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Original Article

Impact of MALDI-TOF MS identification on anaerobic species and genus diversity in routine diagnostics

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ABSTRACT

Objectives: Introduction of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized bacterial identification in the last decade. In 2013, MALDI-TOF MS was implemented for the identification of anaerobic bacteria at our laboratory. This study analyzed the impact of MALDI-TOF MS on the number of different anaerobic genera and species identified in diagnostics.

Methods: 155 anaerobic human clinical isolates, representing the most frequently isolated anaerobic species at our laboratory, were identified by conventional biochemical methods and by a Bruker MALDI Biotyper (Bruker Daltonics, Bremen, Germany). Discrepancies were resolved by partial 16S rRNA gene sequence analysis. In addition, we compared the frequencies of anaerobic genera and species prior to the implementation of MALDI-TOF MS from 2008 to 2012 to the frequencies of anaerobes from 2013 to 2020 when MALDI-TOF MS was used for identification.

Results: The diversity of anaerobic bacteria increased from 12 genera and 20 species in 2012, before the introduction of MALDI-TOF MS, to 16 genera and 31 species in 2013 and to 20 genera and 41 species in 2020 when MALDI-TOF MS was used as primary identification method. MALDI-TOF MS allowed species assignment within closely related species such as the *Bacteroides fragilis* group in accordance with 16S identification, and correctly identified newly described anaerobic species.

Conclusion: Introduction of MALDI-TOF MS identification increased genus and species diversity of the reported anaerobes at our laboratory. Updates to the MALDI-TOF MS database and new species descriptions will further increase the diversity of anaerobic bacteria isolated from infectious processes.

1. Introduction

The isolation and identification of anaerobic bacteria from human clinical specimens is cumbersome and time-consuming due to slow growth, and special nutritional and atmospheric requirements of these microorganisms. Traditionally, anaerobes were identified by morphological and biochemical methods, and by gas liquid chromatographic analysis of metabolic fatty acids [1,2]. The advents of new sequencing technologies led to the description of new anaerobic genera and species, and to the reclassification of existing taxonomic entities [1,3]. Today, 16S rRNA gene sequence homology analysis is considered gold standard for the identification of bacteria including anaerobes; it is however cost- and labor-intensive and not readily available in many diagnostic laboratories [4,5]. Conventional phenotypic methods often fail to identify newly described anaerobes at the species level [1,3,6]. Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) has revolutionized the identification of bacteria in recent years and made identification of anaerobic bacteria easier [5,7–10]. Several studies have evaluated the use of the two most widely used MALDI-TOF MS identification systems, the Bruker MALDI Biotyper and Vitek MS (bioMérieux, Marcy l'Etoile, France) for the identification of anaerobes [11–13]. A recent meta-analysis evaluated 28 studies and showed high reliability and effectiveness of MALDI-TOF MS identification of anaerobic bacteria [14].

Our laboratory introduced MALDI-TOF MS identification of anaerobic bacteria by the Bruker MALDI Biotyper system in 2013.

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Before, anaerobes were primarily identified by conventional biochemical methods. The aim of the present study was to investigate the influence of the implementation of MALDI-TOF MS identification on the number of different anaerobic species and genera in our diagnostic microbiological laboratory. We have evaluated the identification of anaerobes in the pre- and post-MALDI era of our laboratory from 2008 to 2020.

2. Materials and methods

Bacterial isolates and culture conditions. At our laboratory at the Institute of Medical Microbiology of the University of Zurich, Zurich, Switzerland, anaerobic cultures of human clinical specimen are grown on Brucella agar (in-house prepared sheep blood agar plates with hemin and vitamin K; Brucella Medium BBL 211086, Becton Dickinson, Franklin Lakes, NJ) for at least 48 h at 37 °C in an anaerobic chamber (Whitley anaerobic workstation MG1000, Don Whitley Scientific, West Yorkshire, Great Britain). In addition, phenylethyl alcohol agar (BBL 211539, Becton Dickinson) and thioglycollate enrichment broth (BBL 211720, Becton Dickinson) are used depending on the specimen type. As per our institute's algorithm, single anaerobic isolates are identified at the species level whenever possible. Mixed populations of anaerobes are not differentiated and reported as "mixed anaerobic culture". Until 2012, identification of anaerobic bacteria relied on morphological and biochemical characteristics [2,3]. From 2013 on, a Bruker MALDI Biotyper was used for the identification of anaerobes. If no identification was achieved by conventional methods and MALDI-TOF MS, isolates were identified by partial 16S rRNA gene sequence analysis throughout the study period from 2008 to 2020 [3,15,16].

The MALDI-TOF MS validation study comprised 155 clinical anaerobic isolates of our strain collection (Supplementary Table 1). The strains were selected to comprise the anaerobes recovered at our laboratory. Conventional morphological and biochemical identification of the strains was done at the time of primary isolation [2]. The strains were stored in chopped meat carbohydrate broth (in-house prepared enrichment and cultivation broth for anaerobic microorganisms) and subcultured under anaerobic conditions on Brucella agar at 37 °C for 48 h for MALDI identification. Discrepant identifications between conventional methods and MALDI identification were resolved by repeating the conventional identification and by partial 16S rRNA gene sequence analysis that was considered gold standard [3,15,16].

Morphological and biochemical identification. Between 2008 and 2012, anaerobic bacteria were identified primarily by Gram staining, morphological characteristics, conventional biochemical reactions, such as, indole production, nitrate reduction, catalase test, and growth in the presence of vancomycin, colistin, and kanamycin (special potency disks). In addition, gas chromatographic analysis of metabolic fatty acids formed by glucose fermentation was done for all isolates. Additional biochemical tests (e.g. CAMP test or oxidase test), or commercial anaerobic identification systems, such as API 20A (bioMérieux) and RapID ANA II (ThermoFischer Scientific (formerly Remel), Waltham, MA) were added for further differentiation when needed. Group assignment was reported for closely related species (e.g. *B. fragilis* group and *Cutibacterium avidum/granulosum*); when species differentiation could not be achieved [2,3].

16S rRNA gene sequence analysis. Molecular identification of the anaerobic bacteria was done by partial 16S rRNA gene sequence analysis in agreement with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [16], and as described previously [3,15].

MALDI-TOF MS identification. Bacterial isolates were identified by a Bruker MALDI Biotyper as described elsewhere [17]. Briefly, bacterial samples were prepared by the direct colony transfer procedure with or without on-target formic acid extraction or by the manufacturer's tube-based ethanol-formic acid extraction protocol. Direct colony transfer with on-target formic acid extraction was chosen for routine identification of anaerobes due to optimal time-performance ratio [17]. Bruker standard reference database versions V.3.1.2.0 (3995 entries) to V.9 (8468 entries) were used for identification. Database updates were installed regularly (usually annually) by Bruker. Data interpretation was done in line with Bruker's standard criteria; a species cutoff score value of 2.0 and a genus cutoff score value of 1.7 were applied. MALDI-TOF MS species identifications were confirmed by 16S rRNA gene sequence analysis of the corresponding isolate, when appearing for the first time.

Database search and statistical analysis. Laboratory statistic data were extracted from the laboratory information system of our institute (M/Lab, Dorner, Müllheim, Germany) for the years 2008–2020. Statistical calculations were performed with MS EXCEL 2016 (Microsoft, Redmond, WA) and IBM SPSS, version 26 (SPSS Inc., Chicago, IL). Overall differences between the three sample preparation methods were assessed using the Friedman test. The Wilcoxon signed-rank test was used for pairwise comparison of the three methods. A P value of <0.05 was considered statistically significant; a Bonferroni correction was applied (0.05/3) across pairwise comparison.

3. Results

Anaerobic bacterial cultures. In 2008, out of all bacteriological samples 8903 (17.3%) were submitted for anaerobic cultures. The samples included primarily sterile human specimens, particularly aspirates, biopsies, tissue, deep wound swabs, abscesses and cerebrospinal fluid (Table 1). The number of anaerobic cultures increased up to 16711 in 2020 (24.2% of all bacteriological samples). The positivity rate of anaerobic cultures was in the range of 9.3%–12.9% for the years 2008–2020.

Comparison of conventional and MALDI-TOF MS identification. Prior to the implementation of the Bruker MALDI Biotyper for the identification of anaerobic bacteria in 2013, we retrospectively evaluated 155 clinical anaerobic isolates from our institute's strain collection. The analysis included 41 species from 19 genera, among them the most frequently isolated anaerobes at our laboratory, i.e., *Bacteroides* spp., *Clostridium* spp., *Cutibacterium* spp., *Eggerthella lenta*, *Finegoldia magna*, *Parvimonas micra* and *Peptoniphilus* spp. (Table 2, Supplementary Table 1). Applying direct colony transfer sample preparation and Bruker's standard interpretation criteria, i.e., a species cutoff of 2.0 and a genus cutoff of 1.7, a species identification rate of 52.9% (82 out of 155 isolates) was achieved. Twenty-seven out of 155 isolates (17.4%) were identified at genus level only, and 40 out of 155 (25.8%) isolates were not identified by MALDI-TOF MS. Incorrect species assignment by MALDI-TOF MS was observed for six isolates (3.9%): one *Anaerococcus vaginalis* isolate was misidentified as *Anaerococcus hydrogenalis*, one *Bacteroides faecis* isolate as *Bacteroides thetaiotaomicron*, one *Bacteroides xylanisolvens* isolate as *Bacteroides ovatus*, one *Peptoniphilus indolicus* isolate as *Peptoniphilus harei*, and two isolates of *Veillonella dispar* were misidentified as *Veillonella parvula*. Misidentification on genus level was not observed.

By conventional, biochemical identification methods, 122 out of 155 isolates (78.7%) were identified to species level (data not shown), and 33 out of 155 isolates (21.3%) were assigned to genus or group level only, i.e., as *B. fragilis* group (n = 9), *B. ovatus/thetaiotaomicron* (n = 7), *Bacteroides stercoris/eggerthii* (n = 1), *Clostridium* sp. (n = 3), *C. avidum/granulosum* (n = 1), *Peptostreptococcus* sp. (n = 3), *Prevotella* sp. (n = 1), *Varibaculum* sp. (n = 1), *Veillonella* sp.

Table 1
Summary of anaerobic cultures performed at our laboratory from 2008 to 2020.

Year	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	2020
Anaerobic cultures (total)	8903	9484	10088	10887	10762	10164	10686	11796	12840	15622	16100	17276	16711
Positive anaerobic cultures (%)	1150 (12.9)	1086 (11.5)	1219 (12.1)	1227 (11.3)	1087 (10.1)	1125 (11.1)	1230 (11.5)	1224 (10.4)	1292 (10.1)	1459 (9.3)	1814 (11.3)	1793 (10.4)	1783 (10.7)
No. of different species/genera	17/12	16/12	19/12	20/12	20/12	31/16	35/19	38/19	31/20	36/19	38/20	50/25	41/20
Single isolate identifications	442	428	496	532	471	621	661	713	675	816	1069	1072	1071
N = 1 isolate per specimen (%)	440 (38.3)	418 (38.5)	483 (39.6)	520 (42.4)	461 (42.4)	537 (47.7)	593 (48.2)	652 (53.3)	600 (46.4)	739 (50.7)	946 (52.1)	965 (53.8)	954 (53.5)
N = 2 isolates per specimen (%)	1 (0.1)	5 (0.5)	5 (0.4)	6 (0.5)	5 (0.5)	42 (3.7)	31 (2.5)	29 (2.4)	36 (2.8)	37 (2.5)	54 (3.0)	47 (2.6)	45 (2.5)
N = 3 isolates per specimen (%)			1 (0.1)				2 (0.2)	1 (0.1)	1 (0.1)	1 (0.1)	5 (0.3)	3 (0.2)	9 (0.5)
N = 4 isolates per specimen (%)												1 (0.1)	
Anaerobic mixed cultures (not further identified) (%)	709 (61.7)	663 (61.0)	730 (59.9)	701 (57.1)	621 (57.1)	546 (48.5)	604 (49.1)	542 (44.3)	655 (50.7)	682 (46.7)	809 (44.6)	777 (43.3)	775 (43.5)
Negative anaerobic cultures (%)	7753 (87.1)	8398 (88.5)	8869 (87.9)	9660 (88.7)	9675 (89.9)	9039 (88.9)	9456 (88.5)	10572 (89.6)	11548 (89.9)	14163 (90.7)	14286 (88.7)	15483 (89.8)	14928 (89.3)

Table 2
Retrospective identification of 155 anaerobic clinical isolates by MALDI-TOF MS applying direct transfer sample preparation.

Organism	No. (%) of isolates	No. (%) of isolates with MALDI-TOF MS identification level			
		Species identification	Genus identification only	No identification	Misidentification[a]
Bacteroides spp.	32	29 (90.6)	1 (3.1)		2 (6.3)
Clostridium spp.	31	23 (74.2)	5 (16.1)	3 (9.7)	
Cutibacterium spp.	21	5 (23.8)	6 (28.6)	11 (52.4)	
Eggerthella lenta	10	2 (20)	2 (20)	6 (60)	
Finegoldia magna	10	3 (30)	5 (50)	2 (20)	
Parvimonas micra	11	4 (36.4)	4 (36.4)	3 (27.3)	
Peptoniphilus spp.	10	8 (80)		1 (10)	1 (10)
Rare Genera[b]	30	8 (26.7)	4 (13.3)	14 (46.7)	3 (10)
Total	155 (100)	82 (52.9)	27 (17.4)	40 (25.8)	6 (3.9)

[a] The following misidentifications by MALDI-TOF MS on species level were observed (score value ≥ 2.0): *A. vaginalis* was misidentified as *A. hydrogenalis* (n = 1), *B. faecis* as *B. thetaiotaomicron* (n = 1), *B. xylanisolvens* as *B. ovatus* (n = 1), *P. indolicus* as *P. harei* (n = 1), *V. dispar* as *V. parvula* (n = 2).
[b] Rare genera with less than ten isolates.

(n = 5), anaerobic Gram-positive coccus (n = 1), and anaerobic Gram-positive rod (n = 2) (Supplementary Table 2). Of these 33 isolates, 26 isolates were identified at species level by MALDI-TOF MS, and four isolates were assigned to genus level only. For three isolates no identification was achieved by MALDI-TOF MS. The MALDI identifications were confirmed by 16S rRNA gene sequence analysis; except for four isolates for which a discrepancy at species level was observed (*B. thetaiotaomicron* vs. *B. faecis* (n = 1), and *V. parvula* vs. *V. dispar* (n = 3)).

The use of direct colony transfer with on-target formic acid extraction and tube-based ethanol-formic acid extraction significantly increased the species identification rate from 52.9% to 68.4% ($Z = -4.782$, $P < 0.0001$) and to 81.3% ($Z = -6.097$, $P < 0.0001$), respectively (Table 3, Supplementary Table 1). 17.4% of the isolates were identified at genus level only by both direct colony transfer methods; this percentage decreased to 7.7% for tube-based ethanol-formic acid extraction. The rate of non-identified isolates decreased from 25.8% to 9.7% for direct colony transfer with on-target formic acid extraction and to 6.5% for the tube-based extraction method. The rate of misidentification was in the range of 3.9%–4.5% for all three sample preparation methods.

Diversity of anaerobes. In 2008, anaerobic bacteria were isolated in a total of 1150 clinical specimen including particularly aspirates, biopsies, tissue, deep wound swabs, abscesses and cerebrospinal fluid. For 440 (38.3%) specimens a single anaerobic species was identified, and for one (0.1%) specimen two anaerobic species were identified. For 709 (61.7%) specimens we reported a mixed anaerobic culture that was not further identified (Table 1). Until 2020, the number of positive anaerobic cultures increased to 1783. Thereby, 954 (53.5%) specimens with a single anaerobic isolate, 45 (2.5%) specimens with two anaerobic isolates, nine (0.5%) specimens with three anaerobic isolates, and 775 (43.5%) specimens with an anaerobic mixed population (not further identified) were reported.

For the years 2008–2012, anaerobic isolates of 16–20 different species from 12 genera were isolated per year. In 2013, 31 anaerobic species from 16 genera were recovered. This increase was associated with the implementation of MALDI-TOF MS for the

Table 3
Comparison of MALDI-TOF MS sample preparation methods for 155 anaerobic clinical isolates.

Level of identification	Direct colony transfer [No. of isolates (%)]	On-target formic acid extraction [No. of isolates (%)]	Ethanol-formic acid extraction [No. of isolates (%)]
Species identification	82 (52.9)	106 (68.4)	126 (81.3)
Genus identification only	27 (17.4)	27 (17.4)	12 (7.7)
No identification	40 (25.8)	15 (9.7)	10 (6.5)
Misidentification[a]	6 (3.9)	7 (4.5)	7 (4.5)

[a] The following misidentifications by MALDI-TOF MS on species level were observed (score value ≥ 2.0): *A. vaginalis* was misidentified as *A. hydrogenalis* (n = 1), *B. faecis* as *B. thetaiotaomicron* (n = 1), *B. xylanisolvens* as *B. ovatus* (n = 1), *P. indolicus* as *P. harei* (n = 1), *V. dispar* as *V. parvula* (n = 3).

Table 4
Summary of the most frequently isolated anaerobic species (>50 isolates) at our laboratory from 2008 to 2020.

Species	No. of isolates 2008–2020 (%)
Cutibacterium acnes	3749 (41.4)
Finegoldia magna	1187 (13.1)
Bacteroides fragilis/fragilis group	666 (7.4)
Cutibacterium avidum	479 (5.3)
Parvimonas micra	432 (4.8)
Staphylococcus saccharolyticus	180 (2.0)
Clostridium perfringens	173 (1.9)
Veillonella parvula	155 (1.7)
Prevotella bivia	153 (1.7)
Peptostreptococcus anaerobius	87 (1.0)
Bacteroides thetaiotaomicron	78 (0.9)
Cutibacterium granulosum	78 (0.9)
Bacteroides vulgatus	68 (0.8)

identification of anaerobic bacteria in 2013. In the following years 2014–2020, we observed a further increase in anaerobic species diversity; a maximum of 50 anaerobic species from 25 genera were recorded in the year 2019 (Table 1, Supplementary Table 3).

Overall, the most abundant anaerobic species with >50 isolates in the years 2008–2020 were *Clostridium perfringens*, *Cutibacterium acnes*, *C. avidum*, *C. granulosum*, *B. fragilis*, *B. thetaiotaomicron*, *Bacteroides vulgatus*, *F. magna*, *P. micra*, *Peptostreptococcus anaerobius*, *Prevotella bivia*, *Staphylococcus saccharolyticus* and *V. parvula* (Table 4). The frequency of the different species per year from 2008 to 2020 is shown in Fig. 1.

Group assignments such as *B. fragilis* group, *B. ovatus/thetaiotaomicron*, and *C. avidum/granulosum* were used if no unambiguous species assignment was achieved. A decline in the use of these group assignments was observed from 2013 on, and by 2020, group assignments are no longer used (Supplementary Table 3).

Recently described new anaerobic species such as *Acidaminococcus intestini* (2007), *Anaerococcus murdochii* (2007), *B. faecis* (2010), *Fusobacterium canifelium* (2004), *Leptotrichia trevisanii* (2002), *Leptotrichia wadei* (2004), *Murdochiella asaccharolytica* (2010), *Peptoniphilus gorbachii* (2010), *Porphyromonas somerae* (2006) and *Prevotella bergensis* (2006) were identified from 2013 on by MALDI-TOF MS (Supplementary Table 3).

4. Discussion

In the present study, we have evaluated the anaerobic species distribution at our clinical microbiology laboratory between 2008 and 2020. In 2013, we observed a sudden increase in the number of identified anaerobic species from 20 to 31. The number of anaerobic genera increased from 12 to 16 in the same time period. This increase in species and genus diversity was associated with the implementation of MALDI-TOF MS identification by the Bruker MALDI Biotyper system in 2013. Before 2013, identification of anaerobes relied primarily on conventional morphological and biochemical methods. Further increase of anaerobic species was observed in the following years up to 41 species from 20 genera in 2020. Early studies on the identification of anaerobes with MALDI-TOF MS reported poor identification for certain anaerobic species and genera due to limited breadth of the reference databases [4,12,14]. To overcome these limitations the European Network for the Rapid Identification of Anaerobes (ENRIA) was initiated with the aim to improve MALDI-TOF MS based identification of anaerobic bacteria [18–21]. A consortium of European expert laboratories collected and characterized clinical anaerobic isolates and particularly included rare and newly described species. Subsequently, additional reference mass spectra of anaerobic bacteria were introduced into the Bruker database version V6 (6903 entries) [10]. This improved version of the database was installed at our laboratory in late 2016, and presumably accounts for the observed gradual increase of anaerobic diversity. However, we cannot exclude that the increased number of anaerobic cultures performed over time and subsequently the increased number of anaerobic isolates contributed to the higher diversity.

The introduction of DNA sequencing techniques for the identification of microorganisms has led to the description of increasing numbers of anaerobic bacterial species in the last decades [1,4,6], and the discovery of new anaerobic taxa is still on-going [22]. The growing diversity of closely related species made the identification of anaerobes in human, clinical microbiology with conventional morphological and biochemical methods more and more difficult, leading to inconclusive identification or even misidentification [1,3,6]. In our retrospective analysis of 155 anaerobic clinical isolates, we found that MALDI-TOF MS identification was able to correctly identify 26 out of 33 isolates on species level that were previously assigned to genus or group level only by conventional biochemical identification methods, and that these MALDI identifications were in agreement with 16S rRNA gene sequencing results (Supplementary Table 2). In addition, assignment of anaerobic isolates to genus or group level (e.g. *B. fragilis* group) only was more frequent in the years 2008–2012, before the implementation of MALDI-TOF MS identification. These findings highlight the potential of MALDI-TOF MS to discriminate between closely related species [10]. A timely addition of spectra of newly described

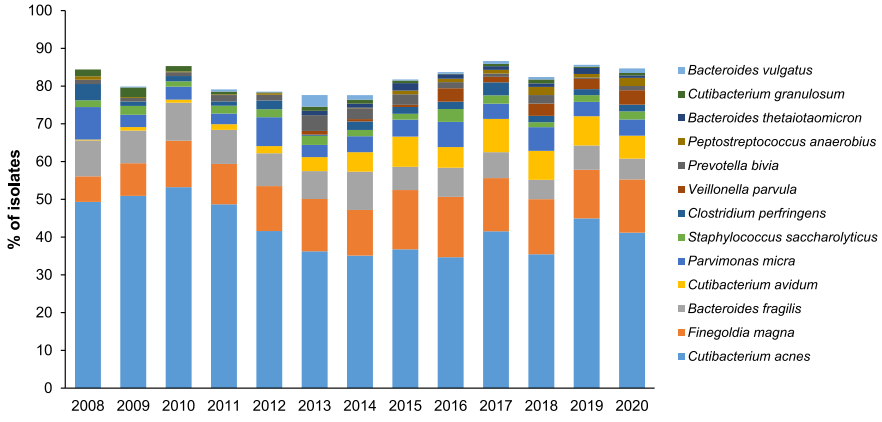

Fig. 1. Frequency of the most prevalent anaerobic species 2008 to 2020 at our laboratory.

anaerobes to the reference databases should be seeked to allow correct species identification [10]. This is underlined by the fact that after the introduction of MALDI-TOF MS in 2013, the detection of recently described new species was achieved; e.g. *M. asaccharolytica* and *P. gorbachii*, both described in 2010.

Discrepancies between MALDI-TOF MS identification and 16S rRNA sequence identification were rare and found primarily for the closely related *B. faecis/B. thetaiotaomicron*, *P. harei/P. indolicus* and *V. parvula/V. dispar* as reported before for the Bruker MALDI Biotyper system [14,18,23–25]. For *Veillonella* spp., sequence heterogeneity within the different 16S rRNA gene copies of a single isolate has been described and accounts for the difficulties in distinguishing *Veillonella* spp. by 16S rRNA sequence analysis [26]. MALDI-TOF MS identification is primarily based on the measurement of ribosomal proteins and therefore possibly also not suited to differentiate *Veillonella* spp. [27].

The most frequently isolated anaerobic species from deep specimen between 2008 and 2020 were *Cutibacterium* spp., *F. magna*, *B. fragilis/fragilis* group and *P. micra*. This is in general agreement with other studies; although a certain variation in the species distribution is observed depending on the pre-selection of the specimen type of the studies [4,14,18]. The high proportion of *C. acnes* may be explained by the association of our laboratory with a large orthopedic university hospital. We have a strong focus on the detection of bone and joint infections, particularly infections with *Cutibacterium* spp. and coagulase negative staphylococci [28,29].

Conclusion. MALDI-TOF MS identification of anaerobic bacteria has proven to be fast, inexpensive and highly-reliable. Implementation of the Bruker MALDI Biotyper system increased the number of different anaerobic genera and species reported from our laboratory. Regular updates to the MALDI-TOF MS databases are needed to keep up with the changing taxonomy of anaerobes and their growing diversity.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.anaerobe.2022.102554.

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