitt B. H., Cunningham S. A., Dailey A. L., Gustafson D. R., Patel R. (2013). Identification of anaerobic bacteria by Bruker Biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry with on-plate formic acid preparation. *J. Clin. Microbiol.* 51, 782–786. doi: 10.1128/JCM.02420-12

Scholz C. F., Kilian M. (2016). The natural history of cutaneous propionibacteria, and reclassification of selected species within the genus Propionibacterium to the proposed novel genera Acidipropionibacterium gen. nov., Cutibacterium gen. nov. and Pseudopropionibacterium gen. nov. *J. Syst. Evol. Microbiol.* 66, 4422–4432. doi: 10.1099/ijsem.0.001367

Shannon S., Kronemann D., Patel R., Schuetz A. N. (2018). Routine use of MALDI-TOF MS for anaerobic bacterial identification in clinical microbiology. *Anaerobe* 54, 191–196. doi: 10.1016/j.anaerobe.2018.07.001

Trevisán M., Areses P., Peñalver M. D., Cortizo S., Pardo F., del Molino M. L., et al. (2012). Susceptibility trends of *Bacteroides fragilis* group and characterisation of carbapenemase-producing strains by automated REP-PCR and MALDI TOF. *Anaerobe* 18, 37–43. doi: 10.1016/j.anaerobe.2011.12.022

Veloo A. C., de Vries E. D., Jean-Pierre H., Justesen U. S., Morris T., Urban E., et al. (2016). The optimization and validation of the Biotyper MALDI-TOF MS database for the identification of Gram-positive anaerobic cocci. *Clin. Microbiol. Infect.* 22, 793–798. doi: 10.1016/j.cmi.2016.06.016

Veloo A. C. M., Jean-Pierre H., Justesen U. S., Morris T., Urban E., Wybo L., et al. (2018). Validation of a for anaerobic bacteria optimized MALDI-TOF MS biotyper database: The ENRIA project. *Anaerobe* 54, 224–230. doi: 10.1016/j.anaerobe.2018.03.007

Xiao Z., Luo Y., Ye L., Wang R., Zhang Y., Zhao Q., et al. (2016). Evaluation of VITEK matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of anaerobes. *Microbiol. Immunol.* 60, 477–482. doi: 10.1111/1348-0421.12393

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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