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Original article

Quality of MALDI-TOF mass spectra in routine diagnostics: results from an international external quality assessment including 36 laboratories from 12 countries using 47 challenging bacterial strains

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ABSTRACT

Objectives: Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) is a widely used method for bacterial species identification. Incomplete databases and mass spectral quality (MSQ) still represent major challenges. Important proxies for MSQ are the number of detected marker masses, reproducibility, and measurement precision. We aimed to assess MSQs across diagnostic laboratories and the potential of simple workflow adaptations to improve it.

Methods: For baseline MSQ assessment, 47 diverse bacterial strains, which are challenging to identify by MALDI-TOF MS, were routinely measured in 36 laboratories from 12 countries, and well-defined MSQ features were used. After an intervention consisting of detailed reported feedback and instructions on how to acquire MALDI-TOF mass spectra, measurements were repeated and MSQs were compared.

Results: At baseline, we observed heterogeneous MSQ between the devices, considering the median number of marker masses detected (range = [2–25]), reproducibility between technical replicates (range = [55%–86%]), and measurement error (range = [147 parts per million (ppm)–588 ppm]). As a general trend, the spectral quality was improved after the intervention for devices, which yielded low MSQs in the baseline assessment as follows: for four out of five devices with a high measurement error, the measurement precision was improved (p-values <0.001, paired Wilcoxon test); for six out of ten devices, which detected a low number of marker masses, the number of detected marker masses increased (p-values <0.001, paired Wilcoxon test).

Discussion: We have identified simple workflow adaptations, which, to some extent, improve MSQ of poorly performing devices and should be considered by laboratories yielding a low MSQ. Improving MALDI-TOF MSQ in routine diagnostics is essential for increasing the resolution of bacterial identification by MALDI-TOF MS, which is dependent on the reproducible detection of marker masses. The heterogeneity identified in this external quality assessment (EQA) requires further study. **Aline Cuénod, Clin Microbiol Infect 2022;•:1**

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Introduction

Matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS) is a commonly used method for microbial species identification in modern diagnostic laboratories [1–3] due to its minimal hands-on and short turnaround time, cost-efficiency, and high accuracy [4,5].

Multiple studies have shown the improved resolution gained by using marker-based analytical approaches [6–9] compared to pattern matching approaches. This insight has led to the development of marker-based databases for bacterial identification [8,10], such as the PAPMID database (Mabritec AG, Riehen, Switzerland) [11]. In such approaches, specific peaks of interest, whose presence is associated with a species [12], lineage [13], or even mobile genetic elements [14,15], are queried in the acquired mass spectrum in order to increase specificity and resolution. Many of the peaks, which can be reproducibly detected in MALDI-TOF mass spectra, correspond to protein subunits of the bacterial ribosome [16]. A high MALDI-TOF mass spectral quality (MSQ) is required in order to reproducibly detect marker peaks.

Despite the success of MALDI-TOF MS for routine microbial species identification, multiple clinically-relevant species are currently not distinguished by commonly used databases using

pattern matching approaches. Possible reasons for this are that (a) the databases are incomplete, (b) the species of interest resemble closely other species in the databases, and (c) MALDI-TOF mass spectra are of low quality. We previously compiled a diverse set of 47 bacterial strains, representing 39 species and 15 genera, which are difficult to be identified at a species level for the above-mentioned reasons [17]. In this previous publication [17], we defined the following five important spectrum features as good proxies for MSQ: (a) the number of ribosomal marker peaks detected, (b) the median relative intensity of ribosomal marker peaks, (c) the sum of the intensity of all detected peaks, (d) a high measurement precision, and (e) reproducibility of peaks between technical replicates. Determining these MSQ features, we previously assessed the performance of different sample preparation protocols on different bacterial groups and consequently proposed to use the formic acid overlay protocol for unknown samples and group specific protocols for highest MSQ [17]. Whether the proposed protocols can effectively increase MSQ of these challenging strains in routine settings has yet to be evaluated.

The aim of this study was therefore to assess (a) the MSQ obtained in routine diagnostics, (b) whether there are routine practices associated with an increased MSQ, (c) whether the MSQ can be improved using the protocols proposed, and (d) compile a reference

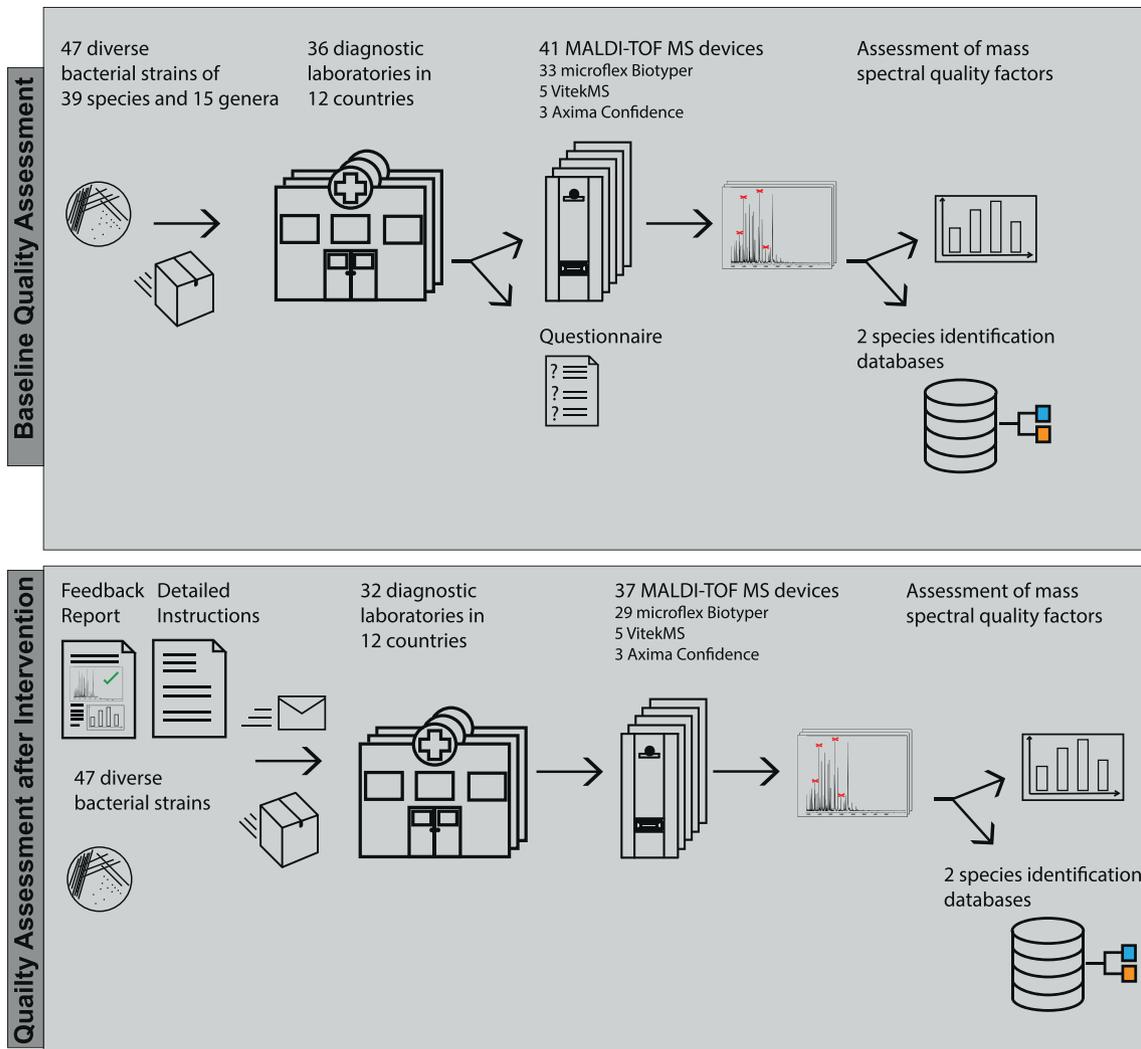


Fig. 1. Overview on the workflow of the study. The upper panel shows the baseline quality assessment including 36 participating laboratories and the lower panel shows the post-interventional quality assessment including 32 laboratories using the same bacterial strains. MALDI-TOF, matrix assisted laser desorption ionization–time of flight; MS, mass spectrometry.

dataset of MALDI-TOF mass spectra including technical replicates, matching genomic sequences, and extensive metadata.

Methods

Design of the external quality assessment (EQA)

Fig. 1 provides an overview over the workflow of this study.

Bacterial strains

The bacterial strains used in this study have previously been described [17] and their whole genome sequences (see [Supplementary material, Table S1](#)), as well as the previously predicted masses of the ribosomal subunits, are publicly available (<https://osf.io/ksz7r/>). See [Supplementary Methods](#) for more detail on these strains and the participating laboratories.

Baseline MALDI-TOF MSQ assessment

The participating laboratories were asked to culture the bacterial isolates and acquire MALDI-TOF mass spectra according to their

routine diagnostic procedures, which may vary between the laboratories. Each laboratory was asked to fill out a questionnaire on routine laboratory practice.

Intervention

Each participating laboratory received a feedback report on the MALDI-TOF mass spectra acquired for the baseline quality assessment (example in [Supplementary File 1](#)) and instructions on how to acquire MALDI-TOF mass spectra in subsequent measurements of the same strains, aiming to improve the MSQ using a standardised approach (see [Supplementary File 2](#)).

We provided the following two different sets of protocols: (a) a simple ‘generic protocol’ (i.e., ‘formic acid overlay’) for all samples and (b) group-specific sample preparation protocols, aiming at highest MSQ [17] (see [Supplementary File 2](#) for more detail).

MALDI-TOF mass spectra processing

Peaks were picked from raw spectra using default settings by the software included in the microflex Biotyper or the VitekMS/Axima Confidence system (see [Supplementary Methods](#) for more detail).

The raw data acquired on each device, the processed peak list and the species identification results of all databases used can be accessed via the Open Science Foundation (<https://osf.io/ae2nk/>).

We queried each spectrum for the following features to assess the MSQ: (a) the number of ribosomal marker peaks detected, (b) the median relative intensity of ribosomal marker peaks, (c) the sum of the intensity of all detected peaks, (d) the measurement precision, and (e) reproducibility of peaks between technical replicates. As factors (a) through (c) often correlate [17], we have focused on factors (a), (d), and (e) in the main text and figures of this study (see [Supplementary Methods](#)).

Scripts used for spectra evaluation and data visualization can be accessed via GitHub (<https://github.com/appliedmicrobiologyresearch/MALDI-TOF-MS-EQA>).

Databases used for species identification

Each spectrum acquired on a Bruker device was compared to the MALDI Biotyper database (MALDI Biotyper Compass Library, Revision E (v8.0, 8468 MSP, RUO, Bruker Daltonics, Bremen, Germany). Spectra acquired on a Axima Confidence or VitekMS device were analysed with the VitekMS database (v3.2, bioMérieux, Marcy-l'Étoile, France).

Furthermore, we compared each spectrum to a ribosomal marker-based database, either PAPMID™ or PAPMID™ subtyping modules (both Mabritec AG, Riehen, Switzerland, henceforward be referred to as PAPMID).

In the main text of this manuscript, we report the species identification by the PPDIM™ database, as this database (a) allows species identification from spectra acquired on devices of different manufacturers and (b) includes all species represented by our strain set.

To evaluate species identification, we classify the results of the PAPMID™ database into the following accuracy categories: (a) the correct species unambiguously receives the highest score ('correct identification'), (b) the correct species and other species receive the highest score i.e., the identification is correct but ambiguous ('correct multi-species identification'), (c) the score is below the identification threshold and no species identification is possible ('no identification possible'), and (d) the identified species is unambiguously wrong ('wrong identification').

More details about database scores and their interpretations can be found in the [Supplementary Methods](#).

Statistical analysis

We used paired Wilcoxon rank tests when comparing spectra acquired from the same strains and excluded spectra of strains which were missing in one of the sets of interest. We used unpaired Wilcoxon rank tests (Mann Whitney U tests) when comparing spectra acquired from different strains. The nomenclature 'median (lower bound of the interquartile range (IQR), upper bound of the IQR)' was used when referring to data in the running text throughout the study.

All analyses were performed in R (v4.0.3) using the ggpubr (v4.0), the rstatix (v0.7) package, and visualised using ggplot2 (v3.3.5) [20].

Results

Access to data

All MALDI-TOF mass spectra acquired for this study, including peaklists and species identification from three different databases can be downloaded from (<https://osf.io/ae2nk/>). The filled-

out questionnaire (see [Supplementary material, Table S3](#)) provides valuable metadata. The bacterial strains included in this study have previously been whole genome sequenced and the raw reads are publicly available (see [Supplementary material, Table S1](#)).

Heterogeneity in MSQ across diagnostic laboratories

For the baseline quality assessment, we received 5035 spectra measured on 41 devices from the 36 participating laboratories. We observed differences between the devices in MSQ considering the number of marker masses detected (e.g., device 7: median = 25; IQR = [20, 28]; device 32: median = 5; IQR = [3, 14]) (Fig. 2A).

The heterogeneity of MSQ was reflected in varying accuracy in species identification (see [Supplementary material, Table S4](#)). Over all bacterial strains and using a marker-based species identification, the fraction of spectra, which were correctly and uniquely identified to the species level, ranged from 22.5% (18/80 spectra, device 9) to 78.2% (147/188 spectra, device 35). We observed no difference in MSQ between the different MALDI-TOF MS manufacturers and an increasing accuracy of species identification with increasing MSQ (Fig. 2A).

The MSQ differed between bacterial groups with a lower MSQ observed for spectra of gram-positive isolates compared to spectra of gram-negative isolates (marker masses detected: median = 16; IQR = [12, 20] vs. median = 18; IQR = [15, 22]; $p < 0.0001$) (Fig. 2B).

We observed a four-fold difference in measurement error when comparing the most and least precise measurements (device 11: 147 parts per million (ppm), IQR, 109 ppm–192 ppm vs. device 41: 588 ppm, IQR, 533 ppm–631 ppm) (Fig. 2C).

These differences in MSQ are mainly represented by a few participating laboratories, while the data from most laboratories cluster around the overall median = 16; IQR = [13, 19] marker masses detected and 280 ppm, IQR, 177ppm–426 ppm in measurement error.

Routine laboratory practices are associated with MSQ

Every participating laboratory filled out a questionnaire on laboratory practices (see [Supplementary material, Table S3](#)). As in each laboratory different combinations of practices apply, some of which are not reflected in this questionnaire, we cannot identify causative factors of laboratory practices on spectral quality. However, we observed that certain practices correlated with improved MSQ represented by an increased number of ribosomal subunits detected detailed as follows: (a) acquisition of spectra on steel targets compared to disposable targets (median = 17; IQR = [13, 20] vs. median = 13; IQR = [10, 16]; $p < 0.0001$), (b) cleaning steel target plates with 'methanol-acetone' protocol compared to other cleaning protocols (median = 23; IQR = [18, 26] vs. median = 16; IQR = [12, 18]; $p < 0.0001$), (c) regular hardware services by the MALDI-TOF MS provider (median = 17; IQR = [13, 20] vs. median = 15; IQR = [11, 18]; $p < 0.0001$), (d) working with a MALDI-TOF MS workstation (i.e., for a certain period, one or more member of staff are responsible for all MALDI-TOF MS measurements) (median = 17; IQR = [14, 21] vs. median = 16; IQR = [12, 18]; $p < 0.0001$), (e) replacing the matrix solution after 7 or less days (median = 17; IQR = [13, 20] vs. median = 15; IQR = [11, 17]; $p < 0.0001$), and (f) sub-culturing isolates on agar plates after defreezing, or culturing the isolates on agar plates from the ESwab transport medium, compared to strains which were measured directly after culturing on agar plates from frozen stocks (median = 17; IQR = [13, 20] vs. median = 11; IQR = [6, 15.8]; $p < 0.0001$) (see [Supplementary material, Fig. S3](#)) (Fig. 3).

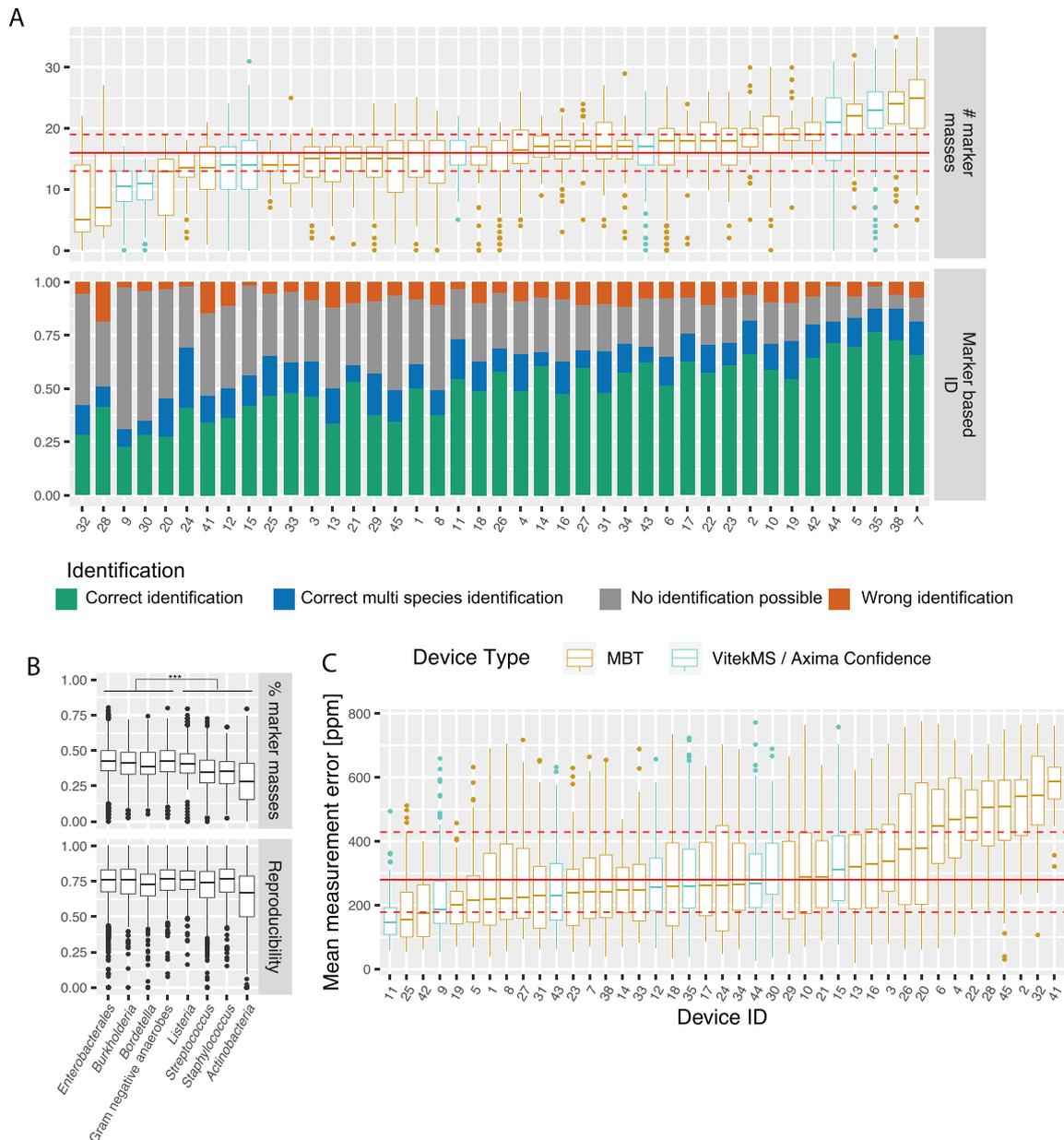


Fig. 2. (A) Number of ribosomal marker masses detected in matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra acquired on 41 devices (upper row) and the evaluation of the species identification results using a marker-based approach (lower row). (B) Relative number of marker masses detected per phylogenetic group (upper row) and reproducibility between technical replicates (lower row). (C) Measurement errors of the different devices. Boxplots: The middle line corresponds to the median, the lower and the upper hinge depict the first and the third quartile, whereas the whiskers extend from the hinge no further than 1.5 times the interquartile range. Data points beyond this range are depicted as individual points. MBT, microflex biotyper.

Effect of standardized protocols on MALDI-TOF MSQ

Calibration

We asked all participating laboratories to calibrate the devices (i.e., mass-axis calibration) before acquiring the second set of MALDI-TOF mass spectra [21]. In the calibration process, the time of flight of proteins with known mass is measured. From this, the conversion from time of flight to mass is calculated and reset for the following measurements. The measured time of flight of a protein can change with external factors such as (a) the temperature and thus the length of the flight tube, (b) the thickness of the sample, and (c) the curvature of the target. Compared to spectra acquired for the baseline quality assessment, the measurement error was significantly lower for the spectra acquired in this

second round in 14 of 36 devices, no significant change was observed in 11 of 36, and a significant increase in measurement error was observed on 11 of 36 devices. When focusing on devices, at baseline yielding a measurement error above 500 ppm, we recorded a significant decrease in measurement error in 4 of 5 cases (Fig. 4).

Comparing sample preparation protocols per device

We observed an improved MSQ represented by an increased detection of marker masses using the formic acid overlay protocol compared to spectra acquired for baseline quality assessment in 10 out of 36 devices, and a decrease in 17 out of 36 devices. In 9 out of 36 devices there was no significant change. Of the devices for which the median number of marker masses was lower than 15 for

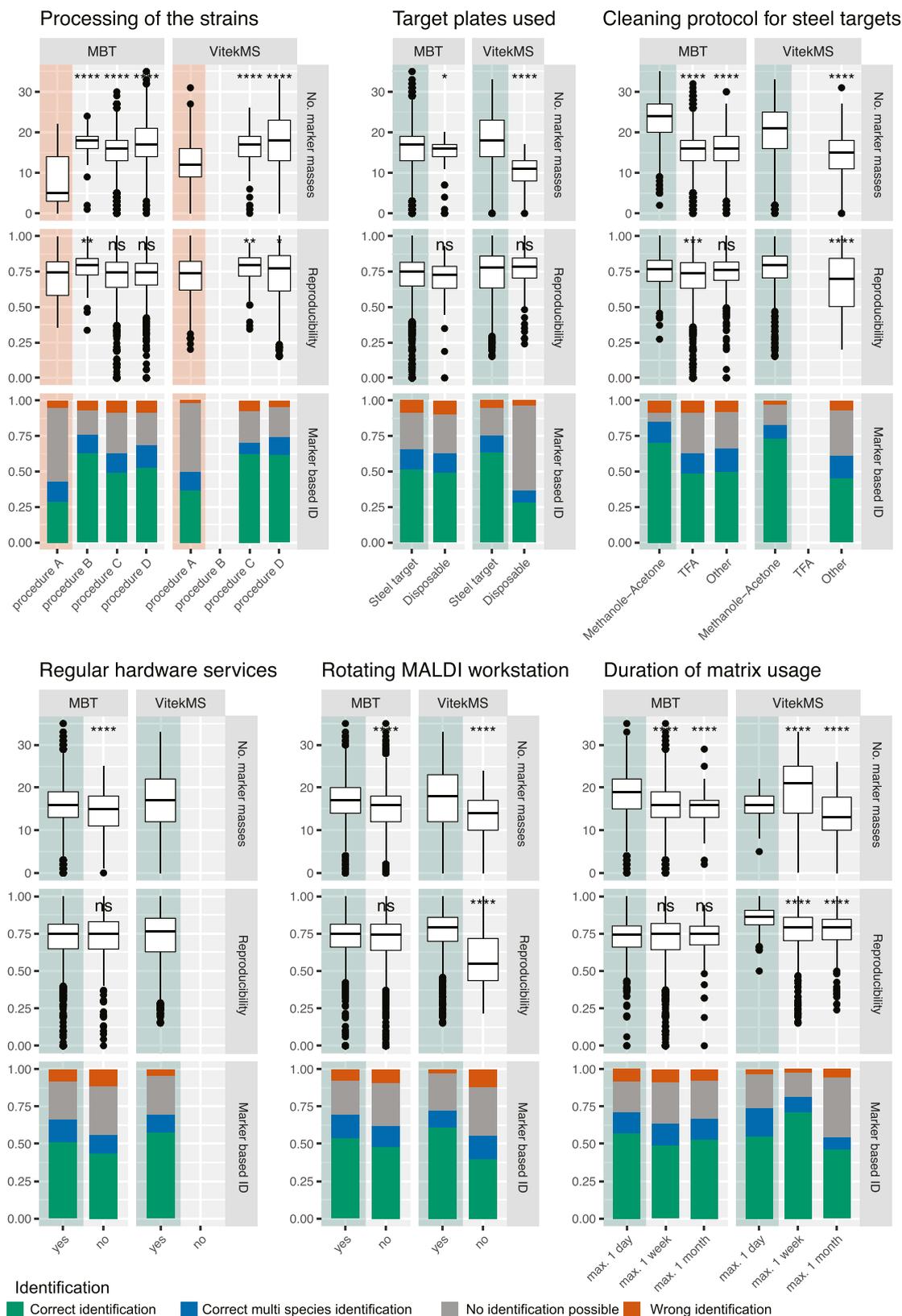


Fig. 3. Mass spectra quality features (a) number of detected marker masses and (b) technical reproducibility of A: strains processed in laboratories using different culturing procedures (procedure A: streaked out from frozen stock; procedure B: streaked out from frozen stock, subcultured once; procedure C: streaked out from ESwab; procedure D: streaked out from ESwab, subcultured once) (left column), using different target plates (middle column) and using varying cleaning protocols (right column). B: performing hardware services or not (left column), working with a MALDI workstation or not (middle column) and keeping the matrix for varying time in the workflow (right column). **** $p < 0.001$, unpaired Wilcoxon-rank test. Boxplots: The middle line corresponds to the median, the lower and the upper hinge depict the first and the third quartile, whereas the whiskers extend from the hinge no further than 1.5 times the interquartile range. Data points beyond this range are depicted as individual points. MALDI, matrix assisted laser desorption ionization; MBT, microflex Biotyper; VitekMS, includes VitekMS and Shimadzu devices.

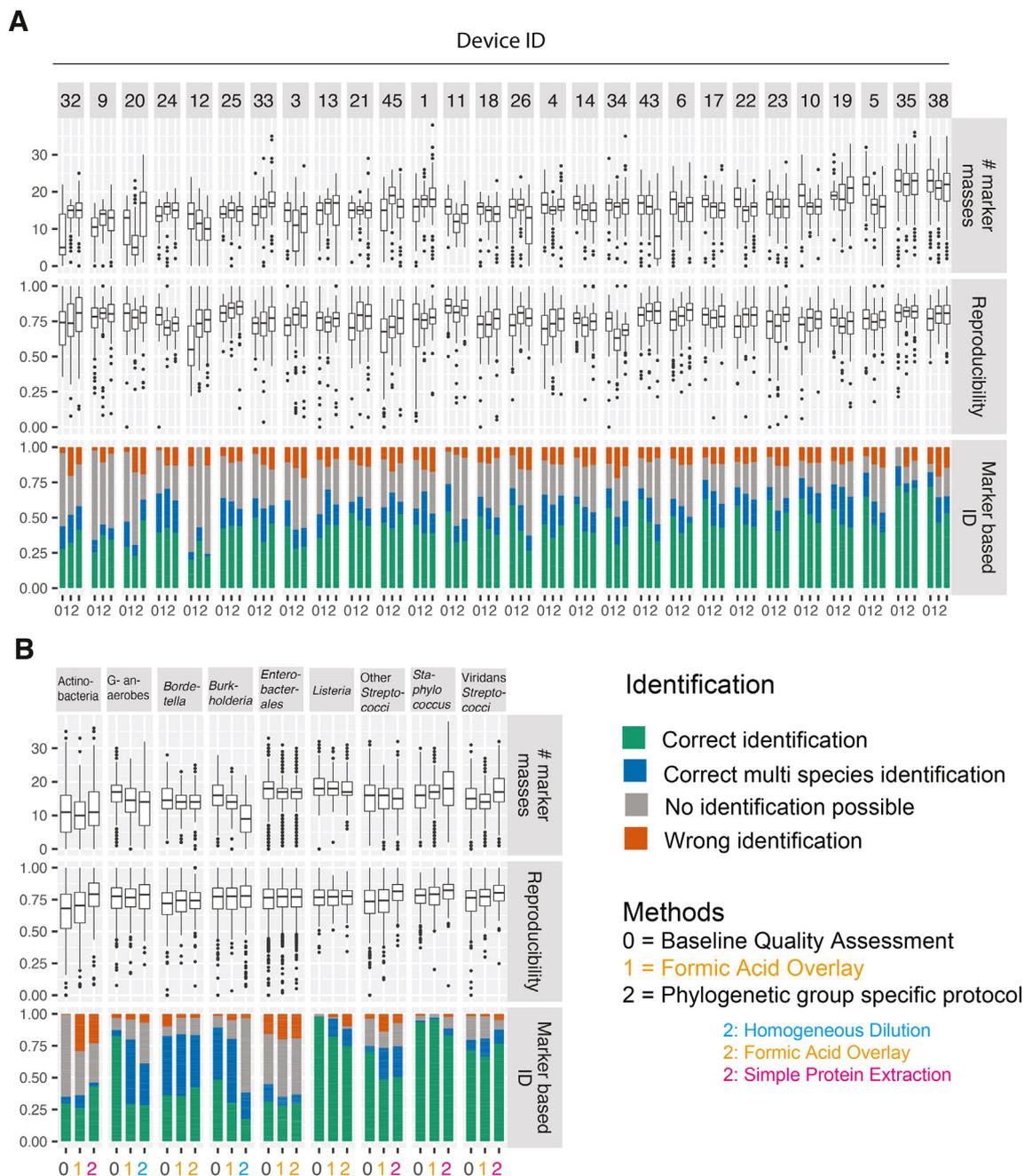


Fig. 5. Effect of different sample preparation protocols on matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectral quality (MSQ) of spectra acquired on 28 MALDI-TOF mass spectrometry (MS) devices (devices on which not all bacterial groups were measured with all three protocols were excluded from this graph). (A) Number of marker masses detected (upper row), reproducibility between technical replicates (middle row), and evaluation of a marker-based species identification (lower row) for spectra acquired with different methods and on different devices. Devices are ordered according to the number of marker masses recorded in spectra acquired for the baseline quality assessment. (B) Number of marker masses detected (upper row), reproducibility between technical replicates (middle row), and evaluation of a marker-based species identification (lower row) for spectra acquired with different methods and from various bacterial groups. Boxplots: The middle line corresponds to the median, the lower and the upper hinge depict the first and the third quartile, whereas the whiskers extend from the hinge no further than 1.5 times the interquartile range. Data points beyond this range are depicted as individual points.

bacterial inoculum used, as this was not indicated precisely enough in the instructions document or (b) differing sensitivities of the MALDI-TOF MS devices used.

We observed the accuracy and resolution of a marker-based species identification mainly following the number of detected ribosomal subunits. There are exceptions to this trend, such as *Staphylococcus aureus* complex mass spectra whose species identification did not improve with a higher median of phylogenetic

marker masses being detected. Possible explanations for this could be (a) the larger scatter of marker masses (i.e., for a larger part of the spectra the discriminatory marker masses were missing) and (b) these spectra were particularly noisy, which led to more false positive marker masses.

Based on the data analysed in this study, we suggest the following practices to be implemented in routine diagnostics: (a) regular assessment of MSQ in diagnostic laboratories, internally

(e.g., weekly), as well as externally (e.g., bi-yearly); (b) frequent calibration of the devices using well defined mass-standards; (c) usage of group specific protocols, whenever routine sample preparation does not yield satisfactory MSQ.

A frequent MSQ assessment could help to notice a drop in MSQ in a timely manner. Depending on the supposed cause for the decreased MSQ, possible responses could be to (a) adjust the devices hardware settings (e.g., the tension of the detector) or (b) refresh the personnel's skills for sample preparation.

We have identified simple workflow adaptations which improve MSQ of poorly performing devices. These implementations could increase the number of reproducibly detected marker masses in routine diagnostics. More reproducibly detected peaks increase the feasibility for MALDI-TOF MS-based typing and might improve the early recognition of spreading clones. The heterogeneity found in this EQA deserves further study in order to optimise MALDI-TOF MS-based routine identification in clinical laboratories.

Author contributions

Planning and conceptualization of the study: A.C. and A.E.; data acquisition: all authors; data analysis: A.C.; writing of the manuscript: A.C. and A.E.; giving relevant feedback throughout the project and on the manuscript: all authors.

Transparency declaration

V.P. is an employee of the company Mabritec AG, Riehen, Switzerland, which commercializes ribosomal marker-based approaches in MALDI-TOF MS data analyses for identification of microorganisms and develops the PAPMID database. The remaining authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cmi.2022.05.017>.

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