In-vitro discrimination of wound associated bacteria by volatile compound profiling using Selected Ion Flow Tube – Mass Spectrometry.

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ABSTRACT

Aims

To determine if bacterial species responsible for clinically relevant wound infection produce specific volatile profiles that would allow their speciation.

Methods and Results

Selected-Ion Flow Tube-Mass Spectrometry in full mass scan mode was used to analyse headspace gases produced by wound-associated bacteria grown *in vitro*, so as to enable identification of bacterial volatile product ion profiles in the resulting mass spectra. Applying multivariate statistical analysis (hierarchical clustering and principal component analysis) to the resultant mass spectra enabled clear speciation. Moreover, bacterial volatile product ions could be detected from artificially contaminated wound dressing material, although the pattern of product ions detected was influenced by culture conditions.

Conclusions

Using selected product ions from the SIFT-MS mass spectra it is possible to discriminate wound-associated bacterial species grown under specific *in vitro* culture conditions.

Significance and Impact of Study

The results of this study have shown that wound associated bacteria can be discriminated using volatile analysis *in vitro* and that bacterial volatiles can be detected from wound dressing material. This indicates that volatile analysis of wounds or dressing material to identify infecting microbes has potential and warrants further study.

KEYWORDS

Bacterial Metabolism, Selected Ion Flow Tube – Mass Spectrometry, Species Discrimination, Volatile Compound, Wound.

INTRODUCTION

Infection of the wound bed is detrimental to healing, resulting in a failure of the wound to move through the phases of healing in a timely manner (Bowler *et al.* 2001; Werthen *et al.* 2010). This has been demonstrated experimentally in animal models (Gurjala *et al.* 2011; Pastar *et al.* 2013). Colonisation and infection of the wound bed develops and changes over time and is dependent on body location; early colonisers are likely to originate from the patient's own skin flora and include coagulase negative staphylococci and *Staphylococcus aureus*. Subsequently, Gram negative rods derived from the gastrointestinal, oral and genitourinary mucosa, and from the local environment may begin to invade (Bowler *et al.* 2001). As wounds mature it is known that anaerobes will play a greater role, as the wound microenvironment is changed by autogenic succession (Bowler *et al* 2001; Daeschlein 2013). Early diagnosis and management of clinically relevant wound infection is essential to avoid complications. Clinicians currently rely on clinical symptoms, signs and non-specific laboratory tests for indicators of infection for early diagnosis. Wound and blood cultures are often utilised, but can take several days to be reported and interpretation in the context of wound infection can be difficult (Macgregor *et al.* 2008; Barajas-Nava *et al.* 2013; Blokhuis-Arkes *et al.* 2015). Burn wounds present additional challenges; the release of high levels of inflammatory mediators result from an altered baseline metabolic profile and a systemic response to injury seen in the burns patient (Greenhalgh *et al.* 2007; Jeschke and Herndon 2014). Standard indicators of infection are therefore difficult to apply and experienced burns specialists are required to attempt to identify subtle signs of infection without definitive point of care testing available (Greenhalgh *et al.* 2007). The World Health Organisation (2014) estimates that 265,000 deaths worldwide occur annually as a direct result of burn wound injuries. Sepsis and other complications resulting from infection are the number one cause of mortality in patients with severe burns (Bowler *et al.* 2001; Church *et al.* 2006; Schultz *et al.* 2013; World Health Organization 2014; Saaiq *et al.* 2015). Loss of the protective skin barrier, often over a large area of the skins surface, results in a high risk of infection, complicated by an induced state of immunosuppression (Church *et al.* 2006; Schultz *et al.* 2013; Saaiq *et al.* 2015). A recent study

based in a major UK regional burn centre identified *Staph. aureus* as the most common organism cultured from burn wound swabs, although several other organisms were also identified including β-haemolytic *streptococcus, Pseudomonas aeruginosa, Escherichia coli* and coagulase negative staphylococci (Alrawi *et al.* 2014). Similarly, these organisms were also among the most commonly isolated from burn wounds in a study carried out in an American hospital, with the exception of β-haemolytic *streptococcus* (DiMuzio *et al.* 2014). A fast, non-invasive method for diagnosing wound infection would benefit patients by allowing clinicians to detect infection at an early stage, enabling appropriate treatment to be promptly administered, reducing the risk of further complications. Furthermore, a UK government commissioned review (O'Neill 2015) highlights the need for rapid diagnostic tests as a key strategy in the battle against the rise of antimicrobial resistance. In particular, to facilitate a reduction in the use of empiric therapy by allowing treatment to be optimised quickly and to promote a transition from the use of broad spectrum agents to targeted antibiotics. Introduction of a rapid test for infection, carried out in the clinic, would also aid the reduction of over use by allowing the implementation of a strategy whereby antibiotic therapy is not administered to patients without a positive bedside test to prove it is required. This may be of particular importance in the burns clinic where symptoms of inflammation are common, but not necessarily associated with the presence of infection.

Production of volatile compounds (VCs) occurs as a result of normal bacterial metabolism; a diverse and complex array of bacterial VCs have been identified, with up to 80 different compounds reportedly produced by a single bacterial species (Schulz and Dickschat 2007; Thorn and Greenman 2012). Characteristic VCs of certain microorganisms have long been recognised by microbiologists, for example the distinctive odour of indole from *E. coli*. Previous work has shown that using selected ion flow tube mass spectrometry (SIFT-MS) analysis, it is possible to detect and quantify the different types and concentrations of VCs produced by a range of bacterial species *in vitro* (Thorn *et al.* 2011) and that by employing appropriate statistical techniques the characteristic VC profiles can be used to discriminate between

bacterial species. In addition, SIFT-MS analysis has been used to investigate bacterial volatiles emanating from a variety of sample types including; blood culture samples (Allardyce *et al.* 2006a; Allardyce *et al.* 2006b; Scotter *et al.* 2006), serum (Spooner *et al.* 2009), urine (Storer *et al.* 2011) and breath (Dummer *et al.* 2013; Gilchrist *et al.* 2013), as well as microorganisms prepared in liquid culture medium (Thorn *et al.* 2011; Shestivska *et al.* 2015).

SIFT-MS technology was originally developed to study the production of molecules that occur in cold interstellar clouds and is described extensively in the literature (Wang *et al.* 2004; Španěl *et al.* 2006; Smith and Španěl 2011; Smith and Španěl 2015). Reagent ions (H_3O^+, NO^+, O_2^+) generated in a gas ion discharge source are selected by a quadrupole mass filter and injected in to a fast flowing carrier gas (usually helium) in the reaction flow tube. Here, the sample gas is introduced via a heated sample inlet and chemical ionisation occurs resulting in the production of characteristic product ions. Downstream reagent and product ions are separated and counted by a further quadrupole mass spectrometer and electron multiplier detector system. Absolute concentrations of trace gases can be quantified based on the ratios of ion count rates and the reaction rate constants determined by detailed studies of the reaction of volatile compounds with the 3 precursor ions.

The main aim of this study is to detect and discriminate wound-associated bacteria, grown *in vitro*, using SIFT-MS coupled with multivariate data analysis. Bacterial species were cultured in complex media; (Tryptone Soya Broth [TSB]) and in simulated wound fluid (SWF) to simulate wound similar conditions (Werthen *et al.* 2010). This approach was developed to determine whether the main bacterial species associated with wound infection produce characteristic volatile profiles, which could be used to develop a diagnostic tool for speciation. Wound dressing removal is associated with increased pain which can cause considerable patient distress, this in itself can contribute to delayed wound healing (Price *et al.* 2008; Upton *et al.* 2012). The ability to assess a wound for infection without removing the dressing, or by direct assessment of discarded dressing material to minimise additional interference with the

wound, would be an advantage. With this in mind, the final aim of this study was to investigate the detection of species specific profiles from sterile dressing material inoculated with wound-associated bacteria. This will help determine if future volatile analysis of real patient discarded dressing material has the potential to be used to diagnose bacterial species present in the wound bed.

MATERIALS AND METHODS

Preparation and maintenance of bacterial cultures.

Bacterial cultures were maintained on beads (Microbank, Pro Lab Diagnostics, Canada) at -80°C, resuscitated as required on Tryptone Soya Agar (TSA) (Oxoid, UK) and incubated aerobically at 37°C, with the exception of *Streptococcus pyogenes* cultures, which were resuscitated on blood agar (Oxoid, UK). All working cultures were stored on sealed plates at 4°C. The following bacterial strains were used during this study; *Escherichia coli* NCTC 10418 (**EC1**), *Escherichia coli* ATCC 10536 (**EC2**), *Escherichia coli* NCTC 12900 (**EC3**), *Escherichia coli* SMD 6099 (clinical isolate) (**EC4**), *Pseudomonas aeruginosa* NCIMB 10548 (**PA1),** *Pseudomonas aeruginosa* ATCC 15442 (**PA2**), *Pseudomonas aeruginosa* NCIMB 8295 (**PA3**), *Pseudomonas aeruginosa* CC197 (clinical isolate) (**PA4**), *Proteus mirabilis* NCIMB 701880 (**PM1**), *Proteus mirabilis* NP1 (clinical isolate) (**PM2**), *Proteus mirabilis* NP4 (clinical isolate) (**PM3**), *Proteus mirabilis* NP6 (clinical isolate) (**PM4**), *Staphylococcus aureus* CC174 (**SA1**), *Staphylococcus aureus* NCIMB 6571 (**SA2**), *Staphylococcus aureus* NTCC 6538 (**SA3**), Methicillin Resistant *Staphylococcus aureus* (Llewelyn; clinical strain) (**SA4**), *Staphylococcus epidermidis* NCTC 11536 (**SE1**), *Staphylococcus epidermidis* NCIMB 12721 (**SE2**), *Streptococcus pyogenes* NCTC 10871 (**SP1**) and *Streptococcus pyogenes* NCTC 10874 (**SP2**).

Liquid broth cultures for head-space analysis.

Overnight plate cultures were used to prepare a test suspension of each microorganism in 10 mL Tryptone Soya Broth (TSB) (Oxoid, UK) adjusted to an OD_{620nm} of 0.20. One millilitre of

the test suspension was used to inoculate 9 mL sterile TSB, aseptically dispensed in to sterile 40 mL glass vials with a PTFE screw cap containing a silicone septum (Supelco, UK), resulting in a final starting OD_{620nm} of 0.02 in each vial. For each bacterial strain used, three separate vials were inoculated, and on each day the experiment was performed, three vials containing 10 mL sterile TSB were incubated as controls. Test and control vials were incubated aerobically at 37°C and 200 rpm (Stuart S150 Orbital Incubator, Bibby Scientific, UK) for 5 hours.

Liquid cultures (simulated wound fluid) for head space analysis.

Overnight plate cultures were used to prepare a test suspension of each microorganism in 10 mL diluent containing 0.85% NaCl with 0.1% Peptone (Oxoid, UK) adjusted to an OD_{620nm} of 0.20. One millilitre of the test suspension was used to inoculate 40 mL glass vials (as specified previously), containing 5 mL Fetal Bovine Serum (FBS) (Sigma Aldrich, USA) and 4 mL sterile diluent; resulting in a simulated wound fluid containing FBS and 0.85% NaCl with 0.1% Peptone at a ratio of 1:1 by volume (Werthen *et al.* 2010) inoculated with the organism of interest at a final starting OD_{620nm} of 0.02 in each test vial. Test vials were prepared in triplicate and incubated for 5 hours prior to analysis as described above.

Wound Dressing Cultures; simulated wound fluid.

Overnight plate cultures were used to prepare test suspensions in 10 mL diluent containing 0.85% NaCl with 0.1% Peptone (Oxoid, UK) adjusted to an OD_{620nm} of 0.20. Ten millilitres of SWF was used to moisten a 10 x 10 cm non-adherent sterile wound dressing, composed of a cellulose pad laminated with a film of polyethylene, housed on a wire support in a sterile incubation chamber. One millilitre of bacterial test suspension was used to inoculate the dressing. Inoculated dressings were incubated in the sealed incubation chambers for 5 hours at 37°C. Throughout the incubation period the dressing samples were slowly perfused with sterile SWF at a rate of approximately 2 mL h^{-1} via a sterile needle, to simulate the conditions of a moderately exuding dressed wound (50 mL in 24 hours: Thomas *et al.* 1996). Following

the incubation period, dressing samples were aseptically removed from the incubation chambers and transferred to individual 1 L gas sampling bags (Tedlar®, Supelco, UK) which were sealed and filled with synthetic air (20% Oxygen, 80% Nitrogen). The gas sampling bags were incubated for a further 1 hour at 37°C to allow the equilibration of the headspace gases.

SIFT-MS Analysis.

Following incubation, samples were analysed by SIFT-MS (Profile 3, Instrument Science Limited, UK) in 'Full Mass Scan' mode using the H_3O^+ precursor ion; each sample was analysed 3 times using repeat scans of 100 seconds, over a spectrum range of 10-200 m/z, to generate a total of 9 scans per bacterial strain from 3 independent replicate samples. Samples were introduced to the instrument by piercing the silicone septum of the sample vial with a sterile needle attached to the SIFT-MS direct sampling inlet, samples were vented with a second sterile needle attached to a 0.2 µM syringe filter (Ministat, Sartorious Stedim Biotech, Germany) to allow the free flow of headspace gases. Similarly, dressing samples were analysed by piercing the silicone septum of the gas sampling bag with a sterile needle attached to the direct sampling inlet of the SIFT-MS instrument.

Data Analysis.

Mean count rates were calculated for each detected mass-to-charge ratio (m/z) product ion across the mass spectrum for each bacterial strain included in the analysis (n=3). Where a product ion was detected in both the headspace of a bacterial culture and the un-inoculated control, t-tests were performed to compare the count rate detected. Only product ions detected at significant levels ($p<0.05$) from the bacterial cultures compared to background control levels were included for further analysis. The mean product ion count rate of background volatiles from the un-inoculated culture media were then subtracted from those detected in bacterial culture head-space, to determine the total count rate attributed to production of volatile compounds in the bacterial culture. Any corrected negative values were disregarded (indicating a reduction significantly below the background levels), as were reagent ion peaks.

A threshold detection signal of 10 counts per second (cps) was applied to identify discriminant volatile compound product ion data. Further selection was required to identify discriminate product ions for the SWF data. To achieve this product ions were disregarded if the 10 cps threshold was met by only a single strain of any species included in the study.

Ward's method of Hierarchical clustering was applied to the resultant data set. This is a data mining technique whereby an algorithm is used to score the dissimilarity between cases in the data set and a matrix constructed. The dissimilarity matrix is visualised through the production of a dendrogram, in which the branching of the clusters groups the cases with the least dissimilarity, and the distance between the nodes indicates the relationship between the clusters. The data were subsequently transformed using Principal Component Analysis (PCA), visualised by constructing plots of the principal components scores for each data set. All data were analysed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, Washington, USA) and IBM SPSS Statistics version 20.0 (IBM Corporation, Armonk, NY, USA). A correction for multiple comparisons was not performed. Using the SIFT-MS kinetics library database, which has been compiled by the instrument manufacturers through detailed studies of the reactions between neutral analyte compounds and the 3 reagent ions, it is possible to make preliminary identifications of the volatile compounds based on the detected SIFT-MS product ions.

RESULTS

Headspace analysis of wound associated bacteria cultured in complex growth medium.

Multivariate analysis of the headspace count rates (log_{10}) of selected mass spectra product ions using Ward's method of Hierarchical clustering analysis is visualised by the dendrogram in figure 1. This shows successful discrimination between *Pr. mirabilis*, *E. coli*, *Staph. aureus* and *Staph. epidermidis* based on the profile of selected volatile compound product ions detected. The data were transformed using principal component analysis to reduce the selected variables to principal components. The first two principal components account for 36.3% and 21.7% of the variability within the data respectively (figure 2a). Plotting the scores

generated by PCA enables the complex data set to be represented in 2-dimensional space and clearly visualises the association of each bacterial strain with the derived principal components and shows that the strains of *Pr. mirabilis* and *E. coli*, occupy discrete regions of the 2-dimensional plot. *Staph. aureus* and *Staph. epidermidis* appear to occupy overlapping regions on this plot, however when the third component is employed (accounting for 10.1% of the total variance) to construct a 3-dimensional plot, these two species separate along the z-axis (not shown). Figure 2b shows the loading of the eigenvectors representing 51 selected product ions based on the selection criteria described in the materials and methods. It should be noted that when the initial t-tests were performed negative product ion count rate values were disregarded since these represent the consumption of substrates in the growth medium by the bacterial species, which was not the focus of this study. The 51 selected product ions constitute the original variables for the principal component analysis shown in figure 2a. This eigenvector plot (figure 2b) shows the contribution of each of the original variables; product ion count rates, to the derived first two principal components and indicates the influence of these variables in determining the position of each test strain. It is important to note that both the absence and presence of product ions dictates the spatial location of the given bacterial strain within the principal component plot. For example, when considering *Ps. aeruginosa* and *Strep. pyogenes* within the principal component, plot the absence of the majority of detected product ions and the presence of product ions m/z 18 and 28 are responsible for the plotted location of these species. This data set shows no significant differentiation between *Ps. aeruginosa* and *Strep. pyogenes* based on the profile of selected volatile compound product ions detected following 5 hour incubation in TSB. This was predominantly due to the absence of product ions detected in the culture headspace, rather than the presence of numerous similar volatile product ions. Although a total of 51 product ions were selected for the analysis of all bacterial species, many of the 51 product ions were absent from the headspace of both *Ps. aeruginosa* and *Strep. pyogenes* strains and this is likely to be the reason for the inability to discriminate between these species. To further assess whether these species could be differentiated based on volatile analysis when cultured in TSB, independent samples were

cultured for 24 hours and the headspace gases analysed by SIFT-MS and Multivariate analysis performed on the data as previously described. Following product ion selection, hierarchical cluster analysis implementing Ward's method resulted in the production of a dendrogram showing the two species separated into discreet clusters, indicating differences between headspace volatile compound product ions of *Ps. aeruginosa* and *Strep. pyogenes* detected following 24 hour incubation (figure 3).

Headspace analysis of wound-associated bacteria cultured in a simulated wound fluid.

Hierarchical clustering analysis of selected product ions implementing Ward's method and using squared Euclidean distance as a measure of resemblance enabled discrimination of *Pr. mirabilis, E. coli, Strep. Pyogenes* and *Ps. aeruginosa* at the species level and staphylococci at the genus level as visualised by the dendrogram shown in figure 4. Principal component analysis was utilised to transform this data set, the first two principal components account for 34.3% and 19.7% of the total variance (figure 5a). It can be seen by plotting the PCA scores that strains of *E. coli* and staphylococci each occupy specific discrete regions of the 2-dimensional plot. Figure 5b shows the loading plot of 26 original variables (product ions) detected by SIFT-MS headspace analysis on the first two principal components, generated by the principal component analysis represented in figure 5a. In figure 5a, strains of *Pr. mirabilis, P. aeruginosa* and *Strep. pyogenes* appear to occupy overlapping regions, however when plotted in three dimensions employing the third principal component (accounting for 9.5% of the total variance), strains of these species are separated along the z-axis (data not shown).

Wound Dressing Cultures; simulated wound fluid.

Figure 6 shows the SIFT-MS product ions detected following analysis of the headspace of wound dressing material inoculated with *Ps. aeruginosa* (PA2)*, E. coli* (EC2) and *S. aureus* (SA3). The plot includes only the volatile compounds detected at levels significantly higher than the uninoculated controls, following background subtraction. High background count rates across the mass spectral range were detected from the uninoculated dressing material.

However, a range of volatile compound product ions were detected above the background count rates from the inoculated dressing samples. Interestingly, these differed from those detected in the headspace of the same species cultured in liquid medium, in both the number and the mass-to-charge ratio of product ions detected. Only 12 product ions were detected in the headspace of the wound dressing material and of these only 6 were common to the product ions detected in the headspace of bacteria cultured in simulated wound fluid.

DISCUSSION

The main study aim was to identify wound associated bacterial species specific volatile profiles *in vitro*. The mass spectral profile of volatile compound product ions derived from the wound-associated bacterial species included in this study varies between species. This also extends, to some strains from a single species, as reported in previous studies (Thorn *et al.* 2011; Shestivska *et al.* 2015). This study has demonstrated that the culture conditions; namely composition of culture media and duration of incubation, influence the range of product ions and count rates of the resultant volatile compound mass spectra. The range and relative concentrations of bacterial VCs under varying physicochemical conditions has been previously observed in other research studies (O'Hara and Mayhew 2009; Chippendale *et al.* 2011; M. E. Dolch *et al.* 2012a; M.E. Dolch *et al.* 2012b). The range and count rates of product ions detected are greater in TSB, a complex nutrient rich commercially available culture media, compared to a simulated wound fluid containing Peptone, NaCl and Fetal Bovine Serum (FBS). A comparison of the discriminant product ions produced when bacteria were grown in either TSB (figure 2) or SWF (figure 5), shows that there are clear differences as well as similarities. For example, m/z 28 is clearly important for the discrimination of Ps. aeruginosa. The differing nutrient sources in the culture media almost certainly results in the upregulation of altered bacterial metabolic pathways, resulting in the production of different volatile metabolites (Audrain *et al.* 2015).

The results from this study demonstrate that, it is possible to discriminate wound associated bacterial species based on the detected volatile compound product ion profiles, whether

cultures are grown within complex medium (TSB) or under wound similar conditions (in SWF). After 5 hours incubation it was possible to discriminate several species based on the profiles using Ward's method of hierarchical clustering analysis; however, it was not possible to discriminate between strains of *Ps. aeruginosa* and *Strep. pyogenes*. Similarly, when the transformed data set was plotted as principal components, *Ps. aeruginosa* and *Strep. pyogenes* occupy closely adjacent regions on the two-dimensional plot. This is the result of the absence of product ions detected in the headspace of either species under these conditions, visualised by comparing the two plots in figure 2. Increasing the incubation period to 24 hours resulted in an increase in the number and count rates of SIFT-MS product ions detected from *Ps. aeruginosa.* This included high count rates for the m/z 18 product ion in all strains and the m/z 28 product ion in two of the four strains analysed. Hierarchical clustering analysis of the data obtained after 24 hours of incubation resulted in successful discrimination between the stains of these two species, based on the clustering shown in the resulting dendrogram. It is possible to make a preliminary identification of the volatile compounds which correspond with the detected SIFT-MS product ions, using predetermined reaction rate constants. When using the H_3O^+ reagent ion, m/z 18 and 28 product ions indicate the presence of protonated ammonia and hydrogen cyanide respectively (Španěl *et al.* 2004; Turner *et al.* 2006). It has been reported elsewhere that both ammonia and hydrogen cyanide production are characteristic of *Ps. aeruginosa* (Nawaz *et al.* 1991; Gilchrist *et al.* 2011). A recent study (Neerincx *et al.* 2015) which investigated the production of these volatiles over time in a number of *Ps. aeruginosa* strains determined that both compounds began to rise above detectable levels only in older cultures, as they began to enter stationary phase. This would explain the presumptive detection of these important volatiles only after 24 hours of culture within this study. It is highly likely that production of these particular volatiles will be important for identification of *Ps. aeruginosa in vivo*, where growth conditions result in a slow growing biofilm state. Indeed, elevated hydrogen cyanide concentrations in nose-exhaled breath have been identified using SIFT-MS as a potential biomarker of *Ps. aeruginosa* infection in adult Cystic fibrosis patients (Gilchrist *et al.* 2013).

The results of this study successfully demonstrated that it is possible to discriminate wound-associated bacterial species based on a profile of selected SIFT-MS product ions when cultured under wound similar conditions. Using Ward's method of hierarchical clustering analysis to determine strain relatedness, four of the six bacterial species were successfully discriminated, based on the clustering shown in the resulting dendrogram. Using PCA and constructing a plot of the first 3 principal component scores resulted in similar discrimination of the bacterial species investigated to the hierarchical clustering analysis, indicating that either of these techniques maybe suitable for modelling this data set. However, the two species of staphylococci could not be discriminated from each other. Further analysis of the headspace of both species of staphylococci following 24 hours of incubation in SWF, also failed to successfully discriminate these species, in contrast to staphylococci cultured in complex culture media (TSB). Future work will assess whether it is possible to discriminate between species of staphylococci based on volatile headspace analysis when these species are grown as biofilm rather than in liquid culture. A biofilm model will simulate more closely the real wound environment (James *et al.* 2008). The altered growth conditions will likely affect the metabolic profile of the organism and result in a change in the volatile metabolites produced.

This study also investigated the detection of species specific profiles from sterile dressing material inoculated with wound associated bacteria; continuously perfused with a simulated wound fluid. A range of SIFT-MS product ions with count rates significantly greater than background control levels were detected from the bacterial species analysed. In addition the different species resulted in the detection of different mass spectra profiles (figure 6). This indicates that volatile analysis of wound dressing material to identify infecting microbes may be possible. Further research is required to validate this approach, and a pilot study is now being undertaken to identify volatile product ion spectra from the gaseous headspace of patient's discarded wound dressings. Ultimately, it will be essential to identify clinically relevant infection as well as the causative organism. However, the ability to identify microbial volatile

compound product ions among the background derived from the dressing, demonstrates that assessment of a dressed wound or discarded dressing may be a valid approach.

As previously discussed, the profile of the product ions of the species grown in wound dressing material was different to those detected from the same species grown using the same medium (SWF) in liquid culture. This suggests that the growth conditions, as well as the medium has an influence over the metabolic state of the organism and therefore the array of volatiles produced. The bacterial cultures were exposed to the same total volume of SWF over the 5 hour incubation period for both culture conditions. The differing traits of bacterial biofilms compared to their planktonic counterparts are now well documented (Cooper *et al.* 2014) and the development of biofilm within the wound dressing material is likely to be a contributing factor to the differing profiles identified. Biofilms occurring in the wound bed *in vivo* are not adhered to a solid surface, such as the fibres of dressing material, but the semi-solid structures that make up the tissue (Cooper *et al.* 2014). Development of a wound biofilm model based on a continuously perfused semi-solid matrix would be an advantage for future research. This would allow volatile profiles of the wound associated organisms to be investigated under conditions that closely simulate wound infection and would further facilitate the development of this approach to wound diagnostics. It is highly likely that unique product ion profiles are produced when these bacterial species cause infection in real wounds, not only as a result of the specific growth conditions but also due to interactions between the bacterial species and the host immune response.

Using the predetermined reaction rate constants presumptive identification of the neutral analyte compounds which correspond to the detected SIFT-MS product ions is possible. However, it is important to be aware that the reaction of a single selected precursor ion with similar volatile compounds or compound fragments can result in the production of the same product ions. For example, utilising the H_3O^+ precursor ion as used within this study, the production of 89 m/z product ion can result from the protonation of a number of different analytes with a molecular weight of 88 Da, including Pentanol ($C_5H_{12}O$), Butyric acid (C_3H_7COOH) and Putrescine ($C_4H_{12}N_2$). Furthermore, if a mixture known to contain more than

one of these compounds were analysed using the H_3O^+ precursor ion in multi-ion monitoring (MIM) mode (not used within this study) it would only be possible to determine the total partial pressure of all compounds resulting in the common product ion (Wang *et al.* 2004). In some cases it may be possible to rule out the presence of certain compounds based on the nature of the sample being analysed. However, Pentanol ($C_5H_{12}O$), Butyric acid (C_3H_7COOH) and Putrescine ($C_4H_{12}N_2$) could all be produced as a result of bacterial metabolism, as products of fermentation and decarboxylation reactions (Schulz and Dickschat 2007; Thorn and Greenman 2012; Audrain *et al.* 2015). Ambiguity in the identification of neutral analyte compounds can potentially be overcome by the analysis of the sample using multiple precursor ions, usually H_3O^+ and NO^+, as the reaction of compounds with different precursor ions can result in the production of different product ions. The compounds may then be identified and quantified using the appropriate precursor ion reaction (Wang *et al.* 2004). This study has demonstrated that using SIFT-MS and multivariate statistical analysis it is possible to discriminate wound-associated bacterial species based on the profile of selected SIFT-MS product ions when these species are grown in complex culture media, both in TSB and SWF. Furthermore, our proof-of-principle experiment has confirmed that it is possible to detect volatile compound product ions derived from bacteria when cultured within wound dressing material using a simulated wound fluid. Future investigations must focus on identifying the compounds present in the headspace of bacterial culture by utilising GC/MS for preliminary exploratory analysis and multiple precursor ions for SIFT-MS studies using MIM mode to accurately quantify the volatiles of interest identified.

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CONFLICTS OF INTEREST

No conflict of interest declared.

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Figures

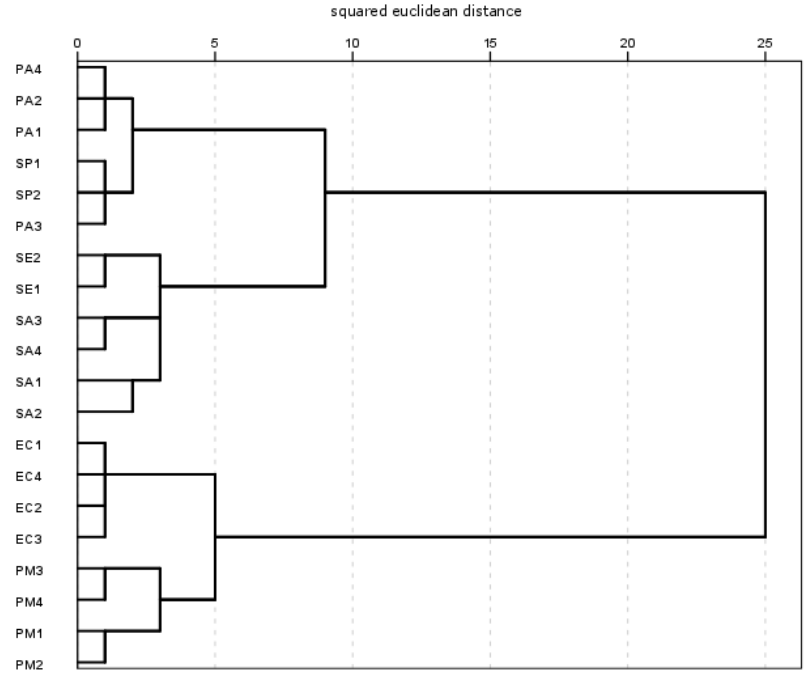

Figure 1 Dendrogram generated by hierarchical cluster analysis (Ward's Method) using selected headspace volatile m/z peaks (Log(cps)), detected by SIFT-MS following 5 hour incubation (n = 3) in TSB. **EC** *E. coli*, **PA** *Ps. aeruginosa*, **PM** *Pr. mirabilis*, **SA** *Staph. aureus*, **SE** *Staph. epidermidis*, **SP** *Strep. pyogenes*.

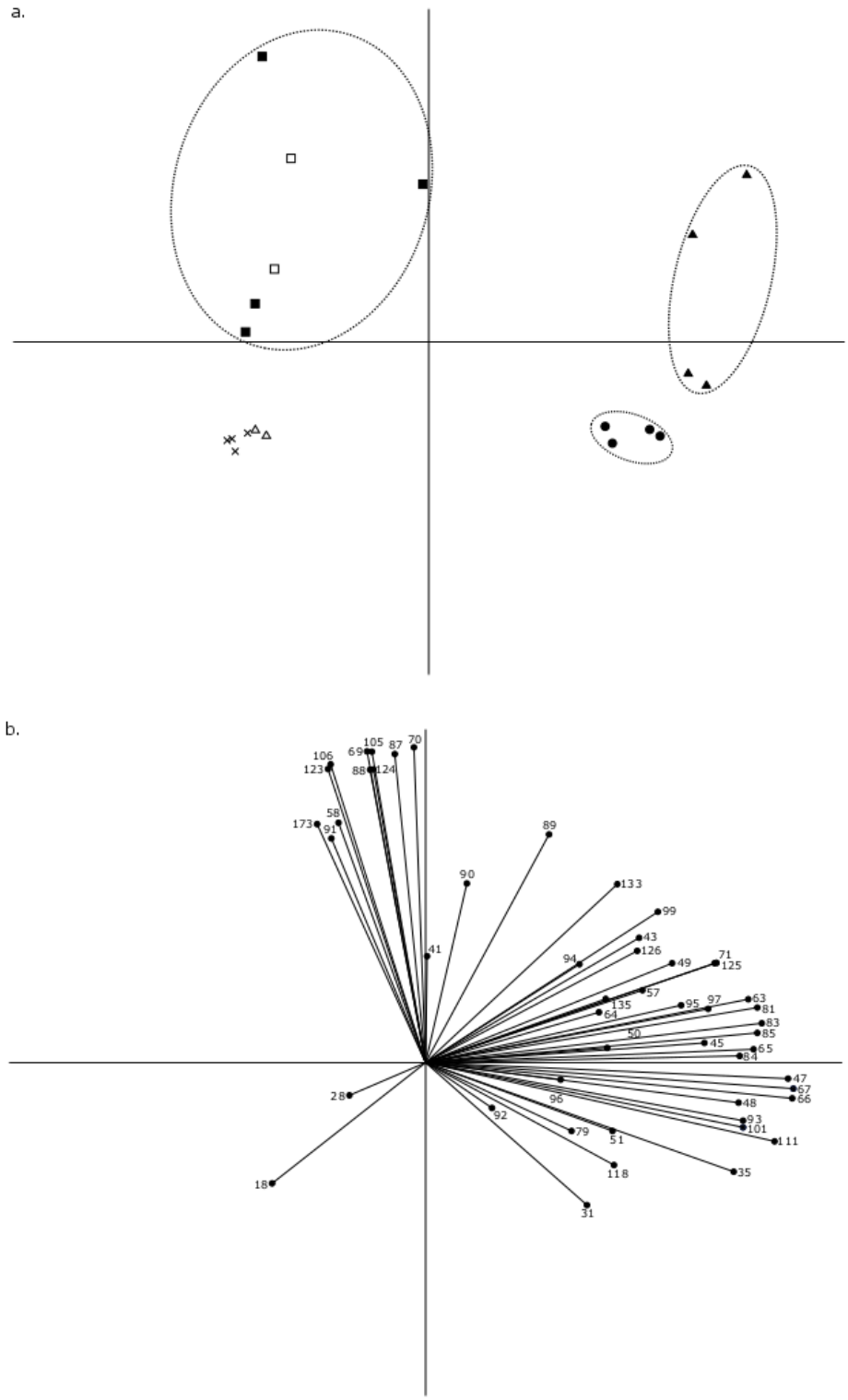

Figure 2 a Plot of the scores of the first two principal components generated by principle component analysis of SIFT-MS concentration data of selected headspace volatiles of bacterial cultures (n=3) incubated for 5 hours in TSB. Principal Component 1 (horizontal axis) accounts for 36.3% of the total variation of the original data set and Principal Component 2 (vertical axis) accounts for 21.7%. Filled circle - *Escherichia coli*, cross - *Pseudomonas aeruginosa*, filled triangle - *Proteus mirabilis*, open square – *Staphylococcus epidermidis*, filled square – *Staphylococcus aureus*, open triangle – *Streptococcus pyogenes*.
b Principal component analysis loading plot –selected m/z peaks detected by SIFT-MS analysis of cultures incubated for 5 hours in TSB. Data points indicate the loading of each m/z peak (variable) on the first two principal components generated from the principal component analysis.

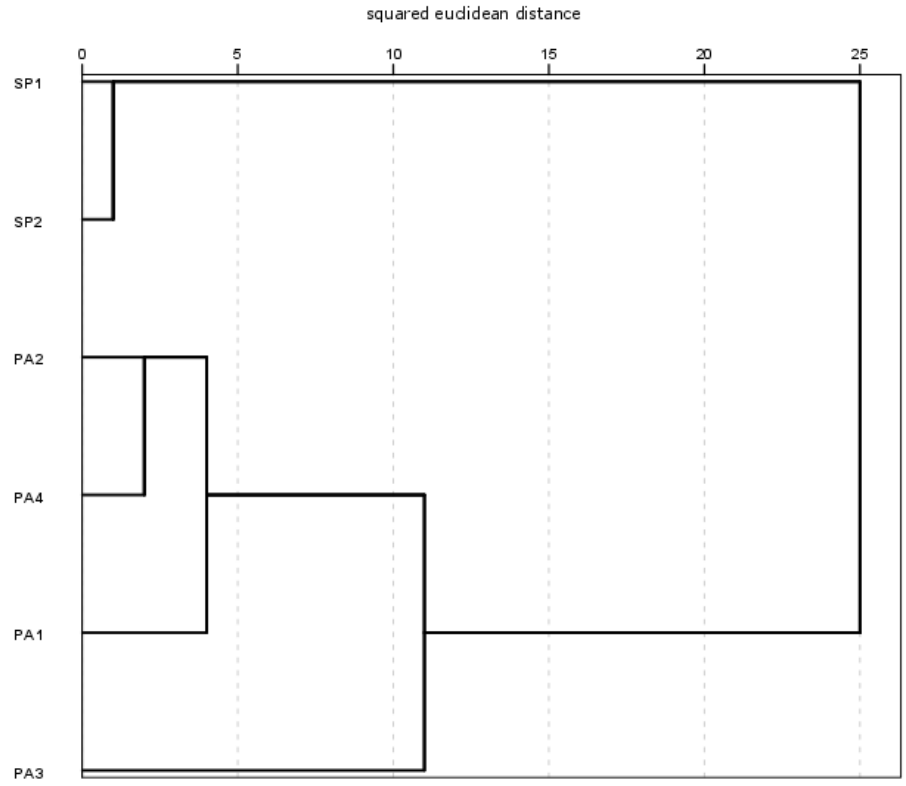

Figure 3 Dendrogram generated by hierarchical cluster analysis (Ward's Method) using selected headspace volatile m/z peaks (Log[cps]), detected by SIFT-MS following 24 hour incubation (n = 3) in TSB. **PA** *Ps. aeruginosa*, **SP** *Strep. pyogenes.*

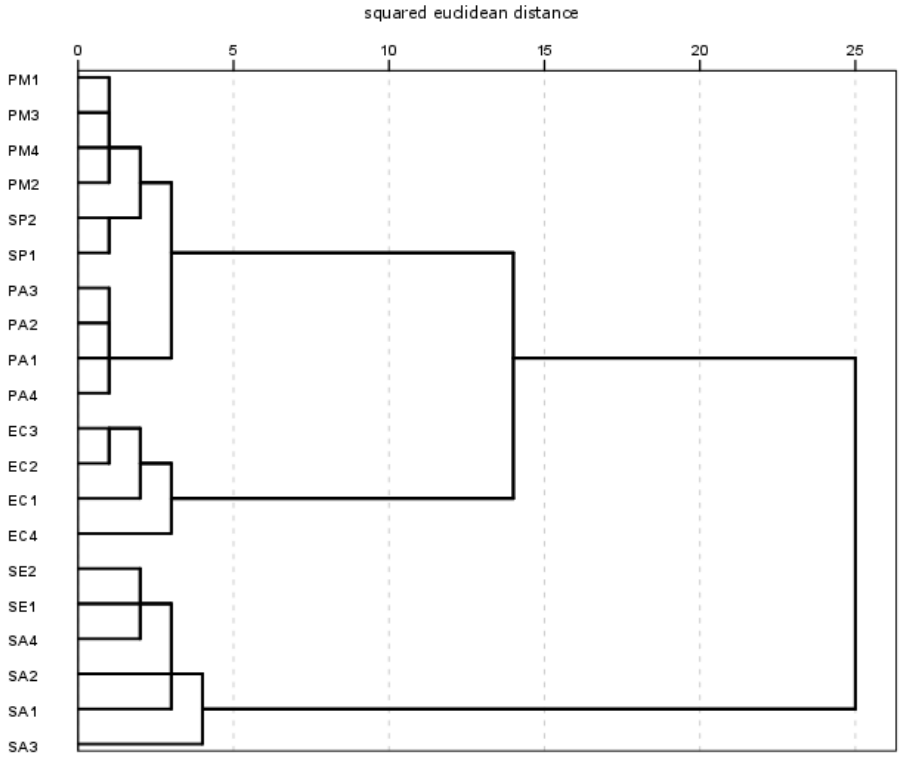

Figure 4 Dendrogram generated by hierarchical cluster analysis (Ward's Method) using 26 selected headspace volatile m/z peaks (Log(cps)), detected by SIFT-MS following 5 hour incubation (n = 3) in SWF. **EC** *E. coli*, **PA** *Ps. aeruginosa*, **PM** *Pr. mirabilis*, **SA** *Staph. aureus*, **SE** *Staph. epidermidis*, **SP** *Strep. pyogenes*.

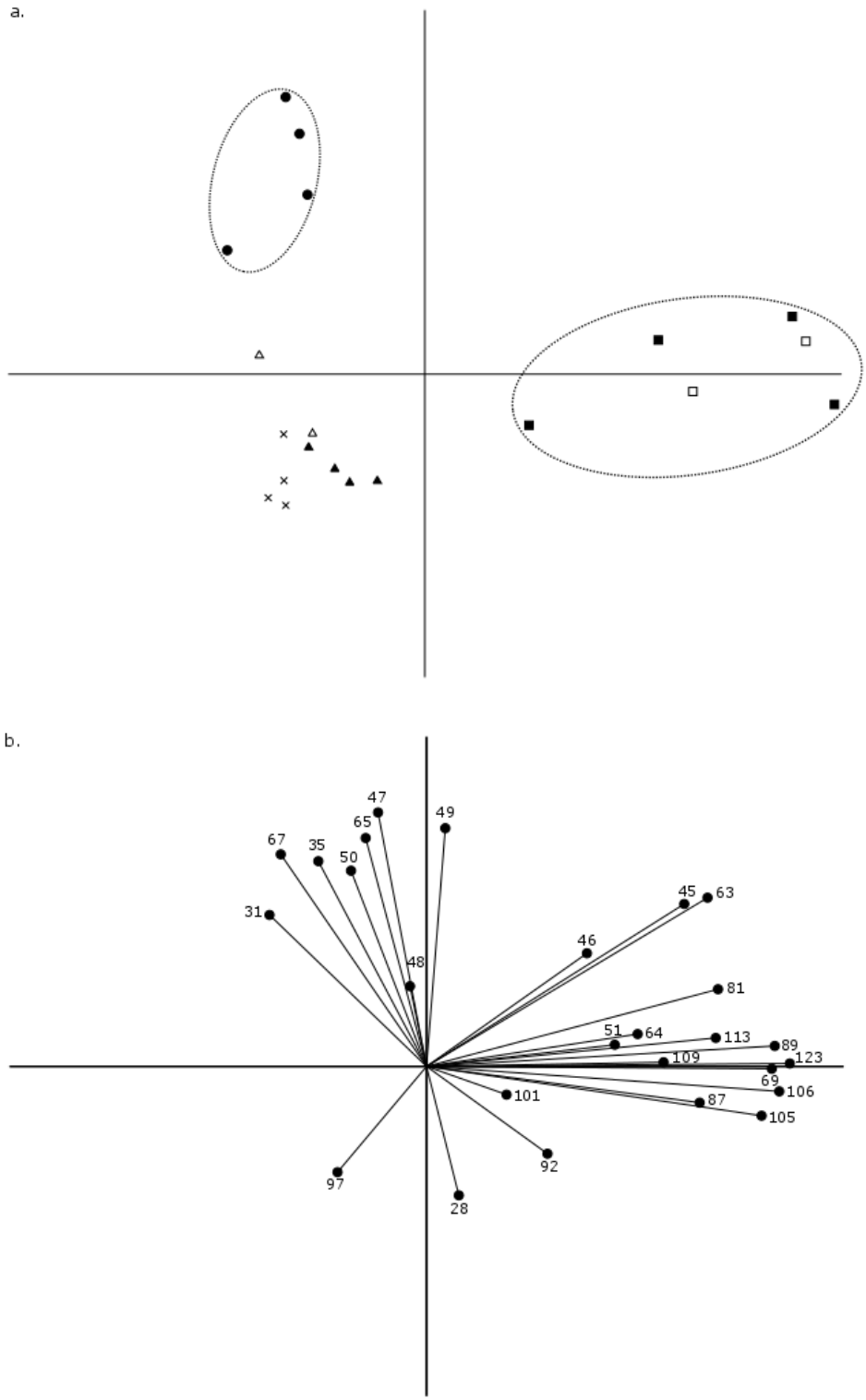

Figure 5 a Plot of the scores of the first two principal components generated by principle component analysis of SIFT-MS concentration data of selected headspace volatiles of bacterial cultures (n=3) incubated for 5 hours in SWF. Principal Component 1 (horizontal axis) accounts for 34.3% of the total variation of the original data set and Principal Component 2 (vertical axis) accounts for 19.7%. Filled circle - *Escherichia coli*, cross - *Pseudomonas aeruginosa*, filled triangle - *Proteus mirabilis*, open square – *Staphylococcus epidermidis*, filled square – *Staphylococcus aureus*, open triangle – *Streptococcus pyogenes*.
b Principal component analysis loading plot –selected m/z peaks detected by SIFT-MS analysis of cultures incubated for 5 hours in SWF. Data points indicate the loading of each m/z peak (variable) on the first two principal components generated from the principal component analysis.

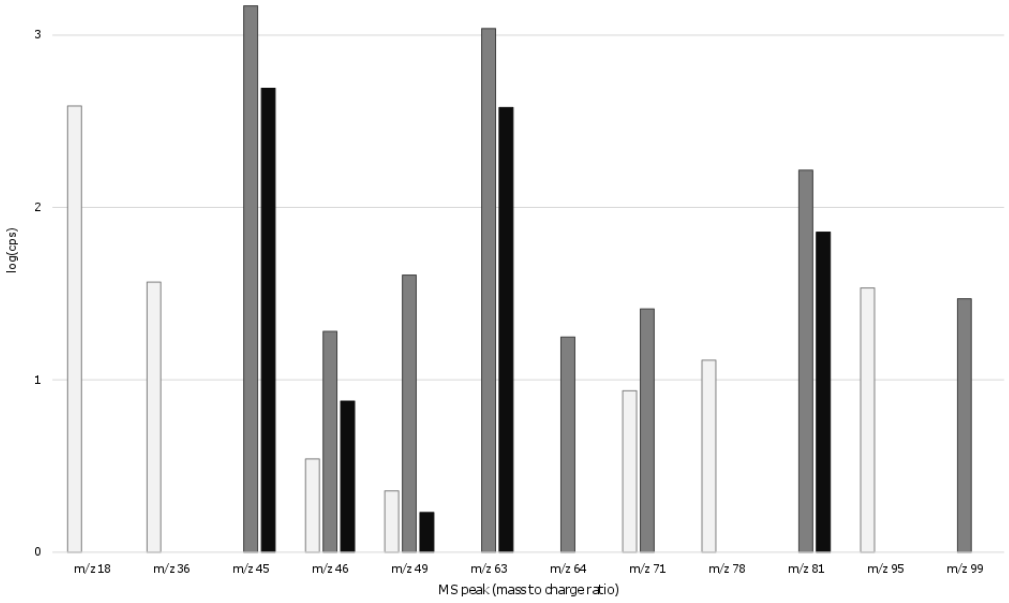

Figure 6 Mass charge peaks detected at levels significantly greater ($p<0.05$) than control levels (SWF only) from dressing material inoculated with P. aeruginosa **PA2** (pale grey), E. coli **EC2** (dark grey) or S. aureus **SA3** (black), incubated for 5 hours at 37°C while continuously perfused with sterile SWF.