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The influence of incubation time, sample preparation and exposure to oxygen on the quality of the MALDI-TOF MS spectrum of anaerobic bacteria

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Abstract

With matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), bacteria can be identified quickly and reliably. This accounts especially for anaerobic bacteria. Because growth rate and oxygen sensitivity differ among anaerobic bacteria, we aimed to study the influence of incubation time, exposure to oxygen and sample preparation on the quality of the spectrum using the Bruker system. Also, reproducibility and inter-examiner variability were determined. Twenty-six anaerobic species, representing 17 genera, were selected based on gram-stain characteristics, growth rate and colony morphology. Inter-examiner variation showed that experience in the preparation of the targets can be a significant variable. The influence of incubation time was determined between 24 and 96 h of incubation. Reliable species identification was obtained after 48 h of incubation for gram-negative anaerobes and after 72 h for gram-positive anaerobes. Exposure of the cultures to oxygen did not influence the results of the MALDI-TOF MS identifications of all tested gram-positive species. *Fusobacterium necrophorum* and *Prevotella intermedia* could not be identified after >24 h and 48 h of exposure to oxygen, respectively. Other tested gram-negative bacteria could be identified after 48 h of exposure to oxygen. Most of the tested species could be identified using the direct spotting method. *Bifidobacterium longum* and *Finegoldia magna* needed on-target extraction with 70% formic acid in order to obtain reliable species identification and *Peptoniphilus ivorii* a full extraction. Spectrum quality was influenced by the amount of bacteria spotted on the target, the homogeneity of the smear and the experience of the examiner.

Keywords: Anaerobic bacteria, identification, MALDI-TOF MS, pre-analytical conditions, quality spectrum
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Introduction

The introduction of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) into routine diagnostic microbiology laboratories has brought a major revolution in the identification of clinically relevant bacteria. The great advantages of this technique are the opportunity to identify bacteria within minutes and the small amount of bacteria that is needed for identification [1]. This especially has an impact on the identification of fastidious, slow-growing and difficult to identify micro-organisms such as anaerobic bacteria [2], for which identification takes days and is not always unambiguous. Also this technique has shown superiority over conventional phenotypic identification methods and was considered to be as reliable as 16S rRNA gene sequencing [3,4].

It is generally advised by the manufacturer to use log phase bacterial cells for identification by MALDI-TOF MS. Three methods are available for the (pre-) treatment of the bacterial

cells: the direct spotting method, the on-target extraction and the full extraction. The extraction methods aim to disrupt the cell wall of the bacterial cells, resulting in better and more reproducible protein spectra. The amount of bacteria needed to obtain a useful protein profile for identification has an influence on the quality of the obtained spectrum [5–7].

It was hypothesized by Johnson *et al.* [8] that anaerobic bacterial species may be sensitive to free oxygen radicals that may damage the cell wall. Therefore, it is possible that the exposure of anaerobic strains to oxygen influences the quality of the spectrum. This was investigated during this study. Because the growth of anaerobic bacteria differs between genera and species, we also aimed to determine the influence of the anaerobic incubation time on the quality of the MALDI-TOF MS spectra. Finally, we tested the reproducibility of the procedure by spotting a set of anaerobic species ten times.

TABLE 1. Anaerobic isolates used to study pre-analytical factors that may influence the quality of the MALDI-TOF MS spectra

Bacterial strain	Origin
Gram-negative bacteria	
Bacteroides thetaiotaomicron	DSM2079
Bacteroides stercoris	Clinical isolate
Parabacteroides johnsonii	Clinical isolate
Prevotella intermedia	ATCC49046
Prevotella oris	Clinical isolate
Alistipes onderdonkii	Clinical isolate
Fusobacterium necrophorum	Clinical isolate
Fusobacterium nucleatum	Clinical isolate
Campylobacter ureolyticus	Clinical isolate
Veillonella parvula	Clinical isolate
Gram-positive bacteria	
Parvimonas micra	DSM20468
Finegoldia magna	DSM20470
Peptoniphilus harei	DSM10020
Peptoniphilus ivorii	DSM10022
Atopobium minutum	DSM20586
Clostridium butyricum	Clinical isolate
Clostridium hathewayii	Clinical isolate
Clostridium ramosum	DSM1402
Actinomyces israellii	DSM43320
Actinomyces graevenitzii	Clinical isolate
Actinomyces meyeri	Clinical isolate
Bifidobacterium dentium	ATCC27678
Bifidobacterium longum	Clinical isolate
Propionibacterium acnes	Clinical isolate
Eggerthella lenta	DSM2243
Collinsella aerofaciens	Clinical isolate

Material and Methods

Bacterial strains

The bacterial species used in this study are presented in Table 1. The identification of the clinical isolates was obtained using MALDI-TOF MS. To study the influence of incubation time, clinically relevant anaerobic strains representing different genera were selected. To determine the reproducibility of spotting, species producing large, medium and small-size colonies were included as well as species producing dry and pitting colonies [9]. Species that had the highest change to be affected by exposure to oxygen, gram-negative anaerobes, gram-positive anaerobes which decolorize easily during gram-staining and gram-positive anaerobes with a thick cell wall were used to study the influence of oxygen [9,10].

Strains were revived from storage at −80°C and subcultured on Brucella Blood Agar (BBA, Mediaproducts, Groningen, the Netherlands), supplemented with hemin (5 mg/L) and vitamin K_1 (1 mg/L). Plates were incubated in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) at 35°C using the Concept 400 anaerobic chamber (Ruskinn Technology, Sanford, ME, USA).

MALDI-TOF MS

MALDI-TOF MS measurements were performed using the microflex MALDI-TOF MS system and the Biotyper 3.0 software (Bruker Daltonik, GmbH, Bremen, Germany). Direct spotting was performed by transferring bacterial cells directly on to the stainless-steel target. After drying at room temperature 1 μL matrix HCCA (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and left to dry at ambient temperature. The on-target extraction was performed by adding 1 μL 70% formic acid to the spotted bacteria. Immediately after drying at ambient temperature 1 μL of matrix was added. For full extraction, bacterial suspensions were prepared by transferring 1 μL loopfull of bacteria in 300 μL sterile distilled water. After obtaining a homogeneous suspension by vortexing or pipetting, 900 μL absolute ethanol was added. The suspension was centrifuged at 13 000 *g* for 2 min and the supernatant was decanted. The centrifugation step was repeated and the remaining supernatant was carefully removed by pipetting. The pellet was resuspended in 30 μL 70% formic acid and 30 μL acetonitrile. After centrifugation at 13 000 *g* for 2 min, 1 μL of the supernatant was spotted on the target. Immediately after drying at ambient temperature the spot was overlaid with 1 μL of matrix. If applicable, ethanol suspensions were stored at −20°C until full extraction was performed.

The instrument was calibrated using a bacterial test standard (BTS, Bruker Daltonik). The identification criteria used were those recommended by the manufacturer (Bruker Daltonik). Log scores of ≥2 were considered reliable species identifications. Log scores of ≥1.7 and <2.0 were defined as reliable genus identification and log scores of <1.7 as no reliable identification.

The reproducibility of spotting

The reproducibility (i.e. variation in spotting) was determined by spotting the same strain 10 times on the target. To

determine inter-examiner variation, the experiment was performed by two examiners, an experienced one and a less experienced one. To increase log scores an on-target extraction with 70% formic acid was performed for *Peptoniphilus ivorii*, *Propionibacterium acnes*, *Bifidobacterium longum*, *Bifidobacterium dentium*, *Atopobium minutum*, *Actinomyces meyeri*, *Actinomyces graevenitzii*, *Actinomyces israellii* and *Eggerthella lenta*. The range of the log scores, standard deviation and the number of strains with no reliable identification (log score ≤1.7), reliable genus identification (log score 1.7–2) and species identification (log score ≥2) were determined.

The influence of incubation time on the quality of MS spectra

Bacterial strains (Table 3) were subcultured on BBA in an anaerobic atmosphere at 37°C. MALDI-TOF MS was performed after 24, 48, 72 and 96 h of incubation. Direct spotting and an on-target extraction were performed at each incubation time. As the identification results are not influenced by exposure to oxygen (see Results section) the spotting was performed on the bench. However, the exposure to oxygen was kept as short as possible. Full extractions were performed if the direct spotting method or the on-target extraction did not yield a log score of ≥2.

The influence of exposure to oxygen on the quality of the MS spectrum

Strains (Table 4) were subcultured on BBA for 48 h in an anaerobic cabinet at 37°C and spotting on the target took place in the anaerobic cabinet (no exposure to oxygen), once by direct spotting and once by on-target extraction with 70% formic acid. If no identification was obtained for gram-positive anaerobic bacteria the ethanol suspension for full extraction was prepared in the anaerobic cabinet. The direct spotting method and on-target extraction were performed after 1, 6, 24 and 48 h of exposure to oxygen at ambient temperature. Full extractions were performed if the direct spotting method or the on-target extraction did not yield a reliable species identification (log score <2).

Results

Reproducibility in spotting

Nineteen species representing 14 different genera were each spotted 10 times on the target and analysed using MALDI-TOF MS by two examiners. The results are summarized in Table 2. For *Bacteroides thetaiotaomicron*, *Fusobacterium necrophorum*, *Veillonella parvula* and *Clostridium butyricum* a log score ≥2 was obtained each time by both examiners. Examiner 2 also repeatedly obtained a reliable species identification for *Parvimonas micra*, *B. longum*, *A. minutum* and *Propionibacterium acnes,* whereas examiner 1 did not always obtain this result. For *B. dentium*, *Fusobacterium nucleatum*, *A. meyeri* and *Prevotella intermedia* both examiners produced similar results (i.e. either a species or genus identification for *F. nucleatum* and *P. intermedia* was produced). In the case of *B. dentium* and *A. meyeri* sometimes no peaks were found. Examiner 2 obtained better results for the identification of *E. lenta*, *A. israellii*, *Finegoldia magna*, *Campylobacter ureolyticus* and *A. graevenitzii*, while examiner 1 obtained better results for *P. ivorii*. The differences

TABLE 2. The range of log scores and identification results of spotting the same strain ten times by two different examiners

	Examiner 1					Examiner 2				
Strain	Range	SD	No reliable ID (*n*)[a]	Genus ID (*n*)	Species ID (*n*)	Range	SD	No reliable ID (*n*)[a]	Genus ID (*n*)	Species ID (*n*)
Gram-negative bacteria										
B. thetaiotaomicron	2.031–2.198	0.052	0	10	10	2.080–2.257	0.052	0	10	10
P. intermedia	1.958–2.121	0.050	0	10	8	1.976–2.092	0.042	0	10	8
F. necrophorum	2.263–2.387	0.050	0	10	10	2.187–2.418	0.072	0	10	10
F. nucleatum	1.596–2.108	0.152	1	9	4	1.192–2.107	0.269	1	9	5
C. ureolyticus	0.962–2.012	0.859	8	2	1	1.700–2.043	0.826	2	8	2
V. parvula	2.306–2.402	0.039	0	10	10	2.182–2.416	0.071	0	10	10
Gram-positive bacteria										
P. micra	1.687–2.338	0.233	1	9	6	2.202–2.399	0.058	0	10	10
F. magna	0.959–2.116	0.344	1	9	5	1.831–2.108	0.075	0	10	7
P. ivorii[b]	1.255–1.743	0.162	7	3	0	1.162–1.637	0.137	10	0	0
A. minutum[b]	1.831–2.528	1.244	4	6	5	2.183–2.481	0.105	0	10	10
C. butyricum	2.054–2.244	0.070	0	10	10	2.009–2.265	0.086	0	10	10
A. israellii[b]	1.114–1.314	0.639	10	0	0	1.968–2.006	0.838	8	2	1
A. graevenitzii[b]	1.338–2.191	0.926	5	5	2	1.851–2.250	1.082	4	6	4
A. meyeri[b]	1.946–2.239	1.116	6	4	3	1.308–2.217	0.961	6	4	2
B. dentium[b]	1.912–2.304	0.908	2	8	7	2.163–2.388	0.971	2	8	8
B. longum[b]	1.780–2.229	0.150	0	10	6	2.018–2.218	0.071	0	10	10
P. acnes[b]	2.014–2.177	0.713	1	8	8	2.058–2.316	0.088	0	10	10
E. lenta[b]	1.263–1.851	0.833	8	2	0	2.104–2.311	0.929	2	8	8

[a]A log score <1.7 or no peaks.
[b]An on-target extraction was performed using 70% formic acid.

between the two examiners are also reflected in the SD values. Further experiments were mostly performed by examiner 2.

The influence of incubation time on identification

For the gram-negative anaerobic bacteria six different species representing five different genera were tested (Table 3). Reliable species identification (log score ≥2) was obtained for four species (60%) after 24 h of incubation using the direct spotting method. After 48 h species identification was obtained for all six tested species, using the direct spotting method. After 96 h of incubation the results did not change dramatically. It can be noted that if no species identification was obtained, the log score was between 1.9 and 2.0.

Fourteen different species representing nine different genera were tested for the gram-positive anaerobic bacteria (Table 3). Overall, direct spotting was inferior to on-target or full extraction at all time-points, although several species could be identified to species level by direct spotting. The majority of strains (86%) were identified by direct spotting after incubation of 96 h. After 24 h of incubation, species identification was obtained for 11 species (79%) by either the direct spotting method, on-target extraction with 70% formic acid or full extraction. At 72 h of incubation 93% of the strains were identified to species level by either direct spotting or full extraction. *A. israellii* was identified at the species level only after 96 h of incubation using the direct spotting method with a log score just above the threshold (log score 2.03). This was also observed for *P. ivorii*, which was identified at the species level after 72 h of incubation (log score 2.02) using the full extraction method, but could only be identified at the genus level at the other three time-points.

TABLE 3. The influence of incubation time on the quality of MALDI-TOF MS spectra. Log score values for species identification are presented, obtained by the direct spotting method. The on-target extraction and the full extraction methods were performed if the direct spotting method yielded a log score < 2

Species	Incubation time (h)			
	24	48	72	96
Gram-negative bacteria				
Bacteroides thetaiotaomicron	2.23	2.21	2.17	2.19
Prevotella intermedia	1.95[a]	2.03	2.27	2.04
Fusobacterium necrophorum	2.27	2.18	2.31	2.29
Fusobacterium nucleatum[b]	2.33	2.19	2.22	2.17
Campylobacter ureolyticus	1.90	2.07	2.01	1.92[a]
Veillonella parvula	2.29	2.45	2.38	2.37
% Reliable species ID direct spotting	60	100	100	83
% Reliable species ID total	60	100	100	83
Gram-positive bacteria				
Parvimonas micra	2.33	2.29	2.30	2.27
Finegoldia magna	2.51[c]	2.43[c]	2.23[c]	2.21
Peptoniphilus harei	2.02[c]	2.23	2.19[c]	2.20
Peptoniphilus ivorii	1.78[c]	1.86[d]	2.02[d]	1.88[d]
Atopobium minutum	1.96[a]	2.26	2.29	2.25
Clostridium butyricum	2.29	2.13	2.17	2.08
Clostridium hathewayii	2.20[d]	2.36	2.26	2.36
Actinomyces israellii	<1.7[e]	<1.7[e]	<1.7[e]	2.03
Actinomyces graevenitzii	2.09[c]	2.36	2.00[d]	2.11
Actinomyces meyeri	2.27	2.15	2.12	2.20
Bifidobacterium dentium	2.30	2.12	2.37	2.28
Bifidobacterium longum	2.18[c]	2.08[c]	2.07	2.01
Propionibacterium acnes	2.14[c]	2.24	2.10	2.16
Eggerthella lenta	2.21[c]	2.20	2.20[d]	2.10[d]
% Reliable species ID direct spotting	29	71	57	86
% Reliable species ID total	79	86	93	93

[a]Both extraction methods yielded a log score lower than the one obtained with the direct spotting method.
[b]The MALDI-TOF MS system identified *F. nucleatum* either as *F. nucleatum* or *F. naviforme*.
[c]On-target extraction with 70% formic acid.
[d]Full extraction.
[e]With all three sample preparation methods no reliable identification or no peaks were obtained.

The influence of pre-treatment on the identification of species belonging to different genera

The same species were used as for determining the influence of incubation time on the quality of the spectra. In order to make a proper comparison only the results after 48 h of incubation (which is the regular incubation time of anaerobic plate cultures in routine laboratories) were taken into account (Table 3). For all tested gram-negative anaerobic bacteria (six species, belonging to five different genera) a log score ≥2 was obtained using the direct spotting method. Of the 14 different gram-positive anaerobic species tested (belonging to nine different genera), 10 could be identified at the species level using the direct spotting method. For the identification of *B. longum* and *F. magna* an on-target extraction with 70% formic acid was needed. *P. ivorii* could only be identified at the genus level using the full extraction method and *A. israellii* could not be identified at all.

The influence of exposure to oxygen on identification

Nine gram-negative species were tested. Exposure to oxygen for up to 48 h did not influence the identification of the tested species using direct spotting at all time-points. *F. necrophorum* could not be identified anymore (no peaks) after 24 h of exposure to oxygen. To confirm this finding more strains of *F. necrophorum* were tested, which all gave the same results (data not shown). *P. intermedia* could not be identified anymore after 48 h of exposure to oxygen. *B. thetaiotaomicron, Bacteroides stercoris*, *Parabacteroides johnsonii*, *F. nucleatum, Prevotella oris*, *Alistipes onderdonkii* and *V. parvula* could be identified at the species level using the direct spotting method, regardless of the time that the cultures were exposed to oxygen for.

Eleven different gram-positive anaerobic species representing eight genera were exposed to oxygen prior to MALDI-TOF MS analyses. No significant influence of exposure to oxygen was noted on the identification of the test species either after direct spotting or after on-target or full extraction, with the exception of *Actinomyces graeventizii* after 48 h. The method

necessary to obtain species identification when spotted in the anaerobic cabinet was not remarkably different to that after exposure of the cultures to oxygen for 48 h (Table 4).

Discussion

The correct identification of bacteria by MALDI-TOF MS depends on two main factors, the quality of the spectrum of the unknown bacterium and the quality and number of reference spectra present in the MALDI-TOF MS database [4]. The latter is in principal determined by the manufacturer of the MALDI-TOF MS system, but individual laboratories can add reference spectra to their database. The quality of the spectrum of the unknown bacterium may be influenced by several factors; for example, the growth phase of the bacterial cells, the thickness of the bacterial cell wall, the extraction method used, the reproducibility of spotting and in the case of anaerobic bacteria the exposure of bacterial cells to oxygen.

TABLE 4. The influence of exposure to oxygen on the quality of the MALDI-TOF MS spectrum. Log score values for a species identification are presented, which were reached by direct spotting. For all strains an on-target extraction was performed and for the gram-positive anaerobic bacteria also a full extraction, if the direct spotting method did not yield a log score ≥2

	Exposure to oxygen (h)				
Species	***0***	***1***	***6***	***24***	***48***
Gram-negative bacteria					
Bacteroides thetaiotaomicon	2.14	2.24	2.10	2.17	2.35
Bacteroides stercoris	2.19	2.14	2.18	2.25	2.27
Parabacteroides johnsonii	2.23	2.32	2.29	2.31	2.28
Fusobacterium necrophorum	2.08	2.30	2.19	<1.7[a]	<1.7[a]
Fusobacterium nucleatum[b]	2.08	2.27	2.32	2.19	2.08
Prevotella intermedia	1.92	1.93	1.86	1.95[c]	<1.7[a]
Prevotella oris	2.08	2.17	2.01	2.35	2.29
Alistipes onderdonkii	2.21	2.24	2.29	2.29	2.21
Veillonella parvula	2.25	2.13	2.2	2.20	2.16
% Reliable species ID direct spotting	89	89	89	78	78
% Reliable species ID total	89	89	89	78	78
Gram-positive bacteria					
Finegoldia magna	2.24	2.05[c]	2.34	2.52	2.50
Peptoniphilus harei	2.15	2.00	2.13	2.23[b]	2.19
Peptoniphilus ivorii	<1.7[d]	1.81[e]	<1.7[d]	1.76[e]	1.80[e]
Clostridium hathewayii	2.36	2.23	2.29	2.07	2.19
Clostridium ramosum	2.03	2.19	2.27	2.06	2.17
Actinomyces graevenitzii	2.05[c]	2.03[e]	2.11	2.06[e]	<1.7[d]
Actinomyces meyeri	2.03	2.27	2.35	2.19	2.25
Bifidobacterium longum	2.00[c]	2.12[c]	1.99[c]	2.15	2.17
Propionibacterium acnes	2.18	2.14[c]	2.12	2.13	2.13
Eggerthella lenta	2.11	2.30	2.27	2.16	2.02[e]
Collinsella aerofaciens	2.26[c]	2.16	2.23[c]	2.32[c]	2.28[c]
% Reliable species ID direct spotting	64	55	73	64	64
% Reliable species ID total	91	82	82	91	82

[a]With direct spotting and on-target extraction, no reliable identification or no peaks were obtained.
[b]The MALDI-TOF MS system identifies *F. nucleatum* either as *F. nucleatum* or *F. naviforme*.
[c]On-target extraction with 70% formic acid.
[d]With all three sample preparation methods no reliable identification or no peaks were obtained.
[e]Full extraction.

The studies performed on the experimental factors influencing the quality of the spectrum all agree on one thing: the quality of the smear is important [5–7]. This means that the amount of bacteria spotted on the target is important. In order to obtain a spectrum of good quality using the full extraction method, the initial solution should contain a minimum of 5×10^6 to 1×10^7 bacterial cells/mL [11]. For the direct spotting the amount of bacteria is less defined. When a too small amount of bacteria is used for the smear, no peaks with sufficient intensity are obtained. A too large amount of bacteria causes saturation of the detector and only prominent peaks are measured. The latter also causes pollution of the MALDI-TOF MS system, which interferes with its accuracy and shortens time between maintenance [12]. The optimal concentration of bacteria in order to obtain a good quality spectrum was determined to be 130 $\mu g/\mu L$ [13]. In general it was concluded that a heavy smear with an on-target extraction with 70% formic acid gave the best results [5–7]. Because it is difficult to define 'heavy or light smear' we have chosen to determine the variation in spotting if one strain is spotted ten times by two examiners, an experienced one (examiner 2) and a less experienced one (examiner 1). Examiner 2 had a more constant way of spotting the strains on the target than examiner 1. Strains with smooth colonies and/or good to normal growth, *B. thetaiotaomicron*, *F. necrophorum*, *V. parvula*, *C. butyricum*, *P. micra*, *B. longum*, *A. minutum* and *P. acnes*, have a small range in the log scores and were identified at the species level (examiner 2). Species with small colonies, rough colonies or a thick cell wall have a larger range of identification scores and are not always identified at the species level. Especially for these strains it is difficult to obtain a smear that is heavy enough and homogeneous.

Fournier *et al.* [14] compared the direct spotting method with the full extraction method for the identification of anaerobic bacteria. Using the full extraction method, 77.7% of the tested strains could be identified at the species level (log score ≥2). With the direct spotting method 64.7% could be identified at the species level. The difference between the two methods was not significant. However, the direct spotting method resulted in lower log scores for the *Clostridium* genus and gram-positive anaerobic cocci. For the identification of gram-positive aerobic cocci it is recommended to use the full extraction method [15]. Schmitt *et al.* [16] evaluated the use of on-target extraction with 70% formic acid for the identification of anaerobic bacteria. Using this simple extraction method they were able to identify 71% of the isolates at the species level and 92% at the genus level. As none of the studies compared the direct spotting method with the two different

extraction methods, we decided to compare all three methods with each other for the quality of identification of the different genera of anaerobic bacteria. For reliable species identification, on-target extraction was necessary for *F. magna* and *B. longum*. Even though a spectrum was obtained for *P. ivorii*, the obtained log score was repeatedly <2.0, and also when the full extraction method was used. It is unclear why *P. ivorii* could not be identified at the species level, because the reference spectrum of the same strain is present in the database.

As MALDI-TOF MS detects mostly 16S ribosomal proteins [11], it is recommended to use bacterial cells in the log phase of their growth in order to obtain a spectrum of good quality. Because the different anaerobic species show different growth rates, for example *Bacteroides fragilis* grows faster than *Bilophila wadsworthia*, we have investigated the influence of the incubation time on the log score. For most tested species, species level identification was obtained after 24 h of incubation, which did not change even after 96 h of incubation. Only one species, *A. israellii*, could be identified only after prolonged incubation (96 h). However, the variation in spotting shows that this species is sometimes correctly identified and at other times no reliable identification was achieved. This is probably caused by the fact that the colony morphology of this species makes it difficult to spot the bacterium evenly on the target or to obtain a homogeneous suspension for full extraction. Prolonging the incubation time for *Actinomyces* sp. in general did not result in a higher identification rate (data not shown). The spotting reproducibility experiment (Table 2) indicated that the non-identification of *A. israellii* after <96 h of incubation is probably a spotting problem. McElvania TeKippe *et al.* [6] determined the optimal conditions for the identification of gram-positive bacteria with MALDI-TOF MS. Among others, they looked at the influence of the incubation temperature on the quality of the spectra. Subsets of common gram-positive isolates were incubated at different temperatures for 5 days. No influence of the incubation temperature was encountered, indicating that it is not necessary for the bacterial cells to be in the log phase of their growth in order to be identified with MALDI-TOF MS. The accompanying paper by Ford *et al.* [5] described that the incubation temperature of gram-negative bacteria does influence the quality of the spectrum. In our study we did not encounter any influence of the incubation time of anaerobic bacteria, either gram-negative of gram-positive, on the quality of the spectrum. This makes it possible to perform MALDI-TOF MS as soon as sufficient growth is obtained, which might be already after 24 h of incubation.

The extraction methods (on target and full extraction) aim to release the present proteins. As it is known that oxygen can damage the cell wall of anaerobic bacteria [8], we determined whether exposure to oxygen has an influence on the quality of the spectra. After 6 h exposure no differences were seen between the methods. Species identification was obtained with either the direct spotting method or the on-target extraction. No full extraction was necessary. Even after 48 h exposure to oxygen most strains could still be identified at the species level. Of all tested species, *P. intermedia* could not be identified anymore (log score <1.7) after 48 h of exposure to oxygen and *F. necrophorum* could not be identified anymore (no peaks) after 24 h and 48 h of exposure to oxygen. We hypothesize that the cell wall of *F. necrophorum* and *P. intermedia* is damaged by the oxygen [8], causing proteins to leak out of the cell, which can therefore not be measured anymore by MALDI-TOF MS.

From this study we can conclude that the incubation time and time of exposure to oxygen do not influence the quality of the spectrum of a wide variety of anaerobic bacteria. A full extraction is in most cases not necessary in order to obtain good species identification. The quality of the spectrum and therefore whether an unknown strain can be identified with MALDI-TOF MS depends mainly on the examiner who is spotting, the type of the colony, the amount of the bacteria spotted and of course whether there are sufficient reference spectra present in the MALDI-TOF MS database.

In order to obtain a reliable species identification for anaerobic bacteria we recommend performing the MALDI-TOF MS measurement after 48 h of incubation in an anaerobic environment, spotting the bacteria on the same day, performing an on-target extraction with 70% formic acid for gram-positive anaerobic bacteria and having the spotting performed by an experienced examiner.

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Transparency Declaration

The authors declare that they have no conflict of interests. A. C. M. Veloo, P. E. Elgersma, A. W. Friedrich and E. Nagy declare that they have no conflict of interests. A.J. van Winkelhoff declares stock ownership of LabOral International

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