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Original Research

Characterization of the Extracellular Volatile Metabolome of *Pseudomonas Aeruginosa* Applying an *in vitro* Biofilm Model under Cystic Fibrosis-Like Conditions

Timo Koehler[1], Jost Wingender[2], Michelle Lueling[2], Sven W. Meckelmann[1], Ursula Telgheder[3,*], Oliver J. Schmitz[1,*]

[1]Applied Analytical Chemistry, University of Duisburg-Essen, 45141 Essen, Germany
[2]Environmental Microbiology and Biotechnology, University of Duisburg-Essen, 45141 Essen, Germany
[3]Instrumental Analytical Chemistry, University of Duisburg-Essen, 45141 Essen, Germany
*Correspondence: ursula.telgheder@uni-due.de (Ursula Telgheder); oliver.schmitz@uni-due.de (Oliver J. Schmitz)
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Abstract

Background: Cystic fibrosis (CF) is an autosomal recessive hereditary disease that leads to the production of thickened mucus in the lungs, favouring polymicrobial infections, such as chronic lung infections with the bacterial opportunistic pathogen *Pseudomonas aeruginosa*. **Method**: A biofilm model in combination with an adapted sampling and GC-MS analysis method were applied to *in vitro* studies on different variables influencing the composition of the extracellular volatile metabolome of *P. aeruginosa*. **Results**: A significant influence on the metabolome could be demonstrated for the culture medium as well as the atmosphere during cultivation (aerobic or anaerobic). Furthermore, a significant influence of the mucoid (alginate-overproducing) phenotype of the bacterium on quantity and composition of volatile organic compounds could be observed. Based on the results a solid culture medium was developed to simulate the nutrient conditions in the lungs of a CF patient. The extracellular volatile metabolome of bacterial strains *P. aeruginosa* ATCC 10145, PAO1 and FRD1 was characterized under CF-like conditions. **Conclusions**: Bacterial strain-dependent metabolites were identified. When *P. aeruginosa* PAO1 and FRD1 clinical isolates were compared, 36 metabolites showed significant variations in intensities. When the clinical isolates were compared with the reference strain (*P. aeruginosa* ATCC 10145), 28 metabolites (*P. aeruginosa* PAO1) and 70 metabolites (*P. aeruginosa* FRD1) were determined whose peaks showed significant deviation ($p > 95\%$) in intensity. Furthermore, the bacterial strains could be differentiated from each other by means of two principal components.

Keywords: *Pseudomonas aeruginosa*; biofilm; metabolomics; volatile organic compounds; thin-film microextraction; thermal desorption gas chromatography-mass spectrometry; cystic fibrosis

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive hereditary disease. The underlying cause are mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a transmembrane channel regulating anion transport [1]. CFTR dysfunction causes the blockage of chloride transport channels across epithelia in the affected organs so that the osmotic balance via the epithelia cannot be maintained. In the respiratory tract, this leads to the secretion and deposition of a highly viscous mucus that impairs mucociliary clearance of inhaled microorganisms. The mucus provides a favourable nutrient source for microorganisms. The CF airway microbiome consists of complex microbial communities [2] and progress of the disease is characterized by the colonization and infection of the respiratory tract with pathogenic microorganisms [1,3]. *P. aeruginosa* is one of the most common opportunistic bacterial pathogens in CF patients. With increasing age of CF patients a higher frequency of chronic infection with *P. aeruginosa* can be observed. The persistence of *P. aeruginosa* is due to the occurrence and growth in biofilms and the formation of biofilm-like cell aggregates inside the mucus [4]. *P. aeruginosa* can actively penetrate the mucus where hypoxic or anoxic conditions prevail, and where the bacteria switch from aerobic respiration outside the mucus to growth via anaerobic respiration with nitrate as the terminal electron acceptor (denitrification) [5,6]. Biofilm formation is the major survival mechanism of *P. aeruginosa* because biofilms show protection against innate and adaptive immune defence mechanisms of the patient and increased tolerance to antibiotics. Although CF patients infected with *P. aeruginosa* are usually treated with antibiotics, a chronic bacterial infection can hardly be prevented. The main cause of premature death of CF patients is failure of lung function due to chronic bacterial infections [1,3,7].

Through adaptive processes of *P. aeruginosa* in chronically infected CF airways diverse non-mucoid and mucoid phenotypes emerge. *P. aeruginosa* can produce at least three distinct exopolysaccharides, namely Psl, Pel, and alginate, that can be expressed in the *P. aeruginosa* aggregates from CF airways [8]. In mucoid strains O-

acetylated alginate is the primary exopolysaccharide that is overproduced as a major exopolysaccharide component of the biofilm matrix. Biofilms of mucoid *P. aeruginosa* variants are of clinical importance in chronic infections because alginate slime layers result in enhanced protection of the bacteria against host immune defences and antibiotics compared to biofilms of non-mucoid bacteria.

Typically, infections of the lung with microbial pathogens are diagnosed by cultivation of respiratory samples such as expectorated or induced sputum, oropharyngeal swabs and bronchoalveolar lavage [9]. However, these methods have been described as disadvantageous due to the invasive approach, the high expenditure of time and the poor specificity and sensitivity [10–13]. Alternatives are time-saving culture-independent molecular methods such as PCR-based techniques. Another approach proposed may be the clinical application of the extracellular volatile metabolome analysis. The non-invasive examination of metabolic volatile organic substances (mVOCs) in exhaled breath has been proposed as an alternative diagnostic method to identify bacterial pathogens [14,15]. However, this requires a preliminary examination of the metabolome of the relevant bacterial pathogens under CF-equivalent conditions.

In recent years, various studies of the intra- and extracellular metabolome of different *P. aeruginosa* strains have been published as summarized in the review of Mielko *et al*. [16]. Various extracellular volatile metabolites of *P. aeruginosa* were detected under laboratory conditions. Volatile metabolites of *P. aeruginosa* identified included acetic acid, acetaldehydes, acetones, 2-butanones, 2-nonanones, 1-undecenes, 2,4-dimethyl-1-heptenes, ethanol, 1-decanol, hydrogen sulphides, dimethyl sulphides, dimethyl disulphides, dimethyl trisulphides, methanethiol and hydrogen cyanides [15,17–21]. Bos *et al*. [14] reported the disadvantage of the limited comparability of the studies published so far, with respect to the different experimental conditions, such as the variation of bacterial strains (genomic variation), cultivation conditions (culture medium, incubation time, and growth temperature), bacterial growth phases, duration of headspace sampling, VOC pre-concentration methods and type of chemical analysis [14].

In vitro studies of volatile organic compounds produced by *P. aeruginosa* were commonly conducted on planktonic cultures in liquid growth media such as LB broth or various media mimicking the nutritional conditions of CF airways such as synthetic CF sputum medium [22] or artificial sputum medium (ASM) in various modifications [23,24].

Based on a standardized methodology, which the authors have published previously [25], the study shown here aims to investigate the influence of cultivation conditions on the metabolome of *P. aeruginosa*. The extracellular metabolome of three strains of *P. aeruginosa*, strain ATCC 10,145 (type strain), strain PAO1 (an acute infection isolate) and strain FRD1 (a chronic infection isolate) cultivated under aerobic and anaerobic conditions were analysed and compared. Furthermore, the influence of the culture medium on the extracellular volatile metabolome was investigated and a culture medium was developed to mimic real conditions as much as possible Using this medium and the previous studies, bacterial biofilms of *P. aeruginosa* were cultured in the laboratory under conditions approximating those in a CF lung. Finally, an analysis of the extracellular volatile metabolome was performed under these conditions.

2. Materials and Methods

2.1 Bacterial Strains

In this study, three strains of *P. aeruginosa* were used for biofilm cultivation, including type strain *P. aeruginosa* ATCC 10145 (DSM 50071), wild-type strain *P. aeruginosa* PAO1 originally isolated from a wound infection [26] and mucoid (alginate-overproducing) strain *P. aeruginosa* FRD1 (mucA22 mutation) isolated from the sputum of a CF patient [27].

2.2 Growth Media

Three agar media were employed for biofilm cultivation. As a non-selective complex growth medium, LB agar was used (35 g L-1 LB agar composed of (per L) 10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar, pH 7.0 $\pm$ 0.2) without KNO_3 (LB). When preparing the agar media for anaerobic cultivation (LBN), potassium nitrate was also added to the 35 g-L 1 LB agar solution. The concentration of potassium nitrate in the LB agar medium was 10.11 $g \cdot L^{-1}$ (final concentration 100 mM) (LBN). As a selective growth medium, Pseudomonas Isolation Agar (PIA) was used composed of (per L) 13.6 g agar, 1.4 g magnesium chloride, 20 g peptone, 10 g potassium sulfate and 0.025 g triclosan, pH 7 $\pm$ 0.2 (25 °C) supplemented with 20 mL glycerol, without (PIA) and with the addition of KNO_3 (final concentration 100 mM) (PIAN). After autoclaving, the agar media (20 mL) were filled into sterile glass Petri dishes (diameter, 90 mm) and allowed to solidify at room temperature.

For simulating growth conditions in the mucus of CF airways, Artificial Sputum Medium (ASM) was used as a solid agar medium based on the formulation of liquid ASM described by Sriramulu *et al*. [23] and Kirchner *et al*. [24]. ASM agar without (ASM) and with added KNO_3 (ASMN) was prepared as follows. 0.8 g DNA, 1.0 g mucin and 1 g casamino acid were dissolved separately in 50 mL and 30 mL autoclaved ultrapure water, respectively, under constant stirring. The solutions were combined and 8 mL of a solution of 3 mg DTPA, 2.5 g NaCl and 1.1 g KCl in 30 mL water were added. For ASMN, KNO_3 was added to a final concentration of 100 mM. After addition of sterile ultrapure water to a volume of 170 mL, the pH value of the solution was adjusted to 6.9 using 1 M Tris buffer and the total vol-

ume was adjusted to 200 mL. Finally, 3 g Bacto agar were added and suspended in the solution. ASM was sterilized by using a microwave autoclave (MediJet500, Phoenix Instrument GmbH, Garbsen, Germany). The procedure consisted of heating the medium to 135 °C within 2 min 40 s, a holding time of 22 s, cooling to 100 °C within 6 min. The medium was further cooled to 50 °C in a water bath for 20 min. 1 mL sterile egg yolk emulsion was added to 199 mL of ASM and after mixing 20-mL portions of the agar medium were dispensed into sterile glass Petri dishes as described above.

2.3 Cultivation of Biofilms

Biofilms were grown as confluent bacterial lawns on the surface of the agar media as an *in vitro* biofilm model of *P. aeruginosa* described before [25]. In brief, single colonies of cultures grown overnight at 37 °C were suspended in 0.14 M NaCl solution. Aliquots of 0.1 mL bacterial suspension were spread-plated on the agar media in glass Petri dishes (diameter, 100 mm) to a concentration of approximately 105 cells cm^{-2} on the medium surface. One PDMS film strip (0.35 mm × 55 mm) was placed in the centre of the glass lids. The inoculated agar plates without added KNO_3 were incubated aerobically and agar plates with added KNO_3 (final concentration 100 mM) were incubated anaerobically at 37 °C for 48 h. Anaerobic cultivation was conducted in anaerobic jars with an oxygen-free atmosphere generated by Anaerocult® A (Merck KGaA, Darmstadt, Germany). All plates were incubated upside down [25].

For biofilm analysis, biomass was scraped from the agar surface using a sterile metal spatula, and suspended in 2 mL deionized water. The suspensions were vortexed for 2 min to disperse the biofilm cells. Serial dilutions of the suspensions were prepared in deionized water. For determination of total cell counts in the biofilm suspensions, bacteria were stained with the fluorochrome 4',6-diamidino-2-phenylindole (DAPI). 1 ml of DAPI solution (25 $\mu g\ mL^{-1}$ in 2% (vol/vol) formaldehyde) was added to 4 mL of cell suspension. After incubation at room temperature for 15 min in the dark the suspension was filtered through a black polycarbonate membrane filter (Millipore, 0.2 μm pore size). Cells on the membrane filters were manually counted under an epifluorescence microscope at 1000-fold magnification. 20 randomly selected fields of view were examined for each filter using a counting grid (100 μm × 100 μm). Results were expressed as cells cm^{-2} of the surface area of the biofilm-covered agar media.

2.4 Sample Preparation

Headspace sampling of the biofilm cultures was performed by TFME using commercial PDMS films (0.35 mm × 55 mm; thickness: 0.45 mm ± 10%; Goodfellow GmbH, Hamburg, Germany). For cleaning of the PDMS films LC-MS grade methanol from VWR (Leuven, Belgium) and 5% aqueous Decon 90 (VWR, Leuven, Belgium) solution was used. The cleaning process is described in [25].

The loaded sorption films were analysed by direct coupling of a thermodesorption system (TDS 3, Gerstel GmbH & Co. KG, Mühlheim a.d.R., Germany) incl. autosampler (TDS-A2, Gerstel GmbH & Co. KG, Mühlheim a.d.R., Germany) with a gas chromatograph (Agilent 6890 GC, Agilent Technologies Inc., Waldbronn, Germany) and a quadrupole mass spectrometer (Agilent 5975 MSD, Agilent Technologies Inc., Waldbronn, Germany). Adsorbed analytes were desorbed in the TDS at 200 °C and transferred to the injector by a helium gas flow (ALPHAGAZ 1, 99.9%, Air Liquide, Düsseldorf, Germany) of 60 $mL{\cdot}min^{-1}$, via a metallic transfer line with an inert surface (Prosteel, deactivated, Gerstel GmbH & Co. KG, Mühlheim a.d.R., Germany) at a temperature of 300 °C. Cold injection system CIS 4 of Gerstel GmbH & CO. KG was used for introduction of the analytes. The injector temperature during thermal desorption is –10 °C to trap the desorbed analytes in the liner filled with deactivated glass wool. The injector is heated to 270 °C with a temperature-programming rate of 10 $°C{\cdot}s^{-1}$ to transfer the analytes from the liner to the GC column. The initial oven temperature is –10 °C (hold time: 2 min), which causes the analytes to refocus at the column head. The GC column was heated to 325 °C (hold time: 8 min) with a temperature programming rate of 10 $°C{\cdot}min^{-1}$. A DB-1 column (Agilent Technologies Inc., Waldbronn, Germany) with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 1 μm was used to separate the analytes. The separated analytes were transferred to the EI-qMS via a transfer capillary heated to 280 °C. Ionization was performed by electron impact ionization at 70 eV. The qMS analysis was performed in scan mode with a scan range of 40–600 Da and a scanning rate of 2.24 $scans{\cdot}s^{-1}$.

2.5 Data Evaluation

The data obtained from the TD-GC-qMS analyses, (Agilent MSD Chemstation data format) were first converted into Agilent MassHunter data format using MassHunter GC/MS Translator software (Agilent Technologies Inc., Santa Clara, USA). Subsequently, a conversion into the ".abf" data format was carried out using the software Reifycs Analysis Base File Converter (Reifycs Inc., Tokyo, Japan). This enables feature analysis using MS-DIAL (version 4.18, RIKEN, Wako, Japan), which performs deconvolution and annotation of the data. The parameters used for deconvolution, feature analysis and annotation are listed in **Supplementary Table 1** . The identification of the analytes based on the obtained EI-MS spectra was performed by using MS databases. The metabolomics specific GC-MS database MassBank of North America (MoNA) by Oliver Fiehn was used and analytes with a similarity score of ≥80% were putatively identified.

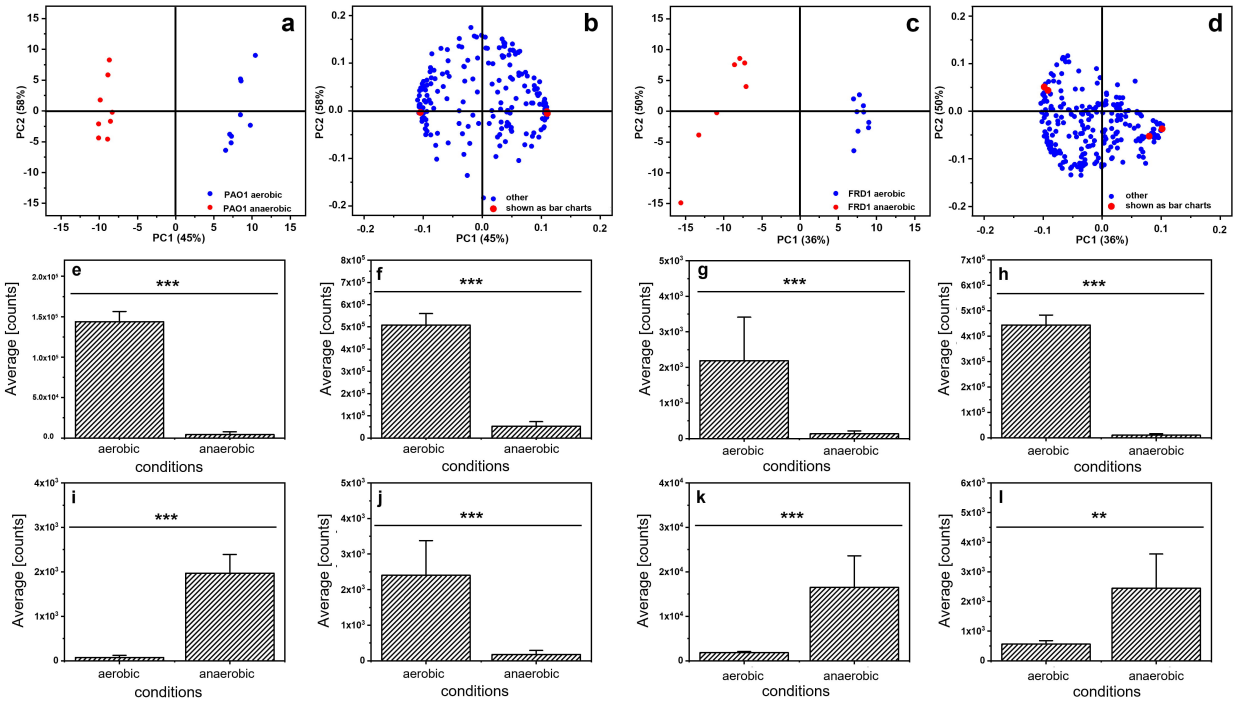

Fig. 1. Graphic representation of the evaluation for the differentiation of cultivation conditions ((aerobic (blue) vs. anaerobic (red) for the bacterial strains *P. aeruginosa* FRD1 and *P. aeruginosa* PAO1 using PCA and *t*-test. The biofilms were cultured aerobically on LB medium and anaerobically on LBN medium for 48 h at 37 °C. The results of the PCA are shown as scores (a: PAO1; c: FRD1) and loadings plots (b: PAO1; d: FRD1). The peak intensities of selected VOCs shown in the bar charts correspond to the mean values from eight biological replicates. The standard deviation of the mean value is shown as an error bar. The substances (peak intensities are shown in the bar charts) (e): 1-tetradecene (PAO1), (f): 3-aminoquinoline (PAO1), (g): dimethyl disulfide (FRD1), (h): 2-aminoacetophenone (FRD1), (i): 2,5-hexadione (PAO1), (j): dimethyl disulfide (PAO1), (k): benzaldehyde (FRD1), (l): 2-hexanone (FRD1). The stars above symbolise the confidence level, where *** represents a *p* value of 0.001, **0.01 and * 0.05 (n = 8).

The statistical evaluation of the deconvoluted data was carried out with a standardized workflow. For this purpose, a peak intensity cut-off of 1000 counts and a similarity score cut-off of 70% was applied. Missing values were not refilled. To reduce the dimensionality of the data and to identify differe nces and similarities, non-supervised Principal Component Analysis (PCA) was performed using SIMCA software (Version: 16.0.2.10561; Satorius AG, Göttigen, Germany).

PCA is an algorithm that helps to reduce dimensionality while retaining most of the variance of the data that contains useful information and is frequently used in omics. Each principal component is identified by the direction and the amount of variance in the data and is a linear combination of the original variables. Each sample can then be represented by relatively few principal components instead of thousands of metabolites. For example, in Fig. 1a, the PC1 axis is explaining the variance between both samples groups which is the growth under aerobic or anaerobic conditions and on the PC2 axis, the variance within each of the sample groups is explained. For further information regarding the interpretation of a PCA, we recommend the publication of Ringnér [28]. Scores and loadings as well as the variance are calculated. Potentially interesting metabolites were further statistically analyzed by parametric significance test (*t*-test; two-sided distribution and heteroscedastic). The significance was evaluated using the p-value and classified into three significance levels, 0.001, 0.01 and 0.05.

With the described materials and methods, both microbiological and analytical sample preparation is possible. The methods used for the characterization of the bacterial biofilms, for the sample preparation using polydimethylsiloxane (PDMS) sorbent films and for the analysis of the extracellular volatile metabolites are described.

3. Results and Discussion

3.1 Effect of Growth Conditions on the Extracellular Eetabolome of P. aeruginosa in the Context of CF Disease

In general, research on the extracellular volatile metabolome of *P. aeruginosa* can be divided into five areas, (1) identification and differentiation of bacterial strains, (2) analysis of metabolic changes due to external factors, (3) identification of unknown metabolites, (4) identification of metabolic profiles using genome structure [16], and (5) effector function of volatiles in intermicrobial interactions such as the recently reported growth inhibition of the

fungus *Aspergillus fumigatus*, relevant in CF airway infections, by *P. aeruginosa* volatiles [29]. Bos *et al.* [14] have already criticized the lack of comparability of published metabolome studies on different bacterial pathogens. Especially metabolome studies in the context of a disease, such as CF, should be performed *in vitro* under conditions like those in the human body. This necessity is due to the variability of the metabolome of bacteria to different external influences, such as nutrient deficiency and oxygen concentration. Furthermore, *P. aeruginosa* is a bacterium that is particularly well adapted to different environmental conditions and is capable of changing the phenotype , e.g., development from a non-mucoid to a mucoid variant during chronic CF airway infections [3]. In the following the influence of the phenotype and growth conditions on the metabolome of *P. aeruginosa* is shown.

An *in vitro* agar plate biofilm model was used throughout the study. The biofilms of *P. aeruginosa* were grown as confluent bacterial lawns directly on the surface of three different solid agar media, unsupplemented (LB, PIA, ASM) and supplemented with 100 mM KNO_3 (LBN, PIAN, ASMN) for cultivation under aerobic and anaerobic conditions, respectively. This type of unsaturated biofilm model has proven useful for studying the morphology and composition of aerobic *Pseudomonas* biofilms such as those of *P. aeruginosa*, including the PAO1 and FRD1 strains used in the present study [30–32]. Recently, this model was used as the basis for a modification of the system suitable for analysing volatile organic compounds in the headspace of aerobically grown *P. aeruginosa* biofilm cultures [25]. In addition to the LB and PIA agar media conventionally used for *P. aeruginosa* cultivation a modified formulation of ASM was included that was originally developed to resemble in its composition (amino acids, mucins, DNA, among other substances) more closely conditions in CF lungs [23,24]. So far, ASM has been described exclusively for the liquid cultivation of *P. aeruginosa* in the context of CF disease. For the cultivation of *P. aeruginosa* biofilms in our *in vitro* biofilm model the preparation of solid ASM was necessary and was accomplished by addition of agar to the liquid medium to obtain the final ASM as an agar medium as described above.

In the present study, monitoring of aerobically and anaerobically biofilm growth at 37 °C for seven days had shown that the stationary growth phase with maximal cell numbers of biofilms were reached after 48 h of incubation. Taken together, the mean cell density values of all strains (*P. aeruginosa* ATCC 10145, PAO1 and FRD1) in the agar-grown biofilms varied between 4.6×10^9 to 7.6×10^9 cells/cm^2 under aerobic conditions and 1.3×10^9 to 6.1×10^9 cells/cm^2 under anaerobic conditionson LB/LBN, between 0.7×10^9 to 2.4×10^9 cells/cm^2 and 3.3×10^9 to 4.0×10^9 cells/cm^2 under aerobic and anaerobic conditions, respectively, on PIA/PIAN, and between 0.8×10^9 to 2.2×10^9 cells/cm^2 and 0.4×10^9 to 3.6×10^9 cells/cm^2 under aerobic and anaerobic conditions, respectively, on ASM/ASMN (mean values of two to four independent experiments, exception for strain PAO1 biofilms on ASM/ASMN with data from only one growth experiment). These bacterial densities indicated the development of multilayer biofilms [33] on the surface of all agar media under both aerobic and anaerobic growth conditions.

Under anaerobic conditions no growth was observed on unsupplemented agar media, while addition of KNO_3 (100 mM) to the agar media allowed bacterial growth, indicating that anoxic growth of *P. aeruginosa* biofilms via anerobic respiration by nitrate reduction (denitrification) occurred in the biofilm model as has been shown previously in anaerobic zones of mucus from CF airways [5] and in growth experiments *in vitro* under controlled laboratory conditions for clinical isolates from CF patients and laboratory strains like non-mucoid strain PAO1 and mucoid strain FRD1 used in the current study [34,35].

3.1.1 Influence of Oxygen Availability on the Extracellular Volatile Metabolome of *P. aeruginosa*

Koehler *et al.* [25] have shown that oxygen availability (aerobic vs. anaerobic growth) affects the metabolome of biofilms of the type strain *P. aeruginosa* ATCC 10145. In the present study, the investigation of the influence of oxygen availability was extended to two different clinical isolates of *P. aeruginosa* with two different phenotypes, the non-mucoid strain *P. aeruginosa* PAO1, and the mucoid strain *P. aeruginosa* FRD1. The analysis of mVOCs was performed by using the *in vitro* model described by Koehler *et al.* [25] on biofilms grown at 37 °C for 48 h aerobically and anaerobically on LB medium or LBN medium, respectively.

Eight biological replicates (biofilm cultures) were used to analyse the mVOCs. The mVOCs of the bacterial strains in the presence or absence of oxygen were identified from the deconvoluted TD-GC-qMS data using the spectra database MONA. In order to differentiate the composition of the atmosphere during cultivation (aerobic and anaerobic) by means of two main components, a PCA was performed. For this purpose, score plots that display the samples according to their position on the PC1 and PC2 (Fig. 1a,1c) as well as loading plots (Fig. 1b,1d) that show the position of the putatively identified metabolites were generated. Furthermore, the significance of the variation in the peak intensity of the metabolites under aerobic and anaerobic conditions was determined using a *t*-test. The mean values of the signal intensity, the associated standard deviations and the confidence level are shown in form of bar charts in Fig. 1e-l.

The score plots of the PCA showed a clear separation between the aerobic and anaerobic growth conditions (Fig. 1a,1c) with many associated metabolites (Fig. 1b,1d). In general, the data suggest a massive change in the volatile metabolome depending on oxygen availability dur-

ing biofilm growth. Moreover, many of these metabolites were significantly different by parametric *t*-test (p values $\geq$ 0.05). This was for example observed for dimethyl disulphide in all investigated strains. Dimethyl disulphide has already been detected in CF sputum *in vitro* metabolome studies as well as in CF-sputum [15,19,21,36]. In total, 50 significantly different metabolites could be determined for strain *P. aeruginosa* PAO and 47 metatabolites for strain *P. aeruginosa* FRD1 (**Supplementary Table 2** and **Table 3**).

These results support the hypothesis that the choice of aerobica *vs*. anaerobic cultivation conditions has a significant influence on the volatile metabolome of *P. aeruginosa*.

Because of this influence, *in vitro* studies of the extracellular volatile metabolome in the context of a bacterial infection in a human disease must adapt the cultivation condition to the disease. Thus, for the *in vitro* investigation of the metabolome of *P. aeruginosa* in the context of a respiratory infection, a CF disease respectively, a microaerophilic or anaerobic cultivation must be chosen, so that from the *in vitro* generated results to the real conditions in the lungs of a CF patient can be concluded.

3.1.2 Investigation of the Extracellular Metabolome from Two *P. aeruginosa* Strains with Mucoid and Non-mucoid Phenotypes

In addition to the cultivation atmosphere, the phenotype of the bacteria may have a significant effect on the metabolome. In cystic fibrosis, the mucoid phenotype is dominant in chronic infections, whereas in initial infections, especially in younger CF patients, non-mucoid strains in the lungs are dominant [3]. A statistical and comparative study of the non-mucoidstrain *P. aeruginosa* PAO1 (acute infection isolate) and mucoid strain *P. aeruginosa* FRD1 (chronic infection isolate) was performed to get more information whether the phenotype has an influence on the extracellular volatile metabolome of *P. aeruginosa*. Cultivation in the *in vitro* biofilm model was performed under anaerobic conditions using the complex LB medium supplemented with potassium nitrate as described by Koehler *et al*. [25]. Eight biological replicates were investigated per bacterial strain.

The comparison of the two phenotypes using a PCA is shown in Fig. 2a. A distinction between the two phenotypes is not possible by means of two main components, since the scatter of results in the scores plot is large (0.2–20, blue dots) for the mucoid strain *P. aeruginosa* FRD1. In contrast, the scatter of results for the strain *P. aeruginosa* PAO1 is small (2–6, red dots), but the results for the mucoid strain are partly within the range for the non-mucoid strain *P. aeruginosa* PAO1. Furthermore, the substance classes are not grouped according to the two phenotypes (Fig. 2b). Therefore, differentiation of the two phenotypes cannot be done at the substance class level. However, the influence of the phenotype, for example the formation of alginate (mucoid phenotype) on the molecular level cannot be excluded. For this purpose, the peak intensities of the identified metabolites were compared with each other and their significance was determined using the *t*-test. Using the example of six metabolites, the difference in mean peak intensities is shown in Fig. 2, with the significance of the difference in peak intensity varying for the selected metabolites.

The peak intensities of certain metabolites were significantly increased in the mucoid phenotype compared to the non-mucoid phenotype (see Fig. 2a–e). However, the opposite trend could be observed for certain metabolites (see Fig. 2f–h). For example, a 4-fold higher peak intensity was observed for benzaldehyde in mucoid strains. The example of the metabolite dimethyl disulphide shows that a significant reduction of peak intensity can also occur in the mucoid phenotype compared to the non-mucoid phenotype. The peak intensity of the mucoid phenotype is reduced by a factor of 13. In total, a significant difference in peak intensity was found for 28 metabolites. The statistical comparison of the mVOCs between the two phenotypes revealed three metabolites whose difference in peak intensity was a confidence level of $p = 0.001$. Eleven metabolites had a confidence level of $p = 0.01$ and fourteen metabolites had a confidence level of $p = 0.05$. A list of these metabolites is shown in **Supplementary Table 4**. Previously, secondary electrospray ionization-mass spectrometry (SESI-MS) was applied to produce fingerprints of the *P. aeruginosa* strains PAO1 and FRD1 directly from the breath of infected mice [37]. It was demonstrated that SESI-MS was capable of differentiating infected versus uninfected mice as well as infections caused by strain PAO1 versus FRD1. In addition, comparison of the *in vivo* volatiles with *in vitro* volatiles from liquid laboratory cultures showed that only 25–34% of peaks were shared between the *in vivo* and *in vitro* fingerprints [37].

In summary, the investigation of the influence of the mucoid phenotype of the bacterium *P. aeruginosa* on its metabolism showed that at the level of substance classes as well as two main components, no distinction was possible in this study. However, differences could be detected at the molecular level. It was shown that, in addition to the cultivation conditions (aerobic and anaerobic), the mucoid phenotype of the bacterial strain must be taken into account in metabolome studies.

3.1.3 Influence of the Culture Media on the Extracellular Volatile Metabolome of *P. aeruginosa*

In addition to the influencing variables already studied and discussed, the culture medium could have an impact on the extracellular volatile metabolome of *P. aeruginosa*. In most metabolome studies published on *P. aeruginosa* the full medium LB-Lennox and bacteria selective growth media are used [14]. Such selective culture media contain only the substances that the respective bacterium needs to grow. Additionally, these culture media contain chemicals that suppress the cultivation of other bacteria.

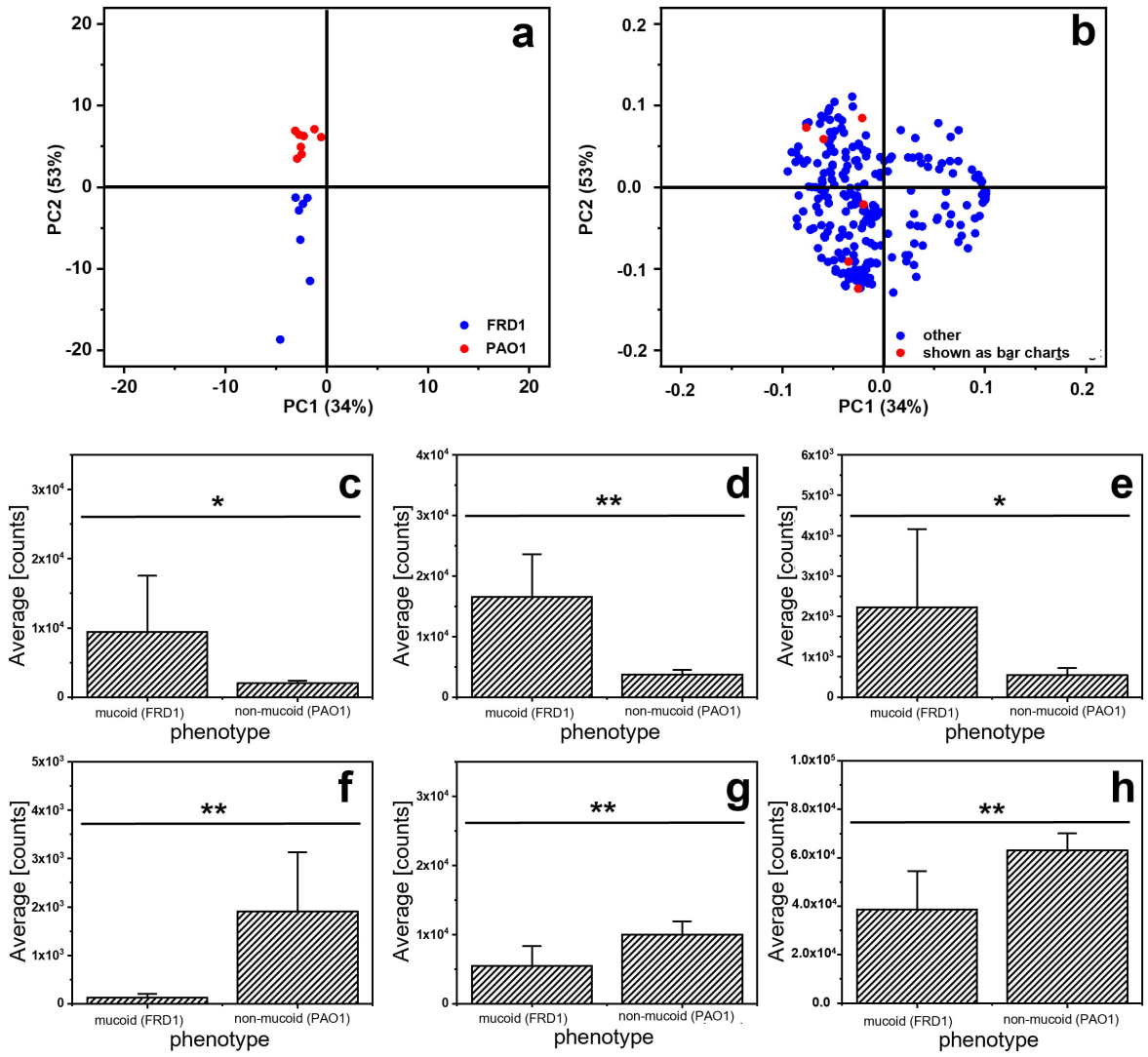

Fig. 2. Comparison of the two strains *P. aeruginosa* FRD1 (blue) and PAO1 (red) with mucoid and non-mucoid phenotype, respectively, by PCA, statistical evaluation by *t*-test. Cultivation in the *in vitro* biofilm model was performed under anaerobic conditions using LB medium supplemented with potassium nitrate for 48 h at 37 °C. Figures a and b: results of the PCA as scores (a) and loadings plot (b). Figures c to h: difference in peak intensity between the two phenotypes considered, using the example of (c): octanol, (d): benzaldehyde, (e): propylbenzene, (f): dimethyl disulfide, (g): 2-undecanone, (h): 4-methylquinoline. The mean values of the peak intensities of the biological replicates produced and their standard deviation as error bars are shown in form of bar charts. The stars above symbolise the confidence level, where *** represents a *p* value of 0.001, ** 0.01 and * 0.05.

The Pseudomonas isolation (PIAN) agar is such a *P. aeruginosa* specific medium. In order to investigate the influence of the culture medium on the metabolome of *P. aeruginosa* in the context of CF disease two clinical strains *P. aeruginosa* PAO1 and *P. aeruginosa* FRD1 were cultivated under anaerobic conditions on both culture media (LBN and PIAN medium). Potassium nitrate was added to both culture media to ensure anaerobic cultivation. Five biological replicates of the strain *P. aeruginosa* FRD1 and three biological replicates of the strain *P. aeruginosa* PAO1 were grown on PIAN medium. The detected extracellular volatile metabolites were compared with those of eight biological replicates of the two strains cultivated on LBN medium. To exclude the influence of the phenotype described in chapter 3.1.2, the comparison of the extracellular volatile metabolites was performed exclusively between cultures with the same phenotype, e.g., FRD1 on LBN *vs*. FRD1 on PIAN.

The scores and loading plots obtained by performing a PCA are presented in Fig. 3a–d. From these results a significant differentiation of the cultivation on both culture media is possible using two main components. To differentiate between the two culture media, only the first principal component is necessary, which explains 84% of the variance for strain *P. aeruginosa* PAO1 and 60% for strain *P. aeruginosa* FRD1. The second principal component only describes the scatter within the group. For PAO1

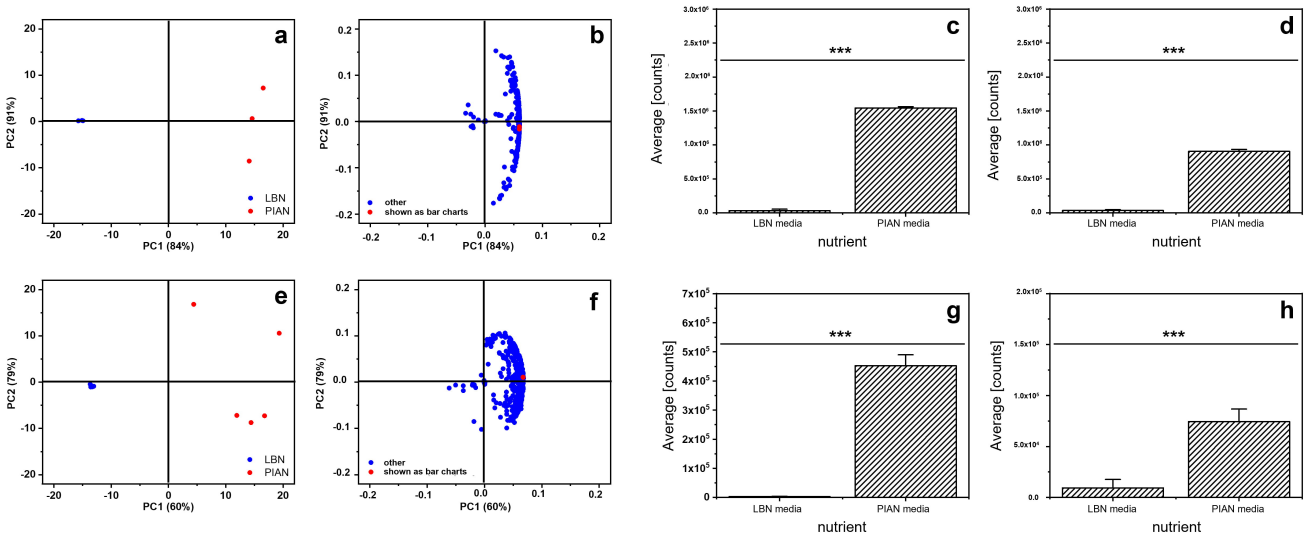

Fig. 3. Comparison of two different culture media (LBN: blue; PIAN: red) in a cultivation of the two strains *P. aeruginosa* PAO1 (a, b) (n = 5) as well as FRD1 (e, f) (n = 3) under anaerobic conditions in an *in vitro* biofilm model by PCA. Cultivation in the *in vitro* biofilm model was carried out under anaerobic conditions in LB medium supplemented with potassium nitrate and in the Pseudomonas isolation agar supplemented with potassium nitrate. The bacteria were cultivated for 48 hours at 37 °C. The mean values of the signal intensities and the corresponding standard deviations are shown as bar graphs. Significance analysis of the difference in signal intensities was performed using a *t*-test (two-sided distribution, heteroscedastic). Metabolites shown are: c = 1-hexadecanol, d = pentadecane, g = 2-nonanone, h = 1-octanol (all ***; $p \geq 0.001$).

this principal component describes 91% variance and for FRD1 79%. Based on the high values, the qualitative observation of the differentiation between the two culture media by means of PCA can be confirmed quantitatively. Considering the corresponding loadings plots, the detected extracellular volatile metabolites can be assigned to the nutrient medium.

Furthermore, the variance between the biological replicates in the LBN medium for the cultivation of *P. aeruginosa* FRD1 (see Fig. 3e) as well as for *P. aeruginosa* PAO1 (see Fig. 3a) is small. A higher variance for both bacterial strains as well as a significantly larger number of extracellular volatile metabolites is achieved by cultivation on PIAN medium. A *t*-test was used to investigate the differentiation of both cultivation media at the molecular level. Fig. 3c–f show two substances for each of the two bacterial strains, whose mean peak intensity is significantly higher when cultivated on PIAN medium (confidence level: $p = 0.001$).

For the detection of the metabolites 2-nonanone and 1-octanol, a higher signal intensity was observed when using PIAN agar (Cultivation of *P. aeruginosa* FRD1, with a statistical significance of 99.9%) (Fig. 3g,3h). Similarly, higher signal intensities were detected for 1-hexadecanol and pentadecane (cultivation of *P. aeruginosa* PAO1 on PIAN agar) with the same statistical significance (Fig. 3c, 3d). Overall, significance analysis of culturing *P. aeruginosa* FRD1 on LBN and PIAN agar revealed 102 metabolites with a significant difference in signal intensity. 21 metabolites could be assigned to the significance level $p = 0.001$, 33 metabolites to the significance level $p = 0.01$, and 48 metabolites to the significance level $p = 0.05$.

The analysis for the bacterial strain *P. aeruginosa* PAO1 revealed 123 metabolites with a significant difference in signal intensity. 25 metabolites could be assigned to the significance level $p = 0.001$, 54 metabolites to the significance level $p = 0.01$ and 44 metabolites to the significance level $p = 0.05$. An overview of the determined metabolites of the two investigated bacterial strains is given in the supplement (*P. aeruginosa* FRD1: **Supplementary Table 5**; *P. aeruginosa* PAO1: **Supplementary Table 6**).

First, this analysis shows that the culture medium has a significant influence on the extracellular volatile metabolome, due to the high number of metabolites with significantly different signal intensities. On the other hand, due to the similar number of metabolites with significant difference in signal intensity, it was shown that the influence of the culture medium is independent of the mucoid phenotype of the bacterium. Analogous to the results of the investigation of the influence of the mucoid phenotype, a difference can be detected on the molecular level.

3.2 Metabolome Study of mVOCs from P. aeruginosa under CF Lung Conditions

The extracellular volatile metabolome of the two clinical strains *P. aeruginosa* FRD1 as well as PAO1 and the reference strain *P. aeruginosa* ATTC 10145 was investigated. The *in vitro* metabolome investigations of the bacterial strains were performed under CF-like conditions us-

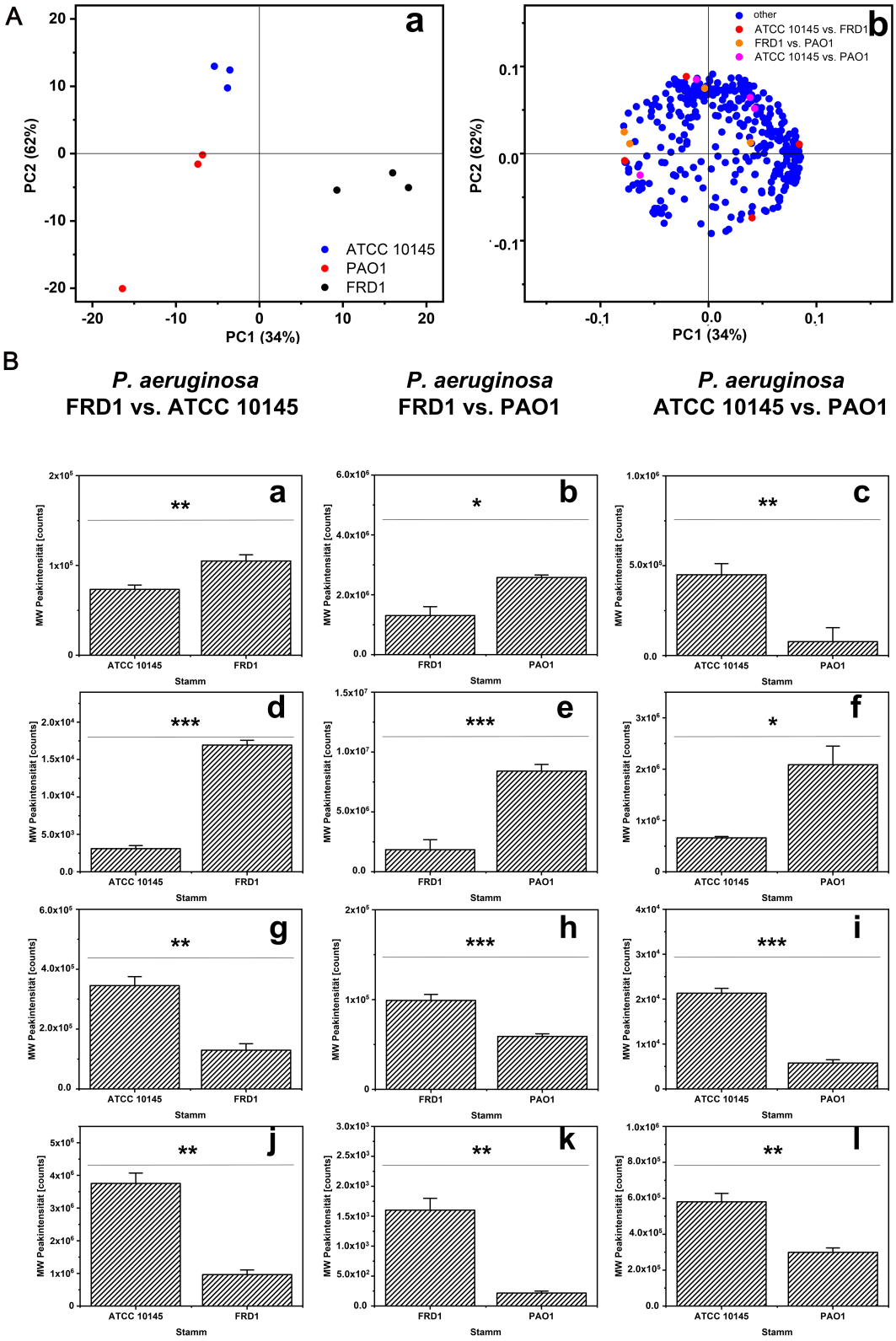

Fig. 4. Comparison of the available *P. aeruginosa* strains (Fig. A: blue = ATCC 10145; red = PAO1; black = FRD1) by means of their extracellular volatile metabolites. The three bacterial strains were cultivated under anaerobic conditions and on ASMN agar using the *in vitro* biofilm model. Comparison was performed using unsupervised PCA. Fig. 4A shows the scores and the loadings plot. In the loadings plot, the metabolites are labeled, which are shown as bar graphs in Fig. 4B (a–l). a = benzophenone, b = tetradecane, c = toluene, d = 2-ethylhexanal, e = 3-methoxypropylamine, f = trans-2-dodecenyl acetate, g = 1-hexadecanol, h = pentadecane, i = methyl benzoate, j = 2-octanone, k = isoprene, l = 1-decanol. * = 0.05; ** = 0.01; *** = 0.001.

ing the solid ASM described above. The mVOCs of three biological replicates of each bacterial strain were identified after deconvolution and feature analysis by the GC-EI-MS specific metabolomics database MoNA. Following data processing and peak identification, the statistical evaluation of the metabolites was carried out performing a PCA following the identification of specific metabolites by evaluating the resulting score and loading plots (see Fig. 4).

Differentiation between the investigated strains of *P. aeruginosa* is possible by the first two principal components, as can be seen from the scores plot in Fig. 4A. Cumulatively, these two principal components explain a variance of 98 %. Furthermore, the first principal component is suitable to explain the difference between the strains *P. aeruginosa* ATCC 10145 and PAO1 and the strain *P. aeruginosa* FRD1. For explaining the difference between *P. aeruginosa* ATCC 10145 and *P. aeruginosa* PAO1 the second principal component can be applied. The variance between the replicates of one strain, except for the replicates of *P. aeruginosa* PAO1, is low compared to cultivation on the other culture media used (see Fig. 4a). In the replicates of the strain *P. aeruginosa* PAO1, only one outlier can be detected. This outlier could be caused on the one hand by irregularities in the analysis or on the other hand by deviation in the sample preparation. The biological variance must also be considered in the discussion, but since only technical replicates were analysed, this can be assumed to be low. Despite this outlier, a clear differentiation between the bacterial strains with the first two principal components as well as an identification of bacterial strain-selective metabolites using the loadings plot (see Fig. 4A,4b) is possible. Based on the scores and loadings plot, it can be assumed that the difference between the bacterial strains *P. aeruginosa* ATCC 10145 and FRD1 as well as between *P. aeruginosa* PAO1 and FRD1 is significantly larger than between *P. aeruginosa* ATCC 10145 and PAO1. This can be explained by the differentiation of the mentioned strains already in the first principal component as well as the number of identified features, shown in the loadings plot (see Fig. 4A,4b). An evaluation of this observation can be carried out with the help of a significance analysis. For this purpose, the variance of the peak intensity of the identified features between all strains was tested using a *t*-test (two-sided distribution, heteroskedastic). By means of the significance analysis, a total of 70 metabolites could be identified that show a significant difference (at least 95%) in peak intensities between the strains *P. aeruginosa* FRD1 and ATCC 10145 (see **Supplementary Table 7**). Analogous to the qualitative observation from the scores and loadings plot, the number of metabolites with a significant difference in peak intensity decreases significantly when comparing the bacterial strains *P. aeruginosa* FRD1 and PAO1 (36 metabolites) and *P. aeruginosa* ATCC 10145 and PAO1 (28 metabolites). In addition to the decrease in total metabolites, the proportion of metabolites with the greatest significance ($p = 0.001$; 99.9%) in the total number also decreases. Both this quantitative and qualitative comparison using scores and loadings plot reveals that *P. aeruginosa* ATCC 10145 and PAO1 are more similar to each other in their extracellular volatile metabolome than *P. aeruginosa* FRD1. This can be attributed to the different phenotype. Furthermore, it can be observed that the difference of *P. aeruginosa* FRD1 to *P. aeruginosa* ATCC 10145 is higher than to *P. aeruginosa* PAO1. This could be explained by different origins of the strains (*P. aeruginosa* ATCC 10145 as a neotype strain of *P. aeruginosa* and *P. aeruginosa* PAO1 as originally a clinical isolate). *P. aeruginosa* PAO1 was originally isolated from a burn wound of a patient and was thus exposed to environmental influences. These environmental influences may have led to mutations that made the P. aeruginosa PAO1 strain resistant to such influences, resulting in the presence of different genes despite the same mucoid phenotype, thereby diverging the phenotypes between the two bacterial strains. Although Kavanagh, who first used *P. aeruginosa* ATCC 10145 in 1947 [38], did not provide any information on the date of origin or the origin of the bacterial strain [38]. Due to this lack of information regarding the original isolation of *P. aeruginosa* ATCC 10145, the explanation is based exclusively on theoretical considerations.

On the molecular level, for example, some molecules of the substance class of methyl ketones (e.g., 2-octanone, 2-hexanone, etc.) could be identified (see **Supplementary Table 7**). Forney *et al.* [39,40] have described the formation of methyl ketones from n-alkanes in bacteria in two publications. Furthermore, Thijsse *et al.* [41] found that *P. aeruginosa* strains accumulate methyl ketones, such as 2-hexanone. This was supported by the research results of Robinson *et al.* [42]. 2-Hexanone and other methyl ketones have already been identified as volatile metabolites in several studies of the metabolome of *P. aeruginosa* [14,18,43–45]. Methyl ketones, such as 2-nonanone [17], have also been detected in CF sputum. However, as Bos *et al.* [14] report, methyl ketones can also be produced by other bacteria such as *S. aureus*. Since *S. aureus* is also a CF-relevant pathogen, the influence of other CF-relevant pathogens must also be taken into account when analysing the extracellular volatile metabolome of *P. aeruginosa* in the context of respiratory infections in CF disease.

4. Conclusions

The influence of all investigated parameters on the extracellular volatile metabolome were demonstrated by the presented results. The influence of oxygen availability and the culture medium is particularly significant. However, at the molecular level, which is important for diagnosis by breath analysis, an influence of the mucoid phenotype is also detectable. Therefore, cultivation of bacterial biofilms under microaerophilic, anaerobic conditions is necessary for robust *in vitro* metabolomic studies of bacterial lung infections in CF disease. In addition, it must be determined

in advance which infection status is to be investigated so that the phenotype of the bacterium can be selected accordingly. The use of a culture medium that best simulates the (nutrient) conditions in the CF lung and mucus is advantageous. Such a culture medium is the artificial sputum medium (ASM).

The investigation of extracellular volatile metabolites of three different strains of the bacterium *P. aeruginosa* have shown that when cultivating the bacterial strains under CF-like conditions, metabolites can be identified whose intensities are significantly increased or decreased compared to the other strains. For example, the following metabolites for the anaerobic cultivation of the mucoid phenotype on artificial sputum medium using the *in vitro* biofilm model have been detected: benzophenone, tetradecane, toluene, 2-ethylhexanal, 3-methoxypropylamine, trans-2-dodecenyl acetate, 1-hexadecanol, pentadecane, methyl benzoate, 2-octanone, isoprene, 1-decanol.

A differentiation of the bacterial strains by the extracellular volatile metabolome could be possible by two principal components of a PCA. Of great importance in CF disease is the bacterial strain *P. aeruginosa* FRD1 due to its mucoid phenotype. Mucoid bacterial strains of *P. aeruginosa* are isolated significantly more often in chronic lung infections, and resistance to antibiotics is significantly increased in such bacteria. To eradicate mucoid bacterial strains, such as *P. aeruginosa* FRD1, early identification of the bacterial strain is necessary. It is possible that the extracellular volatile metabolites identified in this study can be used for such early identification. With regard to CF, it is limiting that only monocultures and CF-like conditions were investigated in this study. A validation of the determined metabolites can be carried out, e.g., by analysing mVOCs of CF-typical bacteria as mono- and co-cultures under CF-like conditions. In this way, non-*P. aeruginosa* selective metabolites as well as deviations in the behaviour of the peak intensities could be determined.

With the presented method volatile metabolites of *P. aeruginosa* as well as different bacteria could be determined as biomarkers. These biomarkers may be detected by a non-invasive "at-bedside" breath target analysis method to detect severe lung infections with *P. aeruginosa* of CF patients at an early stage.

Author Contributions

TK, JW, UT and OJS conceived and designed research study. TK and ML conducted experiments. TK analyzed the data. SWM provided help and advice on performed statistical evaluation. TK wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2705156.

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