Universitätsmedizin Rostock

KLINIK UND POLIKLINIK FÜR ANÄSTHESIOLOGIE UND INTENSIVTHERAPIE

Evaluierung von Messmethodik und Kulturbedingungen

zur Bestimmung volatiler Emissionen von Mykobakterien

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Abkürzungsverzeichnis

GC-MS **G**as**c**hromatografie-**M**assen**s**pektrometrie

in vitro lateinischer Begriff; sinngemäß für "im Reagenzglas"

in vivo lateinischer Begriff; sinngemäß für "am lebenden Objekt"

MAP ***M**ycobacterium **a**vium* ssp. ***p**aratuberculosis*

NTD gepackte Mikroextraktionsnadel, engl.: **n**eedle **t**rap **d**evice

NTME Nadel-Mikroextraktion, engl.: **n**eedle **t**rap **m**icro **e**xtraction

SPME Festphasen-Mikroextraktion, engl.: **s**olid **p**hase **m**icro **e**xtraction

VOC Volatile organische Verbindung, engl.: **v**olatile **o**rganic **c**ompound

1 Einleitung

1.1 Volatile organische Substanzen

Volatile organische Verbindungen, engl.: **v**olatile **o**rganic **c**ompounds (VOCs), können von Lebewesen produziert und über Kontaktflächen wie Haut und Lunge oder Zellwände in die Umwelt abgegeben werden[1,2]. Sie können verschiedenen Substanzklassen, wie z. B. Alkoholen, Aromaten, Aldehyden etc., angehören und ein Produkt physiologischer aber auch krankhafter biochemischer Prozesse im Körper sein. Die Bestimmung von VOCs ermöglicht Rückschlüsse auf diese Prozesse[3]. Das ist sowohl bei Zell- und Bakterienkulturen, als auch bei Pflanzen, Pilzen, Tieren und Menschen möglich[1,4,5,6,7]. VOCs liegen im Konzentrationsbereich von wenigen pmol L^{-1} bis nmol L^{-1} vor. In der menschlichen Ausatemluft macht die Summe aller VOCs im Verhältnis zu Stickstoff und Sauerstoff weniger als ein Prozent aus (siehe Abbildung 1.1).

Abbildung 1.1: Prozentuale Zusammensetzung der menschlichen Ausatemluft. Neben den Hauptbestandteilen sind VOCs in geringen Konzentrationen enthalten («1%).

1.2 Mykobakterien

Die Gattung der Mykobakterien umfasst mehr als hundert Spezies. Einige Mykobakterienspezies können chronische Krankheiten auslösen. Zum Beispiel gilt ***M**ycobacterium **a**vium* ssp. ***p**aratuberculosis* (MAP) als Auslöser der Paratuberkulose bei Wiederkäuern, einer chronischen Enteritis, auch Johne'sche Krankheit genannt. Sie verursacht hohe wirtschaftliche Verluste[8]. Zudem wird eine Beziehung zwischen der Paratuberkulose und entzündlichen Darmerkrankungen, wie z. B. Morbus Crohn, pos-

tuliert[9,10]. Diagnostische Verfahren wie der Erregernachweiß im Kot mittels Polymerase-Ketten-Reaktion (PCR) ist nur ausreichend sensitiv bei Tieren, die zum Zeitpunkt der Probennahme ein große Anzahl an Erregern ausscheiden. Der Nachweis über die kulturelle Anzucht der Erreger ist die bislang sensitivste Methode. Ihr Nachteil liegt in der niedrigen Vermehrungsrate von Mykobakterien. Bis zur visuellen Erkennbarkeit der Kolonien werden 4 bis 18 Wochen benötigt. Die Analyse flüchtiger Substanzen, die von den Bakterien emittiert werden, zeigt Potential zur früheren Erkennung von Mykobakterienwachstum[11,12].

1.3 Von Mykobakterienkulturen emittierte VOCs

Bakterien- bzw. Zellkulturen emittieren ein breites Spektrum von VOCs[2]. VOC-Profile (gemessene VOC-Konzentrationen und Verhältnisse der VOCs zueinander) von Mykobakterien können als Charakterisierungs- und Identifizierungshilfsmittel verwendet werden[11,12,13]. Herkömmliche Methoden wie Färbungen oder Genanalysen beeinflussen oder zerstören die Kulturen. Die VOC-Analyse kann zerstörungsfrei Aufschlüsse über Wachstum und Metabolismus von Zell- und Bakterienkulturen geben[14,15,16,17]. Unterschiedliche Protokolle zur Anzucht von Bakterien oder unterschiedliche Proben-Matrizes (Kot, Flüssig- oder Festmedium) sind die Ursache für hohe Variationen der VOC-Profile. Der Einfluss verschiedener Kultivierungsparameter, wie z. B. Nährmedium, Inkubationszeit, Besiedelungsdichte, auf die VOC-Emissionen ist noch nicht ausreichend untersucht. Um vergleichbare spezies-spezifische VOC-Profile messen zu können, müssen diese Faktoren evaluiert und standardisiert werden.

1.4 Mikroextraktionsverfahren

Die Untersuchung der sehr niedrig konzentrierten VOCs wurde lange mit sehr aufwändigen Präkonzentrationsmethoden durchgeführt. Für eine Reduzierung des Aufwands durch eine Miniaturisierung sind die Festphasen-Mikroextraktion, engl.: **s**olid **p**hase **m**icro **e**xtraction (SPME), und Nadel-Mikroextraktion, engl.: **n**eedle **t**rap **m**icro **e**xtraction (NTME), in Verbindung mit **G**as**c**hromatografie-**M**assen**s**pektrometrie (GC-MS) entwickelt worden. Sie sind heute weit verbreitet für die Analyse von Zell- und Bakterienkulturen sowie für die Untersuchung von Atemgas[18]. Die Erfassung von komplexen VOC-Profilen mit mehr als 50 VOCs kann mit beiden Methoden

durchgeführt werden[11]. Gegenwärtig gibt es nur wenige Studien, die NTME und SPME bezüglich der Analyse aus gleicher Matrix vergleichen. Vergleiche wurden bislang nur anhand eines kleinen Substanzsets durchgeführt[19]. Eine Untersuchung, ob die mit NTME und SPME erfassten VOC-Profile deckungsgleich sind gibt es bis heute nicht.

Needle trap micro-extraction

Die NTME ist ein auf Adsorption beruhendes Verfahren[20,21]. Dafür wird eine gepackte Mikroextraktionsnadel, engl.: **n**eedle **t**rap **d**evice (NTD), verwendet, durch welches das Probenvolumen geleitet wird. Die im Probenvolumen enthaltenen VOCs werden vom Adsorptionsmaterial in der Nadel adsorbiert. Die Überführung der angelagerten VOCs in die GC-MS erfolgt durch Thermodesorption. Durch das Einführen der NTDs in den GC-MS-Injektor sind die VOCs einem plötzlichen Temperaturanstieg um ca. 200 °C ausgesetzt und gehen dabei abrupt in die Gasphase über. Die Sensitivität dieses Verfahrens ist volumenabhängig und kann durch Erhöhung des Volumens, das durch die NTD geleitet wird, verbessert werden.

Solid phase micro-extraction

Die SPME ist ein verteilungsbasiertes Extraktionsverfahren und basiert auf einem Gleichgewicht zwischen dem Probengas und dem Faser-Beschichtungsmaterial[22]. Beim Beproben wird die Faser nach Durchstechen eines Septums aus der Hülse gefahren und der Probe ausgesetzt. Dies ermöglicht die nahezu störungsfreie Beprobung von Gasphasen. Auch hier erfolgt die Übertragung der VOCs in die GC-MS mittels Thermodesorption.

2 Fragestellung

Die vorliegende Arbeit beschäftigt sich mit der Evaluierung und Anwendung analytischer Methoden *in vitro* zur Bestimmung von VOC-Profilen, die von Mykobakterien emittiert werden. Einflüsse der Messmethodik und verschiedener Kulturparameter sollen hierbei untersucht und standardisiert angewandt werden. Folgende Fragestellungen wurden dazu genauer untersucht:

1. Welche Mikroextraktionsmethode (NTME und SPME) ist am besten geeignet für die Analyse bakterieller VOCs?
   a) Wie unterscheiden sich die mittels NTME und SPME über Mykobakterienkulturen gemessenen VOC-Profile?
   b) In wie weit eignen sich NTME und SPME für non-targeted (ungerichtete) Screeningstudien?
2. Welchen Einfluss haben Kulturparameter auf die Emission von VOCs?
   Wie verändern sich von Mykobakterien emittierte VOC-Profile, wenn sie
   a) auf verschiedenen Kulturmedien kultiviert werden?
   b) in unterschiedlicher Zelldichte ausgesät werden?
   c) nach unterschiedlichen Kultivierungszeiten untersucht werden?

3) Ist es möglich, unter standardisierten Kultivierungsbedingungen
   a) verschiedene Mykobakterienspezies anhand ihrer VOC-Profile zu unterscheiden?
   b) VOCs zu bestimmen, anhand derer das Vorhandensein von Mykobakterien erkannt werden kann?

3 Methoden

3.1 *In vitro* VOC-Analyse

3.1.1 Kultivierung von Mykobakterien

Für die Publikationen 1, 2 und 3 wurden die Bakterien nach den Empfehlungen des Referenzlabors für Paratuberkulose im Friedrich-Löffler-Institut Jena kultiviert: Zwei Impfösen, befüllt mit Mykobakterien einer Stammkultur, wurden zur Vermehrung in MB-Bouillon suspendiert und für zwei Wochen bei 37 °C und 70 Umdrehungen pro Minute in einer Schüttelkultur inkubiert. Glasperlen sorgten für die gleichmäßige Verteilung der Bakterien. Nachdem die optische Dichte gemessen und auf 0,6 eingestellt wurde, wurden 100 µl der Kultursuspension in je ein Kulturröhrchen gegeben. Dieses war mit Festmedium gefüllt. Anschließend wurden die Röhrchen elf Wochen bei 37 °C inkubiert. Die Bakterienkulturen wurden im Friedrich-Löffler-Institut Jena angesetzt und zur Analyse per Kurier nach Rostock verschickt.

3.1.2 Probennahme und Präkonzentration von VOCs

Beprobung und Aufkonzentrierung der VOCs aus der Gasphase über den Kulturen erfolgte in den in Publikationen 1, 2 und 3 beschriebenen Studien auf gleiche Weise. Die Probennahme erfolgte mittels Mikroextraktionsverfahren. Der schematische Aufbau beider Mikroextraktionsverfahren ist in Abbildung 3.2 dargestellt. Vor der Probennahme wurden die Bakterienkulturen 20 Minuten bei 37 °C erwärmt.

SPME

In der Studie 1 wurde die verteilungsbasierte SPME eingesetzt. Die Moleküle in der Gasphase der Probe werden auf der SPME-Faser präkonzentriert. Da das Verteilungsgleichgewicht der SPME-Faser zeitabhängig sein kann, wurde für alle Proben dieselbe Expositionsdauer der Faser festgelegt (20 min). In dieser Arbeit wurden SPME-Fasern, bestehend aus Carboxen® und Polydimethylsiloxane (75 µm Siebgröße, Supelco, Bellafonte, USA), verwendet. Zwanzig Minuten vor jeder Probennahme wurde

die Faser bei 290 °C konditioniert. Die Faser wurde anschließend in das Kulturröhrchen eingeführt, 20 Minuten in der Gasphase über den Bakterien equillibriert und dann in den Injektor des Gaschromatografen überführt. Dort erfolgte bei einer Injektortemperatur von 290 °C die Thermodesorption der VOCs von der SPME-Faser in den Trägergasstrom der Gaschromatografiesäule.

NTME

Die NTME wurde in den Studien 1, 2 und 3 angewandt. Diese Verfahren basiert auf der Adsorption der VOCs am Packungsmaterial der NTDs. Dazu wird das zu untersuchende Gas aktiv durch die NTD geleitet. In allen Experimenten erfolgte die Probennahme bidirektional. Das bedeutet, das Probenvolumen wird aus der Probe über die Spitze der NTD in eine Spritze gezogen. Dieses Volumen wird dann in umgekehrter Richtung aus der Spritze durch die NTD wieder in die Probe zurückgegeben. Die verwendeten Nadeln für die NTME waren mit folgenden Materialien gepackt: Divinylbenzen, Carbopack X und Carboxen 1000 (je 1 cm, PAS Technologie, Magdala, Deutschland). Wie bei Trefz et al. beschrieben, hatte sich diese Kombination als optimal für die Extraktion von VOCs vieler unterschiedlicher chemischer Substanzklassen herausgestellt[20]. Vor der Probennahme wurden die Nadeln für 30 Minuten unter stetigem Helium-Fluss bei 250 °C konditioniert, um Rückstände auf den Nadeln zu entfernen. Anschließend wurden die Nadeln mit Teflonkappen verschlossen, um Kontaminationen vor der Probennahme zu vermeiden. Für die Probennahme wurden die Nadeln mit einer Einmalspritze (1 ml, B.Braun, Melsungen AG, Deutschland) verbunden und durch ein Septum in die Kulturröhrchen eingeführt. Schließlich wurde 20 mal ein Milliliter durch die Nadel und wieder zurück gepumpt (= 20 ml bi-direktional). Nach der Probennahme wurden die Nadeln mit Teflonkappen verschlossen und für die weitere Analyse mittels GC-MS gelagert.

Abbildung 3.1: Probennahme bakterieller VOCs mittels NTME. Entnommen aus Publikation 2.

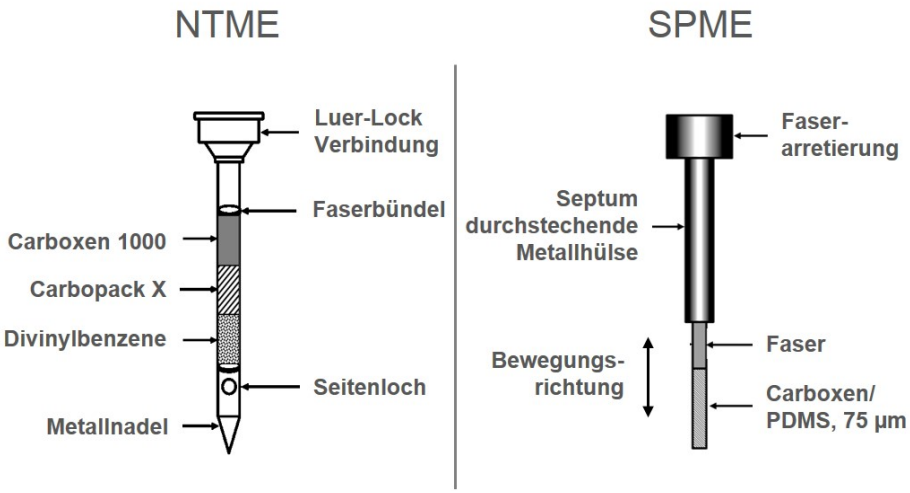

Abbildung 3.2: Schematische Darstellung vom Aufbau eines NTDs für NTME (links) und einer Faser für SPME (rechts).

3.1.3 GC-MS-Analyse

Die GC-MS-Analyse wurde mit einem Gaschromatografen des Typs 7890-A in Verbindung mit einem Quadrupol-Massenspektrometer des Typs 5975C inert XL MSD (beide Agilent, Waldbronn, Deutschland) durchgeführt. Dafür wurden zunächst die SPME-Faser bzw. das NTD in den Injektor eingeführt. Durch den raschen Temperaturanstieg kam es zur Desorption der VOCs vom Absorbtionsmaterial in das Trägergas (Helium) des Gaschromatografen. Die Substanzen wurden durch die Chromatografiesäule aufgetrennt. Anschließend wurden die VOCs durch Elektronenbeschuss (70 eV) ionisiert und fragmentiert. Die ionisierten Fragmente werden im elektrischen Feld des Quadrupol-Massenspektrometers beschleunigt, nach ihrem Masse-zu-Ladungsverhältnis selektiert und schließlich vom Detektor detektiert. Die Analysen für SPME und NTME unterschieden sich im Wesentlichen im Temperaturprogramm und in der verwendeten Säule. Weitere Unterschiede und Gemeinsamkeiten sind in Tabelle 3.1 aufgeführt. Die aus den VOC-Analysen mittels GC-MS ermittelten Massenspektren wurden mit Spektren der Datenbank vom National Institute of Standards and Technology (NIST) verglichen. Dadurch konnten den Spektren vorläufig Substanzen zugeordnet werden. Im Anschluss wurde diese Identifizierung über Massenspektren und die dazugehörigen Retentionszeiten von reinen Referenzsubstanzen verifiziert. Die Referenzsubstanzen wurden in verschiedenen Konzentrationen gemessen, um einen Zusammenhang zwischen gemessener Intensität im Detektor und tatsächlicher Konzentration zu erstellen.

Tabelle 3.1: GC-MS-Einstellungen für die Messung volatiler Verbindungen. Die Tabelle wurde der Publikation 1 entnommen.

Parameter	NTME	SPME
Injektionsmodus/-Zeit	automatisch, 20 s	manuell, 20 min
Injektortemperatur	250 °C	290 °C
Trägergas	Helium	Helium
Gesamtfluss	52.5 mL min^{-1}	52.7 mL min^{-1}
Gesamtdruck	60.305 kPa	64.315 kPa
Septum Spülfluss	1 mL min^{-1}	1 mL min^{-1}
Zeit “Splittless“ Modus	1 min	30 s
Spülfluss “Splitmodus“	50 ml min^{-1}	50 ml min^{-1}
Säulenmaterial	RTX-624	CP-Pora Bond Q Fused Silica
Säulenmaße	60 m x 0,32 mm; 1,8 µm	25 m x 0,32 mm; 5 µm
Säulenfluss	1,5 ml min^{-1}	1,7 ml min^{-1}
Temperaturprogramm	40 °C für 5 min	90 °C für 6 min
	8 °C min^{-1} bis 120 °C für 2 min	15 °C min^{-1} bis 120 °C für 1 min
	10 °C min^{-1} bis 220 °C für 10 min	10 °C min^{-1} bis 140 °C für 7 min
	20 °C min^{-1} bis 240 °C für 4,5 min	15 °C min^{-1} bis 260 °C für 6 min
Elektronen Ionisation	70 eV	70 eV
Ionisationstemperatur	250 °C	230 °C
MS Scanbereich	35-250 Th	35-300 Th
Scanrate	3,31 Scans s^{-1}	2,73 Scans s^{-1}
Quadrupole Temp.	150 °C	150 °C
Lösungsmittel Verzug	1 min	1 min

4 Ergebnisse

Die Ergebnisse der Arbeit werden anhand der folgenden 3 Originalpublikationen in englischer Sprache präsentiert:

Publikation 1 **Evaluation of NTME and SPME: obtaining comprehensive information on volatile emissions from *in vitro* cultures**

Oertel P, Bergmann A, Fischer S, Trefz P, Küntzel A, Reinhold P, Köhler H, Schubert JK and Miekisch W. Journal of Biomedical Chromatography **2018** 2. Mai, e4285, doi: 10.1002/bmc.4285

(Impact-Faktor 2016: 1,613)

Publikation 2 **Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of *Mycobacterium avium* ssp. *paratuberculosis***

Küntzel A, Fischer S, Bergmann A, **Oertel P**, Steffens M, Trefz P, Miekisch W, Schubert JK, Reinhold P, Köhler H. Journal of Breath Res. **2016** 8. September; 10(3):037103. doi: 10.1088/1752-7155/10/3/037103.

(Impact-Faktor 2015: 4,177)

Publikation 3 **Comparative analysis of volatile organic compounds for the classification and identification of *mycobacteria* species.**

Küntzel A, **Oertel P**, Fischer S, Bergmann A, Trefz P, Schubert JK, Miekisch W, Reinhold P and Köhler H. PLoS One **2018** 1. März; doi: 10.1371/journal.pone.0194348

(Impact-Faktor 2016: 2,806)

Im Folgenden werden die Originalpublikationen in deutscher Sprache zusammengefasst. Die vollständigen Ausführungen der Originalpublikationen befinden sich im Kapitel 8.

Publikation 1:

Evaluation of NTME and SPME: obtaining comprehensive information on volatile emissions from *in vitro* cultures.

Oertel P, Bergmann A, Fischer S, Trefz P, Küntzel A, Reinhold P, Köhler H, Schubert JK and Miekisch W.
Biomedical Chromatography **2018** Mai 2
(Impact-Faktor 2016: 1,613)

VOC-Konzentrationen liegen typischerweise im unteren nmol L^{-1} Bereich. Daher werden für die GC-MS basierte Analyse von VOCs geeignete Präkonzentrationsmethoden benötigt. Die vorliegende Studie vergleicht die Effizienz zweier unabhängig voneinander optimierter Mikroextraktionsmethoden, NTME und SPME, für die Analyse komplexer VOC-Profile von Bakterien.
Verglichen wurden SPME-Fasern (75 µm, bepackt mit Carboxen® und Polydimethylsiloxane) und NTME-Nadeln (bepackt mit Divinylbenzen, Carbopack X, und Carboxen 1000). Zunächst wurden beide Methoden mit 72 flüchtigen Reinsubstanzen im Bereich von 0,041 - 62,24 nmol L^{-1} kalibriert und anschließend für die Messung von VOC-Profilen einer MAP-Kultur verwendet.
Die Detektionslimits für NTME reichten von 0,004 bis 3,93 nmol L^{-1} (Median = 0,030 nmol L^{-1}) und für SPME von 0,001 bis 5,684 nmol L^{-1} (Median = 0,043 nmol L^{-1}). Die NTME zeigte Vorteile bei der Messung polarer Substanzen im Besonderen bei Alkoholen. Die SPME zeigte Vorteile bei der Reproduzierbarkeit. Mit beiden Methoden konnten charakteristische VOC-Profile von MAP-Kulturen erfasst werden. Mit SPME konnten 49 VOCs und mit NTME 54 VOCs über MAP-Kulturen erfasst werden. 31 VOCs bildeten dabei die gemeinsame Schnittmenge. Im Vergleich der beiden Methoden stellte sich die NTME als umfassender heraus, was die Erfassung von Verbindungen unterschiedlicher chemischer Klassen angeht. Für eine ungezielte, umfassende Screeningstudie sollten beide Methoden angewandt werden (siehe Abbildung 4.1).

Abbildung 4.1: Schematische Darstellung von VOC-Profilen einer Bakterienkultur gemessen mit NTME und SPME.

Publikation 2:

Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of *Mycobacterium avium* ssp. *paratuberculosis*.

Küntzel A, Fischer S, Bergmann A, **Oertel P**, Steffens M, Trefz P, Miekisch W, Schubert JK, Reinhold P, Köhler H.
Journal of Breath Research **2016** Sep 8
(Impact-Faktor 2015: 4,177)

MAP verursacht chronische granulomatöse Enteritis bei Wiederkäuern. Die zeitaufwändige bakterielle Anzucht aus Proben ist der derzeitige "Goldstandard" der Diagnostik. MAP-spezifische VOCs könnten die kulturelle Diagnostik beschleunigen. Das Ziel dieser Arbeit war die Untersuchung der Beziehung zwischen bakteriellem Wachstum *in vitro* und einer VOC-Profilveränderung. Zudem sollte der Einfluss wichtiger Kulturparameter auf die VOC-Emission bestimmt werden. Folgende Faktoren wurden bei der Kultivierung berücksichtigt und vermessen: Fünf unterschiedliche Kulturmedien, drei verschiedene MAP-Stämme, Beimpfung der Kultur mit unterschiedlicher Bakterienzahl und verschiedene Spannen der Inkubationszeit. NTME mit anschließender GC-MS wurde für die VOC-Analyse verwendet. Alle potentiellen Markersubstanzen wurden mit Reinsubstanzen identifiziert und in verschiedenen Konzentrationen kalibriert.
Mehr als 100 VOCs wurden in der Gasphase über den Bakterienkulturen und puren Medium gemessen. Die Ergebnisse belegen, dass sich die VOC-Profile über den verschiedenen Medien unterscheiden. Hierbei ähnelten sich eihaltige Medien und künstliche Medien untereinander. Dreiundvierzig VOCs konnten als potentielle Biomarker für bakterielles Wachstum auf Herrold's Eigelb-Medium identifiziert werden. Mit diesen Substanzen kann die Abhängigkeit der VOC-Konzentrationen von der Besiedelungsdichte belegt werden. Die VOC-Profile der drei MAP-Stämme unterschieden sich nicht erheblich.
Diese Daten belegen, dass Bakterien unterschiedliche Metaboliten emittieren, wenn sie auf verschiedenen Medien kultiviert werden, dass verschiedene Bakterienstämme sehr ähnliche VOC-Profile zeigen und dass kulturelle Diagnostik von Bakterien unter Berücksichtigung der spezifischen VOC-Profile beschleunigt werden könnte.

Publikation 3:

Comparative analysis of volatile organic compounds for the classification and identification of *mycobacteria* species.

Küntzel A,**Oertel P**, Fischer S, Bergmann A, Trefz P, Schubert JK, Miekisch W, Reinhold P, and Köhler H.
PLOSone **2018** Mar 1
(Impact-Faktor 2016: 2,806)

Mykobakterienspezies können schwerwiegende zoonotische Krankheiten in Säugetieren verursachen. Diagnose und Differenzierung von Mykobakterienspezies werden derzeit durch kulturelle Isolation erreicht. Die etablierten Diagnostikprotokolle beinhalten mehrere Schritte, um die Spezies zu identifizieren. Die Detektion von VOCs über Bakterienkulturen ist eine vielversprechende Möglichkeit, um die Speziesidentifikation zu beschleunigen. Das Ziel dieser Studie war die Analyse von VOCs in der Gasphase über Kulturen von 17 verschiedenen Mykobakterienstämmen. Anschließend sollten spezies-spezifische VOC-Profile sowie ein Substanzenset definiert werden, welches mykobakterielles Wachstum anzeigen könnte. Dazu wurden Bakterienkulturen unter standardisierten Bedingungen kultiviert und die emittierten VOC-Profile mittels NTME-GC-MS erfasst.
Die Mykobakterien emittierten spezies-spezifische VOC-Profile. Als Gemeinsamkeit aller VOC-Profile stellten sich 17 Substanzen als mögliche Biomarker für generelles Mykobakterienwachstum heraus. Davon zeigten acht VOCs (Alkohle, Alkane, Ketone) eine höhere Konzentration und neun (ausschließlich Aldehyde) eine geringere Konzentration als die reinen Mediumproben. Ähnlichkeiten im VOC-Profil entsprachen weniger dem gebräuchlichen Klassifikationsschema über den Phänotyp nach Ernest H. Runyon[23], sondern vielmehr der Zusammensetzung von Zellwandlipiden nach Lechevalier et al.[24]. Die aufgezeigte Möglichkeit, Mykobakterien über VOC-Profile zu detektieren und zu identifizieren, könnte einen weiteren Schritt zur Beschleunigung und Verbesserung der Diagnostik von Mykobakterien-Infektionen darstellen.

5 Diskussion

Der Fokus der vorliegenden Arbeit war die Untersuchung von analytischen Methoden für die Bestimmung von VOC-Profilen *in vitro*. Basierend auf diesen Untersuchungen konnten VOC-Profile über Mykobaktererien erfasst und Kulturbedingungen optimiert werden. Die optimale Verbindung aus geeigneter Mikroextraktionsmethode und einheitlichen *in vitro* Kulturbedingungen konnte eingesetzt werden, um VOC-Profile verschiedener Mykobakterienspezies miteinander zu vergleichen. 17 VOCs konnten als Markersubstanzen für die Anwesenheit von Mykobakterien identifiziert werden.

5.1 Evaluierung von Mikroextraktionsmethoden

NTME und SPME sind etablierte Mikroextraktionsmethoden in der *in vitro* VOC-Analytik[11,17,25]. Der Vergleich beider Methoden erfolgte bisher nur für einzelne Substanzen im Bereich der Umweltforschung[19]. Für *in vitro* Untersuchungen werden beide Methoden zur Erfassung umfangreicher VOC-Profile eingesetzt. In dieser Arbeit wurden erstmals die Sensitivität und Selektivität beider Methoden für eine größere Anzahl an VOCs gegenübergestellt. Bei vergleichbarem Probevolumen (20 ml) ist die Sensitivität von NTME und SPME ähnlich und unterschied sich nur bei vereinzelten VOCs (z. B. Butane, 1-Penten, 2,5-Dimethylfuran, 2-Propanthiol) maßgeblich. Die geringeren relativen Standardabweichungen bei der SPME sind vermutlich darauf zurückzuführen, dass bei der SPME dieselbe Faser verwendet wurde, während bei der NTME verschiedene Nadeln verwendet wurden. Minimale Unterschiede zwischen den Nadeln wurden von Trefz et al. bereits beschrieben[20]. Die NTME zeigte Vorteile bei der Selektivität durch die umfassendere Extraktion von volatilen Alkoholen. Auch in anderen Studien, die das gleiche Faserbeschichtungsmaterial verwenden, wurden mit der SPME keine Alkohole detektiert[11,12]. Dieser markante Vorteil der NTME kann auf der zusätzlichen Verwendung von Divinylbenzol als Absorptionsmaterial beruhen. So wurden in Studien, die zusätzlich zu Carboxen® und Polydimethylsiloxan auch Divinylbenzol verwendeten, mehr flüchtige Alkohole detektiert[26,27].

Neben diesen methodischen Aspekten sind auch applikative Aspekte zu betrachten. Hierbei kann bei der NTME die bessere Lagerungsfähigkeit und Transportierbarkeit der NTDs zu Geltung gebracht werden. Mit der Verwendung mehrerer, bis zur GC-MS-Analyse lagerbaren NTDs können Proben schneller nacheinander genommen werden. Bei der SPME kann die nächste Probennahme aufgrund der schlechteren Lagerungsfähigkeit erst nach dem letzten GC-MS-Durchlauf erfolgen. Dadurch ist das Probennahmeintervall für NTME-Proben kürzer als bei SPME-Proben. Das ist z. B. bei Proben relevant, die sich während der Messperiode verändern können. Ein applikativer Vorteil der SPME ist die geringere Beeinträchtigung der *in vitro* Kultur. Denn im Gegensatz zur NTME muss bei der SPME kein Volumen aktiv durch die NTD hin und her bewegt werden. Dadurch können bei der SPME stabile Druckverhältnisse beibehalten werden.
Als Konsequenz aus den Ergebnissen der Publikation 1 wurde die NTME zur Durchführung der Probennahme in den Studien 2 und 3 ausgwählt. Die umfassendere Detektion aller Substanzklassen für die NTME, im Besonderen von Alkoholen, wurde benutzt, um den Einfluss der Kulturbedingungen auf VOC-Emissionen zu Untersuchen

5.2 Einfluss der Kulturbedingungen von *in vitro* Kulturen auf die VOC-Emission

MAP hat die Fähigkeit, seinen Stoffwechsel an den des Wirtes anzupassen[28]. Bei der *in vitro* Kultivierung von MAP ist dessen primäre Kohlenstoffquelle eine andere als im Wirt. Der hauptsächlich auf Wirt-Lipiden basierende Stoffwechsel wechselt zu Glukose und Glycerol basierten Metabolismus[29]. Diese Fähigkeit könnte ein Grund für die unterschiedlichen VOC-Emissionen bei Kultivierung auf verschiedenen, in dieser Studie untersuchten Medien sein und erklärt die Ähnlichkeit der VOC-Profile über ähnlichen Medien. Basierend auf diesen Ergebnissen ist zu erwarten, dass die Übertragung von *in vitro* VOC-Profilen auf *in vivo* Bedingungen (z. B. in Kotproben) schwierig ist. Die Auswahl des Mediums muss bei zukünftigen Anwendungen der VOC-Analytik in der Diagnostik von MAP-Infektionen berücksichtigt werden.
Unabhängig von der initialen Bakteriendichte bei der Aussaat und dem visuell erfassten Bewuchs der Kulturröhrchen konnten bereits nach vier Wochen Kultivierungszeit signifikante Änderungen zum Medium im VOC-Profil erfasst werden. Da bereits

eine vergleichsweise geringe Anzahl Bakterien detektierbare VOC-Konzentrationen emittierten, ist das ein Vorteil für einen frühzeitigen MAP-Nachweis mittels VOC-Profilen.
Die Kultivierungszeit hatte einen großen Einfluss auf die VOC-Konzentrationen. Eine Zunahme der Bakteriendichte führt zu einer Veränderung der VOC-Profile, (sowohl Zunahme als auch Abnahme einiger VOCs). Diese Variabilität im VOC-Profil kann durch einen einheitlichen Probennahmezeitpunkt nach Beginn der Kultivierung minimiert werden.

5.3 Erfassung spezies-spezifischer VOC-Profile *in vitro*

Dank der Erkenntnisse aus Publikation 1 und 2 konnten in Publikation 3 verschiedene Mykobakterienspezies unter optimalen und einheitlichen Bedingungen kultiviert und analysiert werden. Dadurch war es möglich, spezies-spezifische VOC-Profile zu erfassen. Ähnlichkeiten im VOC-Profil der Mykobakterien entsprachen weniger ihrer phenotypischen Klassifizierung nach Ernest H. Runyon[23], sondern vielmehr der Lipid-Zusammensetzung der Zellwand[24]. In Publikation 3 wurden 17 VOCs evaluiert, die von allen untersuchten Mykobakterien emittiert wurden. Diese waren mitunter schon messbar, obwohl noch kein sichtbares Wachstum feststellbar war. Für die mikrobielle Diagnostik würde eine potentielle Anwendung der VOC-Analyse eine starke Verkürzung der Analysezeit bedeuten[30,31].

6 Zusammenfassung

Volatile organische Verbindungen sind flüchtige Moleküle, die von Lebewesen produziert und über Kontaktflächen wie Haut und Lunge oder Zellwände in die Umwelt abgegeben werden. Die vorliegende Arbeit befasst sich mit der Evaluierung und Anwendung analytischer Methoden zur Bestimmung der Emissionen von Mykobakterienkulturen *in vitro*.

Zwei Mikroexktraktionsmethoden, die Needletrap-Microextraction (NTME) und die Solidphase-Microextraction (SPME), wurden anhand von VOC-Messungen im Headspace von *Mycobacterium avium* ssp. *paratuberculosis* Kulturen miteinander verglichen. Beide Methoden können für die Erfassung komplexer VOC-Profile von Bakterien eingesetzt werden. Die SPME zeigte eine bessere Reproduzierbarkeit der Ergebnisse. Die NTME zeigte Vorteile in der Extraktion von flüchtigen Alkoholen, was in einem umfassenderen VOC-Profil resultierte. Daher wurde die NTME für die Durchführung der weiteren *in vitro* Versuche verwendet.

Nach der Optimierung der Messmethodik wurde der Einfluss verschiedener Kulturparameter evaluiert. VOC-Profile von *Mycobacterium avium* ssp. *paratuberculosis* unterschieden sich signifikant, wenn diese auf verschiedenen Kulturmedien kultiviert wurden. Unterschiedliche Besiedelungsdichten resultierten in unterschiedlichen VOC-Konzentrationen. VOC-Emissionen zeigten einen zeitabhängigen Verlauf während der Kultivierung.

Bei der standardisierten Kultivierung von 13 verschiedenen Mykobakterienspezies wurden 17 VOCs als potentielle Markersubstanzen für die Anwesenheit von Mykobakterien evaluiert. Unter Einbezug weiterer VOCs konnten spezies-spezifische VOC-Profile erstellt werden.

In der vorliegenden Arbeit konnten wichtige Voraussetzungen für VOC-basierte Mykobakterien-Diagnostik *in vitro* erarbeitet werden.

7 Thesen der Arbeit

I. NTME und SPME können für ungerichtete Screeningstudien für komplexe VOC-Profile eingesetzt werden.

II. Die mit NTME und SPME gemessenen VOC-Profile unterschieden sich, besonders bei Alkoholen.

III. Das Kultivierungsmedium und die Bakteriendichte haben einen erheblichen Einfluss auf die VOC-Emissionen von Mykobakterien.

IV. VOC-Emissionen von Bakterien sind zeitabhängig.

V. Siebzehn VOCs konnten als potentielles Markerset für das Vorhandensein von Mykobakterien evaluiert werden.

VI. VOC-Profile können spezifisch für eine Mykobakterienspezies sein

VII. Die kulturelle Diagnostik von Mykobakterien kann durch die VOC-Analytik unterstützt und verkürzt werden.

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8 Originalpublikationen zur kumulativen Dissertation

Die nachfolgenden Seiten umfassen die folgenden Originalpublikationen:

Publikation 1 **Oertel P**, Bergmann A, Fischer S, Trefz P, Küntzel A, Reinhold P, Köhler H, Schubert JK and Miekisch W. *Evaluation of NTME and SPME: obtaining comprehensive information on volatile emissions from in vitro cultures*, Journal of Biomedical Chromatography, **2018** 2. Mai, e4285, doi: 10.1002/bmc.4285
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Publikation 2 Küntzel A, Fischer S, Bergmann A, **Oertel P**, Steffens M, Trefz P, Miekisch W, Schubert JK, Reinhold P, Köhler H. *Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of Mycobacterium avium ssp. paratuberculosis.* Journal of Breath Res. **2016** Sep 8; 10(3):037103. doi: 10.1088/1752-7155/10/3/037103.
(Impact-Faktor 2015: 4,177)

Publikation 3 Küntzel A, **Oertel P**, Fischer S, Bergmann A, Trefz P, Schubert JK, Miekisch W, Reinhold P, Köhler H. *Comparative analysis of volatile organic compounds for the classification and identification of mycobacterial species.* PLoS One. **2018** Mar 20; 13(3):e0194348. doi: 10.1371/journal.pone.0194348. eCollection 2018
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RESEARCH ARTICLE

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Evaluation of needle trap micro-extraction and solid-phase micro-extraction: Obtaining comprehensive information on volatile emissions from *in vitro* cultures

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Abstract

Volatile organic compounds (VOCs) emitted from *in vitro* cultures may reveal information on species and metabolism. Owing to low nmol L^{-1} concentration ranges, pre-concentration techniques are required for gas chromatography–mass spectrometry (GC–MS) based analyses. This study was intended to compare the efficiency of established micro-extraction techniques – solid-phase micro-extraction (SPME) and needle-trap micro-extraction (NTME) – for the analysis of complex VOC patterns. For SPME, a 75 µm Carboxen®/polydimethylsiloxane fiber was used. The NTME needle was packed with divinylbenzene, Carbopack X and Carboxen 1000. The headspace was sampled bi-directionally. Seventy-two VOCs were calibrated by reference standard mixtures in the range of 0.041–62.24 nmol L^{-1} by means of GC–MS. Both pre-concentration methods were applied to profile VOCs from cultures of *Mycobacterium avium* ssp. *paratuberculosis*. Limits of detection ranged from 0.004 to 3.93 nmol L^{-1} (median = 0.030 nmol L^{-1}) for NTME and from 0.001 to 5.684 nmol L^{-1} (median = 0.043 nmol L^{-1}) for SPME. NTME showed advantages in assessing polar compounds such as alcohols. SPME showed advantages in reproducibility but disadvantages in sensitivity for N-containing compounds. Micro-extraction techniques such as SPME and NTME are well suited for trace VOC profiling over cultures if the limitations of each technique is taken into account.

KEYWORDS

bacterial cultures, needle trap micro-extraction, solid-phase micro-extraction, Trace gas analysis, volatile organic compound

1 | INTRODUCTION

Micro-extraction techniques such as solid-phase micro-extraction (SPME) and needle trap micro-extraction (NTME) support trace analysis of volatile organic compounds (VOCs) in various areas, such as the environment (Mesarchaki et al., 2013; Ouyang & Pawliszyn, 2006), food (Tait, Perry, Stanforth, & Dean, 2014a; Warren, Parkinson, & Pawliszyn, 2013), homeland security (Lai, Leung, Magee, & Almirall, 2010) and medical or biological sciences (Eom, Tugulea, & Pawliszyn, 2008; Trefz, Rösner, Hein, Schubert, & Miekisch, 2013). Both techniques enable trace gas analysis with a good sensitivity (Eom et al., 2008; Lord, Zhan, & Pawliszyn, 2010; Wang, Fang, & Pawliszyn, 2005) and do not disturb *in vitro* systems. Depending on the packing material, SPME is mainly based on substance distribution (Boyacı et al., 2015), while NTME is mainly an adsorption-based method (Eom et al., 2008; Trefz et al., 2012). Therefore, in contrast to SPME,

Abbreviations: LCU, liquid calibration unit; MAP, *Mycobacterium avium* ssp. *paratuberculosis*; NTD, needle trap device; NTME, needle-trap micro-extraction; SPME, solid-phase micro-extraction; VOC, volatile organic compound.

the sensitivity of NTME assays can be increased by increasing sample volume (Trefz et al., 2012). Among different applied techniques, gas chromatography–mass spectrometry (GC–MS) has been reported as the 'gold standard' for identifying and quantifying volatiles from ubiquitous sources (Chauhan, Goyal, & Chauhan, 2014).

VOCs emitted from *in vitro* cultures may reveal information on species and metabolism (Hossain, Bojko, & Pawliszyn, 2013; Küntzel et al., 2016; Schulz & Dickschat, 2007; Tait, Perry, Stanforth, & Dean, 2014b). As VOC concentrations in the headspace over these cultures reach the low pmol L^{-1} range, pre-concentration techniques such as SPME or NTME are required for GC–MS analysis. When samples containing a wide range of VOCs from different substance classes are to be analyzed, a compromise between sensitivity and selectivity has to be found. In general, both micro-extraction techniques are suitable for VOC profiling (Trefz et al., 2012; Trefz, Koehler, et al., 2013), but comparison or parallel application of both techniques was done for selected substance sets rather than for entire VOC profiles (Koziel, Odziemkowski, & Pawliszyn, 2001). Hence, this study aimed at a systematic evaluation of NTME and SPME methods, with respect to sensitivity, selectivity and reproducibility when applied to complex samples. In order to demonstrate the performance of both techniques in a real-world application, headspace from cultures of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) were sampled by both techniques. Both methods can be applied with various types of adsorption materials. As both extraction methods require specially adapted materials and appropriate analytical setups we chose adsorption materials and analytical conditions which had already been applied and optimized in other studies (Bergmann et al., 2015; Küntzel et al., 2016; Trefz et al., 2012; Trefz, Koehler, et al., 2013). The following questions were addressed in detail:

- Are sensitivity and selectivity of both techniques comparable?
- Do both techniques allow a bottom-up approach to characterize emitted VOC profiles from bacterial cultures?
- What additional information is revealed by applying both sampling techniques in parallel?

2 | EXPERIMENTAL

2.1 | Selection of packing material and column properties for VOC-profiling

In this study we used micro-extraction devices and chromatographic columns reported to be the best suited for VOC profiling studies using SPME (Bergmann et al., 2015; Trefz, Koehler, et al., 2013) or NTME (Trefz et al., 2012; Trefz, Rösner, et al., 2013). Chromatographic methods were adapted with respect to the applied micro-extraction method. For SPME a Porabond-column, with a high efficiency for highly volatile VOCs, was used. This column is most suitable for separation of these compounds but may get contaminated easily if higher-boiling-point compounds are retained (or irreversibly bound). Therefore, this column cannot be used for NTME. As this technique is extractive, higher-boiling-point compounds would be extracted from the samples and would reach the column. For that reason, different columns were used for the SPME and NTME approaches.

The combination of PDMS and Carboxen is highly efficient for VOC analysis by means of SPME. Needle traps containing the same material combination did not perform well for VOC analysis and suffered from high variations (Trefz et al., 2012). Therefore, we used different materials consisting of three sorbents that were optimally suited for NTME.

2.2 | Needle trap micro-extraction

Triple-bed needle trap devices (NTDs; Shinwa Chemical Industries Ltd., Kyoto, Japan) had an inner diameter of 0.4 mm. NTDs were packed with 1 cm of divinylbenzene (80–100 mesh, ~0.15–0.18mm), Carbopack X and Carboxen 1000 (60–80 mesh, ~0.18–0.25 mm). Prior to measurements, NTDs were conditioned at 250°C for 30 min while being flushed with a constant helium flow. NTDs were sealed with Teflon caps on both ends in order to prevent contamination. Analysis by means of GC–MS was carried out as described in Table 1.

2.3 | Solid-phase micro-extraction

Pre-concentration of VOCs via SPME was carried out in the same manner as described in previous studies (Bergmann et al., 2015; Trefz, Koehler, et al., 2013) using Carboxen®/polydimethylsiloxane (CAR/PDMS)-SPME fibers (75 µm, Supelco, Bellefonte, PA, USA). Cleanliness of the fibers and the GC column was ensured by means of a blank run before the actual measurements. Analysis by means of GC–MS was carried out as described in Table 1.

2.4 | Quantification of VOCs

Seventy-two VOCs were selected by pilot experiments with MAP to be quantified with both methods. For calibrations, eight concentration levels ranging from 0.04 to 62.24 nmol L^{-1} were measured in triplicate. Not all 72 VOCs were calibrated at once. Standard mixtures of up to 18 VOCs were used for calibrations. Distinct concentration levels of liquid reference substances were diluted in water for distinct calibration levels and subsequently nebulized by means of a liquid calibration unit (LCU; Ionicon Analytik GmbH, Austria) in the following way: a small flow of liquid standard (25 µL min^{-1}) was introduced into a nitrogen gas stream (1 L min^{-1}) and then sprayed through a small orifice into an evaporation chamber with a temperature of 37°C, resulting in a humidity of 22 g m^{-3}. Standards with gaseous reference substances were prepared in Tedlar® bags via dilution with moisturized pure nitrogen (purity <99.999%).

Limits of detection (LOD) and lower limits of quantification (LLOQ) were determined from the signal-to-noise ratio (S/N), which was acquired experimentally from 10 blank samples. For NTME, these blanks consisted of NTDs which were similarly conditioned but measured without further sampling. For SPME, vials with pure nitrogen were used as blank samples to acquire the S/N ratio of the instrument and possibly carry-over. The LOD was defined as an S/N of 3 and the LLOQ as an S/N of 10.

TABLE 1 Settings of pre-concentration methods and GC–MS for *Mycobacterium avium* ssp. *paratuberculosis* (MAP) headspace analysis. Two similar GC–MS devices, one for each method, were utilized in this study

	SPME	NTME
Needle/fiber material	1 cm Car/PDMS, 75 µm	DVB, Carbopack X Carboxen 1000, at 1 cm
GC-device	Agilent 7890A	Agilent 7890A
Mode of injection	Manually, 20 min	Autosampler, 20 s
Injector temperature	290°C	250°C
Total flow	52.7 mL min^{-1} at 64.315 kPa	52.5 mL min^{-1} at 60.305 kPa
Septum purge flow	1 mL min^{-1}	1 mL min^{-1}
Time splitless mode	30 s	1 min
Splitmode purge flow	50 mL min^{-1}	50 mL min^{-1}
Carrier gas	Helium	Helium
Column material	CP-Pora Bond Q Fused Silica (25 m × 0.32 mm × 5 µm)	RTX-624 (60 m × 0.32 mm; 1.8 µm)
Column flow	1.7 mL min^{-1}	1.5 mL min^{-1}
Temperature program	90 °C for 6 min 15 °C min^{-1} to 120°C for 1 min 10°C min^{-1} to 140°C for 7 min 15 °C min^{-1} to 260°C for 6 min	40°C for 5 min 8°C min^{-1} to 120°C for 2 min 10°C min^{-1} to 220°C for 0 min 20°C min^{-1} to 240°C for 4.5 min
Electron ionization	70 eV	70 eV
Ionization temperature	230°C	250°C
MS	Agilent 5975C inert XL MSD	Agilent 5975C inert XL MSD
Scan-range	35–300 Th	35–250 Th
Scan-rate	2.73 scans s^{-1}	3.31 scans s^{-1}
Quadrupole temperature	150°C	150°C
Solvent delay	1 min	1 min

2.5 | NTME and SPME sampling procedures of for calibration

For NTME, 20mL aliquots were extracted bi-directionally by means of an 1 mL syringe (Transcoject®) from either the Tedlar® bags or from a T-piece attached to the outlet of the LCU. For SPME, 15mL of gas standards from Tedlar bags or LCU outlet were transferred with a glass syringe into evacuated 20 mL headspace vials. The remaining vacuum in those vials was equilibrated with pure nitrogen. The SPME fiber was automatically cleaned in the hot injector under a constant helium stream and was subsequently pierced through the septum of a vial in order to expose the it to the gas phase for VOC extraction. The loaded SPME fiber was again automatically inserted into a hot injector so that VOCs were released from the fiber and separated and analyzed by the GC–MS system.

2.6 | Bacteria cultivation

Strains from MAP used in this experiment were field-isolated strains 04A0386 (type III, isolation from sheep, strain A) and 05A2431 (type II, isolation from cattle, strain B). Two loops of bacteria, cultured on HEYM-MJ (Herold's Egg Yolk Agar supplemented with amphotericin, nalidixic acid, vancomycin and mycobactin J; Becton Dickinson, Sparks, USA), were suspended in MB-Bouillon and propagated for 2 weeks at 37°C in an incubator shaker (70 rotations per minute) in the presence of sterile glass beads. After measuring the optical density (04A0386 = 0.915 and 05A2431 = 0.014) 100 µL aliquots of the bacterial suspensions were inoculated onto vials with HEYM-MJ slants and incubated for 11 weeks at 37°C. For NTME eight vials were inoculated with strain A and seven with strain B, whereas seven vials for strain A and six for strain B were inoculated for SPME measurements. The vials were sealed with silicone/Teflon septa. Two vials of HEYM-MJ only inoculated with MB-Bouillon but not with bacteria served as controls for each method. Bacterial growth was monitored visually by appraising colonies and bacterial layer. Isolation and cultivation were done at the Friedrich-Loeffler-Institut in Jena according to standard protocols recommended by the National Reference Laboratory for Paratuberculosis. For VOC analysis, the bacterial cultures were sent to the University of Rostock.

2.7 | NTME and SPME sampling procedure from bacterial cultures

VOCs from bacterial cultures were extracted directly from the headspace. Prior to sampling, the culture vials were kept at 37°C for 20 min in a close-fitting metal heating block. For NTME, 20 mL of headspace was sampled bi-directionally using 1 mL syringes (Transcoject®). For SPME, the fiber was introduced into the headspace of the samples, equilibrated for 20 min and manually inserted into the GC–MS system.

3 | RESULTS

Evaluation of NTME and SPME was performed by determining method-specific parameters like LOD, LLOQ or reproducibility for

TABLE 2 Figures of merit for *in vitro* extraction of volatile organic compounds (VOCs) by means of needle-trap micro-extraction (NTME) and solid-phase micro-extraction (SPME)

			SPME					NTME				
	No.	VOC	Range of calibration (nmol L^{-1})	RSD 10.41 nmol L^{-1} (%)	R^2	LOD (nmol L^{-1})	LLOQ (nmol L^{-1})	Range of calibration (nmol L^{-1})	RSD 10.41 nmol L^{-1} (%)	R^2	LOD (nmol L^{-1})	LLOQ (nmol L^{-1})
Alcohols	1	1-Hexanol	–	–	–	>20.474	–	0.04–41.49	3	0.9977	0.02	0.05
	2	1-Octen-3-ol	–	–	–	>20.474	–	0.04–41.49	13	0.9915	0.01	0.01
	3	1-Propanol	–	–	–	>20.474	–	0.04–41.49	8	0.9893	0.4	0.89
	4	2-Methyl-1-butanol	–	–	–	>20.474	–	0.04–41.49	5	0.9961	0.03	0.06
	5	3-Methyl-1-butanol	–	–	–	>20.474	–	0.04–41.49	8	0.9900	0.04	0.08
	6	Ethanol	0.04–41.49	54	0.9711	0.01	0.02	0.04–41.49	8	0.9800	2.88	5.73
	7	Isopropanol	–	–	–	>20.474	–	0.04–41.49	5	0.9932	0.18	0.43
	8	Phenylethyl-alcohol	–	–	–	>20.474	–	0.04–41.49	9	0.9941	0	0.01
Aldehydes	9	2,5-Dimethylbenzaldehyde	0.04–20.75	4	0.9973	0.03	0.06	0.04–41.49	18	0.9929	0.21	0.5
	10	2-Methyl-2-butenal	0.04–41.49	4	0.9974	0.04	0.1	0.04–41.49	3	0.9983	0.05	0.1
	11	2-Methylbutanal	0.04–41.49	6	0.9971	0.01	0.02	0.04–41.49	4	0.9952	0.05	0.12
	12	2-Methylpropanal	0.04–41.49	1	0.9966	0.05	0.12	0.04–41.49	14	0.9871	0.24	0.49
	13	3-Methylbutanal	0.04–41.49	1	0.9988	0.08	0.16	0.04–41.49	6	0.9986	0.13	0.31
	14	Acetaldehyde	0.04–41.49	1	0.9876	5.68	15.18	0.08–41.49	6	0.7860	3.93	9.71
	15	Benzaldehyde	0.04–41.49	4	0.9959	0.51	1.13	0.04–41.49	4	0.9977	0.07	0.12
	16	Heptanal	0.1–18.7	2	0.9892	0.37	0.78	0.04–41.49	5	0.9996	0.01	0.01
	17	Hexanal	0.1–18.7	<1	0.9957	0.22	0.43	0.1–18.7	7	0.9995	0.29	0.6
	18	Methacrolein	0.04–41.49	7	0.9995	0.38	0.97	0.04–41.49	7	0.9987	0.02	0.04
	19	Nonanal	0.04–41.49	14	0.9940	1.07	2.43	0.1–18.7	26	0.9808	1.78	3.85
	20	Pentanal	0.1–18.7	0	1.0000	0.11	0.24	0.1–18.7	5	0.9930	0.13	0.32
Alkanes	21	2,4-Dimethylheptane	0.04–41.49	7	0.9319	0.49	1.34	0.04–41.49	14	0.9989	0.04	0.09
	22	2-Methylpentane	0.4–41.49	17	0.9819	0.01	0.03	0.04–41.49	10	0.9935	0.06	0.13
	23	3-Methylhexane	0.04–41.49	3	0.9999	0.03	0.07	0.04–20.75	4	0.9830	<0.01	0.01
	24	4-Methyloctane	0.04–41.49	3	0.9991	0.21	0.5	0.04–41.49	7	0.9965	0.04	0.07
	25	Butane	0.31–62.24	1	0.9971	0.01	0.03	0.31–62.24	4	0.9842	0.44	0.85
	26	Cyclohexane	0.04–41.49	5	0.9974	2.5	5.59	0.04–41.49	3	0.9952	<0.01	0.01
	27	Heptane	0.04–41.49	2	0.9984	0.08	0.19	0.04–20.75	21	0.9870	0.08	0.18
	28	Hexane	0.31–62.24	2	0.9985	0.04	0.1	0.04–20.75	22	0.9907	0.06	0.1
	29	Methylcyclopentane	0.04–41.49	6	0.9977	3.09	7.92	0.04–41.49	4	0.9942	<0.01	0.01
	30	Octane	0.04–37.34	3	0.9979	0.01	0.02	0.04–20.75	27	0.9707	0.08	0.17
	31	Pentane	0.31–62.24	1	0.9974	0.11	0.29	0.04–41.49	20	0.9929	0.27	0.56
Alkenes	32	1-Heptene	0.04–41.49	11	0.9857	0.15	0.34	0.04–20.75	12	0.9960	<0.01	0.01
	33	1-Pentene	0.04–41.49	6	0.9915	0.01	0.03	0.04–20.75	6	0.9820	0.62	1.3
	34	2,4-Dimethyl-1-heptene	0.04–41.49	4	0.9457	0.32	0.72	0.04–41.49	10	0.9984	0.08	0.18
	35	2-Methyl-1-pentene	0.04–41.49	5	0.9992	0.04	0.09	0.04–41.49	8	0.9986	0.06	0.13
Aromates	36	Benzene	0.1–18.7	1	0.9996	0.01	0.02	0.04–41.49	2	0.9969	0.05	0.11
	37	Ethylbenzene	0.04–41.49	2	0.9943	0.01	0.02	0.04–41.49	8	0.9993	<0.01	0.01
	38	Styrene	0.04–41.49	6	0.9967	0.24	0.48	0.04–41.49	14	0.9863	0.04	0.09
	39	Toluene	0.04–41.49	20	0.9970	0.01	0.03	0.1–18.7	9	0.9968	0.02	0.04
Esters	40	2,2-Dimethylpropionic-acid-ME	0.04–41.49	1	0.9925	<0.01	0.01	0.04–41.49	10	0.9990	0.01	0.02
	41	2-OH-benzoic-acid-ME	–	–	–	>20.474	–	0.04–41.49	14	0.9914	<0.01	0.01
	42	Methylacetate	0.04–41.49	25	0.9955	0.01	0.02	0.04–41.49	24	0.9814	0.1	0.23
	43	Benzoic-acid-ME	0.04–41.49	11	0.9997	0.22	0.47	0.04–41.49	15	0.9977	0.01	0.02
Furans	44	2,3,5-Trimethylfuran	0.04–41.49	2	0.9996	0.02	0.04	0.04–41.49	3	0.9981	<0.01	0.01
	45	2,5-Dimethylfuran	0.04–41.49	2	0.9931	<0.01	0.01	0.04–41.49	11	0.9990	0.34	0.8

(Continues)

TABLE 2 (Continued)

			SPME					NTME				
	No.	VOC	Range of calibration (nmol L^{-1})	RSD 10.41 nmol L^{-1} (%)	R^2	LOD (nmol L^{-1})	LLOQ (nmol L^{-1})	Range of calibration (nmol L^{-1})	RSD 10.41 nmol L^{-1} (%)	R^2	LOD (nmol L^{-1})	LLOQ (nmol L^{-1})
	46	2-Ethylfuran	0.04–41.49	3	0.9995	<0.01	0.01	0.04–41.49	4	0.9996	0.01	0.02
	47	2-Methylfuran	0.04–41.49	3	0.9960	0.03	0.09	0.04–41.49	15	0.9946	0.03	0.07
	48	2-Pentylfuran	0.04–41.49	9	0.9272	0.05	0.1	0.04–20.75	18	0.9752	0.01	0.01
	49	2-Propylfuran	0.04–41.49	1	0.9997	0.01	0.02	0.04–41.49	3	0.9940	0.02	0.03
	50	3-Methylfuran	0.04–41.49	1	0.9997	0.02	0.06	0.04–41.49	4	0.9977	0.02	0.04
	51	Furan	0.04–41.49	2	0.9958	0.09	0.22	0.04–41.49	17	0.9893	0.25	0.53
Ketones	52	2,3-Butadione	0.04–41.49	9	0.9975	0.04	0.12	0.04–41.49	2	0.9863	0.1	0.2
	53	2-Butanone	0.1–18.7	1	1.0000	0.03	0.06	0.1–18.7	5	0.9929	0.04	0.09
	54	2-Heptanone	0.04–41.49	3	0.9986	0.23	0.66	0.04–41.49	2	0.9965	0.02	0.04
	55	2-Hexanone	0.04–41.49	3	0.9979	0.14	0.38	0.04–41.49	3	0.9968	0.02	0.05
	56	2-Pentanone	0.04–41.49	3	0.9964	0.01	0.03	0.04–41.49	11	0.9947	0.02	0.05
	57	3-Methyl-2-pentanone	0.04–41.49	2	0.9985	0.02	0.04	0.04–41.49	4	0.9972	0.02	0.04
	58	3-Octanone	0.04–41.49	2	0.9991	0.11	0.21	0.04–41.49	7	0.9973	0.02	0.03
	59	3-Pentanone	0.04–41.49	1	0.9990	<0.01	0.01	0.04–41.49	7	0.9985	0.02	0.04
	60	Acetone	0.1–18.7	2	0.9980	1.38	3.94	0.04–41.49	11	0.9919	0.79	1.39
	61	Cyclopentanone	0.04–41.49	6	0.9998	0.41	1.01	0.04–41.49	11	0.9971	<0.01	0.01
	62	Methylisobutylketone	0.04–41.49	3	0.9963	0.04	0.09	0.04–41.49	8	0.9913	0.02	0.05
S-cont. Comp.	63	2-Propanthiol	0.04–41.49	13	0.9989	0.13	0.32	0.04–41.49	4	0.8470	1.68	3.54
	64	Dimethyldisulfide	0.04–41.49	4	0.9995	0.01	0.01	0.04–41.49	3	0.9993	<0.01	0.01
	65	Dimethylsulfide	0.04–41.49	14	0.9980	0.01	0.02	0.04–41.49	3	0.9942	0.04	0.08
N-containing compounds	66	1-Methyl-1H-pyrrole	0.04–41.49	5	0.9997	0.02	0.04	0.04–41.49	1	0.9997	0.01	0.02
	67	2,5-Dimethylpyrazine	0.04–41.49	1	0.9991	0.08	0.2	0.04–41.49	11	0.9930	<0.01	0.01
	68	2-Methylbutanitrile	0.04–41.49	<1	0.9984	0.04	0.09	0.04–41.49	6	0.9983	0.02	0.04
	69	3-Methylbutanitrile	0.04–41.49	2	0.9992	0.03	0.05	0.04–41.49	3	0.9910	<0.01	0.01
	70	Trimethyloxazole	0.04–41.49	1	0.9942	<0.01	0.01	0.04–41.49	19	0.9903	<0.01	0.01
	71	Bromodichloromethane	0.04–41.49	7	0.9944	0.12	0.28	0.04–41.49	3	0.9840	<0.01	0.01
	72	Trimethylenoxide	0.04–41.49	29	0.9945	0.55	1.54	0.04–41.49	2	0.9950	0.01	0.02

ME, Methyl ester; N, nitrogen; S, sulfur.

several VOCs from different substance classes (Table 2). Additionally, both methods were applied for headspace analysis of MAP cultures. Figure 1 shows two exemplary total ion chromatograms of VOC measurements in the headspace of MAP cultures by means of SPME-GC–MS and NTME-GC–MS. Figure 2 gives an overview of all analyzed VOCs in the headspace of MAP bacterial cultures assessed with SPME- and NTME-GC–MS.

3.1 | Calibration of VOCs

Calibrations were performed in the range of concentrations relevant for *in vitro* cultures (0.041–62.24 nmol L^{-1}, corresponding to 1–1500 ppbV). For NTME (20 mL bi-directional, DVB, Carbopack X, Carboxen 1000), the LODs ranged from 0.004 to 3.929 nmol L^{-1} (median = 0.034 nmol L^{-1}). Alcohols, ketones, furans and esters predominantly showed LODs <0.041 nmol L^{-1}. The values of R^2 were around 0.988. Only 2-propanthiol and acetaldehyde had an R^2 of 0.847 and 0.786, respectively. Relative standard deviations (RSD) at 10.4 nmol L^{-1} ranged from 1 to 27% (median = 7%). For SPME (PDMS/Carboxen), the LODs ranged from 0.001 to 5.684 nmol L^{-1} (median = 0.043 nmol L^{-1}) and were mainly <0.041 nmol L^{-1} for furans and nitrogen-containing compounds. In the investigated range alcohols could not be detected and quantified by means of SPME, except for ethanol. Also 2-hydroxybenzoic acid methyl ester was not detectable within the desired range. The value of R^2 was >0.990 for most substances. The minimum was reached for 2-pentylfuran with a value of 0.972. The RSD ranged from 1 to 29% (median = 3%). In general, calibrations for SPME resulted in lower RSDs for most VOCs included in the study compared with NTME. Linearity in general was excellent for both methods.

3.2 | VOC profiles from headspace over MAP cultures

Seventy-two substances from different chemical classes in the headspace of MAP cultures were assessed and verified by applying both methods. Among these VOCs, 31 were determined with values above their LLOQs with both methods. Eighteen VOCs were exclusively detected by means of SPME-GC–MS. Another 23 VOCs were only detected by means of NTME-GC–MS.

Despite inoculation with bacteria, some vials did not show visible bacterial growth. These vials were defined as nongrown and are treated as an individual group next to bacteria and media samples.

The heatmap in Figure 2 shows differences and similarities between the VOC patterns from bacteria, media and nongrown samples determined by means of SPME and NTME. The most obvious difference between VOC profiles determined by means of the two micro-extraction methods was the much higher number of alcohols that could be detected by means of NTME. Owing to the low sensitivity of SPME for alcohols, only ethanol could be assessed with both methods.

Nongrown samples were similar to the pure media samples and could be distinguished from MAP culture samples by means of their VOC patterns. This was true for SPME-based as well as for NTME-based analyses.

4 | DISCUSSION

In this study differences in selectivity and reproducibility of SPME and NTME were demonstrated. Typical detection limits of both micro-extraction methods were in the lower pmol L^{-1} range (<0.042 nmol L^{-1}). SPME showed advantages concerning reproducibility. NTME performed better with respect to the determination of alcohols. Complex VOC profiles from the headspace of bacterial cultures could be assessed by means of both techniques.

4.1 | Calibrations by means of SPME- and NTME-GC–MS

SPME and NTME calibration showed high regression coefficients and resulted in low detection and quantification limits for both methods. Only few substances reached LLOQs >0.249 nmol L^{-1}. For most substances, sensitivities of NTME (applying volumes of 20 mL) and SPME did not differ markedly. An increase in sampling volume would further improve the detection limits for NTME but also result in higher sampling preparation times for this technique.

NTME and SPME in principle are applicable with various combinations of packing materials (Piri-Moghadam, Alam, & Pawliszyn, 2017; Trefz et al., 2012). From a principle point of view, SPME is meant to operate mainly on distribution-based and NTME on adsorption-based

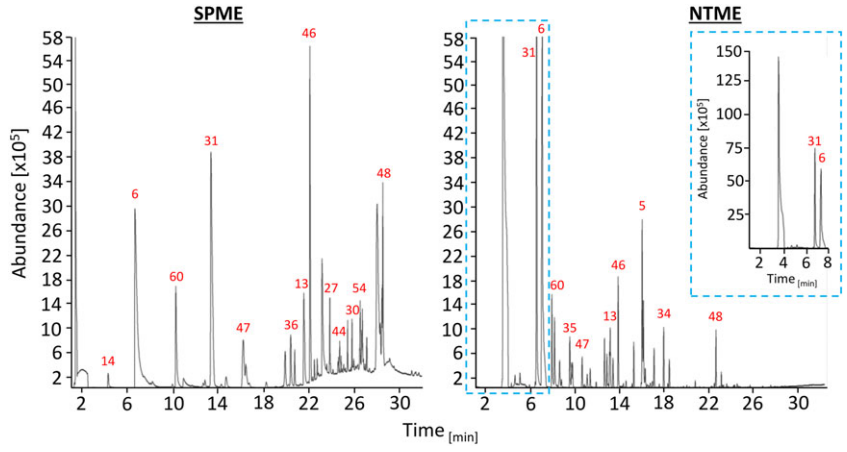

FIGURE 1 Total ion chromatograms of volatile organic compound (VOC) analyses from headspace of *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cultures (strain B). Left, solid-phase micro-extraction (SPME)–GCMS analysis; right, analysis by means of needle-trap micro-extraction (NTME)–GC–MS. Numbers in red correlate to substance numbers listed in Table 2

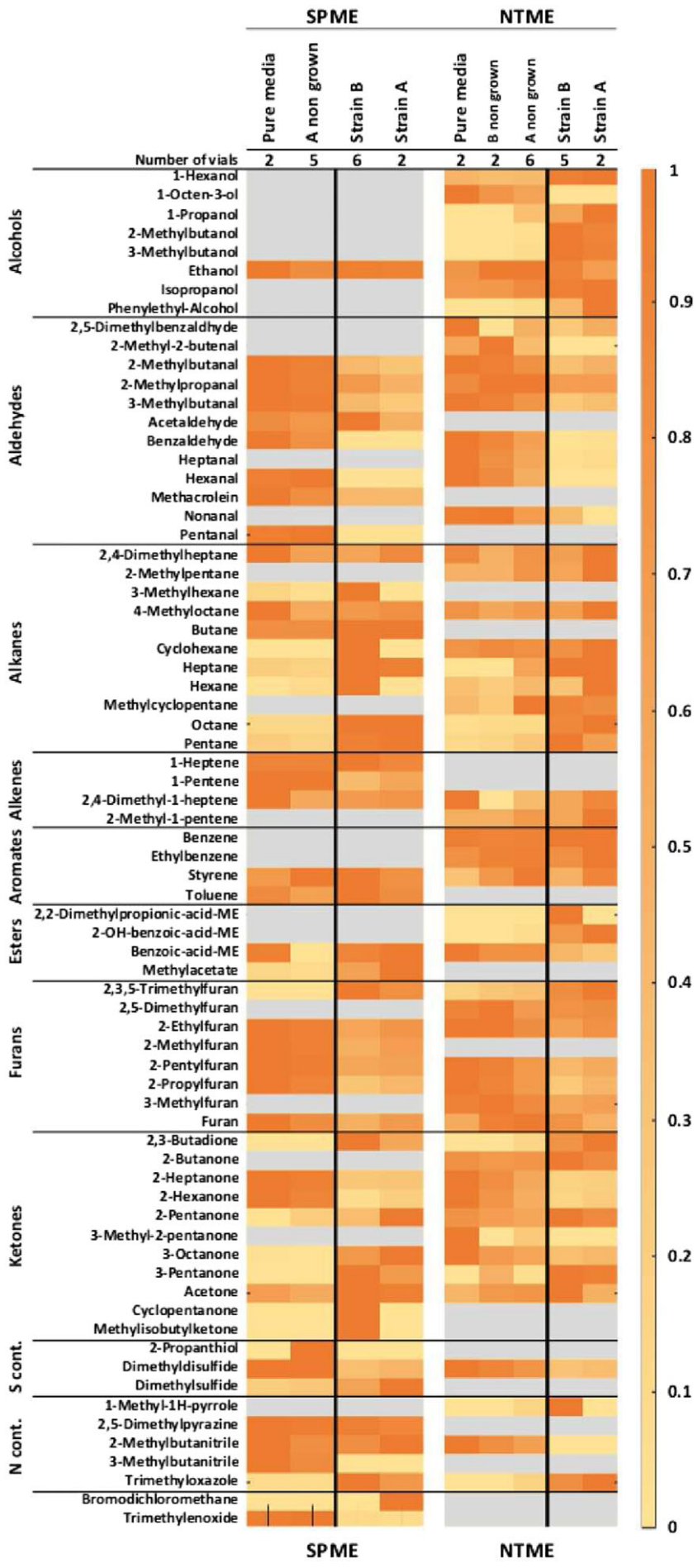

FIGURE 2 VOCs in the headspace of MAP cultures determined by means of SPME- and NTME-GC–MS. Gray color indicates not detected VOCs over MAP cultures. The heatmap shows values which were normalized onto the maximum of each substance for each method. ME, Methylester

mechanisms. According to this basic difference, the properties of the extraction materials and the analytes, efficiency of VOC extraction may differ if different packing materials are applied. In this study packing material was therefore selected with respect to optimal bottom-up properties for VOC profiling validated in previous studies (Bergmann et al., 2015; Küntzel et al., 2016; Trefz et al., 2012; Trefz, Koehler, et al., 2013) rather than to best comparable material combinations. General benefits and drawbacks of both micro-extraction methods are summarized in Table 3.

4.2 | Study limitations

The comparison between SPME and NTME has some limitations. Humid standards were created to mimic headspace humidity in the cultures. For NTME, humidity does not have a significant influence on VOC extraction by means of triple-bed NTDs (Trefz, Rösner, et al., 2013). In contrast, for SPME humidity may affect VOC binding ability (Damerau, Kamlang-ek, Moisio, Lampi, & Piironen, 2014).

Although it is a common method to determine LOD and LOQ from blank samples, this approach may only estimate the lowest detectable or quantifiable signal from analyte and does not give an actually measured limit (Armbruster & Pry, 2008). This has to be kept in mind when comparing the analytical limits of the two methods.

4.3 | Alcohols

SPME showed disadvantages concerning determination of alcohols from pure reference substances as well as from MAP cultures. Ethanol was the only alcohol that was calibrated and found in VOC profiles determined by means of SPME. The fact that NTME is more suitable for the extraction of volatile alcohols compared with SPME is in agreement with studies of Trefz, Koehler, et al. (2013) and Küntzel et al. (2016). According to Tankevièiûtė et al., a better performance of SPME concerning analysis of volatile alcohols may be achieved by using different fiber materials, e.g. Carbowax–DVB (Tankevičiūtė, Panavaitė, Kazlauskas, & Vičkačkaitė, 2004). This is in agreement with other studies of bacterial VOCs where more alcohols were detectable when DVB was included into the fiber material (Chen, Tang, Shi, Tang, & Zhang, 2017; Correa, Coronado, Garrido, Durant-Archibold, & Spadafora, 2017). The LOD of ethanol was higher for NTME compared with other volatile alcohols due to a high background signal.

4.4 | Ketones

Both methods showed low LODs for the investigated ketones, except for acetone. In the case of acetone, Trefz et al. reported a similarly high LOD (1.112 nmol L^{-1}) for the respective NTME material and suggested incomplete desorption from the Carboxen material (Trefz et al., 2012). For SPME, a lower LOD for acetone was achieved by Larroque et al. with the same fiber material (0.005 nmol L^{-1}; Larroque, Desauziers, & Mocho, 2006), probably owing to the longer equilibration time of 6 h, which would not be practical for our type of study.

4.5 | Aldehydes

Aldehydes showed the highest LOD values in both methods. This is most probably due to their reactivity (Fuchs, Loeseken, Schubert, & Miekisch, 2010) resulting in instability during sampling and storage on the adsorbent material. In order to improve aldehyde determination, on-fiber derivatization has often been used such that chemical reaction [e.g. with *o*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine-hydrochloride] prevents the decomposition of aldehydes (Ma et al., 2011; Schmarr et al., 2008).

4.6 | Alkanes and alkenes

LODs were low for both methods. In contrast to NTME, SPME calibration of cyclohexane and methylcyclopentane resulted in comparably high LODs. This could be related to their spatial conformation and thus worse binding ability to the CAR/PDMS fiber compared with linear alkanes. LODs in the same range were reported by Bergmann et al. (2015).

4.7 | Furans and esters

Furans and esters were calibrated with low LODs (median 0.018 and 0.012 nmol L^{-1}, respectively). Similar ranges for SPME and NTME were reported in former studies (Bergmann et al., 2015; Mesarchaki et al., 2013; Trefz, Koehler, et al., 2013). As 2-hydroxybenzoic acid methyl ester has an hydroxyl group, its LOD for SPME is not within

TABLE 3 General aspects of NTME and SPME methods for headspace VOC analysis

		SPME	NTME
Application	Extraction materials	Broad number of combinations commercially available	Any combination with adequate particle size feasible
		Amount of material limited by fiber diameter	Higher amount of packing material possible
	Device stability	Fiber not protected during sampling	In needle (steel) – very robust
	Automated sampling	Longer sampling intervals between vials (equilibration and reconditioning)	Short sampling intervals between vials Bi- and unidirectional sampling possible
	Sample type	Static headspace	Dynamic + static headspace,
	Sampling time	Equilibration based – typically 5–20 min	Volume related – typically 1–5 min
	Automation	Broad number of systems commercially available	Single systems commercially available
	Effects on culture	No impairment	Causes slight vacuum and pressure changes
Methodology	Sensitivity	Depending on equilibration constants of substances	Depending on sampling volume
	Basic working principle	Equilibration based	Exhaustive
	Optimal suited for	Highly volatile nonpolar and semi-polar compounds	Broad range of VOCs – especially for more polar compounds
	Chemical Modifications	On-fiber derivatization	Not yet established

the expected concentration range, corresponding to the LOD of other alcohols assessed with SPME.

4.8 | Nitrogen and sulfur containing compounds

Compared with NTME, SPME shows advantages in reproducibility but disadvantages in sensitivity for N-containing compounds (median RSD: 6% for NTME, 1% for SPME; median LOD 0.005 nmol L^{-1} for NMTE, 0.026 nmol L^{-1} for SPME). The opposite is true for sulfur-containing compounds (median RSD: 3% for NTME, 13% for SPME; median LOD: 0.038 nmol L^{-1} for NMTE, 0.008 nmol L^{-1} for SPME).

4.9 | VOC profiles from MAP cultures

The main advantage of using two instead of one micro-extraction technique is an extended VOC profile. As 18 VOCs out of the 72 volatile substances could be determined by SPME-GC–MS only and 23 VOCs by NTME-GC–MS only, it is apparent that some information may be lost when just one technique is applied, especially in untargeted screening studies. This is most obvious for alcohols, as information on these is mostly lost if only SPME is used. The reason why 36 VOCs (e.g. 2-butanone, heptanal, pentanal or 2-methylfuran and 3-methylfuran) were detected in the headspace of MAP cultures by only one method although calibration was possible by both techniques is not clarified. Some of those differences can be explained by differences in sensitivity [e.g. concentrations of alcohols (other than ethanol), 2,5-dimethylfuran, heptanal, bromodichloromethane in the headspace were below the LOD of one or the other preconcentration method]. As calibrations were performed with a maximum of 18 substances at a time but *in vivo* samples contain up to four times more VOCs, one might assume displacement effects between different VOCs.

5 | CONCLUSION

Preconcentration by means of SPME and NTME enables reproducible and reliable quantitative analysis of volatile organic compounds in the pmol L^{-1} range. Although extraction methods were optimized with respect to adsorption materials, separation and analysis, SPME and NTME showed different sensitivity and selectivity for some VOCs. Hence, for an untargeted screening approach a combination of both methods may be beneficial.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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PAPER

Effects of biological and methodological factors on volatile organic compound patterns during cultural growth of *Mycobacterium avium* ssp. *paratuberculosis*

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Keywords: diagnostic, GC-MS, kinetics, mycobacteria, *Mycobacterium avium* ssp. *paratuberculosis* (MAP), volatile organic compound (VOC)

Supplementary material for this article is available online

Abstract

Mycobacterium avium ssp. *paratuberculosis* (MAP) causes chronic granulomatous enteritis in ruminants. Bacterial growth is still the diagnostic 'gold standard', but is very time consuming. MAP-specific volatile organic compounds (VOCs) above media could accelerate cultural diagnosis. The aim of this project was to assess the kinetics of a VOC profile linked to the growth of MAP *in vitro*. The following sources of variability were taken into account: five different culture media, three different MAP strains, inoculation with different bacterial counts, and different periods of incubation. Needle-trap microextraction was employed for pre-concentration of VOCs, and gas chromatography–mass spectrometry for subsequent analysis. All volatiles were identified and calibrated by analysing pure references at different concentration levels.

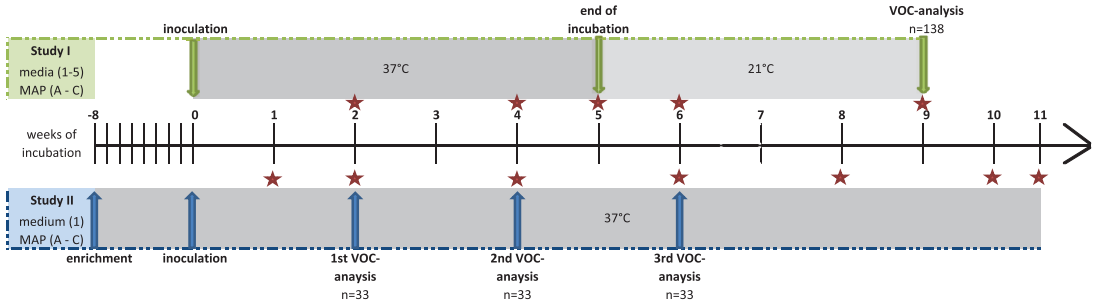
More than 100 VOCs were measured in headspaces above MAP-inoculated and control slants. Results confirmed different VOC profiles above different culture media. Emissions could be assigned to either egg-containing media or synthetic ingredients. 43 VOCs were identified as potential biomarkers of MAP growth on Herrold's Egg Yolk Medium without significant differences between the tree MAP strains. Substances belonged to the classes of alcohols, aldehydes, esters, ketones, aliphatic and aromatic hydrocarbons. With increasing bacterial density the VOC concentrations above MAP expressed different patterns: the majority of substances increased (although a few decreased after reaching a peak), but nine VOCs clearly decreased.

Data support the hypotheses that (i) bacteria emit different metabolites on different culture media; (ii) different MAP strains show uniform VOC patterns; and (iii) cultural diagnosis can be accelerated by taking specific VOC profiles into account.

1. Introduction

Mycobacterium avium ssp. *paratuberculosis* (MAP) causes chronic granulomatous enteritis in ruminants. The corresponding disease, *paratuberculosis* or Johne's disease, occurs worldwide and has strong economic relevance, especially for dairy farms. The pathogen has also been suggested as being involved in Crohn's disease in humans [1]. Unfortunately, the period of incubation can last for years, and there is no effective therapy, while infected animals can shed huge amounts of pathogens, as described by Antagnoli *et al* [2]. To estimate the prevalence and control the disease, it is important to have a sensitive and rapid diagnostic option for each animal.

The most sensitive diagnostic method currently available is direct detection of the bacteria via cultural isolation from feces or tissue samples [3]. Due to the long generation time of 1.3–4.4 d [4] and high requirements for the media [5], cultivation on solid media is very labour-intensive and time consuming,



		media	main ingredient
(1)	HEYM	Herrold's Egg Yolk Medium	egg yolk
(2)	Sto	Stonebrink	whole eggs
(3)	LJ	Löwenstein Jensen	whole eggs
(4)	Du	Dubos	synthetic
(5)	MB	Middlebrook	synthetic

MAP strain		origin	Type
A	reference strain	originated from cattle (2007)	C II
B	field strain	isolated from sheep (2004)	S III
C	fieldstrain	isolated from cattle (2012)	C II

visual assesment of bacterial growth		Score
EK	≤ 20 single colonies	0.5
+	> 20 ; ≤ 50 colonies	1
++	>50; ≤ 100 colonies	2
+++	> 100 colonies	3
R+	thin layer of cultural growth	3
R++	loosened layer of comprehensive growth	4
R+++	concluded layer of comprehensive growth	5

Figure 1. Experimental designs of study I (culture media: $n = 5$, MAP strains: $n = 3$, and duration of incubation: 5 weeks) and study II (one culture medium, MAP strains: $n = 3$, and three durations of incubation, i.e. 2, 4, and 6 weeks). MAP—*Mycobacterium avium* ssp. *paratuberculosis*.

often taking 10 weeks or longer. This determines the risk of contamination with yeast or fungi [3]. Cultivation on liquid media was evaluated for its potential to accelerate diagnosis of MAP. Further improvements were undertaken by defining the readout parameters of bacterial growth such as oxygen consumption or gas pressure; unfortunately, these parameters are non-specific, demonstrating only bacterial metabolism. After direct detection of bacteria, identifying the strain is mandatory, for example via polymerase chain reaction (PCR). There is a need for an accelerated, sensitive and specific diagnostic approach. Thus far, alternative diagnostic procedures have not proved sufficiently sensitive, and the labour-intensive procedure cannot be reduced from a two-step to a one-step method [3].

Volatile organic compounds (VOCs) emitted from the bacteria reflect the metabolome of the bacteria during different processes [6]. Furthermore, a number of different clinical studies linked VOC profiles to infections with specific pathogens [7]. However, before analyzing VOC emissions *in vivo*, precise knowledge is needed about volatiles resulting from pathogens *in vitro* [8, 9]. Within the last decade, a number of *in vitro* studies have been carried out, analysing VOCs from different kinds of bacteria, including MAP. These studies aimed to define specific VOC profiles [10, 11]. The kinetics of bacterial growth and subsequent effects on VOC emissions have been addressed for other bacteria by means of proton transfer reaction–mass spectrometry (PTR-MS) [12, 13] and gas chromatography–mass spectrometry (GC-MS) [14, 15]. Such information, however, is still lacking for MAP. In addition, confounding effects generated by different culture media available for the growth of *Mycobacteria*, and variability between different strains of MAP have yet to be defined. The importance of different ambient and nutrition conditions *in vitro* has already been demonstrated for other bacteria such as *Staphylococcus aureus* [13]. Taking all these aspects into account, the aims of our project were:

(I) to track VOC emissions during cultural growth and correlate them to bacterial replication;
(II) to define a MAP-related VOC profile;
(III) to evaluate factors with a significant impact on the composition of VOC emissions and therefore on a possible diagnostic approach.

Hence, we examined effects of different initial inocula, durations of incubation, different culture media and inter-strain variability.

2. Materials and methods

2.1. Study designs

The following three MAP strains (A, B, C) were included in the project:

(A) MAP 44133 (type-II, reference strain, bovine origin, DSMZ GmbH, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany);
(B) MAP 04 A 0386 (type-III, field isolate from sheep);
(C) MAP 12 MA 1245 (type-II, field isolate from cattle).

Field strains were isolates from tissue or feces, and were isolated and cultivated according to standard protocols recommended by the National Reference Laboratory for Paratuberculosis.

Two independent studies were performed consecutively (figure 1).

Figure 2. Experimental set up for sampling of headspace above culture media. Photo: FLI (W Maginot).

Study I focused on evaluating the influence of culture media on VOC profiles. The following five media (1–5) were included:

(1) HEYM: Herrold's Egg Yolk Medium containing Mycobactin J and amphotericin, nalidixic acid and vancomycin (Becton Dickinson, Sparks, USA);
(2) Sto: Stonebrink Medium with Polymyxin B, Amphotericin B, carbenicillin, trimethoprim, and pyruvate and Mycobactin J (Bioservice Waldenburg GmbH, Germany);
(3) LJ: Löwenstein–Jensen containing Polymyxin B, Amphotericin B, carbenicillin, and glycerol and Mycobactin J (Bioservice Waldenburg GmbH, Germany);
(4) Du: Dubos Oleic Agar with Mycobactin J (produced according to accredited instructions from the National Reference Laboratory for MAP);
(5) MB: Middlebrook 7H10 Medium with Mycobactin J containing oleic acid, albumin, dextrose, catalase and polymyxin B, amphotericin B, carbenicillin and trimethoprim (produced according to accredited instructions from the National Reference Laboratory for MAP).

At least eight vials per medium were used for each of the three MAP strains (A, B, C). In total, 123 vials inoculated with bacteria (about 24 of each medium) were compared to 18 control vials (2–5 of each medium).

For inoculation, three loops of cultured bacteria were added to 4 ml of phosphate buffered saline containing disodium, potassium dihydrogen orthophosphate, and sodium chloride (PBS). The bacterial suspension was thoroughly vortexed and diluted to an optical density of 0.312 ± 0.039, measured via a spectral photometer (Dr Lange Cadas 30, Dr Bruno Lange GmbH, Düsseldorf, Germany), which resulted in a bacterial count of $7.44 \pm 13 * 10^8$ cfu ml^{-1}. Dilutions of 10^{-2}, 10^{-4} and 10^{-6} were then prepared from the original bacterial suspension. Next, 100 μl of the bacterial suspensions of each dilution were inoculated onto each of six slants of each medium. The vials were sealed with Silicone/Teflon septa (Si/PTFE, PAS Technology Deutschland GmbH, Magdala, Germany). Cultures were incubated at 37 °C in a horizontal position for two weeks and then a further three weeks in an upright position. They were then kept at room temperature (21 ± 1 °C) until sampling (see section 2.2).

Study II aimed to assess the time-dependent formation of VOCs during cultivation. The three MAP strains (A, B, C) were cultivated on HEYM only. In total, 71 inoculated vials and 28 control vials were included in this study.

We added 2 ml of original bacterial suspension containing PBS originating from study I to 8 ml of Middlebrook 7H9 liquid medium containing oleic acid, albumin, dextrose, catalase and polymyxin B, amphotericin B, carbenicillin and trimethoprim (MB-Bouillon, produced according to accredited instructions by the National Reference Laboratory for MAP). This suspension was incubated for 54 d at 37 °C in an incubator shaker (70 rotations per min) in the presence of sterile glass beads. The bacterial suspension was thoroughly vortexed and diluted to an optical density of 0.316 ± 0.015, which resulted in a bacterial count of $3.36 \pm 0.14 * 10^5$ cfu ml^{-1}. Dilutions of 10^{-2}, 10^{-4} and 10^{-6} were then prepared from the original bacterial suspension. Next, 100 μl of the bacterial suspensions of each dilution were inoculated onto each of 18 slants of HEYM. The vials were sealed with silicone/teflon septa and incubated at 37 °C in a horizontal position for one week and then further in an upright position. Sampling (see section 2.2) was performed after two, four, and six weeks of incubation. Afterwards, all vials were kept in incubation for 11 weeks in total to be able to assess any growth of MAP.

Vials of each medium inoculated with 100 μl PBS instead of bacteria served as control.

Bacterial growth in all vials was visually assessed at regular intervals until the end of the study (11 weeks in total) as shown in figure 1, and was scored from 0.5 to 5 points.

2.2. Sampling protocol

The headspace above MAP cultures and pure media control slants was pre-concentrated by means of needle trap microextraction (NTME), as described by Bergmann *et al* [16] and Fischer *et al* [17]. The triple-bed needle trap devices (NTDs, Shinwa Ltd, Japan) were packed with divinylbenzene (DVB, 80/100 mesh, 1 cm), Carbopack X (60/80 mesh, 1 cm), and Carboxen 1000 (60/80 mesh, 1 cm). The NTDs were conditioned in a heating device (PAS Technology Deutschland GmbH, Magdala, Germany) at 250 °C for at least 12 h under permanent nitrogen flow (1.5 bar) before first use, and re-conditioned at 250 °C for 30 min before being applied for pre-concentration of the samples. All vials were warmed up in a dry block heating bath (Unitek, Germany) at 37 °C for 20 min immediately before sampling. Needles were pierced through the septum (figure 2) and 20 ml of headspace was conducted through the needle by inflating and releasing a 1 ml disposable syringe (Transcoject GmbH, Neumünster, Germany). Each NTD was sealed using a Teflon cap (Shinwa LTD, Japan/PAS Technology Deutschland GmbH, Magdala, Germany) before and immediately after collecting the sample.

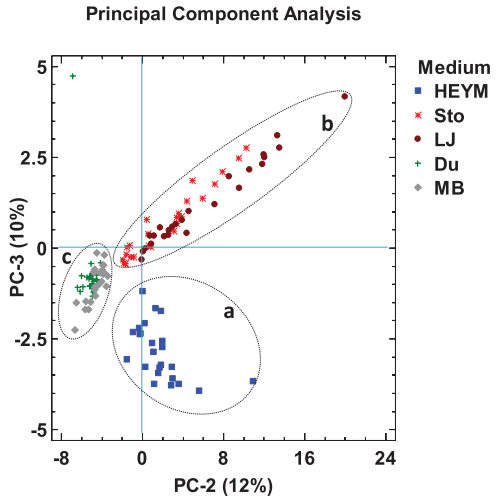


Figure 3. Effects of different culture media on VOC profiles emitted from *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cultures. Main ingredients of media were (a) egg yolk, (b) whole eggs, and (c) synthetic nutrients. HEYM—Herrold's Egg Yolk Medium; Sto—Stonebrink Medium; LJ—Löwenstein Jensen Medium; Du—Dubos Medium; MB—Middlebrook Medium. Illustration is based on a Principal Component Analysis (PCA).

2.3. Identification and quantification of substances

VOC analyses were performed by means of GC-MS. VOCs desorbed from NTDs were separated by GC (Agilent 7890A) and detected by mass selective detector (Agilent 5975C inert XL MSD). This principle has been described previously [10, 16, 18]. VOCs were initially identified via a mass spectral library search (NIST 2005 Gatesburg, PA, USA). Subsequent identification and quantification was established by analysis of pure reference substances (the origin of the chemicals can be found in table S1 (stacks.iop.org/JBR/10/037103/mmedia)) and comparison of GC retention times and mass spectra of all selected marker substances.

Using a liquid calibration unit (LCU, Ionicon Analytik GmbH, Innsbruck, Austria), humidified standards of pure references in different concentration levels were produced for NTME calibration. By measuring 10 blank samples and integrating signal areas of baseline for each marker, the substance limit of detection (LOD, signal-to-noise ratio 3:1) and limit of quantification (LOQ, signal-to-noise ratio 10:1) were calculated. VOC concentrations below LOD were set to zero. Table S2 provides confirmed substances through retention time, mass spectra and quantitative parameters such as LOD and LOQ.

2.4. Selection of VOCs

NTME GC-MS measurements resulted in more than 100 individual substances detected in the headspace of vials and quantified by analysis and calibration of pure reference substances (section 2.3). The total concentrations of the volatiles in the headspace of the vials were considered. To differentiate between VOCs originating from the material or the media, the ones arising or being consumed from MAP cultures, and the ones existing in the surrounding air of the laboratory, we compared results from above cultures, above pure media slants and the laboratory room air. VOCs serving as potential marker substances for MAP growth were selected according to the following criteria. (i) The VOC profile was defined after 6 weeks of cultural incubation, based on the assumption that the bacteria underwent exponential growth throughout this period. (ii) The concentration of VOCs above MAP, disregarding strains and bacterial count, needed to be significantly higher than in the surrounding laboratory room air and needed to differ significantly from VOC emissions above pure media.

2.5. Statistical analysis

All 18 control vials and 123 inoculated vials from study I along with 28 control vials and 71 inoculated vials from study II were included in statistical data analyses. IBM SPSS Statistics 19 (version 19.0, IBM Corporation, NY, USA), Microsoft Excel 2010 (Microsoft Corporation, USA) and STATGRAPHICS Centurion XVI.I (version 16.1.18, Statistical Graphics Corporation) were used. Numerical data are presented as medians and percentiles (25–75%). To identify significant differences between two groups of unpaired data, the Mann–Whitney *U*-test (exact test) was applied. Multifactorial analysis of variance (ANOVA) was used to identify significant influences of methodological effects (different bacterial counts, different duration of incubation) and biological factors (inter-strain variability) on concentrations of selected substances. Values of $p < 0.05$ were considered statistically significant. For visualization, a three-colour heatmap with normalized values was used. To evaluate

the influence of different culture media, a principal component analysis was applied and the scatterplot depicts variations in VOC emissions based on the primary ingredients of the media.

3. Results

3.1. Effects of culture media

Different culture media were associated with remarkable differences in VOC emissions (figure S1) and with significant differences in VOC concentrations.

Substances from all chemical classes were measurable above all five culture media. Some VOCs, like cyclohexane and 3-methylfuran, were emitted from all five culture media, but showed significantly different concentrations. Others were either highly concentrated above egg-containing media (figures 3 and S1) and minimally concentrated above synthetic media, like 3-methyl-1-butanol, or vice versa (e.g. benzene). In contrast, several VOCs were emitted in measurable amounts above some media yet showed concentration values below LOD above other media, for example 2-propylfuran and benzaldehyde. The ingredients of the culture media are listed in table S3. Figure 3 illustrates that the concentration and composition of VOC emissions depended on the ingredients of the media and that score two from the principal component analysis defined a VOC-profile linked to all egg-containing media. Eight VOCs showed significant differences between inoculated and control vials on HEYM, Sto, and LJ, and all inoculated vials had significantly higher or lower VOC concentrations than all individual control vials. The concentrations in MAP-inoculated slopes were lower for 1-propanol, benzaldehyde, and hexanal, and higher for 2-methyl-1-butanol, 3-methyl-1-butanol, 2-hydroxy-benzoate, methyl-acetate, and 3-pentanone.

3.2. VOCs related to MAP

According to the criteria for potential marker substances (section 2.4), 43 VOCs assessed above MAP cultures could be identified as potential biomarkers. These marker substances belonged to the classes of alcohols, aldehydes, esters, ketones, nitrogen compounds, aliphatic hydrocarbons, and aromatic hydrocarbons. Table 1 shows absolute concentrations of selected VOCs. Three alcohols (1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol), two esters (2-hydroxy-benzoate and methyl acetate), two ketones (2,3-butanedione and 3-octanone), and eight hydrocarbons (2,4-dimethyl-1-heptene, 2,4-dimethylheptane, 4-methyloctane, cyclohexane, ethylbenzene, heptane, octane, and styrene) were not detectable above control vials at all, but were highly concentrated above MAP cultures. In contrast, four VOCs (1-octen-3-ol, 2-methyl-2-butenal, heptanal, and 2-methyl-butanenitrile) had no detectable concentration above bacteria after six weeks of incubation, but showed significantly higher concentrations in control vials. Nine volatiles (1-octen-3-ol, 2-heptanone, all nitrogen containing compounds, and six aldehydes) were less concentrated above bacteria than in the headspace above pure media.

3.3. Homogeneity between different MAP strains

Multifactorial ANOVA (table 2) revealed no significant differences between the three MAP strains for 35 of the 43 VOCs, but eight volatiles showed significant differences between different MAP strains. This applied for the reference and the C-type field strain, which showed slightly lower values for some VOCs, for example 2,4-dimethylheptane (figure 4). The lower bacterial density tubes in particular presented higher variation in their VOC concentrations. Figure 4 also illustrates that VOCs above MAP showed significant differences in their concentration compared to control vials, regardless of strain.

3.4. Effects of initial inoculum and duration of incubation

Due to the different dilutions of the original bacterial suspension, an exponentially increasing bacterial count prevailed in inoculated vials which led to significantly different growth: the higher the original bacterial count, the better the bacterial growth. The original bacterial count ensured significant differences for 36 of the 43 VOCs, except for 2-methyl-1-butanol, ethanol, isopropyl-alcohol, 2-methyl-butanenitrile, 2,4-dimethylheptane, and styrene (table 2). The multifactorial ANOVA for the period of incubation resulted in significant concentration differences for 41 of the 43 VOCs, except 1-propanol and 2,4-methyl-1-heptene (table 2). After two weeks of incubation, visually apparent growth was already seen in vials with high bacterial count, while most vials with low bacterial count did not show apparent growth after four or even six weeks of incubation. After two weeks of incubation, VOC concentrations of different bacterial counts were all similar to each other (figure 5(a)), even though vials with high bacterial density showed visible bacterial growth. On the other hand, after four weeks of incubation there were several vials without visual bacterial growth, but significant emissions of 34 VOCs (figure 5(b)).

3.5. Patterns of VOC profiles related to bacterial density

After two weeks of incubation, VOC concentrations of MAP resembled control vials, while most VOC concentrations changed significantly after four weeks of incubation. Three different patterns of MAP-related VOCs were obvious (figure 6). (i) Concentrations of 34 VOCs above MAP were directly related to increasing bacterial density, either by initial inoculum or by bacterial growth over time. Some of these substances decreased after reaching a peak, for example 2-hydroxy

Table 1. Concentration ranges of selected VOC marker substances from all dilutions and all strains to each duration of incubation as well as concentration ranges of VOCs above pure media .

		VOC concnetrations (ppbV)														
		Duration of incubation														
		2 weeks					4 weeks					6 weeks				
		Control vials $n = 9$			MAP inoculated vials n=24		Control vials n=9			MAP inoculated vials n=24		Control vials n=10			MAP inoculated vials n=23	
	VOCs	Median	25%–75%		Median	25%–75%	Median	25%–75%		Median	25%–75%	Median	25%–75%		Median	25%–75%
Alcohols	1-Hexanol	0.00	0.00–0.00		0.00	0.00–0.00	0.00	0.00–1.07		0.00	0.00–17.28	*1.39*	0.97–1.78	*	*16.67*	8.91–26.87
	1-Octen-3-ol	**3.75**	2.95–4.35	*	**2.65**	1.26–3.39	**8.82**	6.12–31.66		**5.81**	0.00–26.17	**15.23**	13.82–17.52	*	**0.00**	0.00–0.00
	1-Propanol	*0.00*	0.00–0.00	*	*56.68*	41.93–66.69	*26.32*	24.73–82.39	*	*197.66*	171.38–257.25	*0.00*	0.00–0.00	*	*312.10*	233.53–365.94
	2-Methyl-1-butanol	0.00	0.00–0.00		0.00	0.00–2.16	*0.00*	0.00–0.00	*	*60.16*	0.00–285.71	*0.00*	0.00–0.00	*	*418.49*	171.10–884.00
	3-Methyl-1-butanol	*0.00*	0.00–0.00	*	*2.27*	0.00–10.75	*0.00*	0.00–0.00	*	*107.69*	24.35–731.12	*0.00*	0.00–0.00	*	*1111.23*	441.55–2128.30
	Ethanol	**151770.88**	108339.85–161897.25	*	**79421.14**	65296.64–90746.79	*130143.41*	118756.59–172184.93	*	*186880.40*	164514.54–204921.97	*106001.65*	97647.12–119652.75	*	*190886.27*	167780.50–222602.97
	Isopropyl alcohol	**2840.89**	2242.78–2933.06	*	**1316.80**	1151.10–1508.69	*2651.11*	2193.44–8384.94	*	*4866.77*	4405.05–5539.28	*2676.13*	2462.09–3042.22	*	*5160.92*	4348.04–5957.95
Aldehydes	2-Methyl-2-butenal	**11.42**	8.82–14.18		**10.28**	0.81–13.15	**15.16**	11.25–53.17		**0.00**	0.00–84.44	**20.38**	19.47–21.40	*	**0.00**	0.00–0.00
	2-Methylbutanal	**509.96**	436.91–762.27	*	**228.53**	145.50–336.23	**798.91**	634.12–880.36		**447.07**	313.42–1349.63	**950.35**	908.34–1008.42	*	**500.32**	247.50–822.20
	2-Methylpropanal	**989.57**	802.37–1284.71	*	**542.00**	173.59–623.19	*1298.88*	903.87–1637.97	*	*2133.89*	1783.07–2825.36	*1564.92*	1366.03–1693.56	*	*2305.64*	1951.82–2949.64
	3-Methylbutanal	**889.73**	828.34–1366.87	*	**491.83**	121.77–725.16	**1368.65**	1105.34–1512.17		**734.33**	412.52–2469.27	**1601.98**	1566.40–1705.13	*	**677.98**	331.05–955.59
	Benzaldehyde	**17.99**	13.64–21.78		**15.07**	7.23–19.17	**33.07**	22.64–77.27		**16.84**	5.56–102.21	**47.17**	43.77–51.91	*	**5.44**	4.76–7.88
	Heptanal	0.00	0.00–0.38		0.00	0.00–0.24	**0.64**	0.47–2.47		**0.53**	0.00–2.49	**1.12**	1.02–1.30	*	**0.00**	0.00–0.29
	Hexanal	**516.70**	442.44–700.70	*	**248.55**	151.90–536.58	**1035.60**	756.99–1613.05		**265.74**	4.39–1927.11	**1293.97**	1222.15–1385.23	*	**39.32**	26.87–47.74
Esters	2-Hydroxy-benzoate	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*0.87*	0.00–2.61	*0.00*	0.00–0.00	*	*1.25*	0.87–1.68
	Methyl acetate	0.00	0.00–0.00		0.00	0.00–12.20	*0.00*	0.00–0.00	*	*68.99*	0.00–278.83	*0.00*	0.00–0.00	*	*242.05*	50.99–324.99
Ketones	2,3-Butanedione	*0.00*	0.00–5.04		*1.43*	0.00–5.62	*0.00*	0.00–5.02	*	*19.39*	0.00–357.66	*0.00*	0.00–5.04	*	*444.40*	69.83–586.68
	2-Butanone	**109.34**	95.54–154.45	*	**85.65**	80.64–92.17	*185.02*	135.25–296.06	*	*495.82*	447.03–647.94	*237.79*	233.95–246.81	*	*791.22*	677.04–889.84
	2-Heptanone	**3.27**	2.97–4.46	*	**2.62**	2.12–3.02	*14.68*	9.35–43.22		*25.77*	7.49–45.91	**37.55**	34.35–43.23	*	**16.97**	13.50–19.73
	2-Pentanone	**2.57**	1.93–2.81	*	**0.90**	0.00–1.73	*4.30*	3.35–7.31	*	*22.58*	3.48–25.56	*9.09*	8.56–10.26	*	*37.53*	31.06–41.00
	3-Octanone	*0.00*	0.00–0.00	*	*6.95*	0.23–9.10	*0.00*	0.00–0.00	*	*38.20*	17.27–92.76	*0.00*	0.00–0.00	*	*28.22*	22.90–48.12
	3-Pentanone	**1.08**	0.00–1.30		**0.00**	0.00–2.29	*1.34*	1.16–2.22		*44.06*	0.00–90.86	*2.38*	1.79–2.87	*	*101.00*	67.95–108.60
	Acetone	**2234.01**	1552.06–2566.38	*	**1158.56**	1018.42–1286.24	*3214.39*	2676.12–5407.98	*	*5855.76*	5046.14–6917.00	*4019.93*	3747.24–4497.59	*	*10488.94*	9711.61–13308.25
NC	2-Methyl-butanenitrile	**6.82**	2.70–9.50	*	**2.81**	1.93–4.09	*9.97*	7.72–13.49		*14.05*	0.00–20.75	**11.59**	11.27–12.00	*	**0.00**	0.00–0.00

(*Continued*)

Table 1. (*Continued*)

		VOC concnetrations (ppbV)														
		Duration of incubation														
		2 weeks					4 weeks					6 weeks				
		Control vials $n = 9$			MAP inoculated vials n=24		Control vials n=9			MAP inoculated vials n=24		Control vials n=10			MAP inoculated vials n=23	
	VOCs	Median	25%–75%		Median	25%–75%	Median	25%–75%		Median	25%–75%	Median	25%–75%		Median	25%–75%
Aliphatic hydrocarbons	2,4-Dimethyl-1-heptene	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*6.04*	0.00–15.39	*0.00*	0.00–0.00	*	*12.30*	0.00–15.84
	2,4-Dimethylheptane	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*6.17*	0.00–8.05	*0.00*	0.00–0.00	*	*6.12*	0.00–6.90
	4-Methyloctane	*0.00*	0.00–1.20		*1.00*	0.00–1.33	*0.00*	0.00–2.87	*	*6.62*	5.69–8.62	*0.00*	0.00–2.40	*	*5.82*	4.97–6.71
	Cyclohexane	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*0.46*	0.38–0.53	*0.00*	0.00–0.00	*	*0.47*	0.41–0.57
	Heptane	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*19.91*	0.00–380.93	*0.00*	0.00–0.00	*	*582.50*	406.91–718.86
	Hexane	**376.45**	235.24–522.10	*	**201.96**	161.19–244.20	*347.25*	331.49–374.18	*	*1437.24*	1170.67–2292.20	*462.43*	408.05–507.96	*	*3476.58*	2792.51–4115.54
	Methylcyclopentane	**1.13**	0.77–1.41	*	**0.71**	0.58–0.81	*1.02*	0.93–1.43	*	*3.92*	3.44–4.63	*1.41*	1.29–1.57	*	*4.63*	3.58–5.23
	Octane	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*24.36*	0.00–191.63	*0.00*	0.00–0.00	*	*274.56*	197.44–359.03
	Pentane	**179.66**	147.42–404.36		**145.74**	87.52–199.31	*475.68*	316.86–991.95	*	*2583.12*	1357.96–20 265.37	*723.46*	661.23–854.86	*	*38 440.38*	13 094.37–49 128.56
Aromatic hydrocarbons	2,3,5-Trimethyl furan	**0.74**	0.69–1.16		**0.00**	0.00–1.11	*1.34*	1.08–2.70	*	*14.37*	11.45–16.67	*1.71*	1.44–1.91	*	*22.23*	19.61–24.02
	2,5-Dimethylfuran	0.00	0.00–0.00		0.00	0.00–6.13	*0.00*	0.00–38.81	*	*71.73*	59.73–77.65	*31.94*	27.83–35.01	*	*101.65*	76.83–115.47
	2-Ehtyl furan	**82.45**	73.65–123.85	*	62.39	50.88–69.42	*243.02*	191.52–444.01	*	*674.86*	555.56–729.66	*457.92*	409.97–494.00	*	*1053.07*	783.05–1256.56
	2-Pentylfuran	**5.44**	4.65–7.76	*	**0.00**	0.00–5.66	*21.84*	15.01–81.39	*	*89.69*	81.57–96.03	*66.09*	58.59–69.86	*	*141.16*	78.77–167.73
	2-Propyl furan	0.00	0.00–0.89	*	0.00	0.00–0.00	*1.98*	1.57–7.46	*	*9.42*	7.76–10.37	*5.78*	5.34–6.40	*	*15.50*	7.16–20.83
	3-Methylfuran	*8.23*	5.99–12.25	*	*81.65*	67.47–98.50	*14.52*	10.06–42.52	*	*608.78*	363.37–702.79	*26.83*	23.38–28.73	*	*673.71*	363.70–899.94
	Furan	**239.66**	168.17–319.91	*	**44.77**	37.71–56.79	*194.94*	141.41–245.70		*234.10*	170.89–291.59	*221.10*	179.77–251.01	*	*411.76*	283.54–495.78
	Benzene	**30.64**	24.52–44.05		**25.04**	21.43–30.62	*49.75*	35.32–131.62	*	*203.18*	186.52–226.85	*70.45*	66.04–75.71	*	*310.80*	274.78–368.08
	Ethylbenzene	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*0.30*	0.27–0.38	*0.00*	0.00–0.00	*	*0.29*	0.24–0.32
	Styrene	0.00	0.00–0.00		0.00	0.00–0.00	*0.00*	0.00–0.00	*	*2.81*	2.61–3.10	*0.00*	0.00–0.00	*	*2.45*	2.04–2.69

VOC—volatile organic compound; *n*—number of slants; ppbV—parts per billion by volume; ∗—indicates significant differences (p-value < 0.05) between inoculated and control vials; NC—nitrogen compound. Italic: MAP > control vials. Bold: MAP < control vials.

Table 2. Effects of inter-strain variability, original bacterial count, and duration of incubation on emitted VOC concentrations above MAP cultures (*p*-values; multifactorial ANOVA).

Chemical class	VOC	Strain	Original bacterial count	Period of incubation
Alcohols	1-Hexanol	n.s.	<0.001	<0.001
	1-Octen-3-ol	n.s.	<0.001	<0.01
	1-Propanol	n.s.	n.s.	n.s.
	2-Methyl-1-butanol	n.s.	<0.001	<0.001
	3-Methyl-1-butanol	n.s.	<0.001	<0.001
	Ethanol	<0.05	n.s.	<0.001
	Isopropyl alcohol	<0.01	n.s.	<0.001
Aldehydes	2-Methyl-2-butenal	n.s.	<0.001	<0.001
	2-Methylbutanal	n.s.	<0.001	<0.001
	2-Methylpropanal	n.s.	n.s.	<0.001
	3-Methylbutanal	n.s.	<0.001	<0.001
	Benzaldehyde	n.s.	<0.001	<0.001
	Heptanal	n.s.	<0.001	<0.001
	Hexanal	n.s.	<0.001	<0.05
Esters	2-Hydroxy-benzoate	n.s.	<0.001	<0.001
	Methyl acetate	n.s.	<0.01	<0.001
Ketones	2,3-Butanedione	n.s.	<0.001	<0.001
	2-Butanone	n.s.	<0.001	<0.001
	2-Heptanone	n.s.	<0.001	<0.001
	2-Pentanone	n.s.	<0.001	<0.001
	3-Octanone	n.s.	<0.01	<0.001
	3-Pentanone	n.s.	<0.001	<0.001
	Acetone	<0.01	<0.01	<0.001
Nitrogen compounds	2-Methyl-butanenitrile	n.s.	n.s.	<0.05
Aliphatic hydrocarbons	2,4-Dimethyl-1-heptene	n.s.	<0.05	n.s.
	2,4-Dimethylheptane	n.s.	n.s.	<0.01
	4-Methyloctane	n.s.	<0.001	<0.001
	Cyclohexane	n.s.	<0.001	<0.001
	Heptane	n.s.	<0.001	<0.001
	Hexane	n.s.	<0.001	<0.001
	Methylcyclopentane	<0.05	<0.001	<0.001
	Octane	n.s.	<0.001	<0.001
	Pentane	n.s.	<0.001	<0.001
Aromatic hydrocarbons	2,3,5-Trimethylfuran	<0.05	<0.001	<0.001
	2,5-Dimethylfuran	n.s.	<0.05	<0.001
	2-Ehtylfuran	n.s.	<0.01	<0.001
	2-Pentylfuran	n.s.	<0.001	<0.001
	2-Propylfuran	n.s.	<0.001	<0.001
	3-Methylfuran	n.s.	<0.001	<0.001
	Furan	<0.001	<0.05	<0.001
	Benzene	<0.05	<0.01	<0.001
	Ethylbenzene	n.s.	<0.05	<0.001
	Styrene	<0.001	n.s.	<0.001

VOC—volatile organic compound; n.s.—not significant (*p*-value $\geqslant 0.05$); strain—*A* reference strain from cattle, *B* field strain isolated from sheep, *C* field strain isolated from cattle; original bacterial count—original suspension $3.36 \pm 0.14 * 10^5$ cfu ml^{-1} and dilutions of 10^{-2}, 10^{-4}, and 10^{-6}; duration of incubation—two, four or six weeks.

benzoate, and hexane (figure 6(a)). (ii) Other VOCs kept increasing without reaching a peak, for example 2-methyl-1-butanol and 2-butanone (figure 6(b)). (iii) In contrast, concentrations of nine VOCs decreased significantly with increasing bacterial density up to a concentration lower than LOD (figure 6(c)). A remarkable pattern was shown by 2-heptanone, which presented decreasing concentrations with increasing

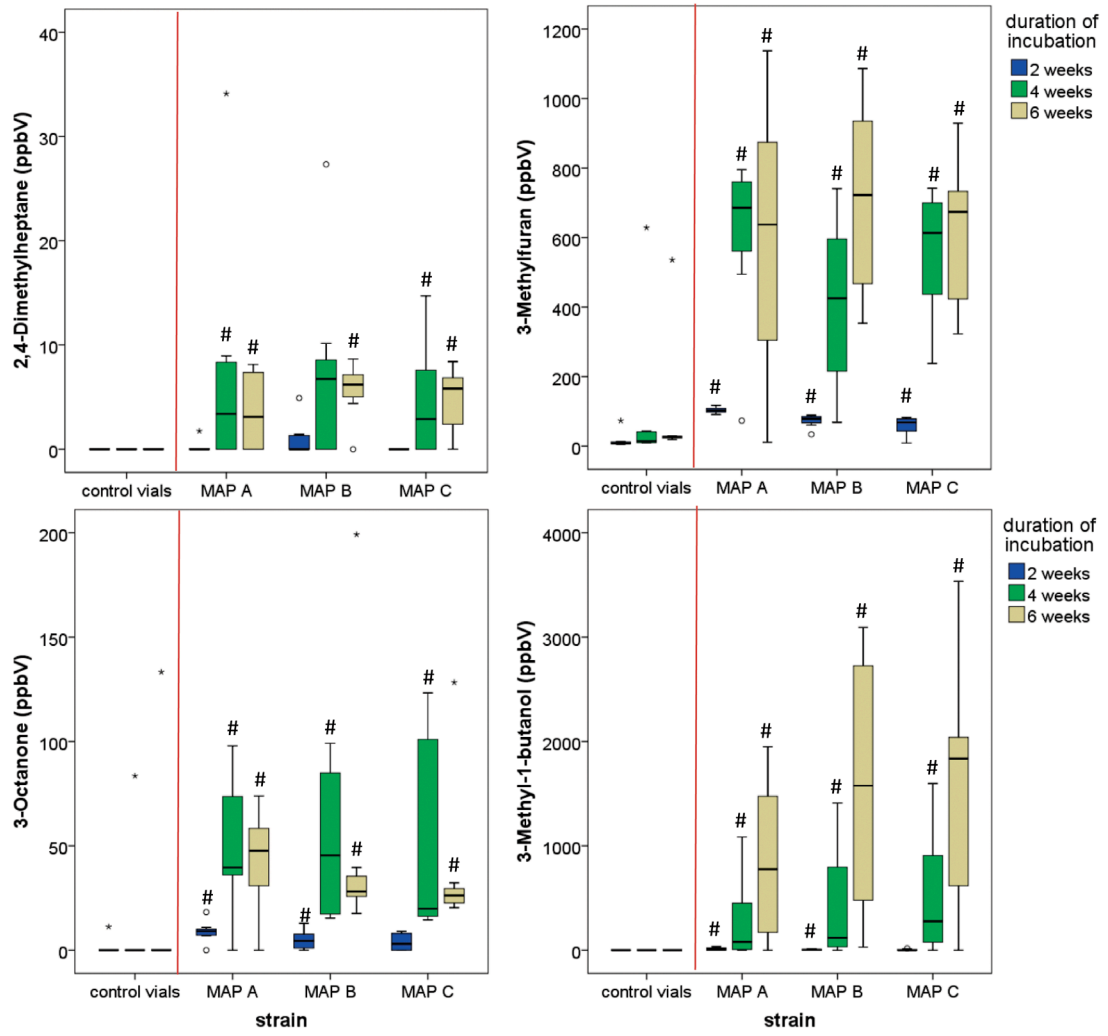


Figure 4. Effects of inter-strain variability on concentrations of emitted VOCs. ppbV—parts per billion by volume; #—significant difference in concentrations between inoculated slants and corresponding control vials (p-value < 0.05); MAP—*Mycobacterium avium* ssp. *paratuberculosis*; A—reference strain (bovine origin); B—field strain isolated from sheep; C—field strain isolated from cattle; °—values are 1,5-3 interquartile ranges (IQR) away from median; ∗—values are beyond 3 IQR.

initial bacterial count, but increasing concentrations within the dilution with increasing duration of incubation. Also, 2-heptanone and six aldehydes (except 2-methylpropanal) represented a special instance of this pattern. VOC concentrations were significantly higher in vials with low bacterial density than controls and decreased further with increasing bacterial density to lower values than controls (figure 6(c), 2-methyl-2-butenal, figure 5(b)).

Remarkable time-dependent changes in the VOC concentrations in the headspace above control slants were also noted. With increasing duration of incubation, all VOCs presenting concentrations higher than LOD increased (figures 5(b) and 6).

4. Discussion

4.1. Metabolism of MAP

As the bacterial density of MAP increased, either by initial inoculum or by bacterial growth over time, the concentrations of the volatiles emitted could be assigned to three different patterns (figure 6). We speculate that VOCs with decreasing concentrations originated from the media and were consumed by replicating MAP. Volatiles with increasing concentrations most likely originated from MAP and could be intermediates or metabolites of several pathways or could function as signaling substances. VOCs which reached a peak had similar origin but either the metabolic pathways stopped due to reduced replication, or synthesis and degradation of substances leveled out, because other metabolic pathways took over. Metabolic regulation always depends on ambient conditions such as available surface area on the medium, nutrient supply, competitors and messenger substances. High levels of emitted aldehydes above vials without visible bacterial growth pointed to the importance of these substances in metabolism of MAP. These could be messenger substances or metabolites in the signaling system for environmental adaptation, comparable with proteins responding to stress like heat, hypoxia or nutrient starvation [19, 20].

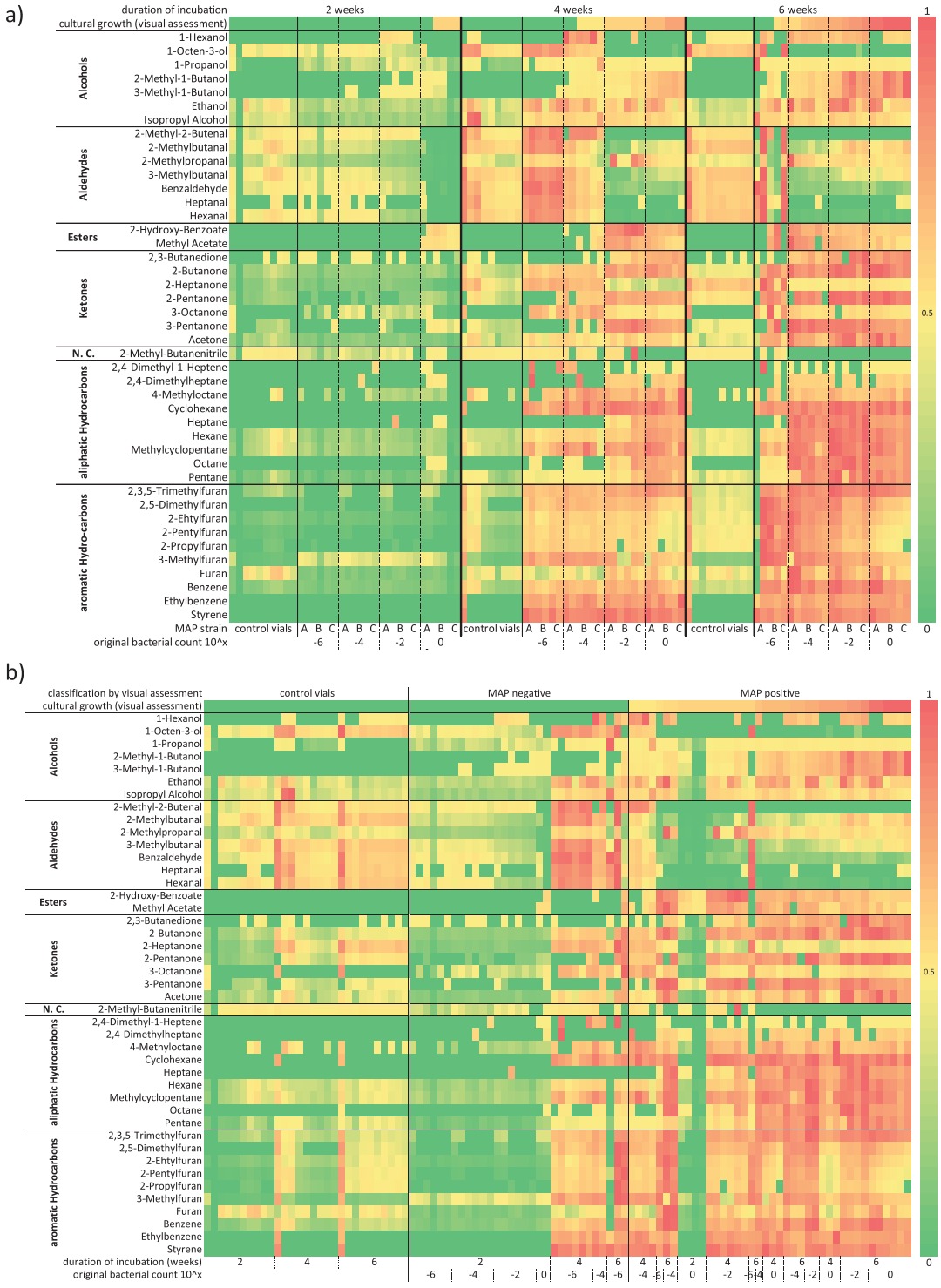


Figure 5. Effects of kinetics illustrated in a heatmap with normalized data of all selected VOCs from *Mycobacterium avium* ssp. *paratuberculosis* (MAP) cultures on Herrold's Egg Yolk Medium as well as from pure media slants pre-concentrated after two, four, and six weeks. Arranged by (a) duration of incubation and (b) visually assessed bacterial growth at time point of sampling; N.C.—nitrogen containing compounds.

The literature contains some studies examining parts of metabolic pathways of *Mycobacteria*. The cell wall of *Mycobacteria* presents many specific molecules. As described by Dhimann *et al* and Appelmelk *et al* [21, 22], lipoarabinomannan (LAM) suppresses the host's macrophage functions. The core of the molecule is linked to α-arabinofuranan and D-galactofuran, and is mainly built during the logarithmic phase of bacterial growth. Furans can be generated during LAM synthesis. Mycolic acids, a formation of long-chain fatty acids, ketones and alcohols [23], are a special feature of the cell wall of *Mycobacteria*. During

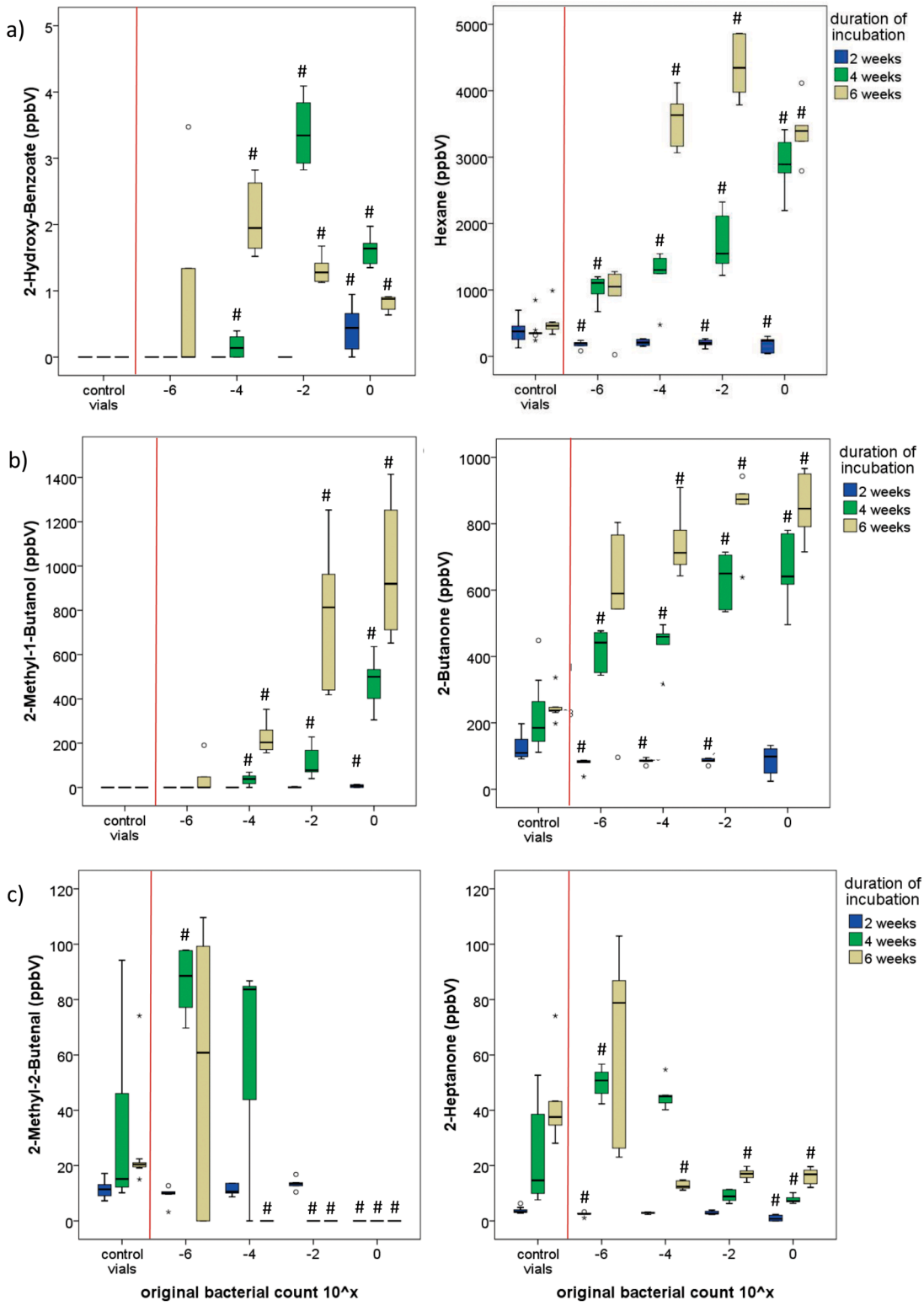


Figure 6. Effects of bacterial density (by original bacterial count and duration of incubation) on concentration of emitted VOCs. ppbV—parts per billion by volume; #—significant difference (p-value $< 0{,}05$) in concentration between inoculated slants and corresponding control vials; °—values are 1,5—3 interquartile ranges (IQR) away from median; ∗—values are beyond 3 IQR.

synthesis of mycolic acids, esters [24, 25] may also be generated. Due to the structure of mycolic acids, branched hydrocarbons may be generated via cleavage. However, the origin of VOCs is mostly unknown and is subject to speculation.

As described by many authors, *Mycobacteria* must adapt metabolically to *in vitro* conditions [26]. Russell *et al* [27] described primary carbon source changes from host-derived lipids *in vivo* to glucose and glycerol *in vitro*. This may explain differences in VOC emissions above different media. Future studies should focus on elucidating the origin of VOCs in metabolic pathways and the effects of different culture conditions.

4.2. VOCs indicating bacterial growth of MAP

For diagnostic purposes, VOCs above MAP need to differ significantly from VOCs above pure media slants. More than 100 different substances were detectable over MAP cultures. After applying the selection criteria, only 43 VOCs remained more concentrated than the surrounding laboratory room air and those differing significantly between inoculated and pure media slants. The concentration above MAP strains could be either higher or lower than those above control slants. Overall, we included 43 volatiles in the VOC profile. Our results agree with those from an earlier study by Trefz *et al* [10]. Both projects resulted in about 40 volatiles belonging to the same chemical classes. About half of these were included in both VOC profiles and showed the same concentration pattern. The variation could be due to numerically larger experimental groups or different culture media and incubation duration. *In vitro* VOC profiles of *M. tuberculosis* described by Syhre *et al* [28] differed from those defined in this project for MAP. To confirm the suitability of VOC analysis for diagnostic applications, more detailed studies comparing many other *Mycobacterium* species are necessary.

4.3. Importance as a diagnostic approach

To display a diagnostic benefit, VOCs need to be independent of biological (inter-strain variability) and methodological factors (culture media, bacterial count, duration of incubation).

4.3.1. Biological factors

For most selected VOCs, we were unable to find significant inter-strain variability between MAP type-II (strain A and C) and type-I/III (strain B). However, the VOC concentrations of the strains originating from cattle (A, C figure 4) seemed to reach slightly lower values than those isolated from sheep (B). This could simply be a sign of weaker growth, which could be partially verified by visual assessment of the slants. On the other hand, strains A and C presented similar VOC concentrations, especially at low bacterial density. A larger number of isolates needs to be investigated to clarify whether the distinct genotypic differences between type-II and type I/III [29, 30] contribute to significant differences in VOC profiles.

4.3.2. Methodological factors

To achieve good sensitivity of bacterial cultivation [5], a few different *culture media* adapted especially to MAP can be used. It is therefore necessary to define a VOC profile which is valid for all these media. Our results suggest this is only possible for egg-containing media or media with mainly synthetic ingredients.

Compared to control slants, the results of this project showed that VOCs were released in detectable and distinguishable amounts after a period of adapting to ambient conditions, regardless of the initially inoculated *bacterial count*. Those used in this project represented bacterial amounts in fecal samples collected from cattle for diagnostic reasons. Animals with about 10^4 colony forming units (cfu) of MAP per gram feces are considered super shedders [31, 32]. This complies with the original undiluted bacterial suspension (10^0) in this project. Hence, the dilutions 10^{-2}, 10^{-4}, and 10^{-6} represent bacterial amounts in samples of low shedding animals without symptoms. Thirty-four of 43 VOCs showed significant emissions above inoculated slants without apparent bacterial growth. VOCs which were not detectable over pure media slants at all require special attention. These 15 VOCs (three alcohols, two esters, two ketones, and eight hydrocarbons) are particularly promising for possible diagnostic use. These volatiles were not emitted from any medium, but were generated by all MAP strains.

Unfortunately, most of these VOCs seem to be emitted only after a particular *incubation period*. Thirteen VOCs displayed significant differences in concentration for all dilution stages after four weeks of incubation. Significant concentration differences compared to control vials, which were independent of bacterial density, are also particularly promising for possible diagnostic use. 1-propanol and 3-methylfuran already showed reliable concentration differences at all dilution stages after just two weeks of incubation. However, at this early stage VOC emissions were low and unstable. Some vials displayed visually detectable growth but no VOC emissions. This could be due to the persistence of MAP while adapting to the medium and ambient conditions, which can lead to reduced metabolism. On the other hand, the discrepancy between visually apparent growth and lack of detectable VOCs indicates a need to adjust the methodology to increase sensitivity by decreasing LOD and LOQ.

5. Conclusions

This study revealed a MAP-related VOC profile that included 43 volatiles. The composition of VOC emissions changed with increasing bacterial density, and therefore displayed different stages of growth and different stages of metabolism. Since each VOC presented unique courses of concentration, a fixed profile is unsuitable for future diagnostic purposes. Instead, a more flexible or floating system which considers different VOC patterns is needed. From a diagnostic perspective, inter-strain variability and initial inoculum are less important or even negligible. In contrast, methodological factors, such as duration of incubation and ingredients of the culture media need to be standardized very carefully because they significantly affect the VOC profile. Hence, different ambient conditions are causing different stages of metabolism and growth of bacteria as well as different VOC emissions. This is important information for future developments towards *in vitro* testing of bacterial growth.

The results also stress the importance of in-depth knowledge about influences on VOC composition

before defining reliable and accurate marker sets for diagnostic purposes for any bacteria.

How conclusive the results are for other *Mycobacteria* than MAP or for MAP infections *in vivo*, respectively, remains to be elucidated in future research.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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Publikation 3

PLOS ONE

RESEARCH ARTICLE

Comparative analysis of volatile organic compounds for the classification and identification of mycobacterial species

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Abstract

Background

Species of Mycobacteriaceae cause serious zoonotic diseases in mammals, for example tuberculosis in humans, dogs, parrots, and elephants (caused by *Mycobacterium tuberculosis*) and in ruminants and humans (caused by *M. bovis* and *M. caprae*). Pulmonary diseases, lymphadenitis, skin diseases, and disseminated diseases can be caused by non-tuberculous mycobacteria (NTM). Diagnosis and differentiation among *Mycobacterium* species are currently done by culture isolation. The established diagnostic protocols comprise several steps that allow species identification. Detecting volatile organic compounds (VOCs) above bacterial cultures is a promising approach towards accelerating species identification via culture isolation. The aims of this project were to analyse VOCs in the headspace above 13 different species of mycobacteria, to define VOC profiles that are unique for each species, and to compile a set of substances that indicate the presence of growing mycobacteria in general.

Materials & methods

VOCs were measured in the headspace above 17 different mycobacterial strains, all cultivated on Herrold's Egg Yolk Medium and above pure media slants that served as controls. For pre-concentration of VOCs, needle-trap micro-extraction was employed. Samples were subsequently analysed using gas chromatography-mass spectrometry. All volatiles were identified and calibrated by analysing pure reference substances.

Results

More than 130 VOCs were detected in headspace above mycobacteria-inoculated and control slants. Results confirmed significant VOC emissions above all mycobacterial species that had grown well. Concentration changes were measurable in vials with visually assessed bacterial growth and vials without apparent growth. VOCs above mycobacterial cultures could be grouped into substances that were either higher or equally concentrated,

lower or equally concentrated, or both as those above control slants. Hence, we were able to identify 17 substances as potential biomarkers of the presence of growing mycobacteria in general.

Conclusions

This study revealed species-specific VOC profiles for eleven species of mycobacteria that showed visually apparent bacterial growth at the time point of analysis.

Introduction

About 150 species belong to the family of Mycobacteriaceae. Some members of the *Mycobacterium tuberculosis* complex (MTC) may cause serious zoonotic diseases in mammals, for example *Mycobacterium tuberculosis* causes tuberculosis in humans, dogs, cats, parrots, and elephants [1–5]. Domestic and wild ruminants and swine serve as vectors for *M*. *tuberculosis* and demonstrate asymptomatic infections [6,7]. Tuberculosis in cattle, sheep, and goats is mainly caused by *M*. *bovis* (MB) and *M*. *caprae* [8], but can be transferred to humans, too. With increasing importance, a high number of non-tuberculous mycobacteria (NTM) can cause pulmonary diseases (which resemble tuberculosis), lymphadenitis, skin diseases, and disseminated diseases [9–13]. NTM cover the *M*. *avium* complex (MAC)—which includes *M*. *avium* ssp. *avium* (MAA), *M*. *avium* ssp. *hominissuis* (MAH), *M*. *avium* ssp. *paratuberculosis* (MAP), and *M*. *intracellulare* (MI)—and other NTMs.

The different species of mycobacteria target different, frequently varying hosts. Colonisation often leads to asymptomatic infections, but can also result in clinical disease. Due to their unique cell wall, which consists predominantly of mycolic acids, mycobacteria have a high tenacity. Some species are obligate parasites, while others are found in the environment. So far, little is known about the prevalence of NTM in livestock herds apart from MAP [14]. Paratuberculosis or Johne's disease is caused by MAP and leads to granulomatous enteritis in ruminants [15,16]. This disease is characterised by intermittently emerging diarrhoea and weight loss. Its enormous economic importance is due to reduced slaughter weight and increased susceptibility to other diseases in infected animals [17]. Paratuberculosis also adversely affects animal's reproduction [18] and milk yield [19–21]. Because of the high tenacity of MAP in the environment [22] and the incidence in raw milk [23,24], it has frequently been discussed as a pathogen with zoonotic potential [15,25]. There are a few case reports of patients with a suppressed immune system, by human immunodeficiency virus [26] or inflammatory bowel disease [27], who have been tested positive for MAP.

The most sensitive diagnostic method currently available is direct detection of the bacteria via cultural isolation from faeces or tissue samples [28]. Due to the long generation time and high requirements for the media [29], cultivation on solid media is very labour-intensive and time-consuming taking several weeks. After direct detection of bacteria, identifying the species is mandatory [30], for example via polymerase chain reaction (PCR) [31]. Thus far, alternative diagnostic procedures have not proved sufficiently sensitive [32,33], and the labour-intensive procedure cannot be reduced from a two-step to a one-step method [28]. There is an urgent need for an accelerated, sensitive, and specific diagnostic approach.

A potential approach to improve and accelerate the detection of growing bacteria could be the analysis of volatile organic compounds (VOC) released by bacterial cultures [34]. Volatiles are not only emitted from anthropogenic sources, but also from every living cell [35]. With

regard to bacterial culture, VOCs can provide information about the presence of growing bacteria and may help to differentiate between bacterial families or even species. This has been shown in a number of studies using different analytical methods: for example, highly selective gas chromatography-mass spectrometry (GC-MS) [36–38] and proton transfer reaction time-of-flight mass spectrometry [39–41] or the less selective simpler techniques such as multi-capillary column–ion mobility spectrometry [42] and differential ion mobility spectrometry [43]. Although these studies have suggested that identification of bacteria and bacterial growth by means of VOC analysis may become feasible [44], only a few studies have targeted VOC profiles from above different species of the same bacterial family, e.g. for Mycobacteriaceae [45–47]. In addition, there are only a few studies that have considered the dependence of VOC profiles on cultivation protocols and conditions [40,48,49].

A lack of knowledge still exists with regard to species-dependent formations of VOCs during bacterial growth of a variety of tuberculous and NTM under standardised conditions of propagation, inoculation, and incubation, as analysed by means of GC-MS.

Therefore, the aims of this study were (i) to prove the presence of growing bacteria of 13 different mycobacterial species by means of VOCs, (ii) to define a core VOC profile for the genus *Mycobacterium*, and (iii) to discriminate mycobacterial species from each other by their VOC profile.

Material and methods

Ethics statement

Statements of animal research ethics committees were not required because this study did not include any animal experiment, anaesthesia or necropsy.

Reference strains of bacteria were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany). Field strains originated from local laboratories for veterinary diagnostic where they had been isolated and cultured before from tissues or faeces of animals in conformity with routine herd diagnostics or animal disease surveillance. All strains were further cultivated according to standard protocols recommended by the National Reference Laboratory for Paratuberculosis.

Study design

Thirteen species were included in this project (Table 1). All 13 species were cultivated on commercial Herrold's Egg Yolk Medium (HEYM) containing mycobactin J, amphotericin, nalidixic acid, and vancomycin (Becton Dickinson, Sparks, USA). In total, 140 inoculated vials and 23 control vials were included in this study.

The isolates were maintained on solid media according to their cultural demands. To prepare the inoculum, three loops of cultured bacteria were added to 10 mL Middlebrook 7H9 liquid medium containing oleic acid, albumin, dextrose, catalase, polymyxin B, amphotericin B, carbenicillin, and trimethoprim (MB-bouillon, produced according to accredited instructions of the National Reference Laboratory for Paratuberculosis). These suspensions were incubated for 7 days at 37˚C in an incubator shaker (70 rotations per min) in the presence of sterile glass beads, except one flask containing *M*. *marinum* that was incubated at 30˚C (MM30). The bacterial suspensions were thoroughly vortexed and diluted with MB-Bouillon to an optical density of 0.306 ± 0.02. Subsequently, nine replicates per strain were generated by inoculating 100 μl of the bacterial suspension onto each of nine HEYM slants. The vials were sealed with Silicone/Teflon septa and incubated at the appropriate temperature (see Table 1), in a horizontal position for one week and then further in an upright position. Sampling was performed after two weeks of incubation for fast-growing, three weeks for intermediate-growing, and

Table 1. Study design and included species.

Abbreviation	*Mycobacterium* species / strain	Strain designation	Origin	Inoculum (cfu)	n	Duration of incubation (weeks)
MB	*M. bovis*	43990	DSMZ GmbH	4.85E+08	3	4
MAP	*M. avium* ssp. *paratuberculosis*	44133	DSMZ GmbH	6.00E+01	3	4
		04A0386	field isolate from sheep faeces	6.55E+02	3	4
MAA	*M. avium* ssp. *avium*	44156	DSMZ GmbH	1.55E+08	8	2
		03A2754	field isolate from cattle faeces	3.77E+07	9	2
MAH	*M. avium* ssp. *hominissuis*	09MA1289	field isolate from swine lymphnode	3.78E+08	9	2
		00A0799	field isolate from cattle lymphnode	2.95E+08	9	2
MI	*M. intracellulare*	43223	DSMZ GmbH	4.00E+06	9	2
		11MA1917	field isolate from lung tissue of rainbow lorikeet	3.30E+06	9	2
MT	*M. terrae*	43292	DSMZ GmbH	8.95E+07	9	3
MM37	*M. marinum*	44344	DSMZ GmbH	9.10E+06	9	3 37˚C
MM30				9.05E+06	9	3 30˚C
MK	*M. kansasii*	43224	DSMZ GmbH	8.65E+06	9	4
MC	*M. chelonae*	43804	DSMZ GmbH	8.80E+04	6	2
MD	*M. diernhoferi*	43524	DSMZ GmbH	8.55E+07	9	2
MF	*M. fortuitum*	46621	DSMZ GmbH	7.55E+07	9	2
MP	*M. phlei*	43239	DSMZ GmbH	1.10E+07	9	2
MS	*M. smegmatis*	43756	DSMZ GmbH	1.28E+07	9	2

cfu–colony forming units; DSMZ GmbH- German Collection of Microorganisms and Cell Cultures, Braunschweig; n–number of replicates.

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four weeks for slow-growing mycobacteria (Table 1). MM30 was incubated at 30˚C due to its adaptation to fish and reptiles [50] and also at 37˚C to be able to compare results to the other species. Vials inoculated with 100 μl MB-Bouillon instead of the bacterial suspension served as controls and were also incubated for two, three, and four weeks. This enabled analysis of and correction for media-derived VOCs.

Bacterial growth was visually assessed at regular intervals until the time of analysis, when it was scored as follows:

0.5 points less than 20 colonies apparent on the slant
1 point between 20 and 50 colonies apparent on the slant
2 points between 51 and 100 colonies apparent on the slant
3 points over 100 colonies apparent on the slant or a thin layer of growth
4 points a loosened layer of comprehensive growth is apparent on the slant
5 points a concluded layer of comprehensive growth is apparent on the slant

Sampling protocol for VOC analysis

Pre-concentration of VOCs from the headspace above the inoculated slants and the pure media control slants were carried out by means of needle trap micro-extraction (NTME), as described by Trefz et al. 2013 [51]. The triple-bed needle trap devices (NTDs, Shinwa Ltd., Japan) were packed with divinylbenzene (DVB, 80/100 mesh, 1cm), Carbopack X (60/80 mesh, 1 cm), and Carboxen 1000 (60/80 mesh, 1 cm). Before first use NTDs were conditioned in a heating device (PAS Technology Deutschland GmbH, Magdala, Germany) at 250˚C for at least 12 h under permanent helium flow (1.5 bar), and re-conditioned at 250˚C for 30 min

before being applied for pre-concentration of the samples. Immediately before sampling all vials were warmed up in a heating block at 37˚C (Unitek, Germany) for 20 min. Needles were pierced through the septum, and 20 ml of headspace was bi-directionally passed through the needle by inflating and releasing a 1 ml disposable syringe (Transcoject GmbH, Neumünster, Germany). Each NTD was sealed using a Teflon cap (Shinwa LTD., Japan/PAS Technology Deutschland GmbH, Magdala, Germany) before and immediately after collecting a gaseous sample. In parallel to this procedure, further NTDs were exposed to laboratory room air (n = 10) to be able to estimate unwanted contaminations of the pre-concentration devices during routine handling.

Identification and quantification of substances

VOC analyses were performed by means of GC-MS. VOCs that thermally desorbed from NTDs were separated by gas chromatography (Agilent 7890A) and detected by mass spectrometry (Agilent 5975C inert XL MSD). For all experiments, a RTX-624 (60 m; 0.32 mm; 1.8 μm film thickness) capillary column from Restek (Bad Soden, Germany) was used. Inlet temperature for desorption was 250˚C and the column temperature program for separation worked as follows—40˚C for 5 min, 8 K/min to 120˚C for 2 min, 10 K/min to 220˚C, 20 K/min to 250˚C for 4 min. Electron ionisation (70 eV at 250˚C) and total ion chromatogram measurements (scan range, 35–250 amu) were applied for all samples. This process has been previously described [51,52]. VOCs were initially identified by a mass spectral library search (NIST 2005 Gatesburg, PA, USA). Analysis of pure reference substances (origin of chemicals in S1 Table) and comparison of GC retention times and mass of all selected marker substances specified subsequent identification and quantification.

For NTME calibration, a liquid calibration unit (LCU, Ionicon Analytik GmbH, Innsbruck, Austria) provided humidified standards of pure references in different concentration levels. The signal-to-noise ratio was used to calculate the substance limit of detection (LOD, signal-to-noise ratio 3:1) and limit of quantification (LOQ, signal-to-noise ratio 10:1). Noise was determined from blank samples (n = 10). VOC concentrations under LOD were set to zero. Supplement S2 Table provides methodological details of identified substances (retention time and quantitative parameters, such as LOD and LOQ).

Selection of VOCs

NTME GC-MS analysis resulted in more than 130 individual volatile substances detected in the headspace of vials and quantified by analysis and calibration of pure reference substances (section 2.3). Values represent the concentrations of the volatile compounds in the headspace of the vials. We compared the VOC concentrations above control slants, above mycobacterial cultures, and from the laboratory room air. This was done in order to differentiate between VOCs originating from the material or the media, those arising from or being consumed by bacterial cultures, and those existing in the surrounding air of the laboratory. The inclusion criterion for a volatile was that its concentration was above that of the surrounding laboratory room air. VOCs that had a higher concentration in the laboratory air or had a high variability above the control slants were excluded.

Statistical analysis

Concentration values of selected VOCs of all 23 control vials and 140 inoculated vials were included in the statistical data analyses. R x64 (version 3.3.1, R Development Core Team, New Zealand) in conjunction with R studio (version 0.99.903, R-Tools Technology Inc., Canada) and Microsoft Excel 2016 (Microsoft Corporation, USA) were used. Numerical data are

presented as medians and percentiles (25–75%). To identify significant differences between groups of data, the Kruskal-Wallis Test followed by the Tukey HSD-test was applied. The Mann-Whitney-U-Test was employed to identify VOCs with significant inter-strain variability in concentration. Values with $p < 0.05$ were considered statistically significant. For visualisation, a three-coloured heat map with normalised values was prepared. A principal component analysis (PCA) was used to convert possible correlated variables into components with the objective of visualising those components and aligning them with different qualities of the samples: classification of mycobacteria, the colony forming units (cfu) of the inoculum, visually assessed bacterial growth, and duration of incubation.

Results

Detecting the presence of growing bacteria via VOCs

The VOC composition in the headspace above slants clearly distinguished between control vials and vials with bacterial growth (Fig 1).

Four species of mycobacteria (i.e. MAP, MB, MT, MM30, and MM37), formed well-defined clouds in a PCA (Fig 1), which separated them from all other species. The clusters of MS, MI, and MD overlapped, as did MF, MAA, MAH, MP (abbreviations in Table 1). The same visualisation with a PCA, but grouped via different qualities of the samples shows that the clusters do not resemble colony forming units (cfu) of the inoculum, bacterial growth or duration of incubation. An exception was the VOC compositions above inoculated slants with poor bacterial growth, i.e. MK and MC (Fig 1), because these vials presented themselves in the same cluster as the control vials. Hence, they were excluded from further investigations regarding species-

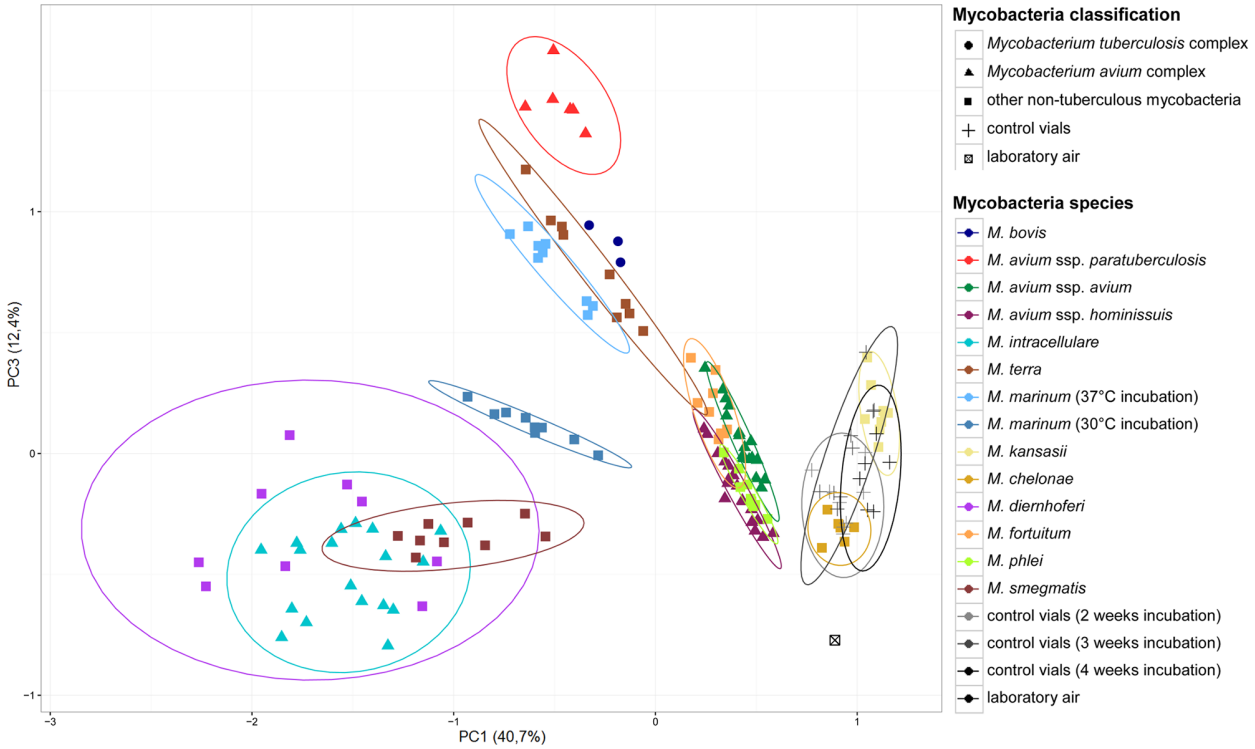

Fig 1. Differentiation of mycobacterial species by VOC profile. Illustration is based on a principal component analysis (PCA).

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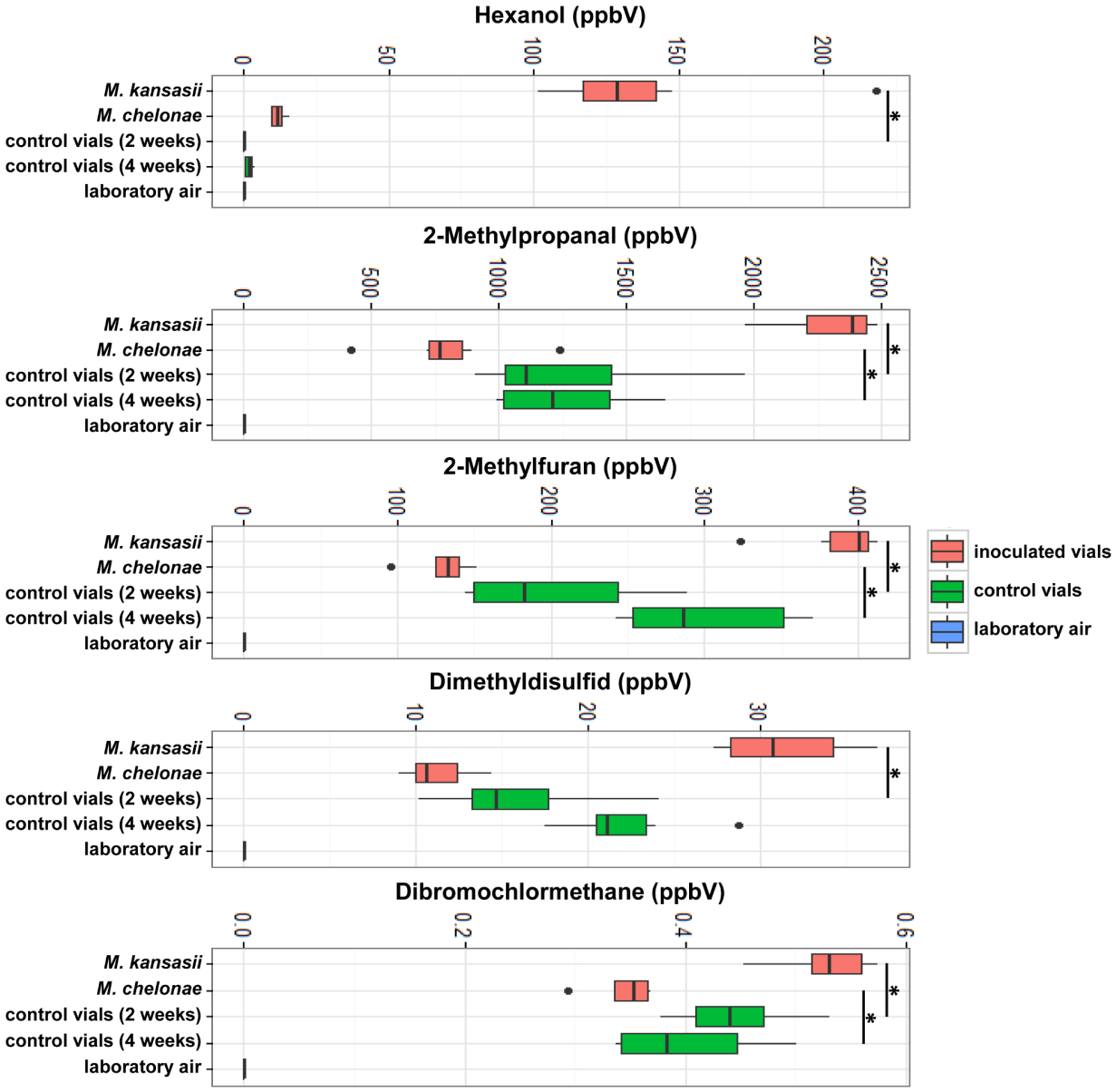

Fig 2. Significant differences in VOC concentration above vials with poor bacterial growth at the time point of analysis and above non-inoculated control vials. *—significant when p-value < 0.05.

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specific VOC profiles and comparison of those. However, they did have significant differences in the concentration of 14 VOCs in the case of MK and six VOCs in the case of MC when compared to control vials (Fig 2).

VOCs were organised into four groups, stated in S3 Table and visualised in Fig 3:

(Ia) n = 13 concentrations of VOCs above inoculated slants were either higher or equal than above control vials

(Ib) n = 16 VOCs were only detectable above inoculated slants not above control vials

(II) n = 12 concentration of VOCs was higher or equally concentrated above control slants than above inoculated ones

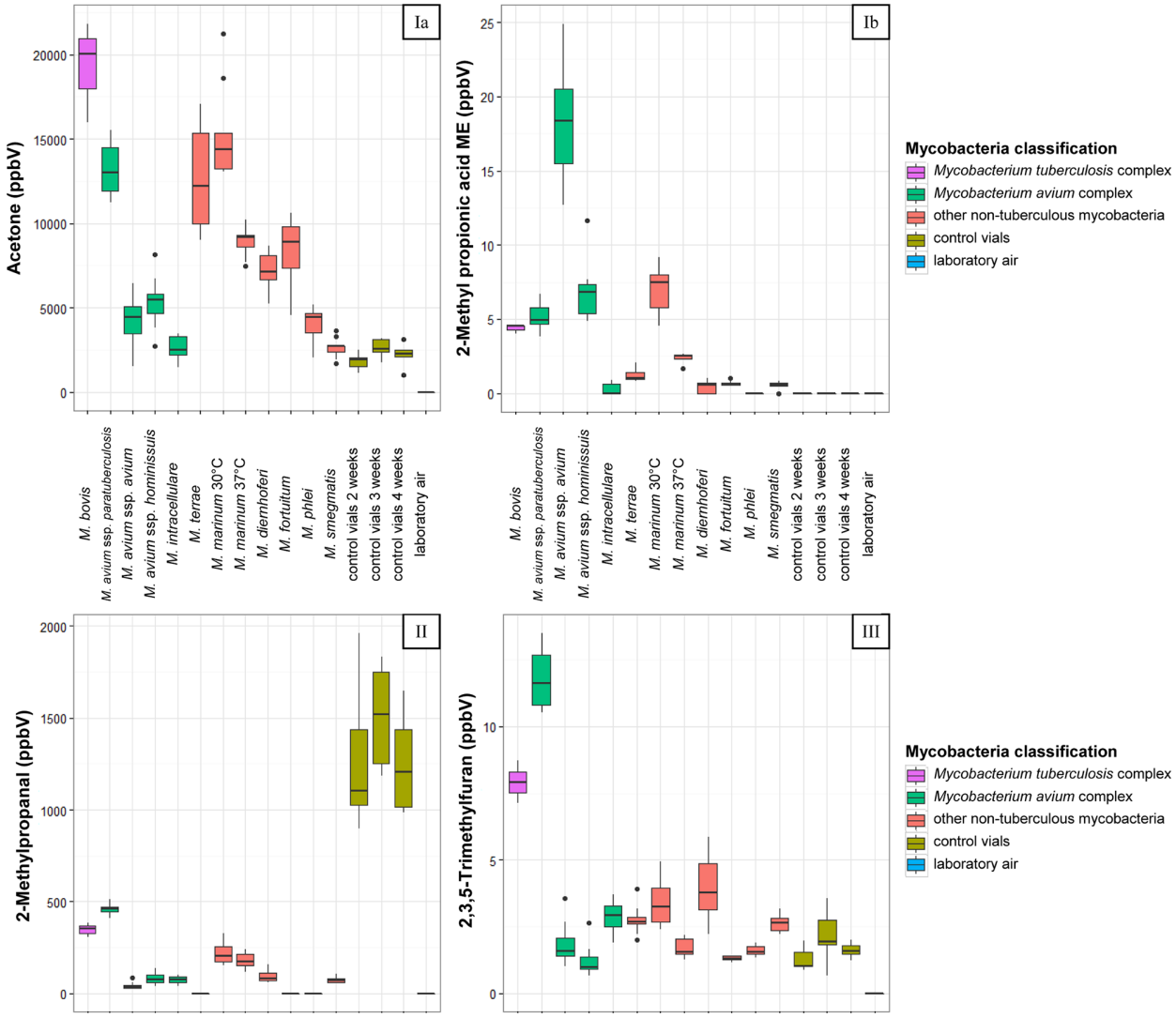

Fig 3. VOC concentrations above different mycobacterial species and pure media control slants inoculated with MB-Bouillon, forming four groups of substances. Ia—VOC concentrations above inoculated slants were higher than or equal to control vials; Ib—VOCs were detectable above inoculated slants only and not above control vials; II—VOC concentrations above inoculated slants were equal to or lower than above control slants; III—VOC concentrations above inoculated slants were higher than, equal to or lower than above control slants; ME–methyl ester.

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(III) n = 13 VOCs with a concentration being higher, equal or lower above inoculated slants than above control slants

All eleven mycobacterial species could be differentiated from control vials by at least 21 VOCs (Table 2).

Core-profile of Mycobacteriaceae

Seventeen substances showed the same tendencies for all mycobacteria. While the inoculated vials had eight volatiles (2-methylpropanol, 2-methyl-1-butanol, pentane, heptane, octane, 2,3-butadione, 3-pentanone, and 3-octanone) that presented higher concentrations than the control slants, they had nine VOCs (acetaldehyde, propanal, 3-methylbutanal,

Table 2. VOC concentration above inoculated slants compared to control vials.

chemical class	volatile organic compound	MB	MAP	MAA	MAH	MI	MT	MM37	MM30	MD	MF	MP	MS
Alcohols	Ethanol	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.
	2-Methylpropanol	↑	↑	n.s.	↑	↑	n.s.	↑	↑	↑	n.s.	n.s.	↑
	3-Methyl-1-butanol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	↑	↑	n.s.	n.s.	↑
	2-Methyl-1-butanol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	↑	↑	n.s.	n.s.	↑
	Phenylethylalcohol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	↑	↑	n.s.	n.s.	↑
	2-Propen-1-ol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.	↑	n.s.	n.s.	↑
	4-Methyl-1-pentanol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	↑	↑	n.s.	n.s.	↑
	3-Methyl-1-hexanol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.	↑	n.s.	n.s.	↑
	Pentanol	↓Ø	↓Ø	↓Ø	↓Ø	n.s.	↓	↓	↓	n.s.	↓	↓Ø	n.s.
	Hexanol	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	n.s.	↑	n.s.	n.s.	↑
	2-Heptanol	↑	↑	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	↑
	3-Octanol	↑	**X**	n.s.	n.s.	↑	n.s.	n.s.	n.s.	↑	n.s.	↑	↑
Aldehydes	Acetaldehyde	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	2-Methylpropanal	↓	↓	↓	↓	↓	↓Ø	↓	↓	↓	↓Ø	↓Ø	↓
	3-Methylbutanal	↓	↓	↓	↓	↓	↓Ø	↓	↓	↓	↓Ø	↓Ø	↓
	2-Methylbutanal	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓Ø	↓
	Benzaldehyde	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø
	Propanal	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø
	Pentanal	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø
	Hexanal	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø
	Heptanal	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø	↓Ø
Hydro-carbons	2,2-Dimethylbutane	n.s.	↑	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	2,3-Dimethylbutane	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	2-Methylpentane	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.
	3-Methylpentane	n.s.	↑	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.
	Pentane	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
	Heptane	↑	↑	n.s.	n.s.	↑	↑	↑	↑	↑	↑	n.s.	↑
	Octane	↑	↑	↑	n.s.	↑	↑	↑	↑	↑	↑	↑	↑
	Nonane	↑	↑	n.s.	n.s.	↑	↑	n.s.	↑	↑	n.s.	n.s.	↑
	Methylcyclopentane	n.s.	↑	n.s.	n.s.	↑	↑	↑	n.s.	↑	n.s.	n.s.	↑
	Hexane	↑	↑	n.s.	n.s.	↑	↑	↑	↑	↑	n.s.	n.s.	↑
Ester	2-Methyl-propionic acid ME	↑	↑	**X**	↑	n.s.	n.s.	↑	↑	n.s.	n.s.	n.s.	n.s.
	3-Methyl-1-butanol acetate	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	↑	↑	n.s.	n.s.	↑
	Benzoic acid ME	n.s.	n.s.	**X**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Furans	Furan	n.s.	n.s.	↓	↓	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	2-Methylfuran	↓	**X**	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	2-Ethylfuran	↓	n.s.	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
	2-Propylfuran	↓	**X**	↓Ø	↓Ø	↓Ø	↓Ø	n.s.	↓Ø	n.s.	↓Ø	↓Ø	↓Ø
	2,3,5-Trimethylfuran	↑	**X**	n.s.	n.s.	↑	n.s.	↑	n.s.	↑	n.s.	n.s.	↑
	2n-Butylfuran	n.s.	**X**	↓Ø	↓Ø	↓Ø	↓Ø	↓	↓Ø	n.s.	↓Ø	↓Ø	↓Ø
	Dibromochloromethane	↓	↓	↓	↓	↓	↓	n.s.	n.s.	n.s.	↓	↓	n.s.
Ketones	Acetone	↑	↑	↑	↑	n.s.	↑	↑	↑	↑	↑	n.s.	n.s.
	2,3-Butadione	n.s.	↑	n.s.	n.s.	n.s.	↑	↑	n.s.	n.s.	↑	n.s.	n.s.
	2-Butanone	↑	↑	↑	n.s.	↑	↑	**X**	↑	↑	↑	n.s.	↑
	2-Pentanone	↑	↑	↑	n.s.	↑	↑	**X**	↑	↑	↑	n.s.	n.s.
	3-Pentanone	↑	**X**	↑	↑	↑	↑	↑	↑	↑	n.s.	↑	n.s.
	Methylisobutylketone	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.	↑	n.s.	n.s.	n.s.
	2-Heptanone	↓	↓	↓	↓	n.s.	↓	↓	↓	n.s.	n.s.	n.s.	↓
	3-Octanone	↑	↑	↑	↑	↑	↑	↑	↑	↑	n.s.	↑	↑

(*Continued*)

Table 2. (Continued)

chemical class	volatile organic compound	MB	MAP	MAA	MAH	MI	MT	MM37	MM30	MD	MF	MP	MS
N-containing compounds	Acetonitrile	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	↑	n.s.	n.s.	n.s.
	2-Methylpropanenitrile	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↑	n.s.	n.s.	↑
	2-Methylbutanenitrile	n.s.	n.s.	n.s.	n.s.	↓Ø	↑	↓Ø	↓Ø	↓Ø	↑	n.s.	↓Ø
	3-Methylbutanitrile	n.s.	n.s.	n.s.	n.s.	↑	↑	↑	↑	↑	n.s.	n.s.	↑
	Dimethyldisulfid	n.s.	n.s.	↓	↓	n.s.	↓	↓	↓	↑	↓Ø	↑	n.s.
	total count of VOCs	30	34	27	24	38	31	37	35	43	24	21	37

MAP—M. avium ssp. paratuberculosis, MAA—M. avium ssp. avium, MAH—M. avium ssp. hominissuis, MI—M. intracellulare, MT—M. terrae, MM37 –M. marinum (37˚C), MM30 –M. marinum (30˚C), MD—M. diernhoferi, MF—M. fortuitum, MP—M. phlei, MS—M. smegmatis, ME—methyl ester, **X** –'Indicator substance' each value of one species is higher than all values of all other species, ↑ –substance concentration above bacteria significant higher than above control slants, ↓ - substance concentration above bacteria significant lower than above control slants, Ø –substance concentration below level of detection, n.s.–not significant.

https://doi.org/10.1371/journal.pone.0194348.t002

2-methylbutanal, pentanal, hexanal, heptanal, benzaldehyde, and 2-methylpropanal) that decreased during the incubation period (see Fig 3II).

Species-specific VOC profiles

'Indicator substances' are characterised by the fact that the concentration value of each individual measurement of a certain VOC above one species were higher than all individual values of all other species. Consequentially, the median concentration of this VOC above this species was significantly higher than median values of the other species (Table 2). MAP posed six indicator substances with the highest concentrations (for example 2,3,5-trimethylfuran (Fig 3III)), while for MAA the indicator substances were 2-methyl propionic acid ME (Fig 3Ib) and benzoic acid ME. Two VOCs were detectable as indicator substances above MM37.

Not only the highest concentration of a substance, but also specific concentration levels of substances indicate the presence of a particular species. For example the median concentration of 2,3,5-trimethylfuran (Fig 3III) was five times higher above MB, but seven times higher above MAP, each compared to the concentration above control vials. Another example was the concentration of 2-butanone, which was three times higher above MM30 and six times higher above MM37 compared to control slants (S3 Table).

All species of mycobacteria could be differentiated from each other by the concentrations of a certain number of VOCs, even though they formed clusters in the PCA (Fig 1). Concerning MI, MD, and MS, the concentrations of nine volatiles above MI were significantly lower than above MD. These were ethanol, 4-methyl-1-pentanol, furan, 2,3,5-trimethylfuran (Fig 3III), acetone (Fig 3Ia), acetonitrile, hexanol, 3-methyl-1-hexanol, and 3-methyl-1-butanol acetate. The concentrations of 2-methyl-1-butanol and octane above MI were significantly higher than above MD and MS. Also, 2-propen-1-ol, 2-methylpropanol, 3-methyl-1-butanol, heptane, 3-octanone, and methylisobutylketone were significantly more concentrated above MI than above MS. For the cluster of MAA, MAH, MP, and MF, three VOCs (2-butanone, 2-pentanone, and 3-pentanone) were significantly more concentrated above MAA than above MP. The substance 2-butanone was significantly higher concentrated above MAA than above MAH, while 2-methyl propionic acid ME (Fig 3Ib), benzoic acid ME—being the indicator substances for MAA -, and 3-pentanone were significantly less concentrated above all three species (MF, MP, and MAH). For these four species (MAA, MAH, MP, and MF), 2-methylpropanol was highest concentrated above MAH.

Table 3. Inter-strain variability of VOCs tested per species by means of Mann-Whitney-U-Test.

	p-value					p-value			
VOC	MAP	MAA	MAH	MI	VOC	MAP	MAA	MAH	MI
2-Methylpropanol	n.s.		n.s.	n.s.	2,2-Dimethylbutane	n.s.			
3-Methyl-1-butanol				n.s.	3-Methylpentane	n.s.			
2-Methyl-1-butanol				n.s.	Pentane	n.s.	n.s.	<0.05	n.s.
Phenylethylalcohol				n.s.	Heptane	n.s.			<0.01
2-Propen-1-ol				<0.001	Octane	n.s.	<0.05		<0.05
4-Methyl-1-pentanol				n.s.	Nonane	n.s.			<0.001
3-Methyl-1-hexanol				n.s.	Methylcyclopentane	n.s.			<0.05
Pentanol	n.s.	n.s.	n.s.	n.s.	Hexane	n.s.			<0.05
2-Heptanol	n.s.				2-Methyl-propionic acid ME	n.s.	n.s.	n.s.	
3-Octanol	n.s.			n.s.	3-Methyl-1-butanol acetate				n.s.
Furan		n.s.	n.s.		Benzoic acid ME		<0.001		
2-Methylfuran	n.s.	<0.01	<0.05	n.s.	Dibromochloromethane	n.s.	n.s.	n.s.	n.s.
2-Ethylfuran		<0.05	n.s.	n.s.	Acetaldehyde	n.s.	n.s.	n.s.	<0.05
2-Propylfuran	n.s.	n.s.	n.s.	n.s.	2-Methylpropanal	n.s.	<0.001	n.s.	n.s.
2,3,5-Trimethylfuran	n.s.			<0.05	3-Methylbutanal	n.s.	n.s.	<0.05	<0.01
2n-Butylfuran	n.s.	n.s.	n.s.	n.s.	2-Methylbutanal	n.s.	n.s.	n.s.	<0.01
Acetone	n.s.	n.s.	n.s.		Benzaldehyde	n.s.	n.s.	n.s.	n.s.
2,3-Butadione	n.s.				Propanal	n.s.	n.s.	n.s.	n.s.
2-Butanone	n.s.	<0.05		n.s.	Pentanal	n.s.	n.s.	n.s.	n.s.
2-Pentanone	n.s.	<0.01		<0.01	Hexanal	n.s.	n.s.	n.s.	n.s.
3-Pentanone	n.s.	<0.05	n.s.	<0.01	Heptanal	n.s.	n.s.	n.s.	n.s.
Methylisobutylketone				n.s.	3-Methylbutanenitrile				<0.05
2-Heptanone	n.s.	<0.01	<0.05		2-Methylbutanenitrile				n.s.
3-Octanone	n.s.	n.s.	<0.01	<0.01	Dimethyldisulfid		n.s.	<0.001	

Only substances, which were included in the species-specific VOC profile, were examined. MAP—M. avium ssp. paratuberculosis, MAA—M. avium ssp. avium, MAH—M. avium ssp. hominissuis, MI—M. intracellulare, n.s.–not significant, ME–methyl ester.

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Homogeneity within species

The strains of each species did not show significant differences in the concentration of most volatiles (Table 3). For MAP, VOCs did not differ significantly from each other. In the case of MAA it was 18 of 27, for MAH 18 of 24, and for MI 24 of 38. Fig 4 shows that the difference in VOC concentration between two strains of the same species was lower than among different species.

Based on the results we propose following VOC profiles for each species (Fig 5).

Discussion

As expected, data support the possibility of distinguishing inoculated slants from pure media control slants by means of VOC analysis, especially for MB, MAP, MAA, MAH, MI, MT, MM, MD, MF, MP, and MS.

Identification of species

The results of the current study suggest that by taking indicator substances into account conclusions about the presence of corresponding *Mycobacterium* species can be drawn. Three of

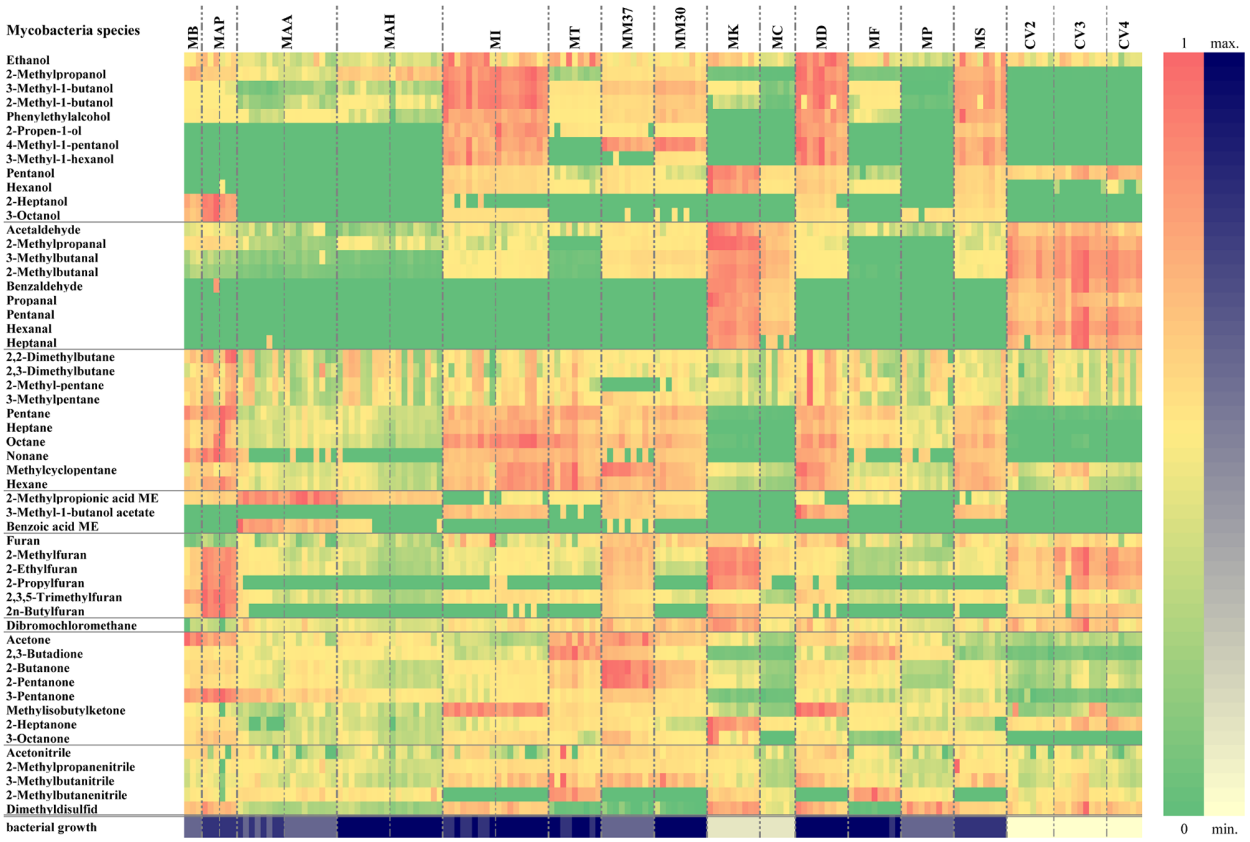

Fig 4. VOC emissions from different mycobacteria. The illustration is a heatmap with normalised data to a maximum of each substance. MAP–*M. avium* ssp. *paratuberculosis*; MAA–*M. avium* ssp. *avium*; MAH–*M. avium* ssp. *hominissuis*; MI–*M. intracellulare*; MT–*M. terrae*; MM37 –*M. marinum* (37˚C); MM30 –*M. marinum* (30˚C); MD–*M. diernhoferi*; MF–*M. fortuitum*; MP–*M. phlei*; MS–*M. smegmatis*; L2 –control vials incubated for 2 weeks; L3 –control vials incubated for 3 weeks; L4 –control vials incubated for 4 weeks; ME–methyl ester.

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the species included in the present study produced at least two of these substances. Combining this with the concentration levels of other VOCs and the complete absence of some other volatiles, unequivocal VOC profiles can be defined for all of the included species (Fig 5). Keeping in mind that these profiles have been defined with only a small selection of specific mycobacteria and measured at specific stages of bacterial growth, further investigations have to address the VOC profiles of these species and other species using different lengths and conditions of incubation.

For MAP, findings of the current study support previously published results [48] where we found 31 VOCs with significant differences compared to control slants after 4 weeks of incubation. In the current study, 34 VOCs had increased or decreased significantly after 4 weeks of incubation. Twenty-one volatiles of the MAP profile defined in the previous study were confirmed in the current study. This time we included 13 additional substances in the MAP-specific VOC profile, which had not been detected before. On the other hand, there were 10 VOCs included in the MAP-specific VOC profile in the previous study, which were excluded from the current study because of the high variability in their concentrations above control vials (see section 2.4 Selection of VOCs). Due to different study designs and the number of comparative groups, different statistical tests had to be used and could, therefore, explain the

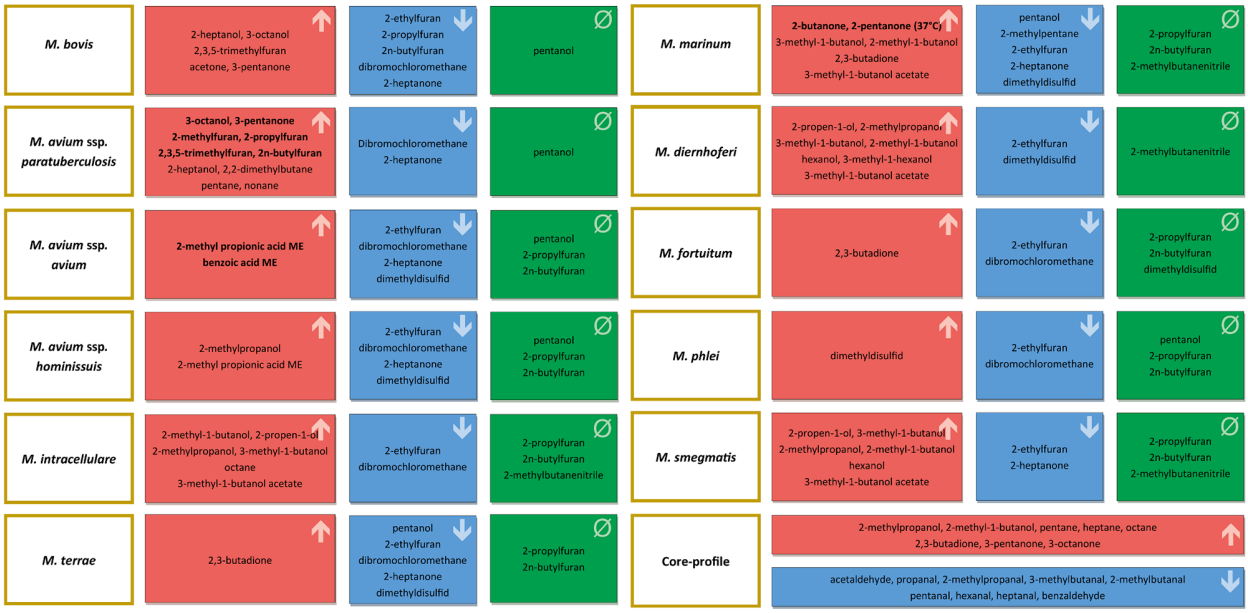

Fig 5. Suggested VOC profile consisting of the most influencing substances for each mycobacterial species. ↑ - substance is significantly higher above bacteria than above control vials; ↓ - substance is significantly lower above bacteria than above control vials; **Ø** –substance is not measurable above bacteria; bold: the values of the indicator substance of a species are higher than all values of all other species; ME–methyl ester.

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differences of the defined MAP-specific VOC profile. Based on the study of Trefz et al. [38], the importance of furans in a MAP-specific VOC profile was assumed. In the current study, the concentrations of four furans were not only significantly higher above MAP than above control, but every sample of MAP inoculated vials showed higher concentrations than all the other samples (see Table 2, 'Indicator substances').

Pentanal was found to be significantly different for each species in the study of Mellors et al. [45], that is *M. avium* (subspecies not designated), *M. bovis* BCG, MI, and *M. xenopi*. Our results show a significant decrease in pentanal concentration above the inoculated slants of all the species, see 3.2 Core-profile. A study by Nawrath et al. [53] incorporated different species of mycobacteria including, MS and MAA amongst others. Compared to their results, only one substance was also found significant in our study for MS: i.e. hexanol. A possible explanation for the different outcomes of the studies is that various incubation protocols were used. Not only do the media and media types (solid compared to liquid) differ from our protocols, but so does the length of incubation. In addition, different methods for pre-concentration and analysis of volatiles have been used. Nawrath et al. [53] used closed-loop stripping analysis combined with GC-MS, while Mellors et al. [45] used solid-phase micro-extraction and analysed via two-dimensional gas chromatography time-of-flight mass spectrometry. Due to different packing materials in the devices used for micro-extraction, the VOCs from different chemical classes bind the trapping devices in variable quality [54].

In a previous study we assessed different culture conditions including bacterial density, duration of incubation, and media type [48]. Here another methodological factor, i.e. incubation temperature, was addressed: MM30 and MM37 were incubated at different temperatures. The VOC patterns above those slants differed significantly even though it was the same species (Figs 1 and 4).

Classification of mycobacteria

The scattering in the PCA (Fig 1) does not reflect the most common classification scheme for mycobacteria. In this scheme developed by Ernest H. Runyon [12], mycobacteria are grouped by their phenotype. This includes pigment production that is dependent on light and bacterial growth rate. Pigments include photochromogens (group I, MK, MM), scotochromogens (group II), and non-chromogens (groups III & IV). Group III contains slow growing mycobacteria (MAA, MAH, MAP, MI, MB) and group IV rapid growing mycobacteria (MT, MC, MD, MF, MP, MS). In addition, there are ungrouped mycobacteria. The overlays also do not correspond to the conventional classification separating MTC, MAC, and other NTMs [55]. The clustering in the PCA corresponds more to the groups classified by lipid composition published by Lechevalier et al. [56]. For example, after the pyrolysis of the mycolic esters of the cell wall of MD and MS in the gas chromatograph, a mixture of mainly unbranched saturated fatty esters with 22 and 24 carbons are released. Other mycobacterial species show a different composition of lipids. Studies addressing the patterns of mycolic acids in the cell wall of mycobacteria suggest using these acids for bacterial classification and identification [57,58]. These studies present the same pattern consisting of alpha, keto mycolic acids, and wax esters for MAA, MAH, and MP, which belong to the same PCA cluster in our study. On the other hand, MI consists of the same mycolic acid pattern, but presents a different result in our PCA. Once again, the impact of culture conditions such as bacterial density, length of incubation, and the stage of bacterial growth are considerable. Since the species were propagated, inoculated, and sampled at different days, methodological factors can be neglected as reason for the clustering in the PCA.

Value for a diagnostic approach

Significant differences among the strains of the same species presented in Table 3 can be partly explained by the different stages of bacterial growth at the time point of analysis, especially for MAA and MI (see Fig 4). A few studies have addressed the kinetics of the volatile profile during different stages of bacterial growth, and have shown that the substance concentrations increase and/or decrease over time [48,49].

Due to poor bacterial growth of MK and MC at the time of VOC analysis, probably because HEYM is not the best-adapted medium for these species, measurements needed to be excluded from further statistical analysis. Even though they showed significant differences compared to control vials, the inclusion criterion required that they exhibit a growth intensity of at least 1 point and 3 points for ideal comparison. Our results confirm an earlier study [48] that volatile emissions from bacteria are measurable long before growth is visually apparent. In both studies, the aldehyde concentrations increased significantly before it decreased, at the time when the cultures first became visible, until the aldehydes above the bacteria were no longer measurable, as compared to control vials (see Fig 4).

Defining a set of volatile substances resembling the presence of any species of mycobacteria could be a helpful tool for future diagnostic application. Other studies have approached this issue as well (e.g. for *E. coli*) [59]. Further investigations are necessary to discriminate these 17 VOCs from substances that indicate the presence of growing mycobacteria in general or display species of the same suborder, for example *Corynebacterineae*.

Conclusions

This study revealed species-specific VOC profiles for eleven mycobacterial species that showed visually apparent bacterial growth at the time point of analysis. We were able to distinguish VOCs above inoculated vials compared to pure media control slants and VOCs that showed

differing patterns above the different *Mycobacterium* species. Therefore, compared to control vials, a core-profile of Mycobacteriaceae could be defined that contains eight increasing and nine decreasing substances. The presence of all 13 species could be proven by means of VOC analysis.

From a diagnostic perspective, inter-strain variability is negligible. VOC emissions seem to correspond strongly with the cell wall structure and particularly the lipid composition of the cell wall. Nevertheless, in comparison to previous studies and the literature, culture conditions and methodological factors seem to have a great impact. This is important information for future developments towards *in vitro* testing of bacterial growth in general. The results indicate that analysis of volatile organic compounds could accelerate and simplify diagnostic methods for Mycobacteriaceae.

How conclusive the results are for other mycobacterial species *in vitro* and for infections *in vivo*, respectively, remains to be elucidated in future research.

Supporting information

S1 Table. Manufacturer of reference substances.
(DOCX)

S2 Table. Reference substances used for identification and quantification of selected VOCs. VOC–volatile organic compound; R2 –coefficient of determination; LOD–limit of detection; LOQ–limit of quantification; ppbV–parts per billion by volume.
(DOCX)

S3 Table. Median and percentiles (0.25 and 0.75) of the concentration of volatile organic compounds in ppbV and Tukey-HSD-test.
(PDF)

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Writing – review & editing: Wolfram Miekisch, Petra Reinhold, Heike Köhler.

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9 Anhang

9.1 Lebenslauf

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9.2 Vollständige Publikationsliste

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- Küntzel A, **Oertel P**, Trefz P, Miekisch W, Schubert JK, Köhler H and Reinhold P. *Animal science meets agricultural practice: Preliminary results of an innovative technical approach for exhaled breath analysis in cattle under field conditions*, Berliner und Münchener Tierärztliche Wochenschrift, **2018** Juni 7, doi: 10.2376/0005-9366-17101
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9.3 Tagungsbeiträge

Der präsentierende Autor ist unterstrichen.

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- Küntzel A., Fischer S., Bergmann A., **Oertel P**., Trefz P., Miekisch W., Schubert JK., Reinhold P., Köhler H.: *MAP-specific volatile organic compound profile: a comparative analysis of three different in vitro studies.* Proceedings oft the International Colloquium of Paratuberculosis (ICP), Juni 2018, Cancun

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- **Oertel P**, Bergmann A, Fischer S, Trefz P, Küntzel A, Reinhold P, Köhler H, Schubert JK and Miekisch W. *Evaluation of SPME and NTME for VOC profiling in bacterial cultures.* Pittcon, März 2017, Atlanta

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- **Oertel P.**, Sardar Pasha SPB, Mike O. Karl, *Retinal cell death is required to induce Müller glia proliferation.* Juni 2013, CRTD sommer conference Dresden.

Drittmittel

- Antrag zur Förderung eines wissenschaftlichen Forschungsvorhabens im Rahmen des Forschungsförderungsprogrammes der Universitätsmedizin Rostock, **FORUN**, *Untersuchung volatiler organischer Verbindungen (VOC) über Stammzellkulturen als Indikator für die Zelldifferenzierung*, **2016**, Phillip Trefz, **Peter Oertel**, Christian Rimmbach

Patentanmeldung

- "Messbox zur Analyse von Substanzen", Aktenkennzeichen: 10 2017 108 022.8., Anmeldetag: 13. April 2017

Eidesstattliche Erklärung

Ich versichere hiermit, die vorliegende Dissertation selbstständig und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt zu haben. Die aus fremden Quellen direkt übernommenen Gedanken und wörtlich entnommenen Stellen sind als solche kenntlich gemacht. Die vorliegende kumulative Dissertation wurde bisher in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Rostock, 20. Mai 2019

Peter Gierschner

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