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Published in:
Environmental Research

DOI (link to publication from Publisher):
10.1016/j.envres.2019.01.008

Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Environmental Research 171 (2019) 558–567

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Microbial species and biodiversity in settling dust within and between pig farms

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ARTICLE INFO

Keywords:
Occupational exposure
Airborne fungi
Bioaerosol
Pig farming
Pathogenic microbes

ABSTRACT

The airborne fungal and bacterial species present in pig farm dust have not been well characterised even though these bioaerosols are known to cause inflammation and other airway maladies. In this study, the microbial species and composition in airborne dust within and between pig farms were investigated. Passively sedimenting dust from six pig farms were collected using electrostatic dust collectors. The bacterial and fungal species were identified using matrix-assisted laser desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and next generation sequencing (NGS).

Dust samples taken within the same stable section revealed high resemblance and stability. Constrained statistical analysis of the microbial community compositions indicated that the types of stable did not appear to have a great effect on the bacterial and fungal β-diversity. In contrast to this, the farm from which samples were taken appeared to have the greatest effect on the bacterial β-diversity, but this trend was not observed for the fungal β-diversity. The most common bacteria and fungi according to NGS data were anaerobes typically associated with the pig intestinal tract and yeasts respectively.

Bacterial sedimentation varied at a rate between 10^3 and 10^9 CFU/m^2/day, with the most common species after aerobic incubation being *Aerococcus viridans* and *Staphylococcus equorum*, while *Clostridium perfringens* and *Staphylococcus simulans* were the most common species after anaerobic incubation. A total of 28 different species of bacteria and fungi were classifiable as pathogens.

In conclusion, the biodiversity in pig farm dust shows a high diversity of bacterial species. However, samples from the same stable section resembled each other, but also different sections within the same farm also resembled each other, thus indicating a high degree of community stability in the dust source. In regards to fungal identification, the biodiversity was observed to be similar between samples from different stable sections and farms, indicating a higher degree of similarities in the mycobiomes found across pig farms studied.

1. Introduction

Farmers working with livestock are on a daily basis exposed to high levels of bacteria and fungi (Eduard et al., 2001). Microorganisms, cell fragments, and metabolites coming from animal dander, faecal matter, and feed materials are typical components found in dust (Donham et al., 1989, 1986). Previous research has shown an association between occupational exposure to increased concentrations of bacterial endotoxin and dust and the development of asthma and other airway maladies (Heederik et al., 1991; Madsen et al., 2015; Zhiping et al., 1996). Of the studies which analysed microbial exposure, many have focused on total bacterial or fungal exposure, rather than analysing the microbial diversity and identifying the fungal and bacterial species. However, in the last years, studies have been conducted on analysing the abundance, genetics, and infections of livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) in farms (Angen et al., 2017; Dahms et al., 2014; Feld et al., 2018; Garcia-Graells et al., 2012; Goerge et al., 2017). Furthermore, the bacterial pathogens *Salmonella, Campylobacter, Listeria, Enterococcus*, and *Clostridium* have been associated with pigs (Baer et al., 2013; Keessen et al., 2011; Nilsson, 2012; Songer, 2010). Members of these genera are known to cause intestinal disease and soft-tissue infections in humans (Swartz, 2002). Recently it

Abbreviations: LA-MRSA, Livestock associated Methicillin Resistant *Staphylococcus aureus*; EDC, Electrostatic Dust Collector; OTU, Operational Taxonomic Unit; ESV, Exact Sequence Variant; NGS, Next Generation Sequencing; MALDI-TOF MS, Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry; NA, Nutrient Agar; DG18, Dichloran Glycerol Agar; RDA, Constrained Redundancy Analysis; TRBA, Technical Rules for Biological Agents; CFU, Colony Forming Unit

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https://doi.org/10.1016/j.envres.2019.01.008
Received 12 October 2018; Received in revised form 30 November 2018; Accepted 4 January 2019
Available online 06 January 2019

was observed that the bacterial diversity found in the anterior nares of pigs resembles that of pig farmers (Kraemer et al., 2018; Weese et al., 2014). This makes it important to obtain knowledge on which fungi and bacteria are present in the air in pig farms.

Different methodological approaches can be used for the identification of airborne fungi and bacteria. One approach, high-throughput next generation sequencing (NGS) has the advantage that both culturable and unculturable microorganisms can be identified. However, these techniques are still inherently biased by primer choice, and the efficiency of DNA extraction, which limit the identification of bacterial and fungal genera (Ghyselinck et al., 2013). Currently, NGS cannot distinguish pathogenic and commensal species from one another in complex environmental samples. Another approach allows for the identification of many culturable bacterial and fungal species down to the species level through the use of matrix assisted laser desportion/ionisation time of flight mass spectrometry (MALDI-TOF MS) after growth on agar plates. This makes it a relevant tool for distinguishing between pathogens, commensals, and other environmental species (Carbonnelle et al., 2011; Madsen et al., 2015; Marklein et al., 2009; Wieser et al., 2012). Therefore, a combination of these two techniques may be required to analyse relevant bacteria and fungi present in complex environmental samples.

In the measurement of potential occupational exposure to microorganisms, samples taken should ideally be representative of the potential exposure that farm workers face. In pig stables, spatial variation (Barber et al., 1991; Wang et al., 2002) and daily variations (Zeitler-Feicht et al., 1991) in the airborne dust have been observed. Pig farmers normally work in different farm sections during a working day, and this is potentially associated with different dust (Mc Donnell et al., 2008) and MRSA exposure levels (Madsen et al., 2018a). However, whether this is also associated with exposure to different airborne microbiota is not yet known. In pig farms, animals of almost similar age and weight, with the exception of farrowing and nursing stables, are kept in the same stables where they are likely to share the same bacterial species (Han et al., 2018).

Using short-term sampling times, e.g. 10 min, a variation in *S. aureus* concentration is observed within the same day and pig farm section, and this could be related to the work-tasks performed (Madsen et al., 2018a). Additionally, farm work may occur both during the day and night. Consequently, a sampling period spanning at least one day and night is preferable. One of the methods used for long-term sampling of airborne settling dust is the electrostatic dust collector (EDC), which has been used for sampling MRSA in pig stables, measuring mite allergen exposure, and bacterial and fungal exposure in indoor environments (Dorado-García et al., 2013; Feld et al., 2018; Frankel et al., 2012; Van Cleef et al., 2015; Zahradnik et al., 2011). The sampler has the advantage that it does not subject the sampled microorganisms to an airflow which is expected to be beneficial for the survival of bacteria.

To help protect workers from potential disease, general knowledge regarding the quantity and identity of bacterial and fungal species present in farm dust is needed. Knowing the species composition and the microbial diversity of dust in pig farms is a vital step towards being able to risk evaluate an exposure in a pig farm.

The aim of this study was to further the understanding of the microbial species and composition in airborne dust within and between pig farms across Denmark. To do this, airborne dust was sampled in different farm sections of LA-MRSA positive pig farms, and for identification a two phasic approach was used. Thus, bacteria and fungi were quantified and identified using MALDI-TOF MS, and Illumina based amplicon sequencing was used to identify the bacterial and fungal diversity. Additionally, statistical analyses were performed to test whether samples taken in different farm sections within a farm resembled each other more than samples taken in different farms with pigs of the same age and vice versa.

Table 1
Overview of the samples taken from farm A to F and stable types and sample name.

Farm	Stable type and sample name	
A	Farrowing A1-A3	Weaning A4-A6
B	Weaning B1-B3	Fattening B4-B6
C	Farrowing C1-C3	Weaning C4-C6
D	Weaning D1-D3	Nursing D4-D6
E	Weaning E1-E4	–
F	Weaning F1-F3	–

2. Materials and methods

2.1. Sampling and storage

Electrostatic dust collectors (EDCs) (ZEEMAN, Holland) were utilised for the collection of bacteria and fungi in settling airborne dust from six conventional swine farms located across Denmark. Samples are referred to as stated in Table 1.

During sampling, EDCs were placed in one to four stable sections and in one or two different stable types (farrowing, nursing, weaning, and fattening). The farrowing section housed sows and piglets of 0–4 weeks, the nursing stable had sick pigs of 3–6 months, the weaning section consisted of pigs of 4–8 weeks, and the fattening section contained pigs up to 6 months. All samples were taken in the winter of 2015/2016. EDCs were left open at a height of 1–1.5 m above the floor with a distance of 2 m between samples for 1–7 days for passive sampling.

2.2. Extraction and cultivation

Dust from EDC cloths was extracted by placing the EDCs in sterile 50 mL tubes with the addition of 15 mL of extraction buffer (0.85% NaCl + 0.05% Tween 80) and shaking at 500 rpm for 15 min. Extracts were aliquoted in 1 mL volumes in cryotubes (Sigma Aldrich, Germany) with 0.5 mL of 85% glycerol. Aliquots were then stored at −80 °C until culturing and sequencing.

For aerobic bacterial and fungal cultivation, a serial dilution series was applied from 10^{-1} up to 10^{-5} with 100 µL of sample being plated on Nutrient Agar (NA) plates (Oxoid, UK) with 1% (wt/vol) cycloheximide (Sigma Aldrich, Germany) and dichloran-glycerol (DG18) agar with chloramphenicol (Oxoid) to select for bacterial and fungal growth respectively. NA and DG18 plates were incubated at 25 °C. The dilution which gave optimal coverage and separation of individual colony forming units (CFUs) was used to represent that sample. Thus, CFUs for identification by matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) ranged between 20 and 200.

Anaerobic growth of bacteria from the resuspended dust samples was performed using a serial dilution series from 10^{-2} to 10^{-3} plated on NA plates. Samples were then incubated in an AnaeroJar (Oxoid) with an AnaeroGen™ (Oxoid) sachet at 37 °C for 3 days. Once the AnaeroJar was opened, the bacterial colonies were immediately prepared for MALDI-TOF MS using the method described below. Only CFUs large enough (Ø > 1.5 mm) for identification were used for identification by MALDI-TOF MS.

2.3. Species identification by MALDI-TOF MS

The MALDI-TOF MS analysis was performed on a Microflex LT mass spectrometer (Bruker Daltonics) using the Biotyper 3.1 software with the BDAL standard library. A bacterial test standard (Bruker Daltonics) was used to calibrate the instrument.

For bacterial identification the extended direct transfer method was used as previously described (Feld et al., 2018). In brief, a sterile toothpick was used to transfer a small amount of bacterial colony onto a

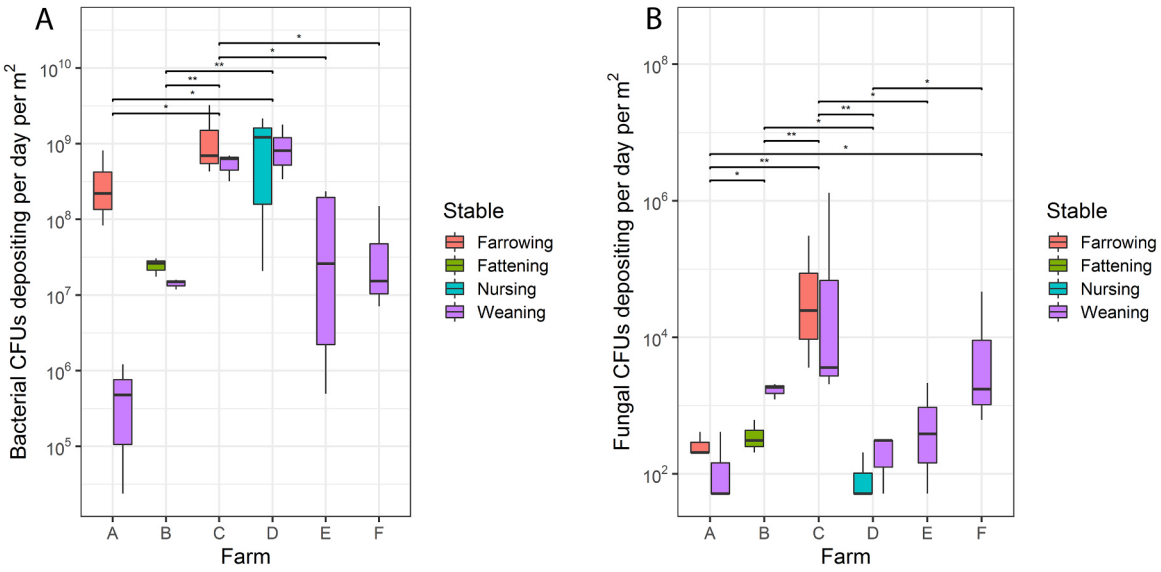

Fig. 1. Sedimentation rate of bacterial (A) and fungal (B) CFUs per m^2 per day in the 6 pig farms studied.

target plate (MSP 96 target polished steel BC, Bruker Daltonics, Bremen, Germany) before the colony was covered with 1 μL of 70% formic acid and left to dry. Once dried, the sample was overlaid with 1 μL of HCCA matrix (α-cyano-4-hydroxycinnamic acid, Bruker Daltonics). For fungal identification, the extended direct transfer method was not sufficient for identification. Those samples from DG18 agar plates were subcultured overnight in Sabouraud growth media (Oxoid) to obtain sufficient mycelium with few to no spores which could affect the MS spectra. The sample was washed twice with water to remove excess growth media before extraction was done using the ethanol extraction protocol (Madsen et al., 2015).

2.4. Next generation sequencing

DNA from resuspended dust samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol with 400 μL of resuspended settling dust used for extraction. Extracted DNA was then amplified in a two-step PCR targeting the V4 variable region of the bacterial 16S rRNA gene and the internal transcribed spacer (ITS) region of fungal ribosomal cistron. The primers targeting the V4 region were: 515F: GTGCCAGCMGCCGCGG TAA and 805R: GGACTACNVGGGTWTCTAAT and for the ITS, the primers used were ITS1F: CTTGGTCATTTAGAGGAAGTAA and ITS1R: GCTGCGTTCTTCATCGATGC (Caporaso et al., 2011; Ghannoum et al., 2010). Amplicon library PCR was performed using 10 ng of extracted DNA as template per 25 μL PCR reaction (400 nM of each dNTP, 1.5 mM $MgSO_4$, 2mU Platinum Taq DNA polymerase High Fidelity, and 1x Platinum High Fidelity buffer (Thermo Fisher, USA) and 400 nM of barcoded library adapter pair (Illumina, USA).

Thermocycler settings included initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s and final elongation at 72 °C for 5 min.

All PCR reactions were run in duplicate and pooled. Amplicon libraries obtained were cleaned using AMPure XP bead protocol (Beckman Coulter, USA) with the following modifications: the sample/bead ratio was 5/4 and the purified DNA was eluted in 23 μL nuclease free water. Library concentrations were measured with Quant-iT HS DNA Assay (Thermo Fisher, USA) and quality was checked using D1000 ScreenTapes (Agilent, USA). Samples were pooled in equimolar concentrations and the library pool was sequenced on the MiSeq platform (Illumina, USA) according to previous published procedure (Caporaso et al., 2012).

Due to PCR amplification issues, sample (B3) was removed from the dataset. All sequenced sample libraries were trimmed and low quality reads were removed using trimmomatic (v0.32) (Bolger et al., 2014). Reads were merged using FLASH (v1.27) (Magoč and Salzberg, 2011) before chimeric sequences were removed and reads were formatted using the UPARSE workflow (Edgar, 2013). Usearch7 was used to dereplicate reads and cluster them into Operational Taxonomic Units (OTUs) at a 97% sequence similarity. Taxonomy was assigned using MiDAS (a manually curated version of SILVA) and UNITE as reference databases for bacterial and fungal taxonomy respectively (Kõljalg et al., 2014; McIlroy et al., 2015). The obtained raw sequence data is available at the European Nucleotide Archive (ENA) under project accession number PRJEB29770.

2.5. Statistics and bioinformatics

All statistical analyses and visualisations were performed in RStudio version 1.1.453 (https://www.rstudio.com/), with R version 3.5.0 (Team, 2015), using the following R CRAN packages: ampvis2 (Andersen et al., 2018), ggplot2 (Wickham, 2009), Phyloseq (McMurdie and Holmes, 2013), and Vegan (Okasanen et al., 2018). Isolates which could not be identified by MALDI-TOF MS were included in the calculation of the sedimentation rates but were not incorporated in the construction of constrained redundancy analysis (RDA) plots. Microbial community composition and structure were explored using heatmaps and RDA plots. Microbial richness and evenness were visualised using Chao1 and CFU counts. Stable section similarity was explored using Jaccard index values. The β-diversity was investigated using RDA plots on Hellinger transformed abundance counts. Statistical comparisons were run between clusters using analysis of similarity (ANOSIM) according to Jaccard index values. Statistical comparisons between sedimentation of bacterial and fungal CFUs from different farms were performed using Wilcoxon signed-rank tests.

3. Results

3.1. Concentrations of bacteria and fungi

Bacterial and fungal concentrations for samples grown aerobically varied between the farms and associated stables. Overall, the concentration of aero-tolerant bacteria in the settling dust amongst all farm sections ranged from 4.1×10^5 to 3.2×10^9 CFUs/m^2/day. Fungal concentrations ranged from below detection to 1.2×10^6 CFU/m^2/day with significant differences between farms (Figs. 1A and 1B).

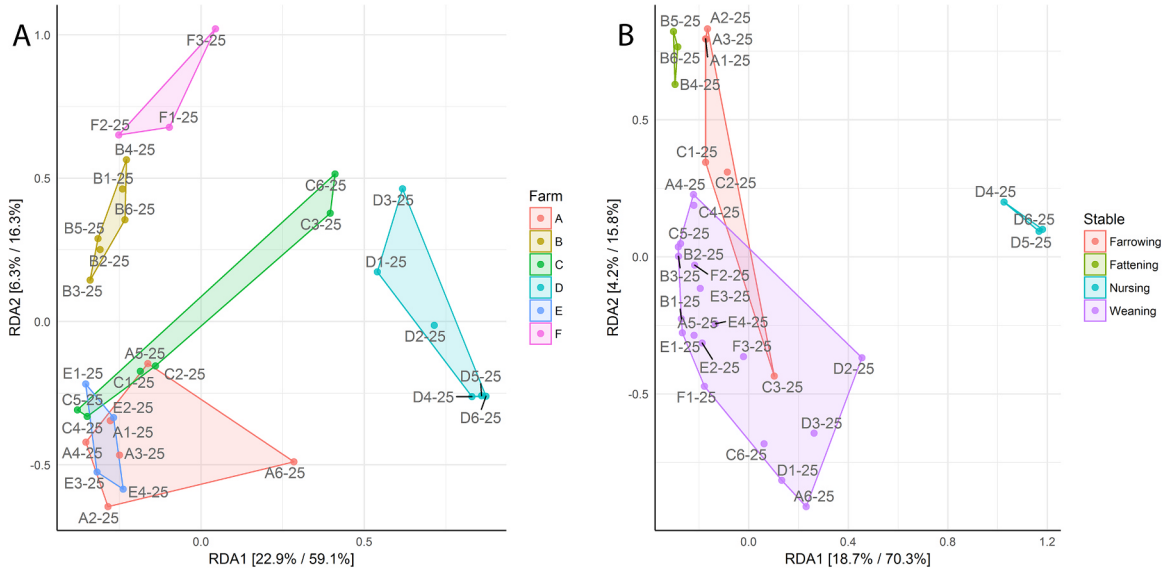

Fig. 2. A and B: RDA plotting of the bacteria cultured at 25 °C and identified by MALDI TOF MS constrained by which farm dust was taken from (A) or based on the stable type (B). Dissimilarity between clusters measured by ANOSIM was observed to be significant ($p < 0.001$) between the Farms (A) while just below significant ($p = 0.055$) between Stable types (B).

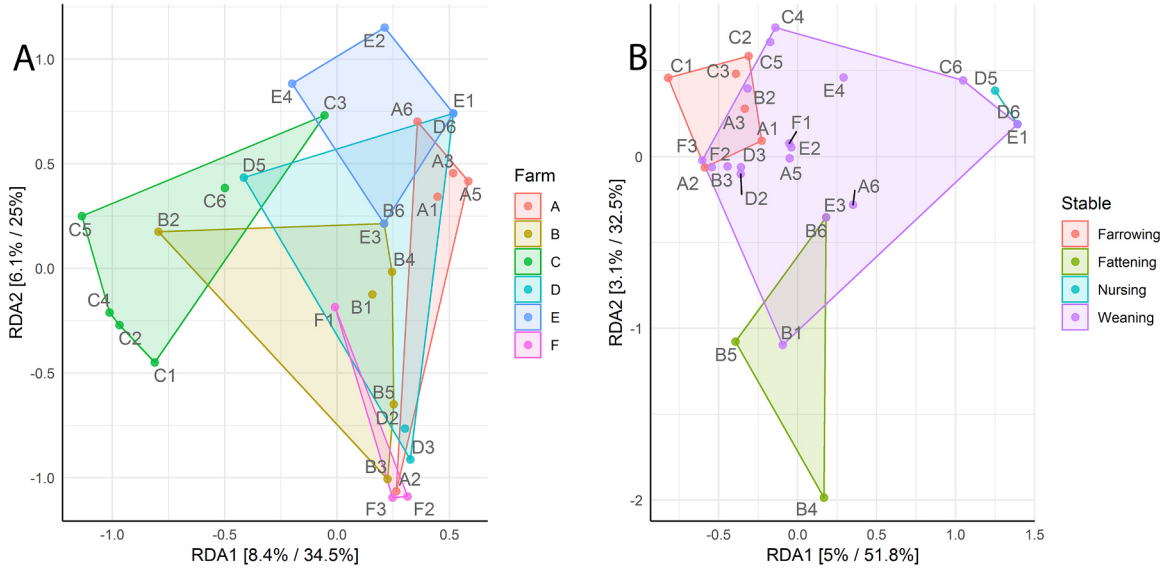

Fig. 3. A and B: RDA plotting of the fungi identified by MALDI TOF MS constrained by which farm dust was taken from (A) or based on the stable type (B). Dissimilarity between clusters measured by ANOSIM was observed to be significant ($p = 0.008$) between the Farms (A) while not significant ($p = 0.353$) between Stable types (B).

3.1.1. Aerobes

After aerobic incubation, a total of 96 bacterial species were identified by MALDI-TOF MS, and 81% of all bacterial isolates were identified to species level. On average 7.7 ± 4.1 different species were observed per sample. Three species were found in nearly each sample: *Aerococcus viridans, Staphyloccus equorum*, and *Leuconostoc mesenteroides*.

RDA plotting of the samples cultured at 25 °C revealed separate clustering based on the farm (Fig. 2A). RDA plotting of the samples constrained based on the stable type (Fig. 2B) showed some separation between the stable types, although it was observed that samples taken from the same farm still clustered closer to one another rather than based on the stable type.

3.1.2. Fungi

Fungal identification revealed 27 mould and yeast species, and 83.5% of all isolates were identified to species level. An average of 2.6 ± 1.7 species were observed in each sample. In some samples (C6, and F3) the yeast species *Filobasidum uniguttulatum* and *Candida famata* respectively dominated. In samples A4, D1, and D4 there were no fungal colonies which formed at any dilution. Constrained analysis of the biodiversity revealed differences in the β-diversity, which were linked to the farm of origin rather than stable type (Fig. 3 A and B).

3.1.3. Anaerobic bacteria

Anaerobic incubation revealed a total of 49 different species of which 24 could not be cultured aerobically. A single sample (D4) did not have any anaerobes. The following genera were not found during aerobic culturing: *Clostridium*, *Lactobacillus*, and *Vagococcus*. In addition to these three genera, several species of *Staphylococcus* were identified, including *S. aureus, S. simulans, S. epidermidis, S. chromogenes, S. pasteuri, S. hominis, S. hyicus,* and *S. haemolyticus.*

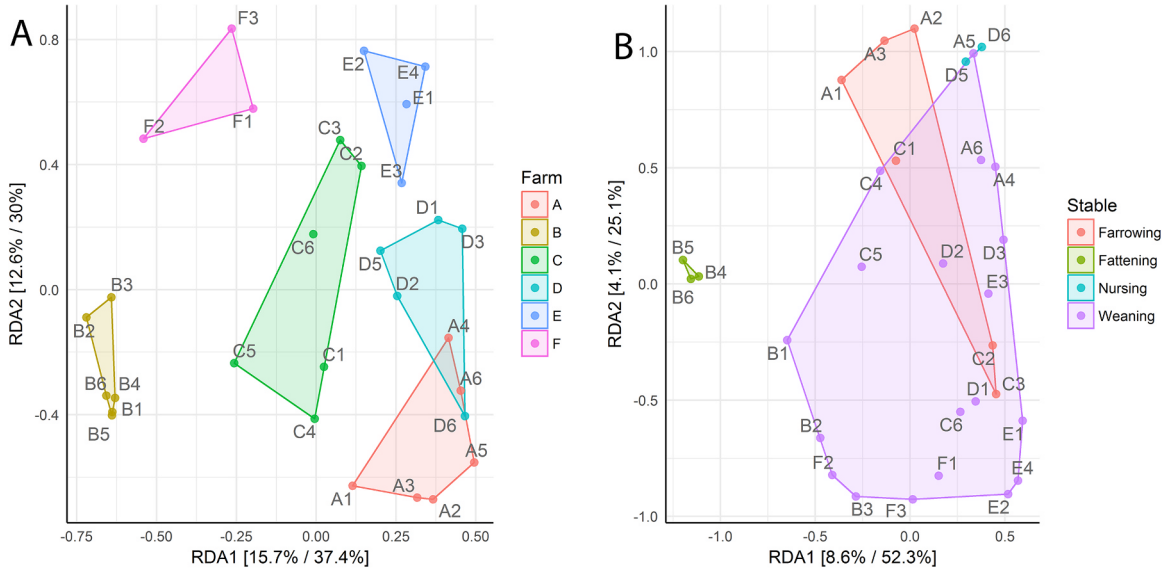

Fig. 4. A and B: RDA plotting of the bacteria cultured at 37 °C anaerobically and identified by MALDI TOF MS constrained by which farm dust was taken from (A) or based on the stable type (B). Dissimilarity between clusters measured by ANOSIM was observed to be significant ($p < 0.001$) between the Farms (A) while not significant ($p = 0.162$) between Stable types (B).

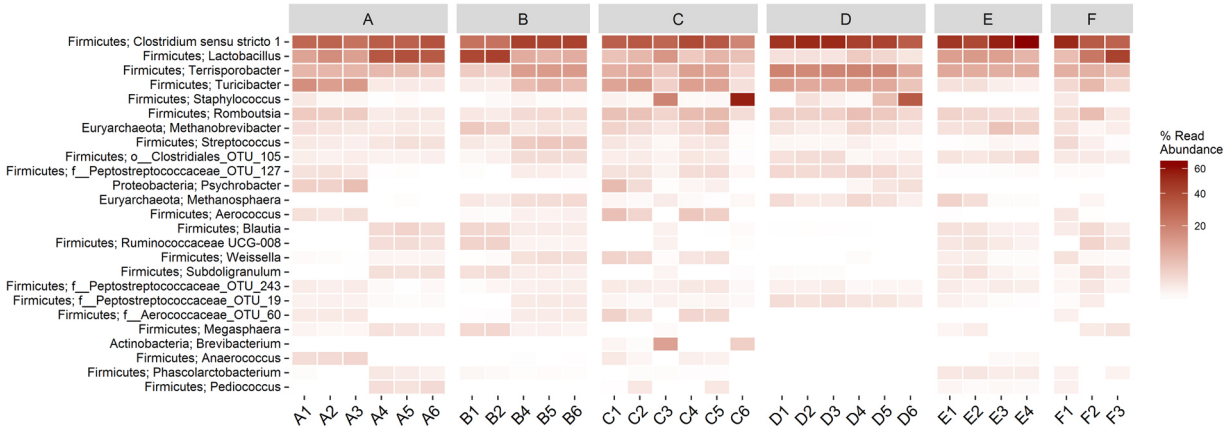

Fig. 5. Heatmap of the top 25 bacterial OTUs identified after targeting the bacterial 16S rRNA V4 region. Samples are faceted by the farm they are taken from. Taxonomy is listed at the phylum and highest taxonomic resolution possible.

Of the *Clostridium* species identified, *C. perfringens* was the most common isolate observed (16/31 samples tested positive for *C. perfringens*) and 11/31 samples tested positive for *S. aureus*. RDA plotting revealed a clear distinction between samples at the farm level (Fig. 4A) rather than based on stable type (Fig. 4B).

3.2. Sequencing

A total of 4.0×10^5 high quality bacterial reads and 1.5×10^5 high quality fungal reads were generated with an average number of 13,340 ± 3704 reads per bacterial sample while fungal samples generated an average number of 5011 ± 2980 reads. The number of reads from each OTU acts as a measure of the relative abundance within each sample and the relative abundance of each OTU varies from sample to sample. Rarefraction curves of the bacterial and fungal OTUs approximated a horizontal line (with the exception of sample B3), indicating that the majority of microbial diversity was captured (Supplementary Figs. 1 and 2).

The most dominant bacterial OTUs were from the genera *Clostridium sensu stricto, Lactobacillus, Terrisporobacter, Turicibacter, Romboutsia, Methanobrevibacter, Aerococcus*, and *Weissella* (Fig. 5), all of which are either facultative or obligate anaerobes. Bacterial α-diversity showed an average richness of 150 OTUs.

Sequencing of fungal DNA showed that the most dominant fungal OTUs were from the genera *Debaryomyces, Cryptococcus, Apiotrichum, Wallemia,* and *Candida*. Validation of the OTUs by BLAST (NCBI) revealed that the OTU classified as *Cryptococcus* was mislabelled and instead belonged to the genus *Filobasidum* and the OTU classified as *Debaryomyces* belonged to the genus *Candida*. A list of the 25 most abundant fungal OTUs observed is shown in Fig. 6.

Community wide analyses were performed on sequencing data using RDA plots to visualise the results and were constrained to identify metadata variables which influenced sample compositions. In Fig. 7A the dataset was clustered based on the farm of origin, and in Fig. 7B the samples were clustered based on stable type. Sequenced samples constrained based on farm of origin clustered closest to samples from the same farm, implying that the bacterial diversity of each farm is unique and has an influence on the bacterial communities present.

Fungal samples constrained based on the farm of origin showed some unique clustering (in particular farms B and E), but the remaining farms had a large overlap implying that the samples are of similar fungal populations, as seen in Fig. 8A and B.

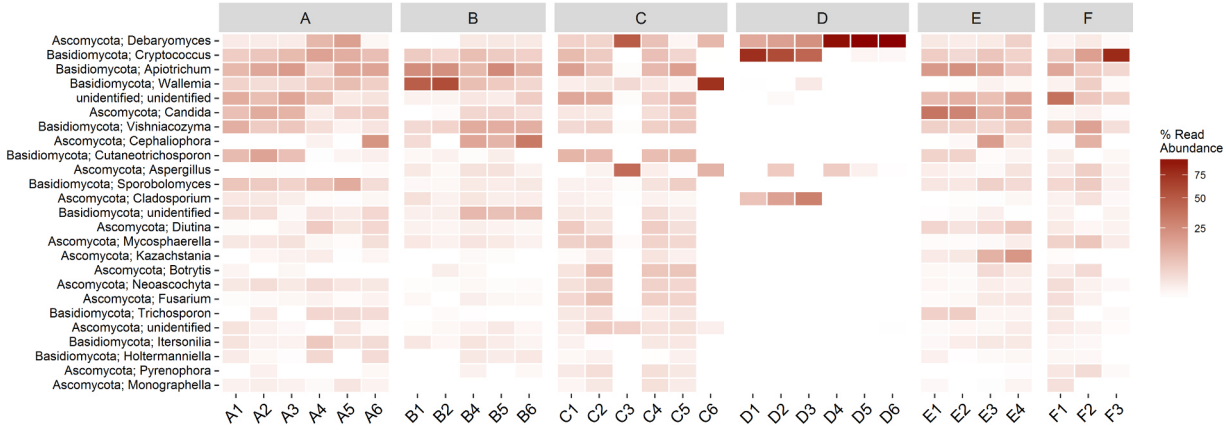

Fig. 6. Heatmap of the top 25 fungal OTUs identified after targeting the fungal ITS region. Samples are faceted by the farm they are taken from. Taxonomy is listed at the highest taxonomic resolution possible.

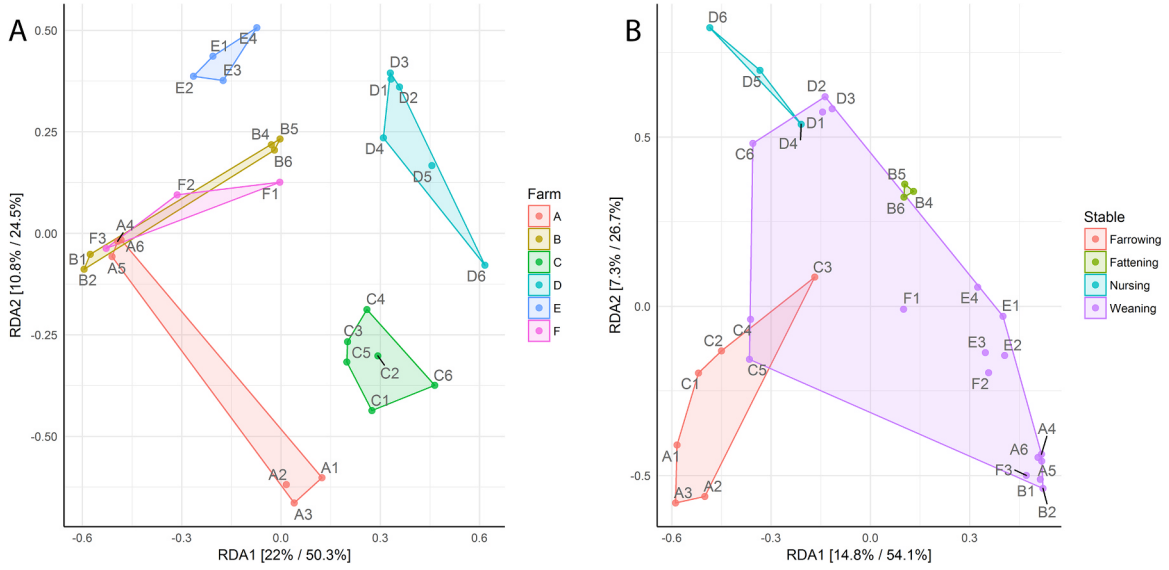

Fig. 7. RDA plotting of the bacterial OTUs identified after sequencing constrained by which farm dust was taken from (A) or based on the stable type (B). Dissimilarity between clusters measured by ANOSIM was observed to be significant between the Farms (A), $p < 0.001$, and between Stable types (B), $p = 0.017$.

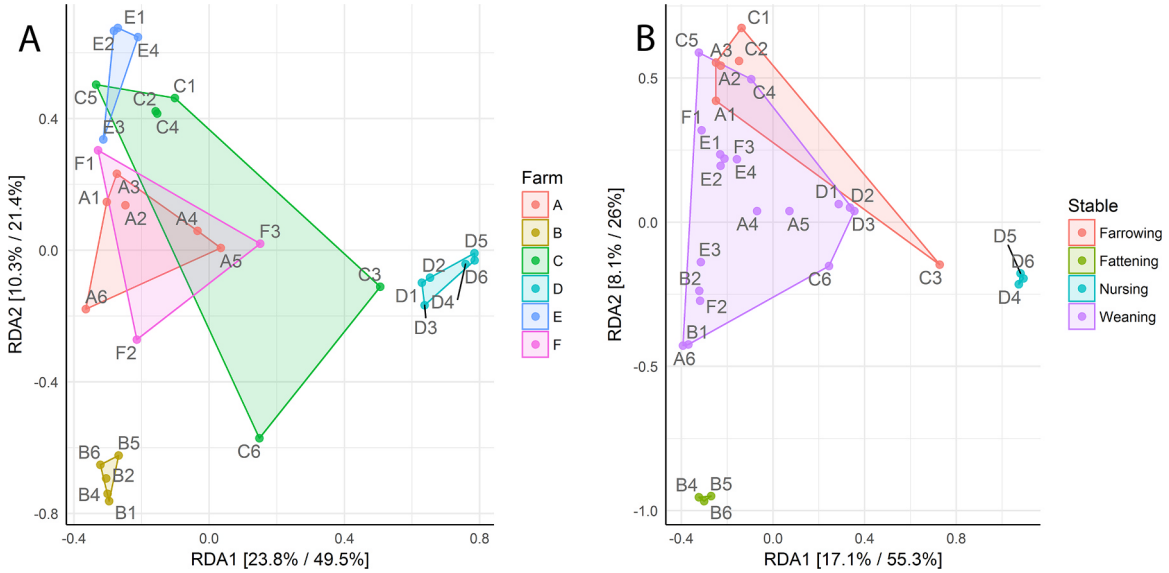

Fig. 8. RDA plotting of the fungal OTUs identified after sequencing constrained by which farm dust was taken from (A) or based on the stable type (B). Dissimilarity between clusters measured by ANOSIM was observed to be significant between the Farms (A), $p < 0.001$, and between Stable types (B), $p = 0.038$.

Table 2
α-diversity indices of each stable type based on species for cultured bacteria and fungi and based on OTU clustering for sequenced data. Significant p-values are written in bold.

	W	Fa	Ft	N	
	Richness				P-value
Aerobes	7.7 ± 3.7	10.8 ± 3.7	13.0 ± 1.7	4.0 ± 3.5	**P = 0.01**
Anaerobes	7.4 ± 4.3	10.7 ± 3.1	8.7 ± 4.7	2.5 ± 0.7	P = 0.10
Fungi	2.9 ± 1.8	3.0 ± 1.7	1.7 ± 1.2	1.0 ± 0	P = 0.31
16S	199.6 ± 34.5	199.3 ± 21.1	238.0 ± 5.6	147.6 ± 10.9	**P < 0.01**
ITS	58.2 ± 24.9	71.8 ± 15.4	91.7 ± 1.5	14.3 ± 6.7	**P < 0.01**
	Shannon-Weaver				P-value
Aerobes	1.2 ± 0.6	1.4 ± 0.2	1.7 ± 0.1	0.7 ± 0.8	P = 0.11
Anaerobes	1.5 ± 0.6	1.6 ± 0.2	1.6 ± 0.7	0.6 ± 0.6	P = 0.14
Fungi	0.63 ± 0.5	0.8 ± 0.6	0.4 ± 0.6	1.0 ± 0	**P = 0.01**
16S	2.99 ± 0.37	3.42 ± 0.18	3.21 ± 0.01	2.73 ± 0.16	**P = 0.01**
ITS	2.3 ± 0.9	2.9 ± 0.9	3.1 ± 0.2	0.1 ± 0.08	**P < 0.01**
	Chao1				P-value
Aerobes	11.4 ± 5.9	17.5 ± 10.2	17.3 ± 3.8	4.0 ± 3.5	**P = 0.03**
Anaerobes	9.8 ± 5.3	15.3 ± 5.4	10.1 ± 6.3	2.5 ± 0.7	**P = 0.03**
Fungi	3.3 ± 2.1	4.0 ± 2.7	2.7 ± 2.9	1.0 ± 0	P = 0.44
16S	252.6 ± 33.5	243.1 ± 25.4	295.5 ± 30.5	192.6 ± 36.4	**P < 0.01**
ITS	68.01 ± 27.7	79.9 ± 18.7	97.2 ± 0.7	22.7 ± 16.3	**P < 0.01**

3.3. Diversity indices

Diversity of the samples was also explored by estimating the α-diversity indices using Shannon-Weaver and Chao1, as shown in Table 2. The α-diversities (richness, Shannon-Weaver, and chao1) were significantly different ($p < 0.001$ to $p = 0.05$) for different farms except for fungal culturing (data not shown). Fungal culturing revealed the lowest level of α-diversity. Amongst the stable types, it was noted that the bacterial diversity was highest as measured using all indices in the fattening stables. Fungal diversity was noted to be highest as measured using all indices in the farrowing stables while both bacterial and fungal diversity were lowest in the nursing stable.

The differences between the stables and farms were explored using the Shannon-Weaver Index to study whether the farm or the stable type had the greatest effect on the diversity. Samples taken from Farm D had a Shannon Index value that was significantly ($p < 0.0001$) lower in the samples which were sequenced targeting the V4 and ITS regions compared to the samples taken from the other five farms. Samples from different stable sections on the same farm were also compared by their Jaccard index to determine whether samples taken from the same stable section resembled each other more compared to the samples taken from different stables and farms. Fungal culturing revealed the lowest level of similarity while bacterial anaerobic culturing revealed the highest level of similarity within each stable section (Supplementary table 1).

3.4. Biological risk group 2 species

In total, 28 species of bacteria and fungi identified by MALDI-TOF MS were categorised under risk group 2 in accordance with the German Technical Rules for Biological Agents (TRBA) 460 (fungi) and 466 (bacteria) as shown in Table 3 (Ausschuss für Biologische Arbeitsstoffe, 2016, 2015).

Table 3
Group 2 microorganisms which can cause disease according to the German Technical Rules for Biological Agents 460 and 466.

Organism(s)	Range in positive samples (CFU/m^2/day)	Disease(s)	Reference(s)
Aerococcus viridans	900–4.05 × 10^6	Urinary tract infections (UTIs), Endocarditis	(Chen et al., 2012; Mohan et al., 2017)
Bacillus cereus	4.5 × 10^3***	Diarrhoea, bacteraemia	(Bottone, 2010)
Clostridium perfringens/bifermentans/ cadaveris/baratii	150–1.76 × 10^5	Enterocolitis, diarrhoea, gas gangrene, necrotic enteritis, bacteraemia, necrotising endometritis, botulism, pneumonia	(Brynestad and Granum, 2002; Hale et al., 2016; Harvey et al., 2002; Lima et al., 2016; Poduval et al., 1999; Songer, 2010)
Enterococcus avium/casseliflavus/durans/ faecalis/villorum/gallinarum/hirae/ faecium	150–4.5 × 10^5	Bacteraemia, UTIs, bacterial endocarditis, and wound infection	(Patel et al., 1993; Reid et al., 2001; Swartz, 2002)
*Filobasidium uniguttulatum**	450–6.8 × 10^4	Cryptococcal meningitis	(Pan et al., 2012)
Leuconostoc mesenteroides sp.	1.5 × 10^4-1.1 × 10^7	Wound infections and other nosocomial infections	(Todorov and Dicks, 2005)
Serratia marcescens	1.5 × 10^3-9.0 × 10^4	UTIs, opportunistic infections	(Passaro et al., 1997)
Staphylococcus aureus/saprophyticus/ pasteuri/haemolyticus/hominis/ hyicus/pettenkoferi/epidermidis	1.5 × 10^3-1.5 × 10^5	UTIs, bacteraemia, sepsis, nosocomial disease	(Allen et al., 2014; Casanova et al., 2011; Gunn and Davis Jr, 1988; Hashi et al., 2015; Lowry, 1998; Palazzo et al., 2008; Petersen et al., 2014; Savini et al., 2009; Schöfer et al., 2011)
*Streptococcus parauberis*** /lutetiensis*	150–7.5 × 10^3	Bacteraemia	(Almuzara et al., 2013)
Vagococcus fluvialis	1.5 × 10^3***	Bacteraemia, bacterial peritonitis	(Teixeira et al., 1997)

* The only class 2 fungal species identified.

** According to the TRBA, although *Streptococcus parauberis* is classified under risk group 2, it is noted to be a pathogen of vertebrates which humans would not normally be infected by, but can be obtained from the environment if a person works in a place with an increase pathogen concentration.

*** Only a single sample contained this pathogen, so no range can be given.

4. Discussion

The bacterial and fungal community structures were used to determine which metadata variable had the greatest influence on the β-diversity seen in the dust samples. In this study, the farm of origin was observed to have the greatest effect on the bacterial β-diversity. This was visualised by samples which clustered closest to other samples from the same farm during plotting. In contrast, the type of stable was not seen to have as great an effect on the bacterial β-diversity. This might partly be due to the uneven number of samples taken from each stable type, but is more likely due to a combination of different environmental factors that are unique to each farm and affect the bacterial microbiota.

On pig farms, pigs living in the same stable are likely to be of almost similar age and weight, with the exception of farrowing and nursing stables, and are likely to have similar microbial profiles. However, in the scope of this project, the effect of stable type on the β-diversity was not observed to fully distinguish between the microbial communities coming from farrowing or weaning stables as the samples consistently had overlapping clustering during constrained plotting. Samples taken from the nursing and fattening stables were noted to have different clustering patterns. However, due to the low number of samples taken (3 from each stable) and that the samples from fattening and nursing stables were only taken from Farm B and D respectively, broad assumptions regarding the overall biodiversity of the microbial communities from these stable types cannot be made. A potential reason for these samples (B4-B6 and D4-D6) being more dissimilar compared to the other stable types could partially be explained by the activity levels and ages of the animals in addition to the dust concentrations within the stables. Interestingly, even though the nursing stable had the highest concentration of bacteria, it was also observed to have the lowest richness amongst the different stables. This was reflected in both the data obtained after MALDI-TOF MS analysis and after bacterial sequencing. Analysis of the Jaccard similarity index values for samples taken from the same stable section revealed that settling dust cultured anaerobically for bacterial species resembled each other more compared to samples taken from different stable sections from different farms. In contrast, the fungal culturing was unable to clearly distinguish between samples coming from the same stable section and samples coming from another stable section on the same farm. This is possibly due to lower diversity in fungal species, but also suggests the possibility that the mycobiomes present in these farms and stables are similar.

In contrast to the trend seen in the bacterial data, the ability to discriminate pig farms from each other was not possible via the identification of the fungal species. In these samples, there was significant overlap in the samples taken from different farms and stables, indicating that the mycobiome found at one pig farm or stable is not unique, but is rather likely to be more similar to the mycobiomes found in other pig farms and stables. Another possible cause for the similar clustering pattern observed after fungal identification by MALDI-TOF MS could be due to several samples with low CFU counts and low diversity.

Culturing of fungi on DG18 plates revealed large variations of CFUs between samples and farms with three samples having no CFUs, to others which contained several hundred. This variation of fungal CFUs between samples taken from the same stable is likely due to the spatial variation of dust, causing more fungal CFUs to settle on one sample compared to another (Barber et al., 1991).

Fungal sequencing identified multiple species of conidia producing fungi, such as *Apiotrichum, Aspergillus*, *Cladosporium,* and *Wallemia*. Although many members of these genera are classified under risk factor 1 in accordance to the German TRBA (Ausschuss für Biologische Arbeitsstoffe, 2016), they may contribute to other respiratory diseases in humans, such as hypersensitivity pneumonitis (Chiba et al., 2009; Dushianthan et al., 2010). Other genera, such as *Kazachstania* and *Candida*, are known members of the pig intestinal mycobiome (Isaacson and Kim, 2012; Urubschurov et al., 2018, 2011) and their presence in high concentrations is consistent with the results obtained regarding the bacterial sequencing where the most dominant OTUs were associated with the gastrointestinal tract. Interestingly, fungal sequencing showed a similar trend to the data obtained after culturing where the fungal β-diversity could not be fully differenciated between samples taken from different farms.

Fungal culturing and subsequent analysis by MALDI-TOF MS was able to identify *Scopulariopsis brevicaulis*, which was not identified during sequencing. This is likely due to the choice of DNA extraction kit, as different kits are known to severely affect the recovery of bacterial and fungal DNA based on the cell wall thickness and a single kit able to extract genomic DNA from all cell types equally still does not exist (Fredricks et al., 2005). However, although identification by NGS and MALDI-TOF MS gave slightly different results, both methods were still able to distinguish samples taken from different farms regarding the bacterial identification using RDA plotting.

The sedimentation rate of aerobic bacteria and fungi varied between stables and farms. However, it is clear that the sedimentation rates of bacteria and fungi exceeded the concentrations observed in the settling dust in home environments by a factor of 10^2–10^5 (Madsen et al., 2018b). Although the majority of the bacterial and fungal species were considered non-pathogenic, high concentrations of these microorganisms can contribute to the inflammatory potential of aerosols. As previous research has shown, increased mould and bacterial concentrations in the air are associated with inflammation of the airways and generalised worsened lung function (Zhiping et al., 1996). In addition, the high concentration of opportunistic pathogens present is concerning as workers can carry these organisms on their clothes and/or airways, before transferring them outside (Angen et al., 2017; Rossi et al., 2017).

During this study, anaerobic incubation and subsequent MALDI-TOF MS analysis allowed for the identification of strict and facultative anaerobes present in the settling dust. In a previous culture-independent analysis of the microbial communities found in pig farms, it was observed that the vast majority of the OTUs found after sequencing were from bacterial genera associated with the intestinal flora of vertebrates, such as *Clostridium, Terrisporobacter,* and *Enterococcus*, many of which are strict anaerobes, which would not be observed using aerobic incubation (Nehme et al., 2008; Pryde et al., 1999; Quan et al., 2018).

From the anaerobic culturing, several species identified by MALDI-TOF MS were previously detected using aerobic incubation. However, there were several strict anaerobes and facultative anaerobes which were not isolated and identified during aerobic incubation. Several of these were members of the genus *Clostridium*. The most commonly isolated *Clostridium* species was *C. perfringens*, which is an opportunistic pathogen involved in soft tissue infections and is associated with diarrhoea in humans (Brynestad and Granum, 2002; Songer, 2010). *Clostridium difficile* was previously detected in air samples of swine containment facilities in the Netherlands (Keessen et al., 2011), but was not observed after anaerobic incubation in this study. Although clostridia are not normally considered to be zoonotic pathogens, there is still the risk of farm workers acquiring *C. difficile* and *C. perfringens* from their work place (Songer, 2010).

The microbial diversity observed in this study is lower compared to other research done comparing the microbial communities found in pig farms (Vestergaard et al., 2018) and comparing the microbial communities found in the anterior nares of pigs, farm workers, and cow farmers (Kraemer et al., 2018). However, this deviation from previous research is potentially due to differences used in dust collection methods and subsequent data treatment. In one study, EDC cloths were allowed to sample dust for 2 weeks uninterrupted and in another study exact sequence variants (ESVs) were applied despite that ESVs tend to overestimate the microbial diversity (Kraemer et al., 2018; Vestergaard et al., 2018). This study has shown that the bacterial species and diversity are, relatively, unique allowing differentiation of pig farms. In addition, this study also compared the fungal species and biodiversity, which were not covered by previous studies.

5. Conclusions

The settling dust from pig farms shows a high diversity of bacterial species with each farm having a unique bacterial fingerprint with regards to the diversity of species and OTUs identified during analyses. However, samples from the same section resembled each other and thus indicate a high degree of community stability in the dust source. In contrast, the same trend was not observed in fungal identification, indicating that the mycobiome found across pig farms may be less diverse. Aerobic culturing revealed a low level of biodiversity within samples (on average 7.7 different species were found per sample) while 96 different species were identified in all samples cultured aerobically. Of the most common species, *A. viridans* and *S. equorum* were observed to be the most abundant after aerobic incubation, while the most commonly identified anaerobic species included *C. perfringens* and *S. simulans*. NGS sequencing revealed that the most common bacterial OTUs were observed to be anaerobes. The most common fungal OTUs were mould and yeast genera which are associated with animal feed and the intestinal flora of pigs. In total, 28 pathogenic species of bacteria and fungi, in concentrations ranging from 1.5×10^2-1.1×10^7 CFUs/m^2/day, according to the German TRBA 466, were observed and they may potentially pose health-risks to workers.

Funding sources

This study was funded by the National Research Centre for the Working Environment, Copenhagen, Denmark and Aalborg University, Denmark.

This study does not involve any samples taken from human subjects.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2019.01.008

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