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Development of a methodological approach for the characterization of bioaerosols in exhaust air from pig fattening farms with MALDI-TOF mass spectrometry

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ABSTRACT

In this paper, we evaluated matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) as a cultivation-independent, routinely applicable approach to identify microbial fractions in bioaerosol emission samples. We developed a streamlined protocol in line with the German state-of-the-art impingement sampling guideline. Following isokinetic sampling, a fast and reliable pretreatment methodology involving a series of cascade filtration steps was implemented, which produced fractions for spectrometric measurement devoid of interfering substances. We sampled the exhaust air from eight pig fattening farms around western Germany, which yielded two sets of samples for both method development and validation. For method development, in total 65 bacterial isolates were produced directly from the exhaust air samples, taxonomically classified by 16S rRNA-Gene sequencing, and subjected to MALDI-TOF analysis. In this way, we could assign fingerprint biomarkers to classified bacterial genera or even species to build up a preliminary reference database. For verification of the novel methodology and application of the reference database, we subjected the second set of exhaust air samples to the developed protocol. Here, 18 out of 21 bacterial species deposited in the database were successfully retrieved, including organisms classified in risk group 2, which might be used to evaluate the pathogenic potential of sampled exhaust air. Overall, this study pursues an entirely new approach to rapidly analyze airborne microbial fractions.

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1. Introduction

Emissions from livestock facilities contain a variety of air pollutants, such as intact cells and cell fragments, plant and animal tissue, which are subsumed under the term “bioaerosol”. Airborne microorganisms (e.g. bacteria and fungi) constitute a large proportion of exhaust emissions from the livestock sector. In particular, intensive stock-rearing of swine and poultry is considered to be the single largest source of airborne microorganisms in the agricultural sector (Gärtner et al., 2016, 2014; Seedorf et al., 1998). The pathogenic potential of bioaerosol is still in dispute (Wielders et al., 2017). Epidemiological exposure-response studies indicate adverse health effects upon exposure of people and animals to higher doses of bioaerosols at workplaces or in the environment (Donham et al., 1989; Douwes et al., 2003; Martin et al., 2015; Pisanic et al., 2015; Walser et al., 2015). Furthermore, the extensive use of antibiotics in intensive meat production facilities has accelerated the selection of antibiotic resistant bacteria, which may increase the risk of critical infectious diseases (Chapin et al., 2005; Gao et al., 2015; Gärtner et al., 2016; Masclaux et al., 2013; Ngbede et al., 2017). Thus, there is an increasing need for efficient monitoring tools capable of capturing and characterizing airborne microbial fractions.

In light of the complex nature of environmental bioaerosol samples, it has been speculated that this could only be achieved by combining various detection methods, thus compensating for the limitations of each individual method (Walser et al., 2015). The

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identification and quantification of microorganisms in bioaerosol samples has traditionally been carried out using culture-based methods (VDI-4253-3, 2008). These approaches are time consuming, and are further limited to the detection of viable microbes. The requirement for microbial cultivation steps can be circumvented by using analytical tools like epifluorescence microscopy, flow cytometry and molecular biological techniques (PCR) (Ghosh et al., 2015). None of these methods are, however, standalone techniques to enable just-in-time measurements of environmental samples in a rapid, routinely applicable manner.

Matrix-assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is considered an attractive screening method due to its minimal workload and excellent sensitivity, which affords the generation of unique biomarker spectra for e.g. specific microorganisms with minimum sample pre-treatment (Madsen et al., 2015; Pineda et al., 2003; Sala-Comorera et al., 2016). Rapid detection and identification of microorganisms in environmental or clinical samples remains, however, a challenging task regardless whether bacterial cultivation is implemented as a pre-measurement step. Bioaerosols may constitute a variety of particulate organic matter, which hinders a clear definition of sample matrices and the formulation of universally applicable strategies to identify inherent microbial fractions.

Recent studies highlight the application of MALDI mass spectrometry for the analysis of bacterial isolates taken from environmental samples like drinking water (Sala-Comorera et al., 2016), and food (Hausdorf et al., 2013). Further, airborne bacteria and fungi present in different environments were cultivated, and subsequently measured by MALDI-TOF-MS (Angelakis et al., 2014; Madsen et al., 2016a,b). As a first step towards cultivation-independent MALDI-based microbial detection methods, clinical blood samples have recently been screened for specific microbial targets (Prod'hom et al., 2010; Thomin et al., 2015; Wattal and Oberoi, 2016).

In this study, we evaluated the potential of MALDI-TOF mass spectrometry as a profiling method for inherent bacterial fractions in bioaerosols. We targeted bioaerosol emissions from intensive pig meat production systems by impingement sampling, and designed a simplified analysis workflow including (a) the development of a protocol for sample pre-treatment without cultivation steps, (b) the selection of suitable MALDI preparation methods and detection parameters, (c) the generation of a reference biomarker ion database by bacteria isolated from sampled bioaerosol emissions, and (d) taxonomic classification of the isolated bacteria by 16S rRNA-Gene sequencing.

2. Materials and methods

2.1. Pig fattening facilities and impinger exhaust air sampling

Eight pig fattening facilities in different regions of North Rhine-Westphalia (NRW, Germany) were selected for bioaerosol emission sampling (see Supporting information for further description of the sampled sites, e.g. regarding stable occupancy data and air sampling volumes per impinger sampling). Selection of facilities and emissions sampling were carried out by the North Rhine-Westphalian Agency for Nature, Environment and Consumer Protection (LANUV NRW), Germany. Bioaerosols are sampled according to the German guideline VDI 4257 Part 2 (VDI-4257-2, 2011) using emission impingers. The guideline describes the procedure of emission sampling under isokinetic conditions using an emission impinger (modified AGI 30; Table S2) located at outside facility chimneys. The setup has been designed for out stack configuration with the sampling device being installed with the entry nozzle, the sampling bend and part of the suction tube right in front of the impinger. A more detailed and visual description of the sampling device and the respective procedure can be found in the supporting information of this paper.

Impingement sampling was carried out isokinetically at exhaust chimneys between March 2014 and February 2015. The sample volume was 52 ml, comprising an impinger solution volume of 32 ml, and 20 ml rinsing liquid. Four samples per sampling day were taken at facilities 1–5 and 7–8. At facility 6, which served as the reference sampling site, eight samples were collected and split into four samples each for MALDI-measurement and taxonomic classification, respectively (backup samples). The samples from facilities 1–5 were used for the development of a sample pre-treatment workflow, whereas samples taken at facilities 6, 7 and 8 served to validate the developed protocol using the established reference database (see Sections 2.2.2, 3 and 5). The database itself was constructed by isolation, taxonomic classification and MALDI measurement of airborne bacteria in emission samples from facility 6. Taxonomic classification was carried out at the Federal Institute for Occupational Safety and Health (BAuA), Berlin (Sections 2.2.2 and 2.2.3).

At each sampling location, a field and a laboratory blank were taken for quality assurance. Phosphate-buffered saline (PBS) (4,3 g/l NaCl, 7,23 g/l $Na_2HPO_4 \times 2\,H_2O$ and 3,56 g/l KH_2PO_4, pH 7) was used as impinger sampling solution (VDI-4257-2, 2011). After each sampling, the impinger liquids were transferred to cooling boxes, and shipped at $5 \pm 3\,^{\circ}C$ by using cooling packs. Samples were transferred to the laboratories in less than 24 h after sampling, and stored in darkness at $5 \pm 3\,^{\circ}C$ until further processing.

2.2. Analysis

2.2.1. Sample pre-treatment for MALDI-measurement

Sample pre-treatment was crucially required to separate and detach the microbial fraction from particles and other interfering constituents usually found in exhaust emissions. Following cascade filtration, two different size fractions ($S_{20\,\mu m}$ and $S_{5\,\mu m}$) were selected for MALDI measurement, which contained the target analyte (microbial fraction) in semi-pure form. This differs from the commonly applied technique of using cultivation-based approaches preceding spot preparation, which usually includes time-consuming incubation steps followed by direct deposition of single bacterial colonies on the target (Ghosh et al., 2015; Wang et al., 2016).

In each case, sample pre-treatment was initiated by pooling the four impinger samples taken on one day (each containing 52 ml). The resulting composite samples (208 ml total volume) were homogenized by shaking, followed by sequential filtration through 70 μm and 40 μm pore size cell strainers (Falcon, Corning Inc. Life Science, Durham, USA). The 40 μm filtrate suspension was centrifuged at $6200 \times g$ for 60 min at 4 °C, and the pellets were resuspended in 3 ml PBS buffer. The resulting suspensions were passed through 20 μm pore size filtration columns (CHROMABOND, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany), and the filtrates were centrifuged at 16,100 x g for 15 min. The pellets were resuspended in 100–200 μl PBS buffer (20 μm fraction; $S_{20\,\mu m}$). For preparation of the 5 μm fraction ($S_{5\,\mu m}$), the 20 μm suspension was filled up to 3 ml with PBS buffer, and filtered through a 5 μm pore size syringe filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany). The filtrates were centrifuged at $16,100 \times g$ for 15 min, and the resulting pellets were resuspended in 10–50 μl PBS buffer. The 20 μm and 5 μm fraction were used for the MALDI sample preparation.

2.2.2. Bacterial isolation from samples of reference facility 6

Isolation of single, cultivable bacterial species from heterogeneous bioaerosol samples was performed using standard

Table 1
Tentatively determined MALDI-TOF biomarker ions of isolated airborne bacteria, including indications whether a biomarker is specific for a species or the superordinate genera. Tolerated deviation: ±5 Da.

I. Peaks masses (Da) of organisms, for which at least one more species belonging to the same genus was identified. Biomarkers were either specific for the genus-level, or the species-level.

Organism	Genus	Species
Aerococcus viridans[1]	**3375**[b], **5610**[a], 6675[a], 6751	
Aerococcus urinaeequi	**3374**[b], **5609**, 6674[a], 6750[a]	
Brevibacterium pityocampae[c]	**3722**[b], **3780**[b], 4365, 7447, **7565**	3459, **3805**, 4509, **4983**, 5141, 5785, 5912, **6348**, 6445, **6518**, **6590**, 7322, **7339**, 7476, 7795, 8978
Brevibacterium yomogidense[c]	**3723**[b], **3779**[b], 4362, 7447, **7562**	7214, 8221, 8543, 8767, 9193, **9497**, 9810, 12940
Corynebacterium ammoniagenes	3015, 4016	4626, 5539
Corynebacterium casei	**4688**[a], 6317[a]	3698, 3769, 5662, 6371[a], 6780
Corynebacterium humireducens	4712[a]	**3235**, **3561**, **3906**, 3945, **4430**, 6305[a]
Corynebacterium stationis	4017, **4689**[a], 6319[a], 6336	6024, **6510**[a], **7166**, 8469[a]
Corynebacterium striatum[1]	6334[a]	3046, 3107, **3677**, **4632**, 4659, 4675, **4752**, 4770, **5423**, **7079**, 7106
Corynebacterium xerosis	3013, 4711[a]	4352
Staphylococcus cohnii	**6382**[a]	**3413**, **4179**, **5633**, 5819, 5998, 6412[a]
Staphylococcus epidermidis[1]	3834, **5303**[a], 5388, 9634[a]	3341, 3953, **4189**[b], **4814**, **5112**, 6663, 7530, **8383**, 9583, 15176
Staphylococcus equorum	3831, **5304**[a], 5390, **5877**[a], **6384**, **6821**[a]	4046, 4231, **4990**, **5193**, **5430**[a], 6066, 6235, 6249, **7520**, 7770, 9163
Staphylococcus pasteuri[1]	**5874**[a], **6820**[a], 9634	**6203**, **6453**, 9117
Staphylococcus saprophyticus[1]	**5877**[a]	6039, 6291, 8068, 8157, 9092, 9652, **9854**

II. Peak masses (Da) of organisms, for which no other species belonging to the same genera were identified.

Organism	Mass (Da)
Brachybacterium nesterenkovii[c]	3383[b], **4142**, 6377, 6576, 6768
Enterococcus avium[2]	3064, 3569, 4613, **4842**, **5685**, 6284
Kocuria palustris	**4203**, **4931**, **5021**, **5162**, 5489, **5507**, 6651, 6670, 6698
Moraxella pluranimalium[1,c]	3135, **3475**[b], **3875**, **4119**, 4447[b], **5990**, 6801, **6950**, 6969, 7436, **7886**, 8899[b], 9414, 10046, 10354
Nocardiopsis listeri[c]	4073, 4173
Rothia endophytica[c]	**3508**, 5496, **5619**, **5674**, **6174**, **6627**

[1] Risk group 2 pathogen according to German TRBA 466.
[a] Signals were assigned to ribosomal proteins using the protein database UniProt. Matches with uncharacterized proteins or ambiguous assignments were not considered.
[b] Putatively doubly charged ion; Masses indicated in bold were successfully retrieved in bioaerosol emission spectra.
[c] Organisms lacking detailed characterization or entries in the UniProt database.

microbiological techniques with six different agar-based media (Supporting Table S2). The four backup impinger samples from facility 6 were pooled (208 ml total volume) and homogenized by shaking. A decimal dilution series was applied from 10^{-1} up to 10^{-7}. Single colonies were picked, plated on fresh agar plates and incubated at 37 °C for 5 days. This step was iteratively repeated (minimally five times) until the presence of contaminants could be excluded by means of microscopic evaluation.

2.2.3. Taxonomic classification

Bacterial isolates were identified according to Martin et al. (2009) based on 16S rRNA-Gene sequence analysis. Briefly, the 16S rRNA fractions were extracted by using the GenElute Plant Genomic DNA Miniprep Kit (Sigma Aldrich, USA) following the manufacturer's descriptions. The genes were amplified using universal primers according to Weisburg et al. (1991). Taxonomic classifications of all bacterial isolates were performed by comparing the obtained 16S rRNA-Gene sequences to available sequences of all currently and validly described bacterial type strains (Euzeby) in public databases (Ribosomal Database or GenBank) (Benson et al., 2013; Cole et al., 2014).

2.2.4. MALDI sample spot preparation and measurement

$S_{20\ \mu m}$ and $S_{5\ \mu m}$ fractions (Section 2.2.1) or bacterial isolates (2.2.2) were spotted on stainless steel MALDI target plates by adopting a protocol based on (Donohue et al., 2006) and (Bizzini et al., 2010) for whole-cell MALDI spot preparation. Slight modifications were implemented to increase the concentration of matrix-embedded intact cells: In case of the $S_{20\ \mu m}$ and $S_{5\ \mu m}$ fractions, the pellets were washed twice with 1 ml ultrapure water, followed by resuspension in small volumes of ultrapure water. Samples were spotted on a stainless steel plate by mixing 10 µl of the resulting suspensions with 1 µl of the matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid (CHCA), in 50% (v/v) acetonitrile (ACN), 47% (v/v) water and 3% (v/v) trifluoroacetic acid (TFA)) (Sigma-Aldrich, St. Louis, MO, USA). For each sample, a total volume of 10 µl was applied in iterative steps à 2 µl and dried using a common hair dryer.

In case of bacterial isolates, 1–3 colonies were resuspended in 1 ml ultrapure water. Cells were pelleted by centrifugation (16100 × *g*, RT, 5 min), and washed twice with ultrapure water. Following final cell re-suspension in 20–200 µl of distilled water, steel target spots were prepared by mixing 2 µl of the bacterial suspensions with 1–2 µl of matrix solution (same as above). Drying was performed as described above. Mass spectra were recorded using an Axima Confidence (Shimadzu Biotech Corp., Kyoto, Japan) MALDI-TOF mass spectrometer equipped with a nitrogen UV laser (337 nm). The system was operated in linear, pulsed ion extraction and positive-ion mode with the following parameters: pulsed extraction, 2100 V; source, 20000 V; linear detector, 2750 V. The firing rate was 10 Hz. A minimum of 200 profiles per sample was generated to obtain satisfactory signal-to-noise ratios in cumulative spectra (five laser shots accumulated to one profile spectrum). The selected mass range was 700–20000 Da. Pulsed ion (delayed) extraction was optimized for ion focusing and transmission at 7800 Da. Peak processing settings were selected as described in the supporting information. External mass axis calibration was performed using spectra of the reference strain *Escherichia coli* DH5α (Bruker Daltonik GmbH, Bremen, Germany). A mass tolerance limit of ±300 ppm was set at all calibration points by mass axis calibration.

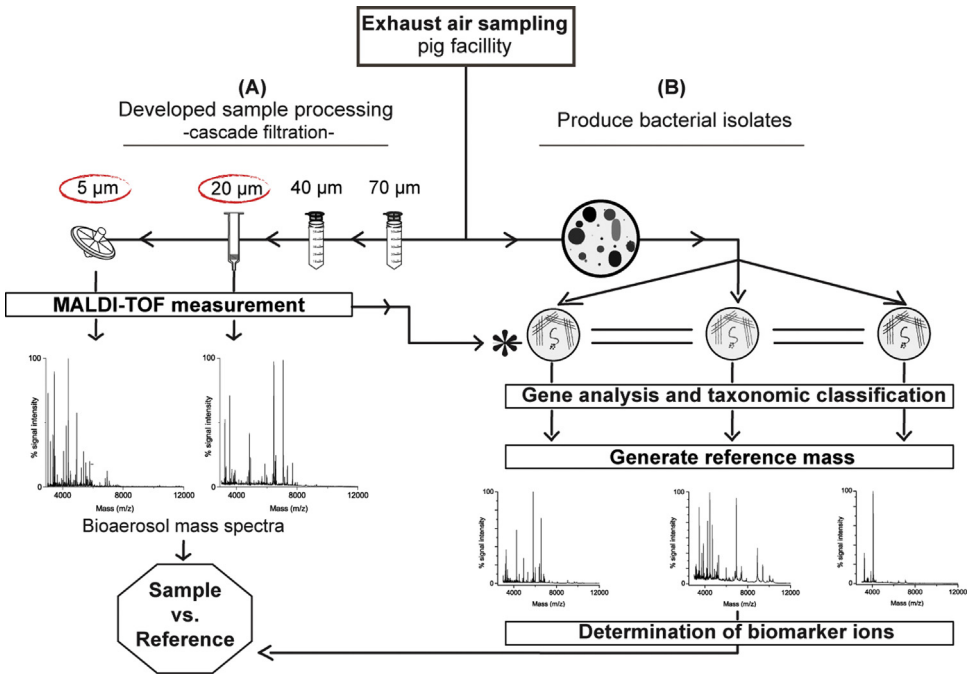

Fig. 1. Sample pre-treatment procedure to characterize bioaerosol fractions containing bacteria by MALDI-TOF, and generation of biomarker ions from bacterial cultures (scheme).

2.2.5. Reference library and protein identification

The reference library (Table 1) was generated using fingerprint ions of bacterial isolates. Peaks with a signal-to-noise ratio (S/N) ≤3 and repetition rate ≤90% in the cumulative fingerprint spectrum of single species were excluded. Unspecific peaks observed in more than one bacterial isolate spectrum were excluded. Determined bacterial biomarkers were, if possible, correlated to published peptide masses using the protein sequence database UniProt (http://www.uniprot.org/proteomes; accessed 2016-04-05) (Apweiler et al., 2004; Pundir et al., 2015; The-UniProt-Consortium, 2017). The maximal deviation for assignment of empirical to associated data was set to ±5 Da. The search was extended to peptide masses corresponding to N-terminal methionine loss in cases where no hit was determined for the unmodified peptide. Other posttranslational modifications, e. g. acetylation and methylation, were not considered.

3. Results

A total number of 36 emission impinger samples taken at real livestock facilities was used to design and subsequently verify a novel MALDI mass spectrometry based bacterial identification methodology. For sample pre-treatment, a streamlined workflow was established to enable facile and rapid separation of the microbial cell fraction from other constituents commonly found in environmental samples. Application of the pre-treatment procedure yielded bacterial fractions devoid of interfering substances and suitable for MALDI measurement.

3.1. MALDI sample pre-treatment and spot preparation

The exhaust air from livestock facilities contains a wide range of particulate constituents which may interfere with sample cleanup and background measurement. Thus, we designed a sample pre-treatment strategy to separate bacterial fractions from complex samples by applying sequential filtration and centrifugation steps. Four different filtrations steps in the size range of 70–5 µm (cut-off) were applied, among which the 20 µm and 5 µm fractions proved suitable for MALDI-TOF analysis (Fig. 1). The quality and reproducibility of generated spectra (5 µm or 20 µm fractions) were further optimized by applying the MALDI spot preparation technique described in Section 2.2.4, including sample embedment in the versatile matrix CHCA dissolved in a defined mixture of acetonitrile, water and trifluoroacetic acid.

Table 2
Number of assigned and total peaks detected in bioaerosol MALDI mass spectra.

Facility	Fraction pore size [µm]	Number of peaks	Number of assigned peaks
6[a]	20	37–38	14
6[b]	5	70–80	26
7[c]	20	47–61	29
7[d]	5	40–52	18
8[e]	20	22–27	7
8[f]	5	19–26	11

Number of prepared MALDI target spots: [a]n = 2; [b]n = 3; [c]n = 3; [d]n = 4; [e]n = 8; [f]n = 8.

3.2. Bioaerosol MALDI mass spectra

In all generated MALDI-TOF mass spectra of samples pretreated in this manner, *m*/*z* values up to 15 kDa were observed, whereas the predominant peak fraction covered the range of 3000–8000 Da. The latter range was, consequently, used for biomarker identification (Fig. 2). Under given conditions, spectra yielded in total between 19 and 80 desorbed and detected molecules (Table 2). Following systematic evaluation, pre-treated impinger samples from livestock facilities 6–8 yielded *m*/*z* values at 4719, 4817, 4846 and 6465 Da in all analyzed spectra regardless of sampling facility or date. The aforementioned peaks were present in all fractions with one exception ($S_{5\ \mu m}$ of facility 7; Fig. 3, green bars). Thus, is was possible to use the four signals not only as specific biomarkers, but also as indicators for reproducibility. In order to evaluate the notion that the four signals could be specific ionized fragments assignable to bacte-

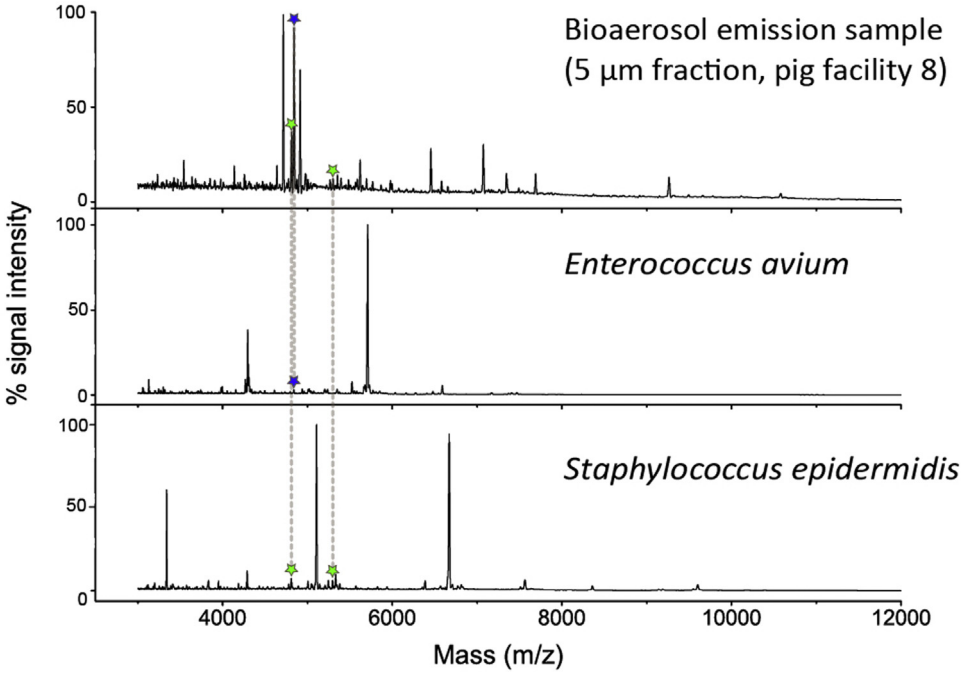

Fig. 2. Comparison of MALDI mass spectra from one bioaerosol sample and two bacterial isolates. Exemplary biomarkers assigned to the spectra are indicated.

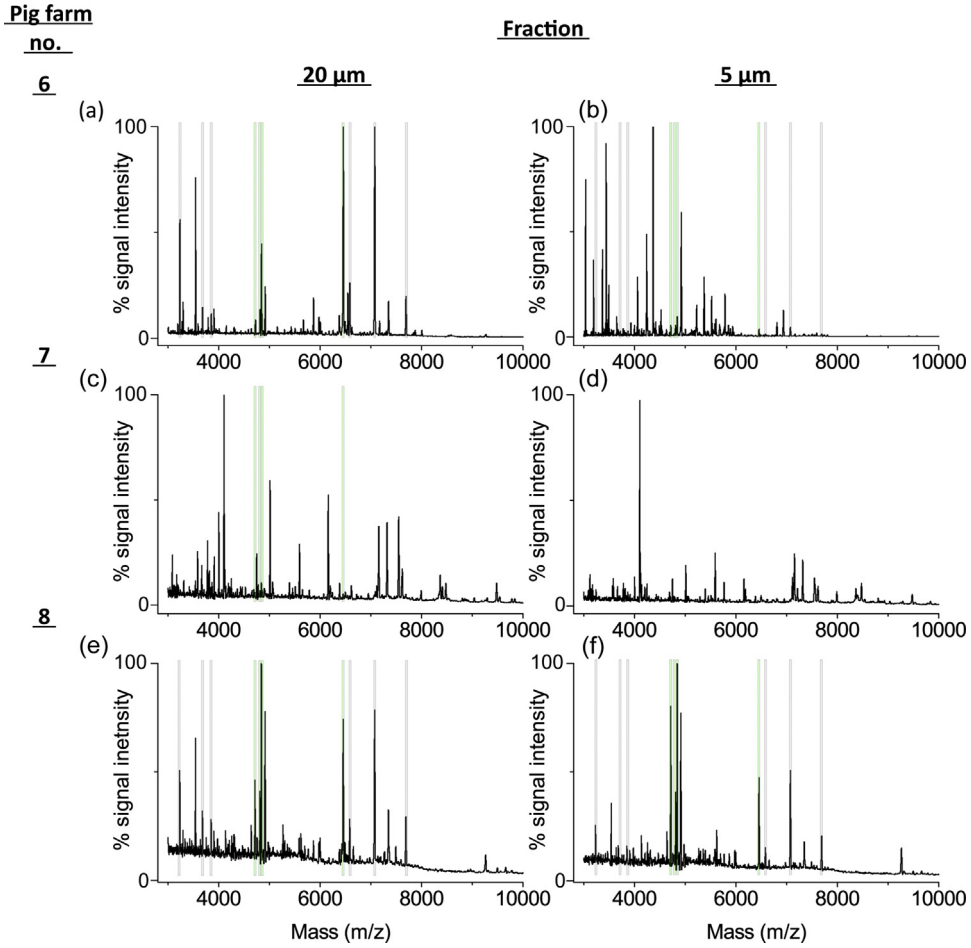

Fig. 3. MALDI mass spectra of 20 µm and 5 µm filtration fractions of bioaerosol emission samples from different pig fattening facilities. Recurring *m*/*z* values are indicated with green/grey bars. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rial species and genera, we designed and constructed a fingerprint mass ion database.

3.3. Reference database

65 aerobic bacterial cultures were isolated, by plating and separation, from four pooled bioaerosol emission samples taken in addition to the four samples used for MALDI at facility 6 (see Section 2.2.2). The produced isolates were taxonomically classified by 16S rRNA-Gene sequencing, and subjected to MALDI-TOF pretreatment and measurement protocols to assign biomarkers ions (Fig. 1 and Table 1). Out of 65 bacterial isolates, a total number of 21 different species belonging to 10 different genera and 3 different phyla were identified by 16S rRNA-Gene sequencing. Among these, seven assignments are closely related to bacteria, which are characterized as biological agents of risk group 2 (TRBA 466, 2015). Reference spectra for all 21 organisms in question were generated, and tentative genus- or species-specific peaks were successfully derived. These biomarkers served to construct the tentative fragment database used in this study (Table 1).

3.4. Database application

As mentioned before, reference bacteria were isolated from an emission impinger sample of one designated livestock facility (see above). Next, verification and application of the database was performed by using samples from different livestock facilities (facilities 6, 7 and 8) to ensure representativeness. Firstly, it was possible to assign two out of four of the aforementioned universally present signals (4719, 4817, 4846 and 6465 Da) to isolated bacteria: peaks at 4817 and 4846 Da matched with fingerprint ions of *Staphylococcus epidermidis* (4814 Da) and *Enterococcus avium* (4842 Da). Since these two species are classified in risk group 2, their finding in all tested bioaerosol samples might be of importance when considering guided parameters for the characterization and comparison of bioaerosol emission samples in general.

Among the reference organisms used to construct the database, different genera and species yielded different amounts of tentative biomarkers in the range of 1–16. The retrieval of one to four fragments could generally be demonstrated in sample spectra (Table 1).

Notably, in several cases we could identify genera or species through assignment of different biomarkers. This result substantiates the approach of assigning more than one peak to each organism, raising the question whether it might be necessary to distinguish between recurring signals, which can be used as key parameters for comparing different bioaerosol emission samples, and variable signals to identify specific organisms.

Since biomarkers were selected based on uniqueness and were only chosen when absent in spectra of other organisms, we considered the finding of one or more genus- or species-level signals as indicative of the presence of the genus or species in question in a given sample. We successfully retrieved 18 out of 21 reference species in one or more emission samples from facilities 6–8, with the exception of *Corynebacterium ammoniagenes*, *Corynebacterium xerosis* and *Nocardiopsis listeria* (Fig. 4). Three reference species, namely *Corynebacterium striatum*, *Moraxiella pluranimalium* and *Staphylococcus epidermidis*, were retrieved in all emission samples regardless of facility or fraction (Figs. 4 and 5). *Aerococcus viridans*, *Aerococcus urinaeequi* and *Corynebacterium casei* have to be considered separately, since their assignment is strictly bound to the genus-level identification (Table 1 and Fig. 4). Among the three livestock facilities tested (6–8), facilities 6 and 8 showed striking similarities: peaks at 3232, 3680, 3852, 6592, 7090 and 7706 Da were, together with the four aforementioned "universal"

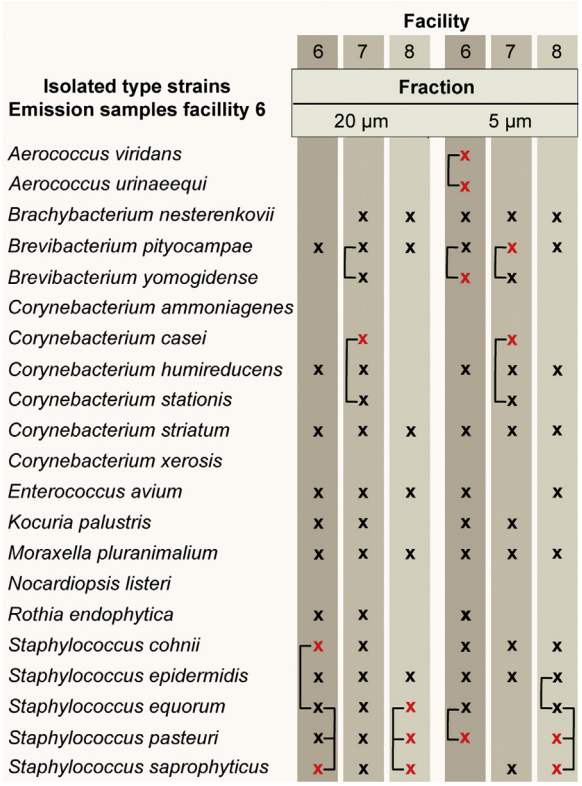

Fig. 4. List of bacterial species successfully assigned to bioaerosol sample spectra on the species- or genus-level using the generated biomarker database. Red x: Genus-specific bacterial biomarker, which was only assigned for the organism in question; Black x: At least one species-specific biomarker ion was assignable for the organism in question. Connecting lines indicate shared biomarker ions (not individually observed). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

peaks, observed in the two filtrate fractions of both facility emission samples (Fig. 3, grey bars). The 7706 and 3852 Da fragments are anticipated to be the singly and doubly charged ions from the same parent molecule, whereas 6592 Da likely corresponds to *Brevibacterium pityocampae* (6590 Da), a risk group 1 organism (TRBA 466, 2015).

4. Discussion

As public awareness of potential risks arising from bioaerosol emissions increases, the demand for innovative and particularly routinely applicable methods for the detection of bacterial fractions in such emissions is growing correspondingly (Jahne et al., 2015, 2016). In this study, we aimed at establishing MALDI-TOF mass spectrometry based techniques as a technology platform for bacterial screening in bioaerosol emission samples with minimal workload. Furthermore, by choosing a German state-of-the-art in situ sampling procedure (VDI-4257-2, 2011) and by not considering artificially designed reference samples in favor of real exhaust air samples, we aimed to design a protocol applicable in line with current guidelines and close to real testing conditions.

A wide range of bioaerosol sampling devices are in use for bioaerosol monitoring. Common procedures used in this regard are impaction, impingement, and filtration. In this study, emission impingement (using a modified AGI 30 impinger) was selected as the most suitable sampling technique to cover a broad spectrum of particle sizes as demonstrated elsewhere (Gärtner et al., 2008a, 1885, 2008b). This approach was deemed crucial in light of the

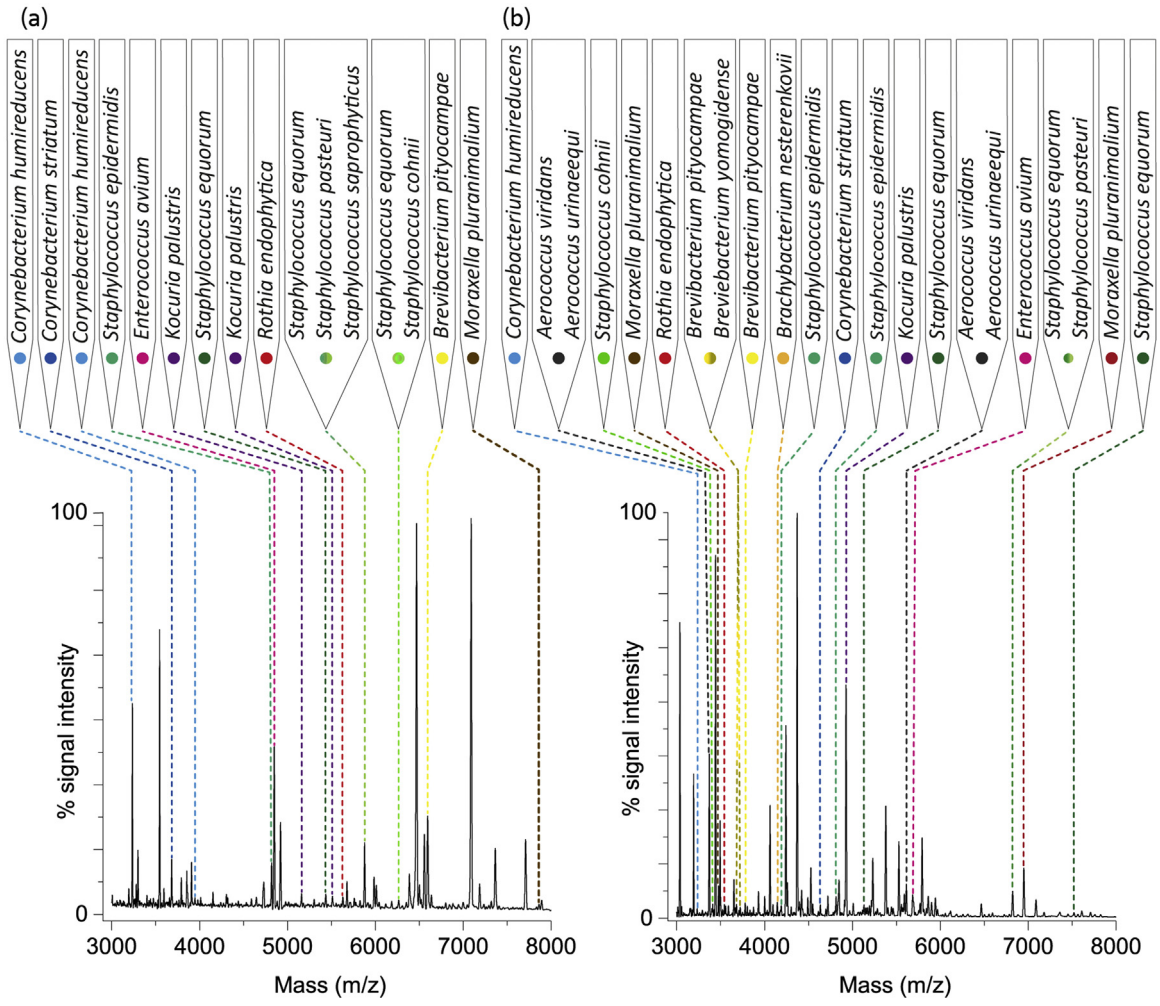

Fig. 5. MALDI-TOF mass spectra generated from (a) 20 μm and (b) 5 μm filtrate fractions of a bioaerosol emission sample taken from pig fattening facility 6. Bacterial biomarkers are indicated by different colours.

ambiguous state of air-suspended microbes being either present in free or particle-associated form (see below).

Bioaerosols are a complex mixture of different components with a size distribution from nanometer scale to particles up to 100 μm in diameter (Adell et al., 2012; Stetzenbach et al., 2004). Among these constituents are water- or solvent-soluble inorganic and organic molecules, prokaryotic and eukaryotic cells, viruses, mold as well as animal and plant fibers and debris (Curtis et al., 1975; Stetzenbach et al., 2004). In this study, initial attempts to measure samples without prior sample cleanup failed (data not shown). Apparently, due to the presence of interfering compounds like fibrous animal or plant material, both the efficiencies of analyte embedment in matrix crystals and the ionization process considerably decrease. Direct application of small pore size filters, on the other hand, removed both interfering constituents as well as the target analyte. In this regard, we anticipate that airborne bacteria mainly occur in the form of aggregates embedded in a surrounding matrix or attached to larger particles rather than suspended as single cells (Lighthart, 1994). This notion is strengthened by the observation that differential centrifugation was not successful in separating the bacterial fraction from inherent particles. Hence, we developed a preparation method based on four-step cascade filtration alternated by washing/centrifugation steps, which enabled the differential removal of larger and medium-sized particles, followed by collection of (semi)pure bacterial cell fractions.

Sample preparation parameters (i. e. when protein extraction, spotting, matrices or solvents are considered) are crucial in case of MALDI-based techniques, and likely influence mass spectra appearance, reproducibility, mass accuracy and method sensitivity (Sedo et al., 2011; Wang et al., 1998). In this study, we used a modified preparation procedure based on (Donohue et al., 2006) and (Bizzini et al., 2010) for whole-cell MALDI preparation. Modifications were introduced to reduce workload and effort in data collection while increasing the yield of intact bacterial material. These improvements further increased the quality and reproducibility of generated spectra. CHCA was chosen as matrix in light of its benefits in forming uniform crystals which increases the reproducibility of profiles, its insensitivity toward sample impurities, and the possibility to measure a wide range of peptides and small proteins (Wang et al., 1998). A mixture of acetonitrile, water and TFA was used as solvent to enhance or facilitate cell disruption of bacteria.

The predominant peak fraction was observed in the range of 3000–15000 Da. The ratio of assignable biomarkers to the total number of peaks was not found to be consistent when different sampling facilities and sample fractions ($S_{20\,\mu m}$ and $S_{5\,\mu m}$) were considered (Table 2). In case of facility 6, the $S_{5\,\mu m}$ fraction produced twice the number of assignable peaks compared

to the $S_{20\ \mu m}$ fraction (see Fig. 5 and Table 2). Different ratios were observed, however, in sample fractions of facilities 7 and 8. Notably, the predominant fraction of bacterial genera and species might have not been captured due to the fact that anaerobic or non-cultivable bacteria were not considered in bacterial isolation trials carried out here. Arguably, the bacterial diversity in given bioaerosol emission samples predetermines the overall signal distribution, and strongly influences the ratio of assignable peaks to the overall number of peaks as well as the probability of individual fragments to be detectable. Further, "spot-to-spot" reproducibility and inter-sample variability introduce common hurdles or errors directly influencing spectrum quality and biomarker detectability (Nishikaze et al., 2012; Toh-Boyo et al., 2012). Based on 16S rRNA gene analysis of isolates, 7 out of 21 bacteria detected in exhaust air from pig fattening farm no. 6 were closely related to species of risk group 2 (according to the German TRBA 466, 2015). Organisms classified in this risk group are opportunistic pathogens with moderate potential to cause infectious diseases, for which usually effective prophylaxis or treatments are available. Nevertheless, their presence or accumulation points to increased pathogenic risk for bioaerosol-exposed citizens and workers. Risk group 1 organisms, in turn, are not associated with infectious diseases, although their monitoring might be of importance to evaluate the feeding state and healthiness of the livestock, and to compare the "well-being" of different livestocks over time.

The total number of signals observed in the range of 3–20 kDa in generated spectra was lower than previously anticipated. It has been described that a single microbial cell constitutes approximately 500–4000 proteins, whereas a typical MALDI spectrum of microorganisms yields only between 10 and 30 peaks (Arnold and Reilly, 1999; Pineda et al., 2003; Ryzhov and Fenselau, 2001). This discrepancy further emphasizes the challenge of comparing MALDI-derived microbial biomarkers when different sampling, pre-treatment and spotting techniques are applied to generate the spectra. Protein databases, in turn, should only be used as supporting tools for a further refined peak characterization.

The general composition of a complex sample predetermines measurability and detection limits as demonstrated in previous studies involving milk and urine specimens, which were subjected to MALDI-TOF MS analysis (Barreiro et al., 2012; Demarco and Burnham, 2014). An important parameter regarding the quality of generated spectra is the concentration of intact cells in investigated samples. In the abovementioned studies, detections limits of 10^5–10^7 CFU/ml for the model bacterium *E. coli* were determined. Bioaerosol emissions from pig fattening facilities contain 10^7–10^8 cells/m^3 (Gärtner et al., 2016). This corresponds to approximately 10^5–10^6 cells per ml impinger sample, and up to 10^8–10^9 cells per ml $S_{20\ \mu m}$ or $S_{5\ \mu m}$ fraction, which in line with previous notions should be sufficient intact cellular material for MALDI-based analyses. Nevertheless, this approach mainly depends on the physiological conditions of the target microorganisms and their ionization efficiency. Future studies need to address potential disturbances by background peaks and artifacts, the influence and interference of different sample constituents, and possible sources of error introduced by different ionization affinities.

As mentioned above, a substantial fraction of peaks could not be assigned to specific organisms deposited in the database (Table 2). Arguably, fragments derived from microorganisms, plant, animal or other organic material could yield background peaks contributing to spectra of complex environmental samples. Further, in previous studies, strains belonging to the genera *Lactobacillus, Clostridium, Terrisporobacter, Turicibacter, Romboutsia*, *Prevotella and Streptococcus* were assigned to the dominant bacterial fraction occurring in bioaerosol emissions from pig fattening facilities (Gärtner et al., 2016; Kim and Isaacson, 2015; Marti et al., 2009; Nehme et al., 2008). Among these are strictly anaerobic bacteria, which are not cultivable using the cultivation conditions applied in this study (Section 2.2.2). Moving forward, it is planned to systematically update the constructed reference database by including these organisms and other species found in real livestock facility bioaerosol samples. It should be noted, however, that due to the presence of microbial consortia, which are difficult or impossible to cultivate, databases in this regard may never be fully exhaustive.

5. Conclusions

Overall, this study represents the first step towards routine sample preparation and analysis of the bacterial consortia present in anthropogenic bioaerosol emissions. A pre-treatment and cleanup protocol for impinger samples taken according to state-of-the-art guidelines without the need for time-consuming cultivation steps has been developed. The measurement setup allows the detection of signals up to 20 kDa, which nicely covers the expected range of bacterial signature biomarkers. Assignment of peaks to specific organisms is demonstrated. In terms of completing the presented database, the range of organisms, which in part is predetermined by the applied cultivation parameters, needs to be expanded, and systematic evaluations regarding the influence of sampling, media, culture conditions, and sample pre-treatment on protein profiles need to be implemented. Further, it may be of general interest to enlarge the scope of the presented protocol by analyzing samples from a wider range of livestock facilities. These implementations will pave the way towards the long-term aim in this regard, which is the development of rapid and reliable screening tools aided by chemometric models like principle component analysis (PCA). To enable complementary PCA analyses, data from a wider range of biological reference methods need to be correlated to implement MALDI-TOF mass spectrometry as a routine bioaerosol emission monitoring method in the field of environmental protection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijheh.2017.05.003.

References

Adell, E., Estelles, F., Torres, A.G., Cambra-Lopez, M., 2012. Morphology, chemical composition, and bacterial concentration of airborne particulate matter in rabbit farms. World Rabbit Sci. 20, 241–252.

Angelakis, E., Yasir, M., Azhar, E.I., Papadioti, A., Bibi, F., Aburizaiza, A.S., Metidji, S., Memish, Z.A., Ashshi, A.M., Hassan, A.M., Harakeh, S., Gautret, P., Raoult, D., 2014. MALDI-TOF mass spectrometry and identification of new bacteria species in air samples from Makkah, Saudi Arabia. BMC Res. Notes 7, 892.

Apweiler, R., Bairoch, A., Wu, C.H., Barker, W.C., Boeckmann, B., Ferro, S., Gasteiger, E., Huang, H., Lopez, R., Magrane, M., Martin, M.J., Natale, D.A., O'Donovan, C.,

Redaschi, N., Yeh, L.S., 2004. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 32, D115–D119.
Arnold, R.J., Reilly, J.P., 1999. Observation of Escherichia coli ribosomal proteins and their posttranslational modifications by mass spectrometry. Anal. Biochem. 269, 105–112.
Barreiro, J.R., Braga, P.A., Ferreira, C.R., Kostrzewa, M., Maier, T., Wegemann, B., Boettcher, V., Eberlin, M.N., dos Santos, M.V., 2012. Nonculture-based identification of bacteria in milk by protein fingerprinting. Proteomics 12, 2739–2745.
Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W., 2013. GenBank. Nucleic Acids Res. 41, D36–D42.
Bizzini, A., Durussel, C., Bille, J., Greub, G., Prod'hom, G., 2010. Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J. Clin. Microbiol. 48, 1549–1554.
Chapin, A., Rule, A., Gibson, K., Buckley, T., Schwab, K., 2005. Airborne multidrug-resistant bacteria isolated from a concentrated swine feeding operation. Environ. Health Perspect. 113, 137–142.
Cole, J.R., Wang, Q., Fish, J.A., Chai, B.L., McGarrell, D.M., Sun, Y.N., Brown, C.T., Porras-Alfaro, A., Kuske, C.R., Tiedje, J.M., 2014. Ribosomal database project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 42, D633–D642.
Curtis, S.E., Drummond, J.G., Grunloh, D.J., Lynch, P.B., Jensen, A.H., 1975. Relative and qualitative aspects of aerial bacteria and dust in swine houses. J. Anim. Sci. 41, 1512–1520.
Demarco, M.L., Burnham, C.A., 2014. Diafiltration MALDI-TOF mass spectrometry method for culture-independent detection and identification of pathogens directly from urine specimens. Am. J. Clin. Pathol. 141, 204–212.
Donham, K., Haglind, P., Peterson, Y., Rylander, R., Belin, L., 1989. Environmental and health studies of farm-workers in swedish swine confinement buildings. Br. J. Ind. Med. 46, 31–37.
Donohue, M.J., Smallwood, A.W., Pfaller, S., Rodgers, M., Shoemaker, J.A., 2006. The development of a matrix-assisted laser desorption/ionization mass spectrometry-based method for the protein fingerprinting and identification of Aeromonas species using whole cells. J. Microbiol. Methods 65, 380–389.
Douwes, J., Thorne, P., Pearce, N., Heederik, D., 2003. Bioaerosol health effects and exposure assessment: progress and prospects. Ann. Occup. Hyg 47, 187–200.
Gärtner, A., Gessner, A., Rabe, R., Mehring, M., 1885. Emissionsmessungen von Mikroorganismen: Entwicklung und Erprobung eines Messverfahrens. VDI-Berichte Nr., 179–199.
Gärtner, A., Gessner, A., Balfanz, J., Jäckel, U., 2008a. Performance characteristics for the measurement of microorganisms sampled with the LANUV-emission impinger. Gefahrstoffe Reinhaltung der Luft 68, 357–360.
Gärtner, A., Molter, L., Gessner, A., 2008b. Determination of the collection efficiency of an impinger for emission measurements of microorganisms. Gefahrstoffe Reinhaltung der Luft 68, 351–356.
Gärtner, A., Gessner, A., Martin, E., Schneider, D., Jäckel, U., 2014. Emissions from poultry houses – Investigation on the composition of bacterial community and antibiotic resistance – Part 2: Results. Gefahrstoffe Reinhaltung der Luft 74, 377–383.
Gärtner, A., Gessner, A., Gromöller, S., Klug, K., Knust, K., Jäckel, U., 2016. Emissions from pig barns – Investigation on the composition of bacterial community and antibiotic resistance. Gefahrstoffe Reinhaltung der Luft 76, 31–38.
Gao, L., Tan, Y., Zhang, X., Hu, J., Miao, Z., Wei, L., Chai, T., 2015. Emissions of Escherichia coli carrying extended-spectrum β-lactamase resistance from pig farms to the surrounding environment. Int. J. Environ. Res. Public Health 12, 4203–4213.
Ghosh, B., Lal, H., Srivastava, A., 2015. Review of bioaerosols in indoor environment with special reference to sampling, analysis and control mechanisms. Environ. Int. 85, 254–272.
Hausdorf, L., Mundt, K., Winzer, M., Cordes, C., Fröhling, A., Schlüter, O., Klocke, M., 2013. Characterization of the cultivable microbial community in a spinach-processing plant using MALDI-TOF MS. Food Microbiol. 34, 406–411.
Jahne, M.A., Rogers, S.W., Holsen, T.M., Grimberg, S.J., Ramler, I.P., 2015. Emission and dispersion of bioaerosols from dairy manure application sites: human health risk assessment. Environ. Sci. Technol. 49, 9842–9849.
Jahne, M.A., Rogers, S.W., Holsen, T.M., Grimberg, S.J., Ramler, I.P., Kim, S., 2016. Bioaerosol deposition to food crops near manure application: quantitative microbial risk assessment. J. Environ. Qual. 45, 666–674.
Kim, H.B., Isaacson, R.E., 2015. The pig gut microbial diversity: understanding the pig gut microbial ecology through the next generation high throughput sequencing. Vet. Microbiol. 177, 242–251.
Lighthart, B., 1994. Physics of microbial bioaerosols. In: Lighthart, B., Mohr, A.J. (Eds.), In Atmospheric Microbial Aerosols: Theory and Applications. Chapman & Hall, London, pp. 5–27.
Madsen, A.M., Zervas, A., Tendal, K., Nielsen, J.L., 2015. Microbial diversity in bioaerosol samples causing ODTS compared to reference bioaerosol samples as measured using Illumina sequencing and MALDI-TOF. Environ. Res. 140, 255–267.
Madsen, A.M., Alwan, T., Orberg, A., Uhrbrand, K., Jorgensen, M.B., 2016a. Waste workers' exposure to airborne fungal and bacterial species in the truck cab and during waste collection. Ann. Occup. Hyg. 60, 651–668.
Madsen, A.M., Larsen, S.T., Koponen, I.K., Kling, K.I., Barooni, A., Karottki, D.G., Tendal, K., Wolkoff, P., 2016b. Generation and Characterization of Indoor Fungal Aerosols for Inhalation Studies. Appl. Environ. Microbiol. 82, 2479–2493.
Marti, R., Dabert, P., Pourcher, A.M., 2009. Pig manure contamination marker selection based on the influence of biological treatment on the dominant fecal microbial groups. AEM 75, 4967–4974.
Martin, E., Kämpfer, P., Jäckel, U., 2009. Identification of bacterial diversity in the indoor air environment. Gefahrstoffe Reinhaltung der Luft 69, 97–101.
Martin, E., Dziurowitz, N., Jäckel, U., Schafer, J., 2015. Detection of airborne bacteria in a duck production facility with two different personal air sampling devices for an exposure assessment. J. Occup. Environ. Hyg. 12, 77–86.
Masclaux, F.G., Sakwinska, O., Charriere, N., Semaani, E., Oppliger, A., 2013. Concentration of airborne Staphylococcus aureus (MRSA and MSSA), total bacteria, and endotoxins in pig farms. Ann. Occup. Hyg. 57, 550–557.
Nehme, B., Letourneau, V., Forster, R.J., Veillette, M., Duchaine, C., 2008. Culture-independent approach of the bacterial bioaerosol diversity in the standard swine confinement buildings, and assessment of the seasonal effect. Environ. Microbiol. 10, 665–675.
Ngbede, E.O., Raji, M.A., Kwanashie, C.N., Kwaga, J.K., 2017. Antimicrobial resistance and virulence profile of enterococci isolated from poultry and cattle sources in Nigeria. Trop. Anim. Health Prod. 49, 451–458.
Nishikaze, T., Okumura, H., Jinmei, H., Amano, J., 2012. Correlation between Sweet Spots of Glycopeptides and Polymorphism of the Matrix Crystal in MALDI Samples. Mass Spectrom. (Tokyo) 1, A0006.
Pineda, F.J., Antoine, M.D., Demirev, P.A., Feldman, A.B., Jackman, J., Longenecker, M., Lin, J.S., 2003. Microorganism identification by matrix-assisted laser/desorption ionization mass spectrometry and model-derived ribosomal protein biomarkers. Anal. Chem. 75, 3817–3822.
Pisanic, N., Nadimpalli, M., Rinsky, J.L., Stewart, J., Wing, S., Love, D.C., Hall, D., Heaney, C.D., 2015. Pig-2-Bac as a biomarker of occupational exposure to pigs and livestock-associated Staphylococcus aureus among industrial hog operation workers. Environ. Res. 143, 93–97.
Prod'hom, G., Bizzini, A., Durussel, C., Bille, J., Greub, G., 2010. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for direct bacterial identification from positive blood culture pellets. J. Clin. Microbiol. 48, 1481–1483.
Pundir, S., Magrane, M., Martin, M.J., O'Donovan, C., UniProt Consortium, 2015. Searching and navigating UniProt databases. Curr. Protoc. Bioinf. 50, 1.27.1–1.27.10.
Ryzhov, V., Fenselau, C., 2001. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Anal. Chem. 73, 746–750.
Sala-Comorera, L., Vilaró, C., Galofré, B., Blanch, A.R., García-Aljaro, C., 2016. Use of matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry for bacterial monitoring in routine analysis at a drinking water treatment plant. Int. J. Hyg. Environ. Health 219, 577–584.
Sedo, O., Sedlacek, I., Zdrahal, Z., 2011. Sample preparation methods for MALDI-MS profiling of bacteria. Mass Spectrom. Rev. 30, 417–434.
Seedorf, J., Hartung, J., Schroder, M., Linkert, K.H., Phillips, V.R., Holden, M.R., Sneath, R.W., Short, J.L., White, R.P., Pedersen, S., Takai, H., Johnsen, J.O., Metz, J.H.M., Koerkamp, P.W.G.G., Uenk, G.H., Wathes, C.M., 1998. Concentrations and emissions of airborne endotoxins and microorganisms in livestock buildings in Northern Europe. J. Agric. Eng. Res. 70, 97–109.
Stetzenbach, L.D., Buttner, M.P., Cruz, P., 2004. Detection and enumeration of airborne biocontaminants. Curr. Opin. Biotechnol. 15, 170–174.
TRBA 466, 2015. Technical Rules for Biological Agents: Classification of Prokaryotes (Bacteria and Archaea) into Risk Groups. Committee on Biological Agents – ABAS, Berlin, pp. 65, 317, 116.
The-UniProt-Consortium, 2017. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 45, D158–D169.
Thomin, J., Aubin, G.G., Foubert, F., Corvec, S., 2015. Assessment of four protocols for rapid bacterial identification from positive blood culture pellets by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Vitek (R) MS). J. Microbiol. Meth. 115, 54–56.
Toh-Boyo, G.M., Wulff, S.S., Basile, F., 2012. Comparison of sample preparation methods and evaluation of intra- and intersample reproducibility in bacteria MALDI-MS profiling. Anal. Chem. 84, 9971–9980.
VDI 4253 Part 3, 2008. Measurement of Airborne Microorganisms and Viruses in Ambient Air; Culture Based Method for the Quantitative Determination of Bacteria in Air; Method After Separation in Liquids. Kommission Reinhaltung der Luft im VDI und DIN – Normenausschuss KRdL, Düsseldorf, Beuth, Berlin, pp. 1–26.
VDI 4257 Part 2, 2011. Bioaerosols and Biological Agents; Emission Measurement; Sampling of Bioaerosols and Separation in Liquids. Kommission Reinhaltung der Luft im VDI und DIN – Normenausschuss KRdL, Düsseldorf, Beuth, Berlin, pp. 1–60.
Walser, S.M., Gerstner, D.G., Brenner, B., Bunger, J., Eikmann, T., Janssen, B., Kolb, S., Kolk, A., Nowak, D., Raulf, M., Sagunski, H., Sedlmaier, N., Suchenwirth, R., Wiesmuller, G., Wollin, K.M., Tesseraux, I., Herr, C.E., 2015. Evaluation of exposure-response relationships for health effects of microbial bioaerosols – a systematic review. Int. J. Hyg. Environ. Health 218, 577–589.
Wang, Z.P., Russon, L., Li, L., Roser, D.C., Long, S.R., 1998. Investigation of spectral reproducibility in direct analysis of bacteria proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy. Rapid Commun. Mass Spectrom. 12, 456–464.
Wang, H., Chen, Y.-L., Teng, S.-H., Xu, Z., Xu, Y.-C., Hsueh, P.-R., 2016. Evaluation of the Bruker Biotyper matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system for identification of clinical and environmental isolates of Burkholderia pseudomallei. Front. Microbiol. 7.

Wattal, C., Oberoi, J.K., 2016. Microbial identification and automated antibiotic susceptibility testing directly from positive blood cultures using MALDI-TOF MS and VITEK 2. Eur. J. Clin. Microbiol. Infect. Dis. 35, 75–82.
Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16 s ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.
Wielders, C.C., van Hoek, A.H., Hengeveld, P.D., Veenman, C., Dierikx, C.M., ZomerT, P., Smit, L.A., van der Hoek, W., Heederik, D.J., de Greeff, S.C., Maassen, C.B., van Duijkeren, E., 2017. Extended-spectrum beta-lactamase- and pAmpC-producing Enterobacteriaceae among the general population in a livestock-dense area. Clin. Microbiol. Infect. 23 (2), 120.e1–120.e8.