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Rapid Discrimination of Bacteria by Paper Spray Mass Spectrometry

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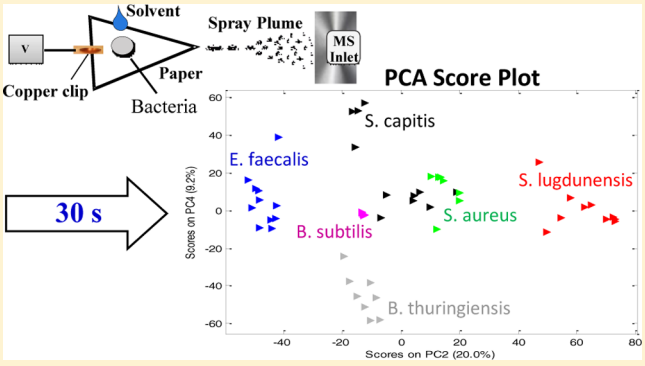
Supporting Information

ABSTRACT: Paper spray mass spectrometry ambient ionization is utilized for rapid discrimination of bacteria without sample preparation. Bacterial colonies were smeared onto filter paper precut to a sharp point, then wetted with solvent and held at a high potential. Charged droplets released by field emission were sucked into the mass spectrometer inlet and mass spectra were recorded. Sixteen different species representing eight different genera from Gram-positive and Gram-negative bacteria were investigated. Phospholipids were the predominant species observed in the mass spectra in both the negative and positive ion modes. Multivariate data analysis based on principal component analysis, followed by linear discriminant analysis, allowed bacterial discrimination. The lipid information in the negative ion mass spectra proved useful for species level differentiation of the investigated Gram-positive bacteria. Gram-negative bacteria were differentiated at the species level by using a numerical data fusion strategy of positive and negative ion mass spectra.



The rapid and accurate identification of microorganisms is relevant to numerous fields, including clinical medicine, public health, biotechnology, and food safety.[1−4] Bacteria discrimination and subsequent identification rely on the observation of endogenous biochemical and/or genetic differences. Biochemical identification of bacteria is commonly performed by integrating information on culture morphology, effects of physical alteration of culture media (e.g., hemolysis), and specific enzymatic reactions (e.g., tryptophanase). The simplicity of these experiments is advantageous; however, the complexity of the biological systems limits the usefulness of simple tests in distinguishing closely related organisms. Enzyme-linked immunosorbent assay (ELISA),[5] polymerase chain reaction (PCR),[6,7] and nucleic acid hybridization[8] are each capable of detecting more subtle genetic differences between bacteria. Thus, the identification of bacteria, sometimes down to the strain level can be achieved by genomic methods but they are often labor-intensive, time-consuming, and expensive.[9−13]

The combined advantages of sensitivity, specificity, and speed make mass spectrometry (MS) an attractive analytical approach for the analysis of intact bacteria.[14−19] Some decades ago, Curie-point pyrolysis MS was shown to be highly discriminatory, but the reduction in molecular complexity at high temperature and the need for pure samples limited the applications of the method.[20−23] Subsequently, fast atom bombardment was used as a rapid method of discrimination.[17,24−28] Thermal degradation combined with in situ chemical derivatization is a powerful discriminatory method that has been widely applied in the context of biological security monitoring.[29,30] Subsequently, measurements of protein profiles have formed the basis of discrimination in electrospray ionization methods.[31−33] Finally and most recently, matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF), also based on protein profiles, has been developed as an important MS method in microbiology.[34,35] Bacterial identification by MALDI-MS offers rapid analysis and accurate identification of bacteria based on comparing a protein fingerprint of the organism tested to a database.[6,34,36−41]

Ambient ionization methods generate ions outside the vacuum of the mass spectrometer and require little or no sample preparation.[42,43] In the exploration of the capabilities of several ambient ionization methods including low temperature plasma (LTP),[44] desorption electrospray ionization (DESI),[45−47] nanospray desorption electrospray ionization (nanoDESI),[2,48−52] direct analysis in real time (DART),[53]

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laser ablation electrospray ionization (LAESI),[54] and rapid evaporative ionization-mass spectrometry (REIMS),[55] bacterial analysis has been examined in a preliminary fashion. In several of these cases, microorganism colonies were examined directly. Paper spray mass spectrometry (PS-MS) has been successful in qualitative and quantitative mass spectrometric analysis of complex mixtures[27−29] but has not been previously applied to bacterial discrimination. This is undertaken in the present study.

Principal component analysis (PCA) is commonly used for exploratory investigation of the complex information contained in full mass spectral data sets, allowing simultaneous consideration of all spectral variables and their intercorrelation.[56] Nearly all of the chemical information content of the original m/z variables is reorganized and compacted into a set of orthogonal principal components (PCs).[57] The projections of the data objects (i.e., the samples) onto the PCs are called scores, while the importance of each original spectral variable in defining a certain PC is given by its loading coefficient. Both scores and loading values can be represented in two-dimensional scatter plots. The relationship between the score and loading plots is evident from the codirectionality of objects and variables in related score and loading plots.[56]

This paper presents the application of PS-MS in the rapid discrimination of bacteria (1−2 min). Due to the large amount of information acquired in full scan mass spectra, multivariate statistical methods are necessary to manage the MS data and extract chemical features from them. In this study, a preliminary PCA was used to explore the differences among bacteria based on PS mass spectra. Then taking advantage of the fact that most of the variance in the data set is available in a few PCs, the generation of which acts as a data reduction technique, linear discriminant analysis (LDA) was applied as a supervised discriminant classification method to quantify the ability of PS-MS chemical signatures in discriminating among bacteria.[58] This approach has been previously reported for other types of samples to tackle classification problems by using PCs scores as variables in LDA.[59,60]

EXPERIMENTAL SECTION

Chemicals and Materials. Methanol (HPLC grade) was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Sodium dodecyl sulfate (SDS), 3-[(3-cholamido-propyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and octyl-β-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO). The culturing medium, trypticase soy agar supplemented with 5% sheep blood (TSAB), was purchased from Remel (Lenexa, KS). Sterile inoculation loops were purchased from Copan Diagnostics, Inc. (Murrieta, CA). Whatman grade 1 cellulose filter paper was purchased from Whatman International Ltd. (Maidstone, England). Copper clips were purchased from McMasters-Carr (Chicago, IL).

Microorganism Culturing. The bacterial isolates, supplied by bioMérieux, Inc. (Hazelwood, MO), were cultured from frozen samples stored at −80 °C on TSAB in cryotubes. Bacteria were aliquoted from frozen storage by sterile wooden splints, streaked on TSAB plates, and incubated at 37 ± 1 °C for approximately 24 h. Bacteria were subcultured from an isolated colony, removing it using a sterile loop, and incubated for an additional 24 h in a VWR forced air incubator (Chicago, IL). Mass spectral analysis was performed on samples taken from the subcultured plates after 24 h, any plates appearing to have two bacterial colony morphologies were excluded from analysis. All bacterial culturing activities were performed in a biological safety cabinet and biohazardous materials were autoclaved. All experiments were performed under Institutional Review Board guidelines IBC protocol #070-004-10 "Novel tissue, Biological fluid and Bacteria Evaluation by Mass Spectrometry" as amended. One strain of 16 clinically relevant bacteria were analyzed; see Table S1 of the Supporting Information. Several of these species were selected for their high level of similarity by either phenotypic or genotypic methods.[35,61] Multiple bacterial strains were not included and are not anticipated to effect species level discrimination, based on genetic homology between strains.[62]

Instrumentation. Mass spectrometric analysis was performed using a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, CA). Spectra were collected with automatic gain control (AGC) activated and a maximum ion trap injection time of 50 ms and three microscans. Full scan negative (−) and positive (+) ion spectra were obtained over the range of m/z 200−2000 in profile mode. The temperature of the MS capillary was set at 275 °C. The tube lens voltage was set at −100 and 0 V in the negative and positive ion modes, respectively. The capillary voltage was maintained at −50 and 0 V in the negative ion mode and positive ion mode, respectively. The voltage used for paper spray ionization was −3.5 kV in the negative ion mode and 2.8 kV in the positive ion mode. Tandem mass spectrometry (MS/MS) was carried out using collision-induced dissociation (CID) for structural elucidation. An isolation window of 1.0−1.8 (m/z units) and normalized collision energy of 15%−25% (manufacturer's unit) were chosen. The identities of highly abundant ions have been confirmed by high resolution mass measurements acquired using an Orbitrap mass spectrometer (Exactive, Thermo Fisher Scientific, San Jose, CA). The Orbitrap instrumental conditions in the negative ion mode were as follows: maximum injection time of 50 ms; two microscans; m/z range of 200−2000; activated AGC; −25 V capillary voltage; and −165 V tube lens voltage.

Paper Spray-MS Methods. A triangular piece of filter paper, 10 mm (height) by 8 mm (base), was held with the apex in line with the inlet of the mass spectrometer at a distance of 5 ± 1 mm from it.[63] High voltage was supplied via the mass spectrometer using copper clips attached to the paper triangles. Approximately 100 μg (≤5 bacterial colonies) of material was selected from the culture plates using sterile inoculation loops and smeared onto the middle of the surface of the paper triangle. Fifteen microliters of solvent was then applied by pipet, with the selections of solvent and volume being guided by evaluations of spray duration and mass spectral quality (Supporting Information, Figure S1). PS-MS spectra were recorded for 1−2 min/sample and are displayed without background subtraction.

With the aim of improving classification, a one-step PS-MS experiment was developed in which positive and negative mode information was collected from the same samples. This was accomplished by switching the polarity of the instrument during data acquisition during a single solvent addition (pure methanol). Signal in this bipolar method was obtained on average for 60 s, negative ion spectra were collected for the first 30 s, and positive ion data were collected for the remaining 30 s.

Multivariate Statistics. PCA and LDA A list of m/z values and corresponding ion abundances from averaged mass spectra were imported into Matlab (MathWorks, Inc., Natick, MA),

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and data processing was performed prior to multivariate statistics using in-house Matlab routines. All spectra were normalized by the standard normal variate (SNV) transform to correct for both baseline shifts and global intensity variations.[64,65]

Gram-positive bacteria PS-MS data consisted of 51 samples examined in the positive ion mode and 53 in the negative ion mode. Each sample was analyzed with at least 6 replicates in the negative ion mode, yielding a data set of 53 samples (see Table S1 for details). Positive mode PS-MS data set yielded a data matrix of 51 rows (samples) and 13 200 columns (m/z values), over a slightly reduced mass range m/z 200−1300. Negative mode PS-MS data matrix consisted of 53 rows and 10 681 columns over a reduced mass range m/z 200−1100. The positive and negative ion matrices of Gram-positive bacteria were further reduced to eliminate unnecessary information, only random noise was detected in this region and it was determined to not affect statistical outcomes.

PS-MS data from Gram-negative bacteria included 68 samples collected in both the positive and negative ion modes. The experimental results were obtained with at least 6 replicates forming the data set. Two matrices were built containing 68 rows with the (+) PS-MS matrix containing 15 600 columns over the mass range m/z 200−1500 and the (−) PS-MS matrix containing 15 481 columns, after removing a m/z 645−655 window to exclude the interfering ion of m/z 649.7, $[CHAPS+Cl]^-$ and related isotopic peaks.

The negative ion PS-MS data matrix of Gram-positive bacteria was analyzed by PCA. LDA was applied after unsupervised data compression by means of PCA, using the PCs as new variables instead of the original MS data set.[59,60] For the Gram-negative bacteria data set, the same strategy of data analysis, based on PCA and then LDA, was applied first to the negative ions. To improve classification of Gram-negative bacteria the positive and negative ion data were used in conjunction via data fusion. The midlevel data fusion strategy used in this study has been presented elsewhere[57] and applied here to investigate whether the combined analysis of positive and negative ions could improve sample characterization and classification of 10 different species of Gram-negative bacteria. Positive ion mode data used in the data fusion strategy was obtained from "bipolar" experiments, whereas negative mode data was obtained in an independent experiment performed using different solvent conditions.

When building a discriminant-classification model, validation is a fundamental step to evaluate the predictive ability of the model. In this study, a cross-validation (CV) strategy was used in LDA and the CV prediction rates (i.e., the percentage of correct predictions on the objects in the CV evaluation sets) were computed.[58] Five CV deletion groups were selected for all LDAs.

RESULTS AND DISCUSSION

Paper Spray Methodology. Optimization of paper size and tip angle was explored based on previous PS-MS analyses of pharmaceuticals in blood spots.[66] The size of the paper was determined to be of minor consequence to signal intensity and stability, but not surprisingly it did alter the amount of solvent that could be absorbed. The tip angle was determined to be a more important parameter as it influenced spectral quality significantly. The tip angle affects the strength of the electric field and the emission of charged droplets as well as the competitive process of gaseous discharge. A more acute angle (30°) resulted in greater currents (>1.5 μA) suggesting that the electric field strength exceeded the threshold required for stable electrospray and resulted in a gaseous discharge. It was determined empirically that such discharges adversely affected spectral quality leading to increased noise and spectral complexity.[67] The less acute angle of 45° yielded spray currents consistent with typical electrospray plume formation (0.1−0.5 μA) and resulted in higher quality mass spectra. Therefore, it was decided that a paper tip angle of 45° was most suitable under the conditions tested.

The addition of compounds which reduce the surface tension (e.g., surfactants) of electrosprayed solvents is known to enhance ionization efficiency.[68] Multiple surfactants were explored including CHAPS, SDS, and octyl-β-D-glucopyranoside. The addition of CHAPS, a zwitterionic surfactant, to the spray solvent (methanol) resulted in stable mass spectral signals of greater intensity compared to other surfactant additives. CHAPS was detectable in both the positive and in the negative mode as the protonated molecule and the chloride adduct, m/z 615.5 and 649.5, respectively. A deleterious effect upon positive mode PS-MS was noted with CHAPS, likely the consequence of charge competition, while negative mode PS performance was improved significantly with <0.1% CHAPS. Therefore, 0.05% CHAPS in methanol (v/v) was chosen as spray solvent in negative ion mode PS-MS experiments unless otherwise mentioned, while methanol alone was always employed in the positive ion mode. The mass spectra of different replicates, obtained from different colonies from a single plate, are obtained in successive fashion with high reproducibility. As illustrated in Figure S2, the variation in relative abundance of the prevalent phospholipid ions observed at m/z 705, 719, 733, 747, and 773 is less than 5%.

Differentiation of bacteria was assessed at the Gram reaction level, then at the genus level and finally at the species level. Ions from various classes of compounds were observed in this study including fatty acids (FAs), phosphatidylglycerols (PGs), phosphatidylethanolamines (PE), lyso-phosphatidylglycerols (LPGs), rhamnolipids, and lipopeptides. These observations correlate to a degree with the bacterial cell membrane composition of PGs, PEs and DPGs.[55]

Gram stain reactivity differentiation multivariate statistical analysis was performed on the PS mass spectra recorded for Gram-positive and Gram-negative bacteria. Separation between Gram-positive (blue triangles) and Gram-negative bacteria (red circles) is apparent in the PC1 versus PC2 score plot in Figure S3a. The PC1 versus PC2 loading plot reveals the contribution of the original variables in computing the two lowest-order PCs. In more detail, in the loading plot (Figure S3b), the negative ion of m/z 719.7, appears to be more abundant in Gram-negative bacteria, whereas the negative ion of m/z 721.7 appears to be more abundant in Gram-positive bacteria. The peaks present at m/z 719.7 and 721.7 are attributed to phosphatidylglycerols $[PG(32:1)-H]^-$ and $[PG(32:0)-H]^-$, respectively, as confirmed by CID, isotopic abundances, and exact mass measurements (Supporting Information, Table S2 and Figure S4). Remarkably, subclusters were noted in the PCA score plot, shown in Figure S3c and d, and they are related to the different taxonomic levels under investigation. This result indicated that the lipid profiles present in bacteria may represent unique fingerprints of bacterial species and can be detected by PS-MS. Further, the spectral fingerprints could be used to construct a database that provides matches, at a definite confidence level, between unknown samples and bacterial

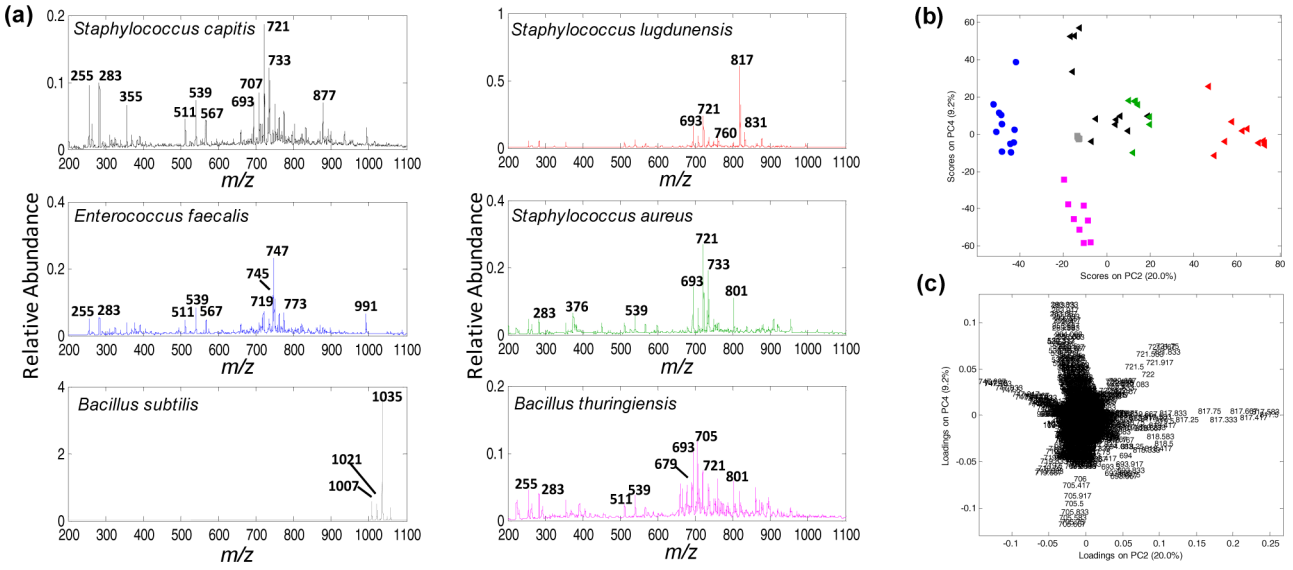

Figure 1. (a) Average mass spectra (normalized to the individual total ion currents) obtained by PS-MS of six different Gram-positive bacteria in the negative ion mode. Fifteen microliters of 0.05% CHAPS in methanol was used as spray solvent. (b, c) PCA of Gram-positive bacteria using negative ion mode information. *S. capitis* (black triangles), *S. lugdunensis* (red triangles), *S. aureus* (green triangles), *E. faecalis* (blue circles), *B. subtilis* (gray squares), and *B. thuringiensis* (pink squares). (b) PC2 versus PC4 score plot. (c) PC2 versus PC4 loading plot.

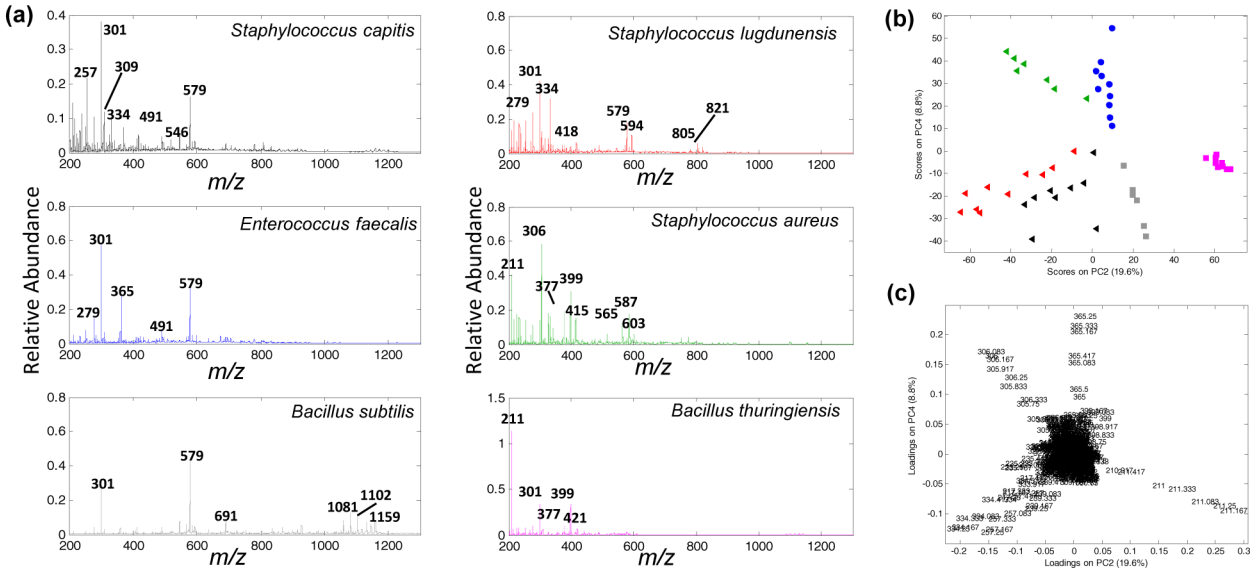

Figure 2. (a) Average mass spectra (normalized to the total ion current) obtained by PS-MS of six different Gram-positive bacteria in the positive ion mode. Fifteen microliters of methanol was used as spray solvent. (b, c) PCA of Gram-positive bacteria using positive ion mode information. *S. capitis* (black triangles), *S. lugdunensis* (red triangles), *S. aureus* (green triangles), *E. faecalis* (blue circles), *B. subtilis* (gray squares), and *B. thuringiensis* (pink squares). (b) PC2 versus PC4 score plot. (c) PC2 versus PC4 loading plot.

species present in the database. PCA was performed again considering Gram-positive and Gram-negative bacteria separately, so that more detailed differences among bacterial taxonomy could be described.

Gram-Positive Bacteria. *Bacillus*, *Enterococcus*, and *Staphylococcus* genera of Gram-positive bacteria were investigated, and average negative mode mass spectra are displayed in Figure 1a.

Visual inspection of the mass spectra indicated differences between bacteria at the genus level, with more subtle differences generally occurring between species. The tentative identifications of the ions at *m/z* 693, 707, 719, 721, 733, 735, 747, and 761 are $[PG(30:0)-H]^-$, $[PG(31:0)-H]^-$, $[PG(32:1)-H]^-$, $[PG(32:0)-H]^-$, $[PG(33:1)-H]^-$, $[PG(33:0)-H]^-$, $[PG(34:1)-H]^-$, and $[PG(35:1)-H]^-$, respectively.[17,46] CID measurements confirmed the identity of the fatty acids of *m/z* 721.4 to be C15:0 (*m/z* 241.2) and C17:0 (*m/z* 269.2), as displayed in Figure S4a.

The PCA score plot displayed in Figure 1b presents the negative ion mode PS-MS data for Gram-positive bacteria. The score plot indicated that the investigated bacteria can be discriminated at the species level using the negative ion mode information.

The separation of *Staphylococcus* genus members (triangles) was confined to the upper-right quadrant of the score plot, along the PC2 axis. *Enterococcus faecalis* (circles) was similarly separated along PC2 occupying the upper-left quadrant, whereas *Bacillus* species (squares) were separated in the

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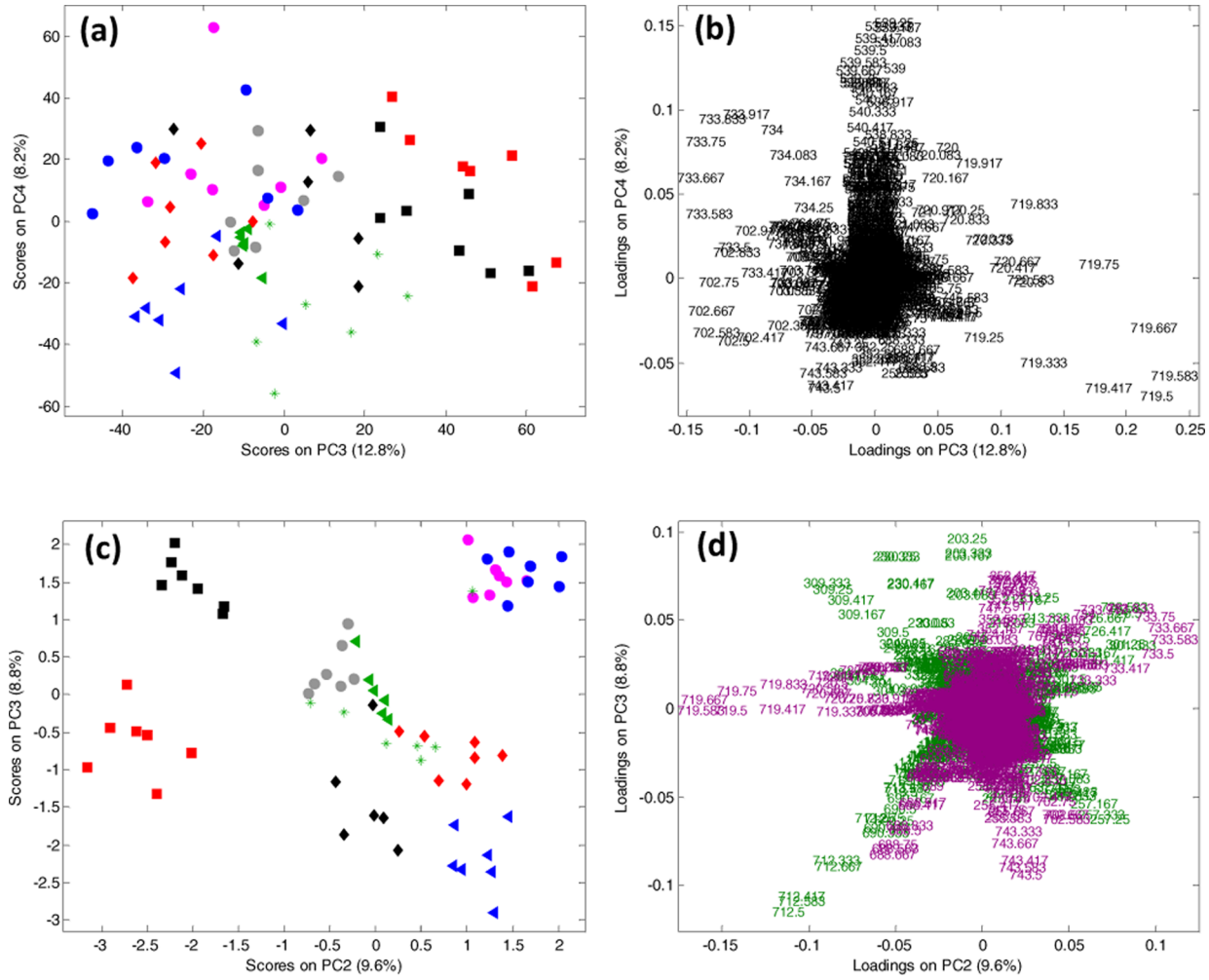

Figure 3. PCA of Gram-negative bacteria: *C. farmeri* (black squares), *C. freundii* (red squares), *E. aerogenes* (gray circles), *E. asburiae* (pink circles), *E. cloacae* (blue circles), *P. penneri* (black diamond), *P. vulgaris* (red diamond), *E. coli* (green stars), *P. aeruginosa* (green triangles), and *P. fluorescens* (blue triangles). Negative ions: (a) PC3 versus PC4 score plot. (b) PC3 versus PC4 loading plot. Fused positive and negative ions: (c) PC2 versus PC3 score plot. (d) PC2 versus PC3 original loading plots labeled in terms of m/z ratio. Violet, negative ions, green, positive ions.

perpendicular direction. In fact, the grouping of scores in Figure 1b indicated greater biochemical similarity between *Staphylococcus capitis* and *Staphylococcus aureus*, in comparison with *Staphylococcus lugdunensis*. This observation corresponds with known genetic differences (as seen in 16S rRNA gene sequencing) among the investigated *Staphylococcus* species.[69] This result suggests that PS-MS is sensitive to differences in genetically closely related species, although the effects are measured indirectly via lipid compositions, as opposed to phenotypic features such as those assayed in biochemical tests.[70] The loading plot displayed in Figure 1c indicated specific ions that were relevant to the separation in PC2 and PC4.

Bacillus subtilis shows an unusual profile in which the C15 cyclic lipopeptide surfactin is clearly evident from the deprotonated molecule at m/z 1035.[46,47,68,71] Surfactin has antimicrobial, antifungal, and antiviral effects, and its secretion is crucial for the survival of *B. subtilis* in harsh environmental conditions.[72−76] Recently, the production of surfactin has been utilized as a distinguishing feature that differentiates various strains of *B. subtilis*.[2] Moreover, surfactin is reported to have surfactant power; it lowers the surface tension of water from 72 to 27 $mN{\cdot}m^{-1}$ at a concentration as low as 20 μM.[68,76] Therefore, the greater abundance of surfactins in comparison with other lipids can be at least partially attributed to its surfactant properties which promote ionization, via the production of smaller secondary droplets and enhanced ion evaporation.[68]

LDA was performed to quantify the separation shown in the PCA score space. LDA was performed after PCA compression by selecting the first five PCs (which explain 90% of total data variability) and five CV-deletion groups following the cross-validation strategy. The prediction rates for all bacterial species and the CV confusion matrix are reported in Table S3 of the Supporting Information. The average successful prediction rate for all the examined Gram-positive bacteria is 98.1%.

The average positive ion spectra of the investigated Gram-positive bacteria are displayed in Figure 2a. The PCA score plot displayed in Figure 2b presents the positive ion mode PS-MS data for Gram-positive bacteria. The loading plot (Figure 2c) indicates which ions are the most relevant for PC2 and PC4 computation.

LDA was performed by selecting the first eight lower-order PCs (that compress 90% of total data variability) and with five CV-deletion groups in cross-validation. The prediction rates for all examined Gram-positive bacteria samples and the CV

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confusion matrix are reported in Table S4 of the Supporting Information. The outcomes essentially confirm the same discrimination capability provided by negative ions (96% average).

Gram-Negative Bacteria. *Citrobacter*, *Enterobacter*, *Escherichia*, *Proteus*, and *Pseudomonas* genera of Gram-negative bacteria were examined in this study, as indicated in Table S1.

Average PS-MS mass spectra were acquired in both negative and positive ion modes, and they are displayed in Figure S5a and b. The mass spectra displayed in Figure S5a show that PG and PE peaks are predominant, which is consistent with data in the literature.[17,45] Peaks at m/z 511, 688, 702, 705, 719, 733, 747, and 761 correspond to $[LPG(18:0)\text{-}H]^-$, $[PE(32:1)\text{-}H]^-$, $[PE(33:1)\text{-}H]^-$, $[PG(31:1)\text{-}H]^-$, $[PG(32:1)\text{-}H]^-$, $[PG(33:1)\text{-}H]^-$, $[PG(34:1)\text{-}H]^-$, and $[PG(35:1)\text{-}H]^-$, respectively.[17,46]

The ion observed at m/z 503 in the negative ion spectra of *Pseudomonas aeruginosa* has been identified as a rhamnolipid with the structure Rha−C10-C10 by CID, as illustrated in Figure S6b. Therefore, the prevalent peaks detected in the m/z 480−640 range were presumed to be rhamnolipids, previously reported to be secreted by *P. aeruginosa*.[2,55] Rhamnolipids act as virulence factors and have antimicrobial activity against a variety of microorganisms such as *Candida albicans*, *Aspergillus niger*, and *Gliocadium virens*.[2,77,78]

The mass spectra recorded in the positive ion mode are rich in information, with sodium and potassium adducts of phosphatidylethanolamines (PEs) being predominant (Supporting Information, Figure S5b). Peaks at m/z 690, 698, 704, 712, 726, 740, 1379, 1401, and 1429 correspond to $[PE(32:1)+H]^+$, $[PE(31:1)+Na]^+$, $[PE(33:1)+H]^+$, $[PE(32:1)+Na]^+$, $[PE(33:1)+Na]^+$, $[PE(34:1)+Na]^+$, $[2PE(32:1)+H]^+$, $[2PE(32:1)+Na]^+$, and $[2PE(33:1)+Na]^+$, respectively.[17,46]

Positive ion mode spectra are dominated by PEs while the corresponding negative ion spectra are dominated by PG ions. Since PEs are expected to have higher proton affinities than the corresponding PGs, positive ion spectra are dominated by the PEs. Whereas it is harder to abstract a proton from PEs than the corresponding PGs, and consequently, PGs dominate the observed negative ion mode phospholipids.[4] This selective ionization phenomenon was previously reported in an electrospray ionization (ESI) study of Gram-negative bacteria extracts.[4] Moreover, several ions were observed in the positive ion spectra of *P. aeruginosa*, m/z 211, 244, and 298, which match literature nominal m/z values of procyanin, hydroxyquinoline, and unsaturated hydroxyquinoline, respectively.[2]

For negative ions, the PC3 versus PC4 score plot shows no clear separation among different bacterial species, as displayed in Figure 3a. Thus, the chemical information provided by (−) PS-MS lipids is not sufficiently specific to represent a unique fingerprint for these sample categories.[35,55] LDA performed on the scores of the first 10 PCs (accounting for about 90% of total data variability) confirms the low predictive ability, with an average successful prediction rate of 70.6% (with 5 CV-deletion groups in cross-validation). The prediction rates for all sample categories and the CV confusion matrix are reported in Table S5 of the Supporting Information. The relatively low prediction rates of the Gram-negative bacteria can be attributed to the significant genetic resemblance of the species of *Enterobacteriacae* family which represent the majority of the investigated samples.[35]

Data Fusion. The strategy of fusing positive and negative ion data has been successfully used to differentiate bovine oocytes and embryos grown in in vivo and in vitro conditions and to provide new insights on chemical features of these microscopic samples.[79,80] Previous studies have demonstrated that fusing the chemical information present in the negative ion mode with that of the positive ion mode increased classification rates.[57] We selected positive ion mode data from bipolar PS-MS experiments and fused with negative mode PS-MS data to demonstrate this potential increase in classification rates for bacteria discrimination. The fusion of the first 10 lower-order PCs from the data sets of negative and positive ions increases the separation among different bacterial species, as visible in the PC2 versus PC3 score plot of Figure 3c and as quantified through LDA. Indeed, the average prediction rate for all sample categories increased from 70.6% to 86.8%. The specific CV prediction rates for all Gram-negative species and the CV confusion matrix are reported in Table S6 of the Supporting Information. Such values indicated that the data fusion strategy significantly increases the classification rate for the categories and allowed more sensitive and specific models to be defined, with lower misrecognition rates. Furthermore, the preliminary PCA exploratory strategy provided information on the intercorrelations among different lipid classes that may be of biological interest. Under the hypothesis that a real data bank should include a much larger number of different species than those considered in this study, the fusion of more complementary chemical profiles, each characterized by specific strengths and limitations, appears to be a potential tool to be used at least to maintain the same classification capability.

Characterization of Bacterial Components in a Mixture. The ability to analyze complex microbial mixtures is invaluable for real-world applications where multiple infections are not rare.[48,81,82] A mixture of *Citrobacter farmeri* and *Citrobacter freundii* (1:1) was prepared by smearing three colonies of *C. farmeri* and *C. freundii* onto the surface of a filter paper triangle. The mixture was analyzed by PS-MS in a typical 60 s spray duration experiment employing bipolar data acquisition and then compared to *C. farmeri* and *C. freundii* data for individual bacterial samples. Negative and positive ion mass spectra are displayed in Figure 4a and b, respectively. The comparison shows that PS-MS is successful in characterizing these closely related bacteria existing in a mixture using both negative and positive ion modes. Prospectively, the contribution of each component can be quantitatively determined through proper methods. This capability will help in the analysis of samples provided by patients suffering from combined infections.[83]

■ CONCLUSIONS

Paper spray mass spectrometry (PS-MS) can be used to generate characteristic, reproducible mass spectra directly from colonies grown on agar. No sample preparation or lipid extraction steps are required prior to analysis. Bacterial discrimination can be achieved in less than 2 min not including any needed culturing time. This method can be applied for differentiation of Gram-positive and Gram-negative bacteria at the species level. Species level differentiation of the included Gram-positive bacteria has been achieved on the set of species examined here with a predicted success rate of 98% based solely on consideration of negative ion mode data. Species level differentiation of Gram-negative bacteria has been achieved with a prediction success rate of 87% when the positive ion and negative ion data are fused using a data fusion strategy.

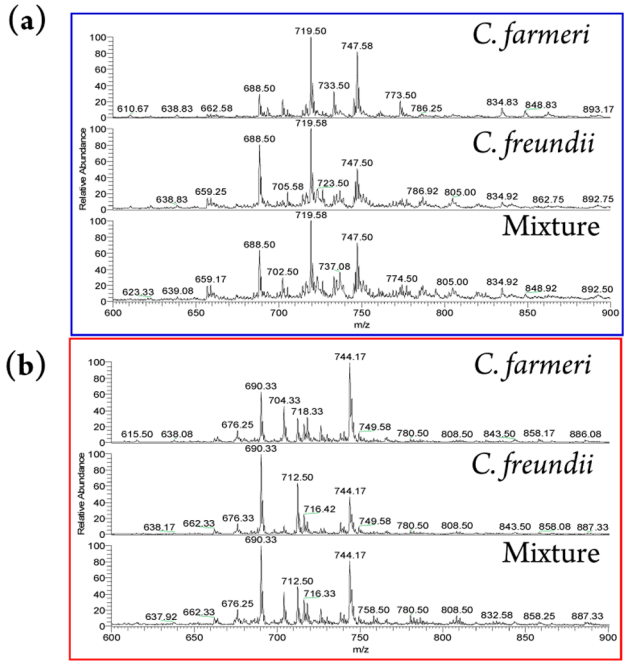

Figure 4. Averaged negative mode (a) and positive mode (b) mass spectra of *C. farmeri*, *C. freundii*, and ~1:1 mixture.

A limited set of microorganisms has been studied by MALDI for comparison with PS-MS, and data are shown in Figure S8, Supporting Information, and briefly discussed there. Although they are based on different types of biomolecules as well as different MS and bioinformatics methods, both approaches are successful in discrimination at the species level.

As stated earlier, alternative approaches for multivariate analysis of either separate or conjoint MS data could be developed and compared with a PCA-LDA strategy to further improve classification capabilities. This initial work presents PS-MS, followed by the simultaneous analysis of all the collected MS data, as a promising strategy to identify bacteria, provided that a sufficiently large database can be created. The future impact of rapid identification of bacteria using reliable methods will be more effective for treatment, faster recovery, and lower cost of treatment as a result of more targeted therapy. The data in this paper establish a foundation for the expansion of the experiment to the development of a large library of microorganisms including a wide variety of species, strains, and culture media.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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