Use of fecal volatile organic compound analysis to discriminate between non-vaccinated and BCG—Vaccinated cattle prior to and after Mycobacterium bovis challenge

Christine K. Ellis  
*United States Department of Agriculture*

Somchai Rice  
*iowa State University, somchai@iastate.edu*

Devin Maurer  
*iowa State University, dmaurer@iastate.edu*

Randal Stahl  
*United States Department of Agriculture*

W. Ray Waters  
*United States Department of Agriculture*

See next page for additional authors

Follow this and additional works at: https://lib.dr.iastate.edu/abe_eng_pubs

Part of the Agriculture Commons, Animal Diseases Commons, Bioresource and Agricultural Engineering Commons, and the Veterinary Infectious Diseases Commons

The complete bibliographic information for this item can be found at https://lib.dr.iastate.edu/abe_eng_pubs/810. For information on how to cite this item, please visit http://lib.dr.iastate.edu/howtocite.html.

This Article is brought to you for free and open access by the Agricultural and Biosystems Engineering at Iowa State University Digital Repository. It has been accepted for inclusion in Agricultural and Biosystems Engineering Publications by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Use of fecal volatile organic compound analysis to discriminate between non-vaccinated and BCG-Vaccinated cattle prior to and after Mycobacterium bovis challenge

Abstract
Bovine tuberculosis is a zoonotic disease of global public health concern. Development of diagnostic tools to improve test accuracy and efficiency in domestic livestock and enable surveillance of wildlife reservoirs would improve disease management and eradication efforts. Use of volatile organic compound analysis in breath and fecal samples is being developed and optimized as a means to detect disease in humans and animals. In this study we demonstrate that VOCs present in fecal samples can be used to discriminate between non-vaccinated and BCG-vaccinated cattle prior to and after Mycobacterium bovis challenge.

Disciplines
Agriculture | Animal Diseases | Bioresource and Agricultural Engineering | Veterinary Infectious Diseases

Comments
This article is from PLoS ONE 12(7): e0179914. https://doi.org/10.1371/journal.pone.0179914.

Authors
Christine K. Ellis, Somchai Rice, Devin Maurer, Randal Stahl, W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyon, Kurt C. VerCauteren, and Jacek A. Koziel

This article is available at Iowa State University Digital Repository: https://lib.dr.iastate.edu/abe_eng_pubs/810
Use of fecal volatile organic compound analysis to discriminate between non-vaccinated and BCG—Vaccinated cattle prior to and after *Mycobacterium bovis* challenge

Christine K. Ellis, Somchai Rice, Devin Maurer, Randal Stahl, W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren, Jacek A. Koziel

1 United States Department of Agriculture, Animal Plant and Health Inspection Service, Wildlife Services, National Wildlife Research Center, Fort Collins, Colorado, United States of America, 2 Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, Iowa, United States of America, 3 United States Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, Iowa, United States of America, 4 United States Department of Agriculture, Animal Plant and Health Inspection Service, Veterinary Services, Wildlife Livestock Disease Investigations Team, Fort Collins, Colorado, United States of America

☯ These authors contributed equally to this work.
‡ These authors also contributed equally to this work.
* Christine.K.Ellis@aphis.usda.gov

Abstract

Bovine tuberculosis is a zoonotic disease of global public health concern. Development of diagnostic tools to improve test accuracy and efficiency in domestic livestock and enable surveillance of wildlife reservoirs would improve disease management and eradication efforts. Use of volatile organic compound analysis in breath and fecal samples is being developed and optimized as a means to detect disease in humans and animals. In this study we demonstrate that VOCs present in fecal samples can be used to discriminate between non-vaccinated and BCG-vaccinated cattle prior to and after *Mycobacterium bovis* challenge.

Introduction

Bovine tuberculosis (bTB), a zoonotic disease affecting international public health, agricultural, wildlife, and trade [1, 2], is caused by *Mycobacterium bovis*. The etiological agent responsible for most of the 9.6 million annual human tuberculosis cases [3] is *M. tuberculosis*; however, approximately 30% of cases may be caused by *M. bovis* infection, especially in developing countries [4] where prevalences of livestock bTB may approach 10–14% [5–8]. The economic costs associated with bTB are significant; from 2008–2009 estimated economic losses globally and in the United States (US) were $3 billion and $40 million, respectively [2].

The US federal bTB eradication program has been successful in decreasing the prevalence of bTB to approximately 0.0006% [2]; however, outbreaks in domestic cattle (*Bos taurus*) and...
captive and farmed cervids continue to occur. Importation or migration of infected animals from other countries and endemically infected feral swine (Sus scrofa; Molokai Island, Hawaii) and white-tailed deer (Odocoileus virginianus; Michigan and Indiana) serve as sources for reintroduction of the disease to domestic livestock [9–15]. Antemortem surveillance tests used for domestic cattle include the caudal fold (CFT) and comparative cervical (CCT) skin tests, and the interferon gamma (IFN-γ) release assay (IGRA; Bovigam, Prions Ag, Schlieren-Zurich, Switzerland). The sensitivity (SN) and specificity (SP) of these tests are reasonable, but all are labor intensive and lack efficient time to results [1, 7]. Other in vitro assays have limitations associated with accuracy and execution that restrict their use in ante-mortem surveillance [7].

Development of an ante-mortem disease monitoring system capable of detecting *M. bovis* infection in cattle, cervids such as white-tailed deer, feral swine, and other wildlife species would improve bTB surveillance by decreasing or eliminating animal handling events, and remove the requirement for specialized training. This system should be highly repeatable, cost efficient, highly sensitive and specific, and provide rapid and early detection of diseased individuals. Evaluation of volatile organic compounds (VOCs) present in breath, biofluids such as urine and blood, and feces have been conducted in an effort to develop a diagnostic modality for identifying disease (including tuberculosis) in humans [16–27]. In cattle, VOC analysis of breath samples has been explored as a method for diagnosis of bovine respiratory disease [28], brucellosis [29], bTB [30–32], Johne’s disease [29, 33], and ketoacidosis [34, 35]. Fecal VOC analysis has been used experimentally to identify goats [33, 36] and cattle [37] infected with *Mycobacterium avium paratuberculosis* (MAP; Johne’s disease).

In previous work we found that fecal headspace VOCs could be used discriminate between non-vaccinated and Bacillus Calmette-Guerin (BCG) vaccinated white-tailed deer prior to and after virulent *M. bovis* challenge with a high degree of SN and SP [38]. The goal of this study was to demonstrate that fecal headspace VOCs could be used to differentiate between non-vaccinated and BCG-vaccinated cattle prior to and post-virulent *M. bovis* challenge. Based upon the results of our analysis, we were successful in achieving this goal. The results of this study, combined with our previous studies [30, 32, 38, 39] provide evidence that breath and fecal VOC analysis have merit for disease surveillance in ruminant species. Our work provides data that adds to existing studies in this area, and encourages future development of VOC analyses for disease diagnosis in human and veterinary medical fields.

**Animals, materials and methods**

**Ethics statement**

All studies were approved by the Institutional Biosafety and Animal Care and Use Committees (IACUC) of the of the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), National Animal Disease Center (NADC), Ames, Iowa, USA (Animal Care and Use Protocol (ACUP) 2688); the USDA, Animal and Plant Health Inspection Service (APHIS), National Wildlife Research Center (NWRC), Fort Collins, Colorado, USA (Protocol QA-2262); and Iowa State University (ISU), Ames, Iowa, USA (IACUC 4-14-7787-B), and were performed within the conditions of the Animal Welfare Act. All animals were housed in appropriate biological containment facilities at NADC under the direct supervision of the institutional clinical veterinarian. Animals were monitored twice daily for overall body condition and well-being, and for any signs of signs of suffering and distress. Strict biosafety protocols were followed to protect personnel from exposure to *M. bovis*, including BSL-3 containment upon initiation of *M. bovis* challenge in animal rooms and standard BSL-3 and BSL-2 laboratory practices for handling *M. bovis* cultures and samples from *M. bovis*-infected animals.
Animals and *Mycobacterium bovis* challenge

One to two day old Holstein bull calves (n = 20) were acquired from a *M. bovis*-free, *M. avium* paratuberculosis-monitored herd, and placed into a NADC BSL-3 agricultural facility for the duration of the study. Cattle were housed under environmental conditions appropriate for normal physiological thermoregulation; in separate biocontainment rooms; with no exchange of air, food, or water occurring between rooms. Diet consisted of alfalfa hay cubes and a commercial pelleted ration provided twice daily, and water *ad libitum*. All calves were castrated at 42–43 days of age (S1 Table). One individual in the BCG treatment group was humanely euthanized prior to the completion of the study due to a non-study related health issue, decreasing the number of individuals in that cohort (n = 9). Given the low dose challenge and relatively short duration of the study, cattle did not develop clinical signs of bTB (cough, dyspnea, anorexia, weight loss) necessitating palliative therapy.

*Mycobacterium bovis* strain 10–7428_CO_Dairy_10-A (*M. bovis* strain 10–7428; USDA, APHIS designation) was used for the challenge inoculum. The inoculum was prepared using standard procedures in Middlebrook 7H9 liquid media (Becton Dickinson, Franklin Lakes, NJ, US) as described by Larsen et al 2005 [40]. Enumeration of *M. bovis* challenge inoculum was performed as previously described [41]. Calves were challenged by aerosol with *M. bovis* using the method described by Palmer et al 2002 [41] at 120–121 days of age.

Diagnostic tests performed

Blood samples were collected from all calves seven days prior to vaccination, 20, 42, and 56 days post-vaccination, nine, 16, and 70 days post-challenge for *in vitro* evaluation of cellular immune responses (CMI) to mycobacterial antigens. The CCT was performed according to USDA, APHIS circular 91-45-01 guidelines [42] on all animals 104 days post-challenge (7 days prior to necropsy). Balanced purified protein derivatives (PPDs) were obtained from the Brucella and Mycobacterial Reagents Section of The USDA National Veterinary Services Laboratory, Ames, IA. Calves were administered 0.1 mL (100 μg) *M. bovis* PPD (PPDb) and 0.1 mL (40 μg) *M. avium* PPD (PPDa) intradermally at separate clipped sites on the side of the neck. Calves were humanely euthanized by intravenous administration of sodium pentobarbital 111 days post-challenge. Necropsy procedures, and gross and microscopic assessment of lesions were performed as described in Palmer et al 2002 [41]. When microscopic lesions consistent with bTB were identified, adjacent sections were stained by the Ziehl Neelsen technique to visualize acid-fast bacilli. Mediastinal, tracheobronchial, medial retropharyngeal, and prefemoral lymph nodes and lungs were sampled for *M. bovis* isolation and identification as previously described [43]. Qualitative assessment of mycobacterial colonization was performed using standard mycobacterial culture techniques [44] with Middlebrook 7H11 selective agar plates (Becton Dickinson, Franklin Lakes, NJ, USA) incubated for 8 weeks at 37˚C. Confirmation of colonies was performed as previously described [45] using IS6110 real time PCR (RT-PCR).

Fecal sample collection

Fecal samples were collected per rectum from all calves one day prior to virulent *M. bovis* challenge and prior to necropsy. Samples were placed in air-tight 50 mL conical centrifuge tubes, and stored on ice until transport to the NADC Tuberculosis Research Laboratory. Fecal sample processing was performed in a Biosafety Class II cabinet (BSC-II). A 1.5 gm fecal aliquot was removed from each sample using a 3.0 mL hypodermic syringe modified by removing the apex of the syringe barrel. Feces were deposited in the bottom of a clean 20 mL amber glass vial (Wheaton, Millville, NJ, USA) and the vial sealed with an air-tight lid containing a PTFE-
lined silicone septum. The outer surface of each vial was decontaminated with tuberculocidal disinfectant (Wexcide, Wexford Labs, Kirkwood, MO USA) and transported in a sealed transfer container to the ISU Atmospheric Air Quality Laboratory for analysis.

**Sample preparation for GC/MS analysis and GC/MS conditions**

Pre-concentration of VOCs was performed using a 2 cm 50/30 μm DVB/Carboxen/PDMS (57348-U, Supelco, Bellefonte, PA, USA) SPME fiber mounted in a CTC Combi PAL™ LEAP GC autosampler (LEAP Technologies, part of Trajan Family, Inc., Carrboro, NC, USA) equipped with a heated agitator (37°C, 250 rpm agitation). Each sample vial was transferred to the heated agitator, thermally equilibrated for 10 minutes, followed by SPME insertion and headspace exposure for 45 min. The SPME fiber was then transferred and inserted into the GC inlet and desorbed with the GC inlet configured in splitless mode at 250°C using helium (He) carrier gas (99.995% purity). Multi-Dimensional Gas Chromatography–Mass Spectrometry (MDGC-MS) analysis was performed on an Agilent 6890 GC with a restrictor guard column, non-polar capillary column (BP-5, 30.0 m x 530 μm inner diameter x 0.5 μm thickness, SGE, Austin, TX, USA) and polar capillary column (BP-20, 30.0 m x 530 μm inner diameter x 0.5 μm thickness, SGE, Austin, TX, USA) connected in series. Sample flow was split 3:1 via open split interface to an olfactometry port (not used in this research) and mass spectrometer, respectively. Flow to the mass spectrometer was determined by the fixed restrictor column inner diameter and excess effluent was directed to the olfactory sniff port. The carrier gas was set to constant pressure of 5.7 psi at the midpoint (connection point of the non-polar and polar column). Oven temperature was programmed as follows: 40°C for 3.00 minutes, ramped to 240°C at a rate of 7°C/min, and held for 8.43 min. Transfer line to the MS was set at 280°C, and heated zones were 150°C for the single quadrupole and 230°C for the source. The MS was operated in electron ionization (EI) mode, with the electron energy set to 70eV, scanning from m/z 34.0–350.0.

**Data analysis**

Files containing GC-MS data for two BCG-vaccinated animals were corrupted, resulting in exclusion of these animals from our analysis. Remaining BCG-vaccinated animals (n = 7) were placed into two samples groups based on post-challenge responses to challenge (Table 1). To keep sample group sizes similar for analyses, four non-vaccinated animals were randomly selected for our analyses. Final sample group designations included BCG-vaccinated virulent *M. bovis* positive calves (BCGprePOS, BCGpostPOS; n = 3); BCG-vaccinated calves with no evidence of virulent *M. bovis* infection (BCGpreNEG, BCGpostNEG; n—4); and non-vaccinated virulent *M. bovis* positive calves (NVpre, NVpost; n = 4).

Baseline-corrected chromatograms were analyzed using the multi-group comparison feature in XCMS Online [46, 47] (www.xcsmonline.scripps.edu) to identify peak ion abundances that differed across sample groups. All remaining statistical analysis were performed using statistical packages available in “R” [48–50]. A principal component analysis (PCA) was performed on the statistically significant ions (α = 0.05, 1.5 fold difference), and the PC scores proportion of variance and cumulative proportion of variance were used to develop five class linear discriminant analysis (LDA) classification models. Training and test datasets containing 70% and 30% of the data were used to produce predicted classifications for each sample, and the results were compared to actual sample group assignment. Misclassification rates were calculated to evaluate the feasibility of using statistically significant ions to discriminate between the sample groups. The number of true positive (infected calves; NVpost, BCGpostPOS); true negative (pre-challenge treatment groups and the post-challenge non-infected...
BCG-vaccinated calves; NVpre, BCGpre, BCGpostNEG); false positive (true negative calves incorrectly classified as infected), and false negative (true positives incorrectly classified as non-infected) animals were summed. Sensitivity was calculated as the total number of true positives divided by the sum of the true positives plus false negatives, and SP was calculated as the total number of true negative samples divided by the sum of the true negatives plus false positives [51]. An agglomerative hierarchical cluster analysis using Ward’s method was performed to further assess use of ion data as a means to discriminate between individual and grouped samples, and to test the robustness of the PCA and LDA classification models.

Statistically significant ion intensities identified by XCMS Online were retention time matched with total ion chromatographic (TIC) peaks using Agilent Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA), and associated peak areas were incorporated into PCA and LDA analyses to assess the feasibility of using the identified peaks to discriminate between sample groups. Group mean peaks areas were calculated for each peak, and between groups mean peak area fold differences \( > = 3.0 \) and biological relevance were used to identify a suite of peaks that best allowed discrimination between the sample groups. This suite of selected peaks was reanalyzed to determine if a minimum number of peaks might be used to discriminate between the sample groups, and whether any peaks might function as biomarkers associated with disease and/or vaccination status. Peaks meeting a minimum spectral match probability \( > = 65\% \) were tentatively identified using the National Institute of Standards and Technology (NIST) W8N08 database (www.nist.gov), the Kyoto Encyclopedia of Genes

| Animal ID | Post-challenge Diagnostic Test Results |
|-----------|---------------------------------------|
|           | Cfu/gm TBLN virulent M. bovis | Gross lesion noted in lung | Gross lesion noted in lymph node(s) | Histopathology | ΔPPDb | RT-PCR | Culture |
| NVpre5 NVpost5 | 2989 | + | + | + | 28 | + | + |
| NVpre88 NVpost88 | 1600 | + | + | + | 62.5 | + | + |
| NVpre91 NVpost91 | 545 | + | + | + | 64.5 | + | + |
| NVpre95 NVpost95 | 82796 | + | + | + | 43.5 | + | + |
| BCG-prePOS8 BCGpostPOS8 | 0 | - | + | + | 54 | + | + |
| BCGprePOS84 BCGpostPOS84 | 1058 | - | - | + | 33.5 | + | + |
| BCGprePOS86 BCGpostPOS86 | 0 | - | - | + | 59 | + | + |
| BCG-pre6 BCGpost6 | 0 | - | - | - | 9.5 | - | - |
| BCGpre76 BCGpost76 | 0 | - | - | - | 33.5 | - | - |
| BCGpre77 BCGpost77 | 0 | - | - | - | 16 | - | - |
| BCGpre85 BCGpost85 | 0 | - | - | - | 20.5 | - | - |

\(^1\) Colony forming units M. bovis cultured per gram tracheobronchial lymph node

\(^2\) Change in response to PPDb pre- vs. post virulent M. bovis challenge

| Table 1. Sample group designation and diagnostic test results for calves. |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|---|
| Animal ID | Post-challenge Diagnostic Test Results |
|-----------|---------------------------------------|
|           | Cfu/gm TBLN virulent M. bovis | Gross lesion noted in lung | Gross lesion noted in lymph node(s) | Histopathology | ΔPPDb | RT-PCR | Culture |
| NVpre5 NVpost5 | 2989 | + | + | + | 28 | + | + |
| NVpre88 NVpost88 | 1600 | + | + | + | 62.5 | + | + |
| NVpre91 NVpost91 | 545 | + | + | + | 64.5 | + | + |
| NVpre95 NVpost95 | 82796 | + | + | + | 43.5 | + | + |
| BCG-prePOS8 BCGpostPOS88 | 0 | - | + | + | 54 | + | + |
| BCGprePOS84 BCGpostPOS84 | 1058 | - | - | + | 33.5 | + | + |
| BCGprePOS86 BCGpostPOS86 | 0 | - | - | + | 59 | + | + |
| BCG-pre6 BCGpost6 | 0 | - | - | - | 9.5 | - | - |
| BCGpre76 BCGpost76 | 0 | - | - | - | 33.5 | - | - |
| BCGpre77 BCGpost77 | 0 | - | - | - | 16 | - | - |
| BCGpre85 BCGpost85 | 0 | - | - | - | 20.5 | - | - |

Paired fecal samples collected from non-vaccinated and BCG-vaccinated calves selected for gas chromatography–mass spectrometry (GC-MS) analysis based on results of diagnostic testing, necropsy, and histopathological results. Samples were collected pre- and post- virulent M. bovis challenge. Each paired sample is identified by vaccination status, pre- or post-challenge time-point, and infection status (example: non-vaccinated pre-challenge = NVpre; BCG-vaccinated pre-challenge (BCGpre); non-vaccinated post-challenge (NVpost); BCG-vaccinated post-challenge not infected (BCGpostNEG); BCG-vaccinated post-challenge infected (BCGpostPOS).
and Genomes database [52, 53], and the Human Metabolome Database [54, 55]. When multiple compounds were tentatively identified at one retention time, all tentative compound identifications were considered based on the possibility of co-elution and likelihood that no reference library contains all known compounds.

**Results**

**Diagnostic tests**

Over the course of the study, no differences in overall health or clinical disease severity were observed among non-vaccinated or BCG-vaccinated calves prior to or after challenge with virulent *M. bovis* (excluding the BCG vaccinated animal euthanized prior to study completion). Results of the CCT skin test and *in vitro* evaluation of cellular immune responses to mycobacterial antigens are published elsewhere [56]. Briefly, all calves were classified as reactors based upon standard interpretation of the CCT skin test [42]. Mean responses of NVpost calves to PPDb were significantly greater than responses observed in the BCGpostNEG and BCGpostPOS calves (S1 Fig). Observed responses to PPDb were significantly greater than the responses to PPDa (S2 Fig), and robust cell-mediated immune responses to mycobacterial antigens were present all calves in all treatment groups [56]. Semi-quantitative scoring of gross lesions in the lungs and lymph nodes (tracheobronchial and mediastinal; TBLN), histopathology, and isolation and identification of mycobacterial isolates were performed at NADC (Table 1) [56]. Gross lesions were noted in the lungs and tracheobronchial and mediastinal lymph nodes of all NVpost calves. Gross lesions were noted in the TBLN of one post-challenge BCG-vaccinated calf (BCGpostPOS8). Granulomas containing acid-fast bacteria were identified histologically in the TBLN of all NVpost and BCGpostPOS calves. Calves in the NVpost and BCGpostPOS sample groups were confirmed positive for virulent *M. bovis* by culture and RT-PCR. There was no evidence of virulent *M. bovis* presence in BCGpostNEG calves.

**Analysis of ion features**

The analysis performed using XCMS Online identified 180 ions, with 105 ions meeting the criteria for statistical significance (\( \alpha = 0.05; \) fold change \( \geq 1.5 \)). Visualization of the characteristics of the statistically significant ion features are presented as a cloud plot (S3 Fig) [57]. The ion PCA classification model was developed using 76 of the 105 statistically significant ions. Twenty-nine ions occurring at late retention times (> 34.0 min) attributed to baseline noise were excluded from the analysis. Results are depicted in a three dimensional (3D) scatterplot (Fig 1). Pre- and post-challenge samples groups are distinctly separate within the three dimensional space. Separation is present between NVpre and all BCG-vaccinated (BCGprePOS, BCGpreNEG) sample groups. NVpost, BCGpostPOS and BCGpostNEG sample groups are distinctly separate as well.

Five class LDA classification models were constructed using two through six PC scores. The model constructed using two PC scores resulted in correct classification of 95% of calves (Table 2). All misclassifications consisted of NVpre calves incorrectly classified as BCGpre calves (i.e., true negatives classed into a different true negative group) which did not adversely affect effect SN:SP (100%; 100%). Classification models constructed using three through six PC scores contained no misclassifications, and calculated SN:SPs of 100%;100%.

The ion cluster analysis shows initial pairing of individual calves within respective sample groups. Subsequent progression places paired individuals into respective pre- and post- virulent *M. bovis* challenge groups (Fig 2). The final clustering produces clear separation between pre- and post-challenge sample groups. Inter-group discrimination is present between NVpre and BCGpre animals. All BCGpreNEG and BCGprePOS samples are found within one cluster,
indicating that these samples are similar prior to virulent *M. bovis* challenge. After challenge, BCGpostNEG and BCGpostPOS samples are found within separate, but closely associated clusters, indicating that while the presence of virulent *M. bovis* in BCGpostPOS animals has induced some difference in those individuals, they still are similar. The location of all NVpost-POS individuals into a distinct cluster indicates that lack of BCG-vaccination prior to challenge creates a strong inter-group variation.

**Analysis of peak areas and compound identification.** Ions identified as statistically significant were GC column retention time matched to 23 chromatographic peaks. Ten peaks were identified by single ion matches, while the remainder (n = 13) were matched to multiple ions. Peak area data were visually inspected for biological relevance, and using these criteria, eight peaks were excluded from consideration. Two additional peaks identified as silane-containing compounds were removed because they were likely contaminants eluted from the GC column or septa material, leaving 13 peaks available for further analysis and tentative compound identification.

The peak area PCA classification analysis was reliably capable of discriminating between the sample groups (Fig 3). Separation of pre- and post-challenge samples is present indicating that exposure to virulent *M. bovis* induces distinct changes in all individuals regardless of vaccination status. Individuals within treatment groups cluster in close association indicating similarity. BCGpreNEG and BCGprePOS animals cluster together indicating that there is little difference between BCG-vaccinated individuals prior to virulent *M. bovis* challenge. The
BCGpre sample group and NVpre sample groups are separate. Post-challenge the BCGpost-NEG and BCGpostPOS groups form separate clusters, indicating that the persistent presence of virulent *M. bovis* in BCGpostPOS animals induces differences between these groups. NVpost individuals form a separate cluster with the exception of one pre-challenge non-vaccinated animal (NVpost91).

LDA classification models were developed using two through four PCs. Models returning the lowest misclassification rates were constructed using two and four PC scores (Table 3). No misclassifications were observed, and corresponding SN:SP were 100%:100%, respectively. The misclassification rate for the model constructed using three PC scores was 17%, with all misclassified samples (100%) consisting of BCGpostPOS calves misclassified as BCGpostNEG (false negative samples). The calculated SN:SP for this model were 83%:100%.

### Table 2. Linear discriminant analysis classification of calves using statistically significant ions.

| Number of Principal Components | 2   | 3   | 4   | 5   | 6   |
|--------------------------------|-----|-----|-----|-----|-----|
| Misclassification Rate (%)    | 5   | 0   | 0   | 0   | 0   |
| Correct Classification Rate (%) | 95 | 100 | 100 | 100 | 100 |

MISCLASSIFICATION

- **False Negative (%)**
  - NVpost misclassified NVpre 0 0 0 0 0
  - NVpost misclassified BCGpre 0 0 0 0 0
  - NVpost misclassified BCGpostNEG 0 0 0 0 0
  - BCGpostPOS misclassified NVpre 0 0 0 0 0
  - BCGpostPOS misclassified BCGpre 0 0 0 0 0
  - BCGpostPOS misclassified BCGpostNEG 0 0 0 0 0

- **False Positive (%)**
  - NVpre misclassified NVpost 0 0 0 0 0
  - NVpre misclassified BCGpostPOS 0 0 0 0 0
  - BCGpre misclassified NVpre 0 0 0 0 0
  - BCGpre misclassified BCGpostPOS 0 0 0 0 0
  - BCGpostNEG misclassified BCGpostPOS 0 0 0 0 0

- **True Negative (%)**
  - NVpre misclassified BCGpre 100 0 0 0 0
  - NVpre misclassified BCGpostNEG 0 0 0 0 0
  - BCGpre misclassified NVpre 0 0 0 0 0
  - BCGpre misclassified BCGpostNEG 0 0 0 0 0
  - BCGpostNEG misclassified NVpre 0 0 0 0 0

- **True Positive (%)**
  - NVpre misclassified BCGpostPOS 0 0 0 0 0
  - NVpost misclassified BCGpostPOS 0 0 0 0 0

Sensitivity (%) 100 100 100 100 100
Specificity (%) 100 100 100 100 100

Five class models were constructed using two through six principal component analysis (PCA) dimensional scores. Models resulting in optimal misclassifications (0%), and SN:SP (100%:100%) were constructed using three through six PC scores. The model constructed using two PC scores returned the highest misclassification rate (5%); however, all misclassifications (100%) consisted of NVpre (true negative) animals misclassified as BCGpreNEG animals (true negatives), which did not affect the SN:SP (100%:100%).

https://doi.org/10.1371/journal.pone.0179914.t002
The peak area cluster analysis shows pairing of individuals within treatment group designation (Fig 4). As the hierarchical model progresses, two BCGpostPOS animals (BCGpostPOS84, BCGpostPOS86) group among the BCGpostNEG animals. The remaining BCGpostPOS animal occupies a separate space in the tree. In the next hierarchical progression, three groups are identified. NVpost animals form a distinct group; all pre-challenge animals (NVpre; BCGpre) fall within a second group; and all post-challenge BCG-vaccinated animals form a third group. The final two groupings consist of one cluster containing all NVpost animals, and another containing all the other animals, indicating that the NVpost cattle are truly unique compared to the other sampled populations. Within the group containing all other treatment groups, NVpre and BCGpre sample groups are distinct from the BCGpostNEG and BCGpostPOS groups. The two misclassified BCGpostPOS animals (BCGpostPOS84, BCGpostPOS86) are identical to those identified in the ion cluster analysis.

Tentative identifications of peaks and associated information are summarized in Table 4. Briefly, compounds tentatively identified using a $> = 65\%$ probability match in the NISTW8N08 library include an alcohol; an aldehyde; an alkane; an imine; one ketone; a pyridine derivative; and a thioether. Five peaks could not be identified using this database. Compounds tentatively identified using the KEGG and HMDB databases that differed from NIST included an organosulfur; an amino acid; an alcohol; a salt of chloric acid; a fatty acid; and a dicarboxylic acid. Five peaks were not identified by KEGG or HMDB. Three compounds could not be identified by any of the databases used.

Fold changes in mean peak area between treatment groups were calculated to identify peaks with greatest potential to perform dependent (pre- vs. post-challenge) and independent (vaccination and infection status) groups comparisons (S2 Table). A mean fold difference $> = 3.0$
was used to identify peaks that best allowed inter- and intra-groups discrimination. The results of this comparison demonstrate that while specific fecal VOC biomarkers indicative of vaccination and/or disease status were not identified, differentiation between groups is still possible using suites of VOCs (Table 5). In the NVpre vs. NVpost comparison, twelve peaks had mean peak area fold differences meeting our selection criteria, with six peaks (2–4, 7, 11–12) having the greatest differences. For the BCGpreNEG vs. BCGpostNEG comparison, 10 peaks were identified, with 7 peaks (1–5, 9, 10) providing best discrimination. Six peaks (1–5 and 10) out of eleven provide best differentiation between BCGprePOS and BCGpostPOS animals. NVpre and BCGpre animals are discriminated by one peak (peak 7). For the NVpre vs. BCGpostNEG groups comparison a suite of 11 peaks (1–10, 13) met our selection criteria, and four peaks (1–3, 7) had the greatest mean fold differences. Four of 11 peaks (1–3 and 7) allow best discrimination between NVpre and BCGpostPOS samples. Discrimination between NVpost and BCGpre animals is best achieved using five (2–4, 11, 12) of 11 peaks. Four (peaks 8, 10–12) of eight peaks are best capable of discriminating between NVpost and BCGpostNEG animals. A suite of seven VOCs was identified in the NVpost vs. BCGpostPOS comparison, with two peaks (8, 12) having the greatest mean fold difference. Two peaks (peaks 11 and 9) comprise the suite of VOCs best capable of discriminating between BCGpostNEG and BCGpostPOS animals. While we were unable to identify a specific biomarker indicative of vaccination status or presence of virulent M. bovis infection, the results of our analysis do demonstrate the merit in evaluating multiple VOCs as a means of identifying animal cohorts.
Potential sources, and cellular, biological or metabolic functions associated with each tentatively identified compound were explored using KEGG, HMDB, and literature searches [32, 38, 53–55, 58–77] (Tables 6 and 7). Extensive metabolic information relative to \( \text{M} \). \text{bovis} \) is not published; therefore, we referred to information published for \( \text{M} \). \text{tuberculosis} \) when necessary. Eight tentatively identified compounds could be associated with mammalian systems. Another eight compounds could be associated with microbial cellular function. Seven compounds appear associated with mycobacterial metabolism, seven have been identified in cattle, four appear to be involved in non-mycobacterial microbial metabolism, and two are associated with immunological function. Overlap occurs among multiple compounds. For example, citrulline is associated with macrophage metabolism and function; is produced as by-product of the urea cycle, and is utilized by rumen microflora to convert urea to ammonia.

### Table 3. Five way linear discriminant analysis classification of calves using 13 peaks.

| Number of Principal Components | 2   | 3   | 4   |
|-------------------------------|-----|-----|-----|
| Misclassification Rate (%)    | 0   | 17  | 0   |
| Correct Classification Rate (%) | 100 | 83  | 100 |

### Misclassification

- **False Negative (%)**
  - Nvpost misclassified Nvpre
  - Nvpost misclassified BCGpre
  - Nvpost misclassified BCGpostNEG
  - BCGpostPOS misclassified Nvpre
  - BCGpostPOS misclassified BCGpre
  - BCGpostPOS misclassified BCGpostNEG
  - Nvpost misclassified BCGpre

- **False Positive (%)**
  - Nvpre misclassified Nvpost
  - Nvpre misclassified BCGpost POS
  - BCGpre misclassified BCGpost NEG
  - BCGpre misclassified BCGpostPOS
  - BCGpre misclassified BCGpostNEG
  - BCGpre misclassified BCGpostPOS

- **True Negative (%)**
  - Nvpre misclassified BCGpre
  - Nvpre misclassified BCGpostNEG
  - BCGpre misclassified Nvpre
  - BCGpre misclassified BCGpostNEG
  - BCGpre misclassified BCGpostNEG
  - BCGpre misclassified Nvpre

- **True Positive (%)**
  - Nvpost misclassified BCGpostPOS
  - BCGpostPOS misclassified Nvpost

| Sensitivity (%) | 100 | 83  | 100 |
| Specificity (%) | 100 | 100 | 100 |

Models resulting in lowest misclassification (0%) and optimal SN:SP (100%:100%) were constructed using two and four PC scores. The model developed using three PC scores returned a misclassification rate of 83%. All misclassifications occurring (100%) in this model were false negatives (BCGpostPOS animals misclassified BCGpostNEG).

https://doi.org/10.1371/journal.pone.0179914.t003
Discussion

We were successful in discriminating between sample groups using statistically significant ion data extracted from our raw data by XCMS Online. The PCA performed on these data resulted in correct classification of all animals to their respective dependent (pre- and post- virulent *M. bovis* challenge) and independent (vaccination and infection) groups. The LDA classification models resulted in correct classification rates of 95–100% (0–5% misclassification). The cluster analysis misclassified two BCGpostPOS animals as BCGpostNEG (false negatives). The best use of the ion data was to evaluate the raw data in a preliminary format. For example, in this study, the number of statistically significant ions associated with a specific peak inconsistently ranged from a single ion to six ions, potentially biasing the analysis toward over or under representation of certain chromatographic peaks. Because ion count data do not reflect peak area, ion intensity does not equate to differences in VOCs between individuals or treatment groups. The intensity of a single ion may not represent the parent ion for a compound, and may result from the ionization of multiple co-eluting compounds that share a common ionization fragment. Finally, relative to the intended purpose of our research (assessment of host-pathogen interactions), associated peaks need to be biologically logical in occurrence. This cannot be ascertained using ion data.

The results generated by the peak area classification models are epidemiologically acceptable. The calculated SN:SP of our optimal LDA classification models were 100%:100%, and 83%:100% for the model that performed poorly. In comparison, the SN:SP of the CFT, comparative CCT, and the IFN-γ assay have been reported to range from 68–96.8%:96–98%; 55.1–93.5%:88.8–100% [2]; and 73–100%:87.7–99.2% [78], respectively. These results could be adequately visualized in the PCA scatterplot. The cluster analysis misclassified two BCGpostPOS animals as BCGpostNEG (false negatives). An advantage of the cluster analysis was its ability to independently group data, eliminating potential selection bias on the part of the researcher, unlike LDA which requires that the groups be defined prior to analysis. As such, we found the cluster analysis a helpful tool to evaluate the robustness of our other analyses.
Table 4. Tentative compound identification of chromatographic peaks.

| Peak | Retention Time | Tentative Compound Identification | Database Identification Number | Compound Class |
|------|---------------|----------------------------------|---------------------------------|----------------|
| 1    | 3.32          | Dimethyl sulfide                 | NIST W8N08                      | Thioether      |
|      |               | 62(999) 47(953) 45(408) 46(361) 61(333) | HMDB02303                      |                |
|      |               | 3-methylthiophene                | KEGG00580                       |                |
|      |               | 97(999) 98(530) 45(174) 53(88) 39(81) | HMDB33119                       | Thiophene      |
| 2    | 15.60         | Benzaldehyde                     | NIST W8N08                      | Aldehyde       |
|      |               | 77(999) 106(935) 105(889) 51(495) 50(296) | KEGG 00261                      |                |
|      |               | Dimethyl sulfone (methylsulfonylmethane, MSM) | HMDB0611                       |                |
|      |               | 79(999) 15(830) 94(477) 81(49) 43(45) | KEGG11142                       | Organosulfur (sulfone) |
| 3    | 16.02         | Unknown                          |                                 |                |
| 4    | 18.64         | Methoxy-phenyl-oxime             | NIST W8N08                      | Imine          |
|      |               | 133(999) 151(649) 135(262) 73(167) 42(165) | HMDB31019                      |                |
| 5    | 18.81         | 2-Methylpyridine (2-picoline)    | NIST W8N08                      | Pyridine derivative |
|      |               | 93(999) 66(520) 92(288) 65(212) 78(206) | HMDB01142                      |                |
| 6    | 18.94         | Citruline                        | KEGGC00327                      | Amino acid     |
|      |               | 69(999) 43(663) 56(638) 30(570) 28(459) | HMDB00904                      |                |
| 7    | 19.14         | Unknown                          |                                 |                |
| 8    | 25.31         | Unknown                          |                                 |                |
| 9    | 28.19         | 2-dodecanone                     | NISTW8N08                       | Ketone         |
|      |               | 58(999) 43(752) 59(308) 71(289) 49(169) | HMDB31019                      |                |
|      |               | 2-chloro-1-propanol              | HMDB31335                       | Alcohol        |
|      |               | 31(999) 58(270) 27(177) 29(151) 62(132) | HMDB06827                      |                |
| 10   | 28.61         | Chlorate                         | KEGG01485                       | Inorganic non-metallic compounds containing a chlorate as the largest oxoanion |
|      |               |                                  | HMDB02036                       |                |
| 11   | 28.68         | 1H-Indole                        | NISTW8N08                       | Indole         |
|      |               |                                  | HMDB00738                       |                |
|      |               |                                  | KEGG00463                       |                |
| 12   | 29.44         | 2, 6, 10, 14-tetramethylhexadecane (phytane) | NIST W8N08                      | Diterpenoid alkane |
|      |               | 57(999) 71(712) 43(612) 85(349) 41(313) | HMDB16741                      | Fatty acyl (amino acid derivative |
|      |               | 5-hydroxy-lysine                 | KEGGC01389                      |                |
|      |               | 70(999) 82(830) 43(684) 42(473) 56(469) | HMDB06827                      |                |
| 13   | 30.13         | 3, 7, 11, 15-tetramethyl-2-hexadecene-1-ol (phytol) | NISTW8N08                      | Diterpene alcohol |
|      |               | 71(999) 43(361) 57(333) 55(299) 69(278) | HMDB02019                      |                |
|      |               | Succinic acid (butanedioic acid) | KEGG00042                       | Dicarboxylic acid and derivative |
|      |               | 55(999) 45(842) 74(753) 27(636) 100(606) | HMDB00254                      |                |

Chromatographic peaks were tentatively identified using three databases. Statistically significant ions identified by XCMS were included in identification of compounds. The first five significant ion fragments as identified by compound matching are noted for reference.

https://doi.org/10.1371/journal.pone.0179914.1004
We were successful in identifying associations between VOCs and vaccination status and/or the presence of *M. bovis* infection. While individual VOCs did not appear to be exclusively associated with sample cohorts, use of inter- and intra-group mean peak area fold differences did identify suites of VOCs that allowed discrimination between independent and dependent treatment groups. An analogy would be use of a blood chemistry analysis to assess the overall function of all of an animal’s health, followed by evaluation of specific suites of individual blood chemistries to assess renal, hepatic or pancreatic function.

These findings may infer that the changes noted are associated with host immunological function; however, given that the BCG-vaccine is comprised of attenuated *M. bovis* that can persist in host tissues [79], and the pathological persistence of virulent *M. bovis* in infected hosts, it is plausible that some VOC changes reflect microbial and/or host metabolic, homeostatic, or physiological processes. In this study, five of our tentatively identified compounds have been associated with *M. tuberculosis* metabolism. Because reference libraries are not exclusive or complete, and the likelihood that biological samples contain unknown compounds, it is important to consider all potential chemical or metabolic matches [80]. Attempting to identify unknown compounds, or those with a low probability matches using a battery of analytical standards is cost prohibitive and was not performed in this study.

We can state with confidence that our findings demonstrate it is possible to differentiate between non- and BCG-vaccinated Holstein cattle before and after virulent *M. bovis* challenge using fecal VOCs collected by methods similar but more refined than those used in a similar study of white-tailed deer [38]. Two compounds identified by both studies (2-dodecanone, 2-methyl pyridine) shared the same trend in peak area fold difference between treatment groups (cattle: NVpost > BCGpostPOS > BCGpostNEG > BCGpre > NVpre; white-tailed deer: NVpost > BCGpostNEG > BCGpre > NVpre, there were no BCGpostPOS deer in this study). A third compound (1H-indole) followed the described sample progression pattern in white-tailed deer, and progressed by treatment group in cattle as NVpost > BCGpostPOS > BCGpostNEG > NVpre = BCGpre, results that are similar and warrant further exploration. None of the compounds found in the suite of breath VOCs used to discriminate between healthy and virulent *M. bovis* infected cattle in our pilot study were identified here [32].

### Table 5. VOC suites allowing discrimination between sample groups.

| Group Comparison          | Peaks                                                                 |
|---------------------------|----------------------------------------------------------------------|
|                           | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| NVpre vs. NVpost          | 12 | 6 |
| BCGpreNEG vs. BCGpostNEG  | 10 | 7 |
| BCGprePOS vs. BCGpostPOS  | 11 | 6 |
| NVpre vs. BCGpre          | 1  | 1 |
| NVpre vs. BCGpostNEG      | 11 | 4 |
| NVpre vs. BCGpostPOS      | 11 | 4 |
| NVpost vs. BCGpre         | 10 | 5 |
| NVpost vs. BCGpostNEG     | 8  | 4 |
| NVpost vs. BCGpostPOS     | 7  | 2 |
| BCGpostNEG vs. BCGpostPOS | 2  | 2 |

Peak area mean fold differences were calculated for dependent (pre- vs. post-challenge) and independent (vaccination and infection status) treatment group comparisons. Suites of peaks with fold differences meeting a minimum selection criteria (>= 3.0) were identified for each comparison. Peaks with greatest mean fold differences are denoted in black. Peaks with lower mean fold differences are noted in gray. Peaks that do not meet the selection criteria are denoted in white.

https://doi.org/10.1371/journal.pone.0179914.t005
Table 6. Potential sources of peak compounds, and order of sample group mean peak areas.

| Peak | Tentative Compound Identification | Tentative Associations [32, 38, 54, 55, 58–77][85, 86] | Order of Mean Peak Area Fold Difference |
|------|----------------------------------|--------------------------------------------------------|----------------------------------------|
| 1    | Dimethyl sulfide                 | • Found in culture headspace of *M. bovis* BCG          | BCGpostPOS = BCGpostNEG > NVpost > NVpre = BCGpre |
|      |                                  | • Produced by bacterial metabolism of methanethiol       |                                        |
|      |                                  | • *M. tuberculosis* and other mycobacteria possess the mddA gene, which encodes a methyltransferase that generates dimethyl sulfide via methylation of methanethiol |                                        |
|      | 3-methylthiophene                | • Rumen byproduct                                        |                                        |
| 2    | Benzaldehyde                     | • Found in some plant material                           | NVpost > BCGpostPOS = BCGpostNEG > BCGpre > NVpre |
|      |                                  | • Found in feces of humans and animals infected with *Clostridium difficile* or *Campylobacter jejuni* |                                        |
|      | Dimethyl sulfone (methylsulfonylmethane, MSM) | • Byproduct of intestinal bacterial metabolism             |                                        |
|      |                                  | • Occurs in some plants                                   |                                        |
|      |                                  | • Normal concentrations in human plasma and CSF          |                                        |
|      |                                  | • VOC associated with rumen metabolism                   |                                        |
| 3    | Unknown                          |                                                         | BCGpostPOS = BCGpostNEG > NVpost > BCGpre > NVpre |
| 4    | Methoxy-phenyl-oxime             | • Imines are common in nature. Example: Vitamin B6 promotes the deamination of amino acids via the formation of imines | NVpost > BCGpostPOS = BCGpostNEG > BCGpre > NVpre |
| 5    | 2-Methylpyridine (2-picoline)    | • May have some inhibitory function against mycobacterial pantothenate synthetase | BCGpostPOS > BCGpostNEG > NVpost > BCGpre > NVpre |
| 6    | Citrulline                       | • Produced by ornithine and carbamoyl phosphate in a central reaction of the urea cycle. | NVpre > BCGpre > BCGpostPOS = BCGpostNEG |
|      |                                  | • Produced as a byproduct during nitric oxide synthesis from arginine. Nitric oxide is synthesized by macrophages from extracellular arginine during early infection, generating citrulline as a byproduct. If extracellular arginine is depleted, reverse synthesis of arginine from citrulline sustains NO production via arginosuccinate synthase. Macrophages lacking this enzyme do not salvage citrulline and are ineffective in controlling mycobacterial infection. |                                        |
| 7    | Unknown                          |                                                         | NVpre > BCGpostPOS > BCGpostNEG = NVpost > BCGpre |
| 8    | Unknown                          |                                                         | NVpost > BCGpostPOS > BCGpostNEG > NVpre = BCGpre |
| 9    | 2-dodecanone                     | • Found in alcoholic beverages                           | NVpre > BCGpre > NVpost > BCGpostPOS > BCGpostNEG |
|      |                                  | • Found in rue, hop, and tomato leaf oils.               |                                        |
|      |                                  | • Identified in feces of white-tailed deer.              |                                        |
|      |                                  | • VOC produced by cattle.                                |                                        |
| 10   | Chlorate                         | • Term includes salts of chloric acid. Natural deposits are found in nature. | BCGpostPOS = BCGpostNEG > NVpost > BCGpre = NVpre |
|      |                                  | • Various microorganisms are capable of reducing chlorate to chloride. Perchlorate reducing bacteria utilize chlorate as a terminal electron acceptor. |                                        |
|      |                                  | • Selective inhibitor of PAPS, which is required by HEVs in lymphoid tissues which support lymphocyte extravasation from blood. |                                        |
|      |                                  | • Can be present in food, chlorinated water, or in some disinfectants |                                        |

(Continued)
| Peak | Tentative Compound Identification | Tentative Associations [32, 38, 54, 55, 58–77]85, 86 | Order of Mean Peak Area Fold Difference |
|------|----------------------------------|---------------------------------------------|--------------------------------------|
| 11   | Indole                           | • VOC produced by cattle. • Produced by bacteria as a part of tryptophan metabolism. • Regulates various aspects of bacterial physiology such as spore formation, plasmid stability, drug resistance, biofilm formation and virulence. The tryptophan biosynthetic pathway is integral to M. tuberculosis survival. | NVpost > BCGpos > NVpre = BCGpre = BCGpostNEG |
| 12   | 2, 6, 10, 14-tetramethylhexadecane (phytane) | • Derivative of chlorophyll. • Found in bovine liver, heart, muscle, fat. • Has been described as a compound capable of binding to CYP124, a catabolic P450 enzyme involved in M. tuberculosis biosynthesis and metabolism. | NVpost > BCGpos > BCGpostNEG > NVpre = BCGpre |
|      | 5-hydroxylysine                  | • Amino acid derivative of lysine found in collagenous tissue including bovine conglutinin, a serum protein capable of binding to immune complexes via complement component C3bi. • Lysine is converted to hydroxylysine in the biosynthesis of mycobactin, a high density lipid hexdentate iron ligand utilized by M. tuberculosis to scavenge host non-heme iron for DNA synthesis and respiration. | |
| 13   | 3, 7, 11, 15-tetramethyl-2-hexadecene-1-ol (phytol) | • Liberated from ruminant gut fermentation of plants, converted to phytic acid and stored in fats. • Decomposition product of chlorophyll. • Found in tocopherol (Vitamin E), and phylloquinone (Vitamin K1). • Has been observed to act as a ligand and will bind with PKS 18 and AccDS receptors of M. tuberculosis. • Phytanic acid is bound by M. tuberculosis CYP124 receptors. | NVpost = BCGpos > BCGpostNEG > BCGpre > NVpre |
|      | Succinic acid (butanedoic acid)  | • Occurs naturally in plants, animals, soils. • Anion (succinate) is a component of the citric acid cycle. • Succinate dehydrogenase (SDH) is important in mitochondrial function (part of respiratory chain and Krebs cycle). • Produced by fermentation of glucose. • Succinic acid producing bacteria have been isolated from the rumen of cattle. • M. tuberculosis utilizes the anion (succinate) to sustain membrane potential, ATP synthesis, and anaplerosis, and can remodel the tricarboxylic acid cycle to increase production of succinate in response to hypoxia. • Methyl-nicotinate has been reported as a potential biomarker for M. tuberculosis. Nicotinamide is a compound with known tuberculocidal activity. | |

Metabolomics data base and literature searches were utilized to identify potential sources and associations of the VOCs identified. Sample groups are arranged in descending order of mean peak area values to demonstrate how each VOC may be used to identify sample groups.

https://doi.org/10.1371/journal.pone.0179914.t006
Table 7. Potential sites of origin; cellular and biofluid locations; and biofunctions of tentatively identified volatile organic compounds.

| Peak | Tentative Compound Identification | Origin | Cellular Location | Biofluid or Tissue | Biofunction |
|------|-----------------------------------|--------|-------------------|-------------------|-------------|
| 1    | Dimethyl sulfide                  | • Endogenous • Microbial | • Cytoplasm      | • Blood • CSF • Feces • Urine • Fat • Intestine • Kidney • Liver | • Osmolyte • Enzyme cofactor • Signaling • Waste products • Sulfur metabolism |
| 2    | Benzaldehyde                       | • Endogenous | • Blood • Feces • Saliva |                          | • Toluene degradation |
|      | Dimethyl sulfone (methylsulfonylmethane, MSM) | • Endogenous • Microbial | • Cytoplasm | • Blood • CSF • Urine • Saliva | • Osmolyte • Enzyme cofactor • Signaling • Sulfur metabolism • Waste product • Methanethiol metabolism |
| 3    | Unknown                            |        |                  |                   |             |
| 4    | Methoxy-phenyl-oxime              |        |                  |                   | • Imines are common in nature. Example: Vitamin B6 promotes the deamination of amino acids via the formation of imines |
| 5    | 2-Methylpyridine (2-picoline)     |        |                  |                   |             |
| 6    | Citrulline                         | • Endogenous | • Mitochondria | • Blood • CSF • Saliva • Urine • Epidermis • Fibroblasts • GIT • Kidney • Liver • Neural Tissue • Placenta • Platelet • Prostate | • Byproduct of Urea Cycle • Arginine biosynthesis. • Aspartate, alanine and proline metabolism. • Biosynthesis of amino acids |
| 7    | Unknown                            |        |                  |                   |             |
| 8    | Unknown                            |        |                  |                   |             |
| 9    | 2-dodecanone                      | • Endogenous • Food | • Membrane | • Feces • Saliva | • Nutrient |
|      | 2-chloro-1-propanol               | • Endogenous • Food | • Cytoplasm • Extracellular |                         | • Nutrient |
| 10   | Chlorate                           | • Endogenous |                  |                   | • Osmolyte • Enzyme cofactor • Signaling |

(Continued)
factors such as the sample (breath vs. feces); VOC collection method (sorbent tubes vs. SPME); and differences in GCMS analysis methods may have contributed to this disparity. Five identified compounds (1H-indole, 2-methyl pyridine, benzaldehyde, dimethyl sulfide, dimethyl sulfoxide) have been associated with normal ruminant physiology in cattle [61, 81]. Dimethyl sulfide has been previously reported in the literature as a compound associated with *M. bovis* cultures [58]. Eight of our tentatively identified compounds have not been previously reported in our prior studies or elsewhere.

We were not successful in detecting a specific candidate VOC biomarker to indicate BCG vaccination or pathogenic *M. bovis* exposure/infection. Tentative biomarkers associated with *M. tuberculosis* infection have been suggested [19, 58, 82, 83]; however, because of the

| Peak | Tentative Compound Identification | Origin | Cellular Location | Biofluid or Tissue | Biofunction |
|------|----------------------------------|--------|-------------------|--------------------|-------------|
| 11   | Indole                           | • Endogenous  
      |        | • Membrane        | • Epidermis        | • Tryptophan metabolism  
      |        |                  | • Feces            | • Phenylalanine, tyrosine, tryptophan biosynthesis  
      |        |                  | • Saliva           | • Protein digestion and absorption  
      |        |                  | • Urine,          |                                                |
|      |                                  | Microbial | Membrane          | • GIT               |                                                |
|      |                                  |          |                   | • Neural Tissues    |                                                |
| 12   | 2, 6, 10, 14-tetramethylhexadecane (phytane) | Endogenous | Cytoplasm         | Blood, Urine       | • Protein synthesis  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Amino acid biosynthesis  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Lysine degradation  
      |        |                  |                    |                                                |
| 13   | 5-hydroxylysine                  | Endogenous | Cytoplasm         | Blood, Urine       | • Protein synthesis  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Amino acid biosynthesis  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Lysine degradation  
      |        |                  |                    |                                                |
| 13   | 3, 7, 11, 15-tetramethyl-2-hexadecene1-ol (phytol) | Food | Extracellular      | Fibroblasts        | • Nutrient  
      |        |                  | • Membrane         |                                                |
|      |                                  |          |                   |                    | • Stabilizer  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Surfactants  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Emulsifier  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Cell signaling  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Fuel and energy storage  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Fuel or energy source  
      |        |                  |                    |                                                |
|      |                                  |          |                   |                    | • Membrane integrity and stability  
      |        |                  |                    |                                                |
| Succinic acid (butanedioic acid) | Endogenous  
      |        | Extracellular      | Blood, CSF, Feces,  
      | Microbial | • Membrane         | Saliva, Urine, Fat,  
      |          | • Mitochondria     | • Mitochondrial electron transport chain  
      |          | • Endoplasmic reticulum | • Citrate cycle  
      |          | • Peroxisome       | • Glucagon signaling pathway  
      |          |                  | • Liver            | • Succinate dehydrogenase component  
      |          |                  | • Muscle           | • Nicotinate and nicotinamide metabolism  
      |          |                  | • Pancreas         |                                                |
|      |                                  |          |                   |                    | • Placenta  
      |          |                  | • Spleen           |                                                |
|      |                                  |          |                   |                    |                                                |

Metabolomics data base and literature searches were used to identify biological sites of origin, and physiological locations and biofunctions of the tentatively identified VOCs.

https://doi.org/10.1371/journal.pone.0179914.t007
influence of diet, behavior, and other factors, identification of one or two compounds as specific indicators of infection by a unique pathogen is difficult. For example, methyl-nicotinate, a compound proposed as a *M. tuberculosis* biomarker, can be found in the breath of smokers [84], is used as a flavoring ingredient, and is present in coffee, various nuts, alcoholic beverages, and fruits [54, 55]. Instead, we demonstrate that a suites of VOCs may be used to discriminate between unique treatment groups prior and after challenge with virulent *M. bovis*. If the VOCs identified in this study are associated only with non-specific host adaptive or immune responses to pathogen presence, comparative research must be conducted exploring the suites of VOCs produced by other infectious diseases of cattle.

Strengths of our study include analysis of samples from physically similar Holstein steers housed in controlled environmental and husbandry conditions, and exposed to a known concentration of *M. bovis* inoculum. Weaknesses include changes in our collection and sample analysis method which were intentional and done with the understanding that retrospective comparison to previous results may be compromised. The small number of samples available for analysis was unavoidable given the expense of housing large ungulates in confinement; however, this study represents the third time we have been able to discriminate between non-infected and *M. bovis* infected animals using VOCs present in breath [32] or feces [38]. Repeatability infers reliability, and repeated studies will allow compilation of a library of identified VOCs that will better define suites of VOCs appropriate for identification of disease or vaccination status.

Developing a disease surveillance modality that uses a readily accessible biological sample (feces) collected directly from domestic livestock would improve surveillance strategies by reducing or eliminating animal handling events, decrease animal stress, allow better opportunity for retesting and storage of samples, eliminate the need for specialized laboratory training, and improve test turnaround time. Use of feces collected from environments where wildlife reservoirs of *M. bovis* or other significant pathogens reside would vastly improve the ability of wildlife managers to assess the health status of wildlife, develop wildlife management strategies enhancing control of agriculturally important diseases, and provide public health support. The capability to differentiate between BCG-vaccinated and non-vaccinated animals prior to and after exposure to *M. bovis* would provide a crucial surveillance modality to control and eradicate bovine tuberculosis in domestic animal and wildlife reservoir populations. Continued use and refinement of our sampling method and analysis is therefore designed to lead toward development of a portable, labor and cost efficient tool that can accurately identify non-vaccinated and/or vaccinated domestic and wild animals infected with *M. bovis* or other pathogens of zoonotic or agricultural importance.

Supporting information

**S1 Table.** Study timeline from initiation of study to completion. This table documents all diagnostic and fecal sampling time-points, age at vaccination and *M. bovis* challenge, and all other procedures pertinent to the study beginning with purchase of calves through euthanasia and necropsy. (TIF)

**S2 Table.** Comparative differences in mean peak areas of sample groups. Differences in mean peak area were calculated for dependent (pre- vs. post-challenge) and independent (vaccination and infection status) comparisons. A minimum fold difference < 3.0 criteria was used to identify suites of VOCs useful in discriminating between sample groups. (TIF)
S1 Fig. Response to PPDb by calves 104 days after virulent *M. bovis* challenge. Blue bars indicate skin thickness (millimeters) prior to injection of PPDb. Skin thickness 72 hours post-PPDb injection, mean, and standard deviation of measurements for non-vaccinated (NVpost) BCG-vaccinated *M. bovis* positive (BCGpostPOS); and BCG-vaccinated virulent *M. bovis* negative calves (BCGpostNEG) are indicated by red, purple and orange bars, respectively. All calves were classified as reactors based on standard interpretation of the CCT [42]. Mean responses for NVpost calves were greater than those of BCGpostPOS and BCGpostNEG calves [56].

(TIF)

S2 Fig. Responses to PPDa and PPDb by calves 104 days after virulent *M. bovis* challenge. Blue bars represent changes in skin thickness (millimeters) 72 hours post-administration of PPDa. Differences in response to PPDb 72 hours post-administration by NVpost; BCGpostPOS; and BCGpostNEG cattle are indicated by red, purple, and orange bars, respectively. Difference in skin thickness in response to PPDa are lower than the changes noted in skin thickness in response to PPDb in all calves. Responses to PPDb are significantly greater in NVpost calves [56].

(TIF)

S3 Fig. XCMS analysis sample groups using 105 statistically significant ions. Sample chromatograms are aligned and overlaid onto the x-axis. Features with high m/z ratios are represented by the dots farthest above the x-axis. Size of circles equates to degree of fold change (features with greatest fold change have the largest radii). Color intensity of the circles corresponds to the statistical significance (p-value) of the fold change as calculated by a Welch t-test with unequal variances (darker color = lower p-value).

(TIF)

Acknowledgments

The authors would like to thank the Dr. Rebecca Cox for excellent clinical veterinary care of the calves and guidance/oversight of biosafety and security procedures within the BSL-3 animal facility. Special thanks to the Animal Care staff at NADC for their assistance with all animal sampling. We would like to thank Nathan Snow, Allen Franklin and Susan Shriner for their assistance in our statistical analysis.

Author Contributions

**Conceptualization:** Christine K. Ellis, Somchai Rice, Devin Maurer, W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren, Jacek A. Koziel.

**Data curation:** Christine K. Ellis, Somchai Rice, Devin Maurer.

**Formal analysis:** Christine K. Ellis, Somchai Rice, Randal Stahl, Jacek A. Koziel.

**Funding acquisition:** W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren.

**Investigation:** Christine K. Ellis, Somchai Rice, Devin Maurer, W. Ray Waters, Mitchell V. Palmer.

**Methodology:** Christine K. Ellis, Somchai Rice, Randal Stahl.

**Project administration:** Christine K. Ellis, Somchai Rice, Devin Maurer, W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren, Jacek A. Koziel.
Resources: Christine K. Ellis, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren.

Software: Christine K. Ellis, Somchai Rice, Randal Stahl.

Supervision: Christine K. Ellis, W. Ray Waters, Mitchell V. Palmer, Jacek A. Koziel.

Validation: Christine K. Ellis, Somchai Rice, Devin Maurer.

Visualization: Christine K. Ellis, Devin Maurer.

Writing – original draft: Christine K. Ellis, Somchai Rice, Devin Maurer.

Writing – review & editing: Christine K. Ellis, Somchai Rice, Devin Maurer, Randal Stahl, W. Ray Waters, Mitchell V. Palmer, Pauline Nol, Jack C. Rhyan, Kurt C. VerCauteren, Jacek A. Koziel.

References

1. Biet F, Boschirolli ML, Thorel MF, Guilloteau LA. Zoonotic aspects of Mycobacterium bovis and Mycobacterium avium-intracellulare complex (MAC). Vet Res. 2005; 36(3): 411–436. Epub 2005/04/23. https://doi.org/10.1051/vetres:2005001 PMID: 15845232.

2. Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, et al. Bovine Tuberculosis: A Review of Current and Emerging Diagnostic Techniques in View of their Relevance for Disease Control and Eradication. Transbound Emerg Dis. 2010; 57(4): 205–220. https://doi.org/10.1111/j.1865-1682.2010.01148.x PMID: 20561288

3. Organization WH. Global tuberculosis report 2015: World Health Organization; 2015.

4. Müller B, Dürr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, et al. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect Dis. 2013; 19(6): 899–908. https://doi.org/10.3201/eid1906.120543 PMID: 23735540

5. Cosivi O, Grange J, Daborn C, Raviglione M, Fujikura T, Cousins D, et al. Zoonotic tuberculosis due to Mycobacterium bovis in developing countries. Emerging infectious diseases. 1998; 4(1): 59–70. https://doi.org/10.3201/eid0401.980108 PMID: 9452399

6. Cleaveland S, Shaw DJ, Mfinanga SG, Shirima G, Kazwala RR, Eblate E, et al. Mycobacterium bovis in rural Tanzania: Risk factors for infection in human and cattle populations. Tuberculosis. 2007; 87(1): 30–43. https://doi.org/10.1016/j.tube.2006.03.001 PMID: 16618533

7. de la Rua-Domenech R, Goodchild AT, Vordermeier HM, Hewinson RG, Christiansen KH, Clifton-Hadley RS. Anti-mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Res Vet Sci. 2006; 81(2): 190–210. http://dx.doi.org/10.1016/j.rvsc.2005.11.005. PMID: 16513150

8. Grange J. <i>Mycobacterium bovis</i> infection in human beings. Tuberculosis. 2001; 81(1): 71–77.

9. Waters W, Stevens GE, Schoenbaum MA, Orloski KA, Robbe-Austerman S, Harris NB, et al. Bovine tuberculosis in a Nebraska herd of farmed elk and fallow deer: a failure of the tuberculin skin test and opportunities for serodiagnosis. Vet Med Int. 2011; 2011.

10. Palmer MV, Whipple DL, Waters WR. Tuberculin skin testing in white-tailed deer (Odocoileus virginianus). J Vet Diagn Invest. 2001; 13(6): 530–533. https://doi.org/10.1177/104063870101300614 PMID: 11724147

11. Cosgrove MK, Campa H, Schmitt SM, Marks DR, Wilson AS, O’Brien DJ. Live-trapping and bovine tuberculosis testing of free-ranging white-tailed deer for targeted removal. Wildlife Res. 2012; 39(2): 104–111.

12. Miller RS, Sweeney SJ. Mycobacterium bovis (bovine tuberculosis) infection in North American wildlife: current status and opportunities for mitigation of risks of further infection in wildlife populations. Epidemiol and Infect. 2013; 141(7): 1357–1370.

13. Schmitt SM, O’Brien DJ, BRUNING-FANN CS, Fitzgerald SD. Bovine tuberculosis in Michigan wildlife and livestock. Ann N Y Acad Sci. 2002; 969(1): 262–268.

14. Palmer MV. Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife. Wildlife and Emerging Zoonotic Diseases: The Biology, Circumstances and Consequences of Cross-Species Transmission: Springer; 2007. pp. 195–215.

15. O’Brien DJ, Schmitt SM, Fierke JS, Hogle SA, Winterstein SR, Cooley TM, et al. Epidemiology of Mycobacterium bovis in free-ranging white-tailed deer, Michigan, USA, 1995–2000. Prev Vet Med. 2002; 54 (1): 47–63. PMID: 12062519
16. Liddell K. Smell as a diagnostic marker. Postgrad Med J. 1976; 52(605): 136–138. https://doi.org/10.1136/pgmj.52.605.136 PMID: 1264934

17. Garner CE, Smith S, Bardhan PK, Ratcliffe NM, Probert CSJ. A pilot study of faecal volatile organic compounds in faeces from cholera patients in Bangladesh to determine their utility in disease diagnosis. Trans R Soc Trop Med Hyg. 2009; 103(11): 1171–1173. http://dx.doi.org/10.1016/j.trstmh.2009.02.004. PMID: 19268999

18. Probert C, Ahmed I, Khalid T, Johnson E, Smith S, Ratcliffe N. Volatile organic compounds as diagnostic biomarkers in gastrointestinal and liver diseases. J Gastrointestin Liver Dis. 2009; 18(3): 337–343. PMID: 19795029

19. Phillips M, Basa-Dalay V, Bothamley G, Cataneo RN, Lam PK, Natividad MPR, et al. Breath biomarkers of active pulmonary tuberculosis. Tuberculosis. 2010; 90(2): 145–151. http://dx.doi.org/10.1016/j.tube.2010.01.003. PMID: 20189456

20. Chen S, Zieve L, Mahadevan V. Mercaptans and dimethyl sulfide in the breath of patients with cirrhosis of the liver. Effect of feeding methionine. J Lab Clin Med. 1970; 75(4): 628–635. PMID: 5444348

21. Kaji H, Hisamura M, Saito N, Murao M. Gas chromatographic determination of volatile sulfur compounds in the expired alveolar air in hepatopathic subjects. J Chromatogr. 1978; 145(3): 464–468. PMID: 659533

22. Leopold DA, Preti G, Mozell MM, Youngentob SL, Wright HN. Fish-odor syndrome presenting as dysosmia. Arch Otalaryngol Head Neck Surg. 1990; 116(3): 354–355. https://doi.org/10.1001/archotol.1990.01870030180222. PMID: 2306356

23. Phillips M, Boehmer JP, Cataneo RN, Cheema T, Eisen HJ, Fallon JT, et al. Heart allograft rejection: detection with breath alkanes in low levels (the HARDBALL study). J Heart Lung Transplant. 2004; 23(6): 701–708. http://dx.doi.org/10.1016/j.healun.2003.07.017. PMID: 15366430

24. Phillips M, Cataneo RN, Ditkoff BA, Fisher P, Greenberg J, Gunawardena R, et al. Prediction of breast cancer using volatile biomarkers in the breath. Breast Cancer Res Treat. 2006; 99(1): 19–21. https://doi.org/10.1007/s10549-006-9176-1 PMID: 16502014

25. Phillips M, Gleeson K, Hughes JMB, Greenberg J, Cataneo RN, Baker L, et al. Volatile organic compounds in breath as markers of lung cancer: a cross-sectional study. Lancet. 1999; 353(9168): 1930–1933. http://dx.doi.org/10.1016/S0140-6736(98)07552-7. PMID: 10371572

26. Shirasu M, Touhara K. The scent of disease: volatile organic compounds of the human body related to disease and disorder. J Biochem. 2011; 150(3): 257–266. https://doi.org/10.1093/jb/mvr090 PMID: 21771869

27. Weidong M, Clement B, Klemm W. Cyclic changes in volatile constituents of bovine vaginal secretions. JChem Ecol. 1995; 21(12): 1895–1906.

28. Burciaga-Robles LO, Holland BP, Step DL, Krehbiel CR, McMillen GL, Richards CJ, et al. Evaluation of breath biomarkers and serum haptoglobin concentration for diagnosis of bovine respiratory disease in heifers newly arrived at a feedlot. Am J Vet Res. 2009; 70(10): 1291–1298. https://doi.org/10.2460/ajvr.70.10.1291 PMID: 19795945

29. Turner C, Knobloch H, Richards J, Richards P, Mottram TTF, Martin D, et al. Development of a device for sampling cattle breath. Biosyst Eng. 2012; 112(2): 75–81. https://doi.org/10.1016/j.biosystemseng.2012.03.001.

30. Ellis CK, Stahl RS, Nol P, Waters WR, Palmer MV, Ryhan JC, et al. A Pilot Study Exploring the Use of Breath Analysis to Differentiate Healthy Cattle from Cattle Experimentally Infected with Mycobacterium bovis. PLoS One. 2014; 9(2): e89280. https://doi.org/10.1371/journal.pone.0089280 PMID: 24586655

31. Parkhurst R, Köhler H, Liebler-Tenorio E, Meyer M, Becher G, Kikowatz A, et al. Chronic intestinal Mycobacteria infection: discrimination via VOC analysis in exhaled breath and headspace of feces using differential ion mobility spectrometry. J Breath Res. 2011; 5(2): 027103. https://doi.org/10.1088/1752-7155/5/2/027103 PMID: 21512209

32. Elliott-Martin R, Mottram T, Gardiner J, Hobbs P, Bartlett P. Preliminary investigation of breath sampling as a monitor of health in dairy cattle. J Agr Eng Res. 1997; 67(4): 267–275.

33. Mottram TT, Dobbelaar P, Schukken YH, Hobbs PJ, Bartlett PN. An experiment to determine the feasibility of automatically detecting hyperketonaemia in dairy cows. Livest Prod Sci. 1999; 61(1): 7–11. http://dx.doi.org/10.1016/S0301-6226(99)00045-7.
36. Bergmann A, Trefz P, Fischer S, Klepik K, Walter G, Steffens M, et al. In vivo volatile organic compound signatures of Mycobacterium avium subsp. paratuberculosis. PLoS One. 2015; 10(4): e0123980. https://doi.org/10.1371/journal.pone.0123980 PMID: 25915653

37. Knobloch H, Köhler H, Commander N, Reinhold P, Turner C, Chambers M, editors. Volatile organic compound (VOC) analysis for disease detection: proof of principle for field studies detecting paratuberculosis and brucellosis. OLFACTION AND ELECTRONIC NOSE: Proceedings of the 13th International Symposium on Olfaction and Electronic Nose; 2009: AIP Publishing.

38. Stahl R, Ellis C, Nol P, Waters W, Palmer M, Vercauteren K, editors. Head space analysis to non-invasively distinguish between vaccinated and bovine tuberculosis-infected white-tailed deer. Meeting Abstract; 2014: Abstract.

39. Bayn A, Nol P, Tisch U, Rhyán J, Ellis CK, Haick H. Detection of Volatile Organic Compounds in Brucella abortus-Seropositive Bison. Anal Chem. 2013; 85(22): 11146–11152. https://doi.org/10.1021/ac403134f PMID: 24156543

40. Larsen MH, Biermann K, Jacobs WR. Laboratory Maintenance of Mycobacterium tuberculosis. Current Protocols in Microbiology: John Wiley & Sons, Inc.; 2005.

41. Palmer M, Ray Waters W, Whipple D. Aerosol delivery of virulent< i> Mycobacterium bovis to cattle. Tuberculosis. 2002; 82(6): 275–282. PMID: 12623270

42. USDA. Bovine Tuberculosis Eradication Uniform Methods and Rules 602 (APHIS 91-45-011). US Government Printing Office, Washington, DC. 2005; 603: 1–29.

43. Palmer MV, Thacker TC, Waters WR, Robbe-Austerman S. Oral Vaccination of White-Tailed Deer (Odocoileus virginianus) with Mycobacterium bovis Bacillus Calmette-Guerin (BCG). PLoS One. 2014; 9(5): e97031. https://doi.org/10.1371/journal.pone.0097031 PMID: 24804678

44. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CFC, Estes DM, et al. Efficacy and immunogenicity of Mycobacterium bovis ΔRD1 against aerosol M. bovis infection in neonatal calves. Vaccine. 2009; 27(8): 1201–1209. PMID: 19135497

45. Thacker TC, Harris B, Palmer MV, Waters WR. Improved specificity for detection of Mycobacterium bovis in fresh tissues using IS6110 real-time PCR. BMC Vet Res. 2011; 7(1): 50.

46. Rinehart D, Johnson CH, Nguyen T, Ivanisevic J, Benton HP, Lloyd J, et al. Metabolomic data streaming for biological-dependent data acquisition. Nature Biotechnol. 2014; 32(6): 524–527.

47. Tautenhahn R, Patti GJ, Rinehart D, Siuzdak G. XCMS Online: a web-based platform to process untargeted metabolomic data. Anal Chem. 2012; 84(11): 5035–5039. https://doi.org/10.1021/ac300698c PMID: 22533540

48. Varmuza K, Filzmoser P. Introduction to Multivariate Statistical Analysis in Chemometrics. 2009. CRC, Boca Raton, FL.

49. Venables W, Ripley B. Modern Applied Statistics Using S. Springer, New York, NY, USA; 2002.

50. Kaufman L, Rousseeuw PJ. Finding groups in data: an introduction to cluster analysis: John Wiley & Sons; 2009.

51. Fletcher RH, Fletcher SW, Fletcher GS. Clinical epidemiology: the essentials: Lippincott Williams & Wilkins; 2012.

52. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000; 28 (1): 27–30. PMID: 10592173

53. Aoki KF, Kanehisa M. Using the KEGG Database Resource. Current Protocols in Bioinformatics: John Wiley & Sons, Inc.; 2002.

54. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, et al. HMDB 3.0—The Human Metabolome Database in 2013. Nucleic Acids Res. 2012. https://doi.org/10.1093/nar/gks1065 PMID: 23161693

55. Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, et al. HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res. 2009; 37(suppl 1): D603–D610.

56. Waters WR, Maggioli MF, Palmer MV, Thacker TC, McGill JL, Vordermeier HM, et al. Interleukin-17A as a Biomarker for Bovine Tuberculosis. Clin Vaccine Immunol. 2016; 23(2): 168–180.

57. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012; 13(4): 263–269. https://doi.org/10.1038/nrm3314 PMID: 22436749

58. McNerney R, Mallard K, Okolo PI, Turner C. Production of volatile organic compounds by mycobacteria. FEMS Microbiol Lett. 2012; 328(2): 150–156. https://doi.org/10.1111/j.1574-6988.2011.02493.x PMID: 22224870

59. Nakajima K. Amelioration effect of a tertiary sulfonium, dimethylsulfiniopropionate in green algae on Ehrlich ascites tumor, solid tumor and related diseases. Anti-cancer drugs from marine origins New York: Springer Publishing. 2014.
60. Carrión O, Curson A, Kumaresan D, Fu Y, Lang A, Mercadé E, et al. A novel pathway producing dimethylsulphide in bacteria is widespread in soil environments. Nat Commun. 2015; 6; 6579 https://doi.org/10.1038/ncomms7579 PMID: 25807229

61. Cali L, Koziel JA, Davis J, Lo Y-C, Xin H. Characterization of volatile organic compounds and odors by in-vivo sampling of beef cattle rumen gas, by solid-phase microextraction, and gas chromatography–mass spectrometry–olfactometry. Anal Bioanal Chem. 2006; 386(6): 1791–1802. https://doi.org/10.1007/s00216-006-0799-1 PMID: 17069001

62. Engelke UF, Tangerman A, Willemse MA, Moskau D, Loss S, Mudd SH, et al. Dimethyl sulfone in human cerebrospinal fluid and blood plasma confirmed by one-dimensional 1H and two-dimensional 1H-13C NMR. NMR Biomed. 2005; 18(5): 331–336. https://doi.org/10.1002/nbm.15996001

63. Hung AW, Silvestre HL, Wen S, George GP, Boland J, Blundell TL, et al. Optimization of inhibitors of Mycobacterium tuberculosis pantothenate synthetase based on group efficiency analysis. ChemMedChem. 2016; 11(11): 38–42. https://doi.org/10.1002/cmdc.201500414 PMID: 26486566

64. Qualis JE, Subramanian C, Raff W, Smith AM, Balouzas AA, et al. Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. Cell Host Microbe. 2012; 12(3): 313–323. https://doi.org/10.1016/j.chom.2012.07.012 PMID: 22980328

65. Oyarzún M, Palma R, Alberti E, Hormazabal E, Pardo F, Birkett M, et al. Olfactory response of Haematobia irritans (Diptera: Muscidae) to cattle-derived volatile compounds. J Med Entomol. 2009; 46(6): 1320–1326. PMID: 19960676

66. Lee J-H, Lee J. Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev. 2010; 34(4): 426–444. https://doi.org/10.1111/j.1574-6976.2009.00204.x PMID: 20073074

67. Shen H, Wang F, Zhang Y, Huang Q, Xu S, Hu H, et al. A novel inhibitor of indole-3-glycerol phosphate synthase with activity against multidrug-resistant Mycobacterium tuberculosis. FEBS J. 2009; 276(1): 144–154. https://doi.org/10.1111/j.1742-4658.2008.06783.x PMID: 19032598

68. Avigan J, Milne GWA, Hight J.R. The occurrence of pristane and phytane in man and animals. BBA-Lipid Lipid Met. 1967; 144(1): 127–131. http://dx.doi.org/10.1016/0005-2760(67)90084-7.

69. Johnston JB, Kells PM, Podust LM, de Montellano PRO. Biochemical and structural characterization of CYP124: A methyl-branched lipid u-hydroxylase from Mycobacterium tuberculosis. Proc Natl Acad Sci. 2009; 106(49): 20687–20692. https://doi.org/10.1073/pnas.0907398106 PMID: 19933331

70. Lachmann PJ, Müller-Eberhard HJ. The demonstration in human serum of "conglutinogen-activating factor" and its effect on the third component of complement. J Immunol. 1968; 100(4): 691–698.

71. Lee Y-M, Leiby KR, Allar J, Paris K, Lerch B, Okarma TB. Primary structure of bovine conglutin, a member of the C-type animal lectin family. J Biol Chem. 1991; 266(5): 2715–2723. PMID: 1993651

72. Madigan CA, Cheng T-Y, Layre E, Young DC, McConnell MJ, Debono CA, et al. Lipidomic discovery of deoxysiderophores reveals a revised mycobactin biosynthesis pathway in Mycobacterium tuberculosis. Proc Natl Acad Sci. 2012; 109(4): 1257–1262. https://doi.org/10.1073/pnas.1109958109 PMID: 22232695

73. Verhoeven N, Wanders R, Soudubray J-M, Jakobs C. The metabolism of phytanic acid and pristanic acid in man: a review. J Inherit Metab Dis. 1998; 21(7): 697–728. PMID: 9819701

74. Devi CA. Docking study on mycobacterium tuberculosis receptors AccD5 and PKS18 with selected phytochemicals. IOSR-JPBS4. 2012; 4: 1–4.

75. Brière J-J, Favier J, Ghouzzi VE, Djouadi F, Benit P, Gimenez A-P, et al. Succinate dehydrogenase deficiency in human. Cell Mol Life Sci. 2005; 62(19–20): 2317–2324. https://doi.org/10.1007/s00018-005-5237-6 PMID: 16143825

76. Raja S, Dhanasekar R. Succinic acid production from bovine rumen–isolation and optimization. Int J ChemTech Res. 2011; 3(4): 1926–1931

77. Eoh H, Rhee KY. Multifunctional essentiality of succinate metabolism in adaptation to hypoxia in Mycobacterium tuberculosis. Proc Natl Acad Sci. 2013; 110(16): 6554–6559. https://doi.org/10.1073/pnas.1219375110 PMID: 23576728

78. Bass K, Nonnecke B, Palmer M, Thacker T, Hardegger R, Schroeder B, et al. Clinical and diagnostic developments of a gamma interferon release assay for use in bovine tuberculosis control programs. Clin Vaccine Immunol. 2013; 20(12): 1827–1835. https://doi.org/10.1128/CVI.00519-13 PMID: 24132602

79. Palmer M, Thacker T, Waters W, Robbe-Austerman S, Lebepe-Mazur S, Harris N. Persistence of Mycobacterium bovis Bacillus Calmette-Guérin in White-Tailed Deer (Odocoileus Virginianus) after Oral or Parenteral Vaccination. Zoonoses Public Health. 2010; 57(7–8): e206–e12. https://doi.org/10.1111/j.1863-2378.2010.01329.x PMID: 20707863

80. Tsugawa H, Tsujimoto Y, Arita M, Bamba T, Fukusaki E. GC/MS based metabolomics: development of a data mining system for metabolite identification by using soft independent modeling of class analogy
81. Shaw SL, Mitloehner FM, Jackson W, DePeters EJ, Fadel JG, Robinson PH, et al. Volatile organic compound emissions from dairy cows and their waste as measured by proton-transfer-reaction mass spectrometry. Environ Sci Technol. 2007; 41(4): 1310–1316. PMID: 17593735

82. Phillips M, Cataneo RN, Condos R, Ring Erickson GA, Greenberg J, La Bombardi V, et al. Volatile biomarkers of pulmonary tuberculosis in the breath. Tuberculosis. 2007; 87(1): 44–52. https://doi.org/10.1016/j.tube.2006.03.004 PMID: 16635588

83. Nawrath T, Mgone GF, Weetjens B, Kaufmann SH, Schulz S. The volatiles of pathogenic and non-pathogenic mycobacteria and related bacteria. Beilstein J Org Chem. 2012; 8(1): 290–299.

84. Scott-Thomas A, Syhre M, Epton M, Murdoch DR, Chambers ST. Assessment of potential causes of falsely positive Mycobacterium tuberculosis breath test. Tuberculosis. 2013; 93(3): 312–317. https://doi.org/10.1016/j.tube.2013.01.005 PMID: 23499413