

Multiple Reaction Monitoring Profiling (MRM-Profilng) of Lipids to Distinguish Strain-Level Differences in Microbial Resistance in *Escherichia coli*

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ABSTRACT: The worldwide increase in antimicrobial resistance is due to antibiotic over-use in agriculture and over-prescription in medicine. For appropriate and timely patient support, faster diagnosis of antimicrobial resistance is required. Current methods for bacterial identification rely on genomics and proteomics and use comparisons with databases of known strains, but the diagnostic value of metabolites and lipids has not been explored significantly. Standard mass spectrometry/chromatography methods involve multiple dilutions during sample preparation and separation. To increase the amount of chemical information acquired and the speed of analysis of lipids, multiple reaction monitoring profiling (MRM-Profilng) has been applied. The MRM-Profilng workflow includes a discovery stage and a screening stage. The discovery stage employs precursor ion (PREC) and neutral loss (NL) scans to screen representative pooled samples for functional groups associated with particular lipid classes. The information from the first stage is organized in precursor/product ion pairs, or MRMs, and the screening stage rapidly interrogates individual samples for these MRMs. In this study, we performed MRM-Profilng of lipid extracts from four different strains of *Escherichia coli* cultured with amoxicillin or amoxicillin/clavulanate, a beta-lactam and beta-lactamase inhibitor, respectively. T-tests, analysis of variance (ANOVA) and receiver operating characteristic (ROC) curves were used to determine the significance of each MRM. Principal component analysis (PCA) was applied to distinguish different strains cultured under conditions that allowed or disallowed development of bacterial resistance. The results demonstrate that MRM-Profilng distinguishes the lipid profiles of resistant and non-resistant *E. coli* strains.

Annually there are over 1.2 billion health care visits in the United States of which 12.6% result in ~154 million antibiotic prescriptions for bacterial infections.¹ Urinary, respiratory and skin infections are most common.² The top pathogens responsible for 80 to 90% of infections are *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, the latter two of which are part of the ESKAPE group of pathogens which also include *Enterococcus faecium*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.³⁻⁵ Infections are especially common in certain care settings and patient populations where they cause high recurrence rates and great severity of infections.⁶⁻⁸ Potential infections are frequently treated prior to confirmation of the presence of pathogens, leading to over-prescription which is strongly associated with the development of antibiotic-resistance and may eradicate potentially commensal microbiome populations, resulting in dysfunction against future pathogens.⁹⁻¹² High rates of infection recurrence and the emergence of pathogens with antimicrobial resistance are found among patients with frequent antibiotic dosing.^{11,13,14} There therefore remains a need for rapid bacterial identification to help suppress this process.

1 In current clinical settings, the identification of bacteria is usually determined based on phenotype and/or
2 genotype. Culture-based phenotyping methods are commonly used in clinical laboratories.^{15,16} Molecular
3 diagnosis, including sequencing of ribosomal genes,¹⁷ whole genome sequencing,^{18,19} and amplification
4 methods,²⁰ represents an alternative strategy for bacterial typing. Current MS-based typing methods are largely
5 focused on the protein profile with an emphasis on ribosomal proteins.¹⁶ In the last decade, matrix assisted
6 laser desorption/ionization coupled with time-of-flight mass spectrometry (MALDI-TOF MS) has been applied
7 successfully using relatively simple sample preparations to acquire fingerprint mass spectra and accomplish
8 identification by database searches.^{21–23} Several commercial platforms have emerged and profoundly changed
9 the state of clinical testing. In addition, tandem MS with or without liquid chromatography has also been used
10 with great success.^{24–26}

13 Metabolomics, which covers a wide range of small molecules but is not widely applied to bacterial
14 identification, is an appealing and emerging field of omics in biomarker discovery by MS.^{27–30} Lipids are believed
15 to play an indispensable metabolic role in many essential biological processes. In microorganisms, the diversity
16 in structures and the complexity of biosynthesis pathways of bacterial lipids enable bacterial cells to adjust their
17 lipid profiles in response to environmental changes, including antibiotic treatment.^{31–33} Conventional lipid
18 pathway analysis involves extensive sample preparation and complex protocols for the characterization of
19 potential lipid biomarkers.^{34,35} The unclear relationship between lipids and molecular function further slows
20 down the pace of development of analytical methods. To accelerate exploratory lipidomic discovery, a novel
21 two-stage methodology, multiple reaction monitoring profiling (MRM-Profiling), has been developed as a
22 diagnostic/distinguishing factor. It has previously been tested on several model systems, including Parkinson's
23 disease,³⁶ polycystic ovarian syndrome,³⁷ diet compliance,³⁸ colostrum uptake,³⁹ atopic dermatitis,⁴⁰ and embryo
24 metabolism.⁴¹ The results of MRM-Profiling have proven to be broadly consistent with those from pathological
25 or clinical analysis.

28 Here we report a study on the feasibility of using MRM-Profiling to differentiate lipid profile differences among
29 bacterial strains with different antimicrobial resistance. Lipid extracts of bacteria were analyzed using
30 electrospray ionization MS (ESI-MS), screened by precursor ion and neutral loss scans and discriminated using
31 PCA. The experiments focused on glycerophospholipids and sphingolipids. Statistical analytical methods,
32 specifically the t-test and ROC curves, provided details as to which of the MRMs were most informative.
33 Additionally, lipid differences observed among bacteria treated using various concentrations of amoxicillin and
34 amoxicillin/clavulanate indicated that lipid profiles are modulated by environmental changes. The lipid
35 composition of bacterial lysates was found to allow discrimination between resistant and non-resistant *E. coli*
36 strains.

43 **EXPERIMENTAL SECTION**

44 **Chemicals.** All chemicals were all purchased from Sigma-Aldrich Co. LLC (St. Louis, MO) except that clavulanate
45 was purchased from Cresent Chemical (Bangladesh).

46 **Bacterial Culture.** Non-resistant *E. coli* strains (ATCC® 25922™, CDC AR-bank #0077, ATCC® 4157™) and resistant
47 strains with beta-lactam resistance gene CTX-M-14 or 15 (CDC AR-bank #0086, CDC AR-bank #0162, CDC AR-
48 bank #0151) were obtained from the ATCC or the CDC and FDA antibiotic resistance isolate bank. Bacterial
49 stocks, stored at -80°C until usage, were generated from tryptic soy broth (TSB) (Becton Dickinson, Franklin
50 Lakes, NJ) cultures inoculated with a single colony taken from streak plate isolates from the supplier's stock on
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tryptic soy agar (TSA) (Becton Dickinson, Franklin Lakes, NJ). Fresh bacteria were cultured from stock by gentle scraping with a sterile implement off the top of the stock and by streaking onto a fresh TSA plate for single colony growth, which was incubated for 15 hours at 37°C. A single colony was then re-streaked onto a fresh TSA plate and was incubated for 15 hours at 37°C. Bacterial titer was estimated by resuspending morphologically similar colonies into 20mL of fresh TSB and by measuring optical density at 600nm (OD₆₀₀) absorbance readings using a BioTek Synergy HTX multi-mode plate reader (BioTek, Winooski, VT) with McFarland standards (Scientific Device Laboratory, Inc., Des Plaines, IL). Verification of culture concentrations was performed with a ten-fold serial dilution in TSB; a 1.0 µL loopful from each dilution was streaked onto BD BBL™ Trypticase™ Soy Agar plates (Becton, Dickinson and Co., Franklin Lakes, NJ), and colonies were counted after incubation at 37°C for 16 hours.

Minimum Inhibitory Concentration Test. A standard 96-well plate-based minimum inhibitory concentration (MIC) test was used to measure antibiotic efficiency of amoxicillin and amoxicillin/clavulanate combination against bacteria in mono-culture. After overnight growth on the corresponding culture medium and OD₆₀₀ measurement, MIC testing was performed with cation-adjusted Mueller-Hinton broth. In a row of the plate, 11 wells contained a starting culture at 5x10⁵ cfu/mL challenged with decreasing antibiotic concentrations delivered by serial dilution (e.g. 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0 µg/mL); the last well contained blank medium as a sterility control. The lowest antibiotic concentration at which no cell number increase was measured via OD₆₀₀ after a 24-hour incubation was the MIC of the antibiotic for that bacterial strain. All MIC tests were conducted in compliance with Clinical Laboratory Standards Institute (CLSI) procedures of clinical testing. The determined values of MIC for each strain appear in Table S1.

Sample Preparation. Samples of specific types of bacteria and MIC concentrations were taken from culture medium, normalized to 10⁷ cells/mL by OD₆₀₀, and were lysed with sonication. Lysate of bacteria was collected and stored at -80 °C. Lipid extraction was accomplished using the Bligh-Dyer⁴² strategy. After drying, lipid extracts were redissolved in acetonitrile/methanol/300 mM ammonium acetate (3:6.65:0.35, v/v/v) and transferred into autosampler vials ready for analysis. The extract solution was diluted to 2.5x10⁵ cells/µL in vial, which is equivalent to lipid extracts of 2x10⁶ cells in each injection of 8 µL solution.

Multiple Reaction Monitoring Profiling. MRM-Profilng is a two-stage MS methodology that benefits from various scan modes in tandem mass spectrometry—especially PREC and NL. In the discovery stage, PREC, NL, and MRM methods are applied to representative samples, mostly focusing on recognition of functional groups present in glycerophospholipids, acyl-carnitines, ceramides, and acyl residues containing different number of carbons with various degree of unsaturation. Specifically, glycerophospholipids contain glycerophosphate (PA), glycerophosphocholine (PC), glycerophosphoethanolamine (PE), glycerophosphoglycerol (PG), glycerophosphoinositol (PI), and glycerophosphoserine (PS). The PREC and NL scans were used as sources of MRM transitions and this experimental data was supplemented by MRM transitions deduced from data in the online lipid database, LipidMaps. Altogether 32 NL scan methods, 33 PREC scan methods, and 13 MRM transitions, as listed in Table S2 were used to survey representative samples from *E. coli* strains CDC 0077 and CDC 0086 cultured without amoxicillin. Experiments were carried out using a triple quadrupole mass spectrometer, Agilent QQQ 6410 (Santa Clara, CA), equipped with Agilent G1367A 1100 series autosampler (Santa Clara, CA). Samples were introduced into the mass spectrometer by direct flow injection ESI, i.e. without chromatographic separation. The ESI voltage was -3.5 kV. Dwell time was 25 ms. Collision energy (in manufacturer's unit) and other scan method details are in Table S2. For each scan method, 8 µL of sample was injected at a flow rate of 20 µL/min. After data acquisition, transitions that were at least 30% higher in ion

counts than blank samples were selected. Other values of threshold or data filtering concepts could have been employed but the tradeoff between chemical information and total instrument time required had an effect on the next stage, therefore we chose to be conservative to avoid losing chemical information. In addition, since representative samples were used, which could not reflect the scope of lipid profiles of all strains, keeping more transitions is beneficial to retaining as much chemical information as possible. In total, 2,329 transitions, of which 1,900 were in the positive ion mode and 429 in the negative ion mode, resulted from the discovery stage. The distribution of MRMs among different classes of lipids or functional groups are shown in Figure S1 and Figure S2 for positive and negative ion modes, respectively. This list served as a pool of candidates for building up MRM methods for the next Screening stage. Selected transitions were then arranged into 9 MRM methods (7 in positive ion mode, 2 in negative ion mode) to investigate individual samples using the same instrument parameters as used in the Discovery stage. Given that the screening time for each method is 3 minutes, this results in a total analysis time of 30 minutes to screen each sample.

Statistical Analysis. Raw data were processed using Proteo-Wizard to extract the ion signals of each transition. The lowest ion count was regarded as the noise level of instrument. The cutoff for the screening stage data was chosen as $3\times$ noise intensity to remove uninformative transitions with low ion intensities. The resulting data was represented as a table of ion counts, in which columns were samples and rows were features (MRMs in this case). T-test, analysis of variance (ANOVA), PCA, and ROC curves were calculated and plotted using MetaboAnalyst. For PCA, ANOVA, and t-test, the original data were scaled by mean-centering and dividing the individual intensities by the standard deviation of each MRM. For the ROC curve, no scaling was applied and the area under the curve (AUC) was calculated for each MRM. The cutoff applied to the t-test and ANOVA was a false discovery rate (FDR) adjusted p -value (or q -value) of 0.05 throughout the data processing.

RESULTS AND DISCUSSION

Lipid Analysis of Non-resistant Strains. Lipid analysis was carried out for non-resistant strains (ATCC 25922 and ATCC 4157) to investigate the effect of antibiotic application. Both strains were cultured under five conditions: no amoxicillin or amoxicillin/clavulanate, amoxicillin at half MIC, amoxicillin at MIC, amoxicillin/clavulanate at half MIC, and amoxicillin/clavulanate at MIC. MRMs were filtered by ion counts and FDR adjusted p -value in ANOVA, and then analyzed by PCA. Figure 1 shows the PCA scores plots of both strains. In the PCA scores plot, each point represents an individual lipid extract sample from the cultured bacteria and the elliptical shaded area is the calculated 95% confidence region for each group. In the case of ATCC 25922 as shown in Figure 1(a) where 197 MRMs were used, bacteria treated with amoxicillin or amoxicillin/clavulanate at MIC are well separated along principal component (PC) 1 from bacteria cultured at half MIC or without amoxicillin. PC1 and PC2 contribute 76% to the total variance. This separation can be attributed to the nature and the amount of the antibiotic, which leads to bacterial growth inhibition and hence the observed changes in lipid profiles.

Similar separation due to treatment differences are observed in the non-resistant strain ATCC 4157 in Figure 1(b). With the same data filters, 157 MRMs were selected. PC1 and PC2 cover 74% of the entire variance observed. Different from ATCC 25922, the group grown with amoxicillin at MIC clusters away from the other groups on the PC1 dimension in the score plot. Groups treated with amoxicillin/clavulanate tend to overlap on PC1, while control and half MIC amoxicillin are closer. The MIC amoxicillin/clavulanate group is differentiated by PC1 to be closer to groups treated with low concentration of amoxicillin, instead of merging with the MIC amoxicillin group as in ATCC 25922.

Lipid Profile Differences Between Non-Resistant and Resistant Bacterial Strains. The results of attempts to distinguish non-resistant and resistant strains are promising. The purpose of carrying out statistical analysis of different strains exposed to the same treatment is to identify transitions that are responsible for the separation between strains. Two non-resistant strains (ATCC 25922 and ATCC 4157) and two resistant strains (CDC 0162 and CDC 0151) were cultured without amoxicillin or amoxicillin/clavulanate as controls or with amoxicillin/clavulanate at half MIC. Transitions related to acyl-carnitines were found to be inconsistent (data not shown) and were removed from the statistics to better concentrate on the pattern of significant phospholipid transitions. Initially, for the control group, 86 MRMs were used for the statistical analysis, from which 38 MRMs were then selected using the FDR adjusted p -value in ANOVA. From the PCA scores plot of these transitions shown in Figure 2(a), PC1 and PC2 contribute 57% to the total variance. The major separation occurs along PC1. Statistical analysis was also carried out in terms of distinguishing non-resistant and resistant strains by regrouping samples into two sets. Because PCA is an unsupervised method, the PCA scores plot remains the same regardless of the labels of each sample if the same set of data is used. We note that, in cases where no antibiotic stress was applied, resistant and non-resistant strains can be well differentiated by PCA. T-tests and ROC curves can provide more insight into the differences between strains. T-test results (Figure 2(b)) imply that 20 transitions, all of which show up in the 35 MRMs in the previous analysis, are significant for the differentiation. PC1 and PC2 take up 67% of the variance. ROC curves can be used to determine important transitions that are potentially characteristic for each set. As shown in Figure S3, there are seven transitions with AUC above 0.9, including three from saturated fatty acyl chain residues, two from PS, one from PE, and one from PG. Transitions selected from each analysis and their corresponding functional group or lipid class are tabulated in Table S3.

Turning to antibiotic treated bacteria, after elimination of transitions from acyl-carnitines and using the same data filter, 86 MRMs were left. ANOVA and t-tests produced sets of 34 and 12 transitions with the same cutoff applied, respectively. From the PCA results in Figure S4, transitions selected from ANOVA do not provide perfect separation between ATCC 25922 and CDC 0162. Transitions from t-tests offer similar separation along PC1 and its first two PCs explain 82% of the total variance. However, the number of features is fewer than in the control set. Among the 12 significant MRMs, three MRMs are found to have AUC above 0.9 from ROC curve, including one from PE, one from 26:0 fatty acyl chain residue, and one from PG, as shown in Figure S5. Details of transitions from each analysis are listed in Table S4. Bacteria treated with amoxicillin/clavulanate at half MIC and the controls have similar lipid transitions determined to be responsible for the separation.

Lipids associated with each MRM are postulated from the characteristic PREC and NL scans, as tabulated in Table S5. Although the exact structure of the lipids associated with each transition could not be decided merely by the MS/MS experiment, it is the pattern of the entire set of selected transitions spread over nine methods that provides reliable differentiation between strains from multiple classes of lipids. The distribution among the lipid classes of the transitions selected from t-tests is shown in Figure 3. Application of amoxicillin/clavulanate resulted in an increase in the number of informative transitions in unsaturated fatty acyl chain residues and a decrease in those in saturated fatty acyl chain residues. The number of informative transitions from PS dropped significantly after antibiotic treatment. Additionally, the total number of significant transitions from glycerophospholipids is larger when no amoxicillin or amoxicillin/clavulanate are applied, implying that more glycerophospholipids are detected. This is consistent with previous research.⁴³ In spite of these successes at differentiation, samples containing both resistant and non-resistant *E. coli* may be more difficult to distinguish.

Different from exact mass searches in the database as is done in proteomics, MRM-Profiling offers opportunity to investigate changes in functional groups with more confidence. The low resolution of the instrument and the use of direct analysis with MRM methods means that it is still possible that isomeric ion pairs exist. For example, PG (35:1) and PGo (36:1) have monoisotopic masses with close values at 762.5411 ($C_{41}H_{79}O_{10}P$) and 762.5775 ($C_{42}H_{83}O_9P$), respectively. In the MRM method, with NH_4Ac introduced into the spray, the ammonium adducts are predominant for PG, making the corresponding transitions appear as 780.5→591.5 for both molecules. Nevertheless, when analyzing a lipid extract, the neutral loss of 189 from the head group in PG is what one should focus on because both candidate ion pairs are PG. Although the number of antibiotics types is limited in current study, MRM-Profiling might be more sensitive and efficient than existing methods as there are more lipid molecules per cell and analysis of small molecules has advantages in ion transmission and ionization efficiency. This preliminary study has demonstrated the feasibility of distinguishing bacterial strains by carrying out MRM-Profiling to characterize lipid patterns.

CONCLUSIONS

We have demonstrated that MRM-Profiling can be applied to distinguish four different strains of *E. coli*, including two non-resistant and two resistant strains. Different strains are well differentiated by PCA using significant MRMs acquired from t-test. This indicates that the lipid profiles not only serve to characterize bacterial species, but they also change in resistant strains compared to non-resistant strains and they are sensitive to antibiotic challenge. Lipid biomarker candidates are also determined, and this tool can help zeroing in on the biomarkers most significant for the separation. Based on previous studies in other complex biological systems, MRM-Profiling could expedite and improve the identification of lipids related with resistance, virulence, and viability. As such it will help benchmark the gene pathway involved in the phenotypes as a novel approach to identify unique resistance genes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Table S1, MIC information; Table S2, details of each scan method in discovery stage; Table S3, MRMs selected from control set; Table S4, MRMs selected from Amox/Clav set; Table S5, postulated formula of MRMs; Figure S1, distribution of positive mode MRMs; Figure S2, distribution of negative mode MRMs; Figure S3, ROC curves of control set; Figure S4, PCA of Amox/Clav set; Figure S5, ROC curve of Amox/Clav set.

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Notes

The authors declare no competing financial interest.

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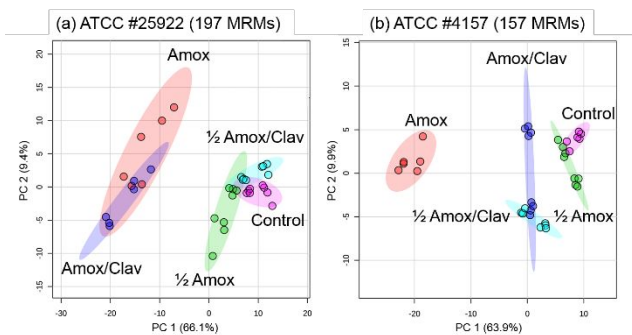


Figure 1. PCA scores plots of non-resistant bacterial strains a) ATCC 25922 and b) ATCC 4157 after various antibiotic treatments. The color-shaded areas are the calculated range with 95% confidence. Amoxicillin and clavulanate are abbreviated as Amox and Clav, respectively.

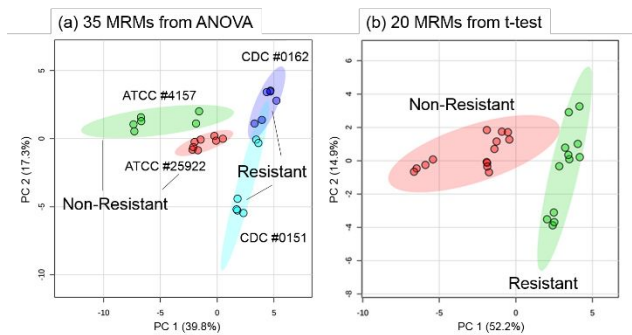


Figure 2. PCA scores plots of control samples (no amoxicillin or amoxicillin/clavulanate applied) using transitions selected from a) ANOVA and b) t-test. Color-shaded areas are the calculated range with 95% confidence.

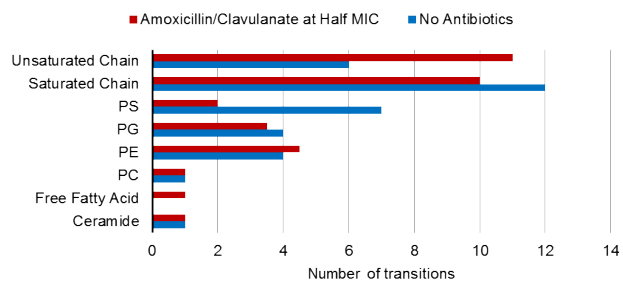
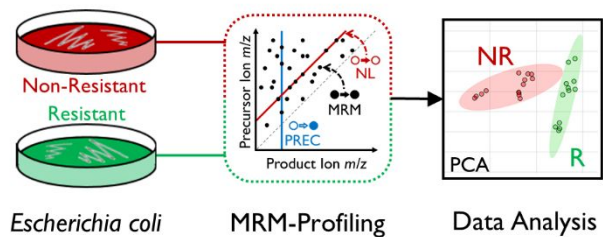
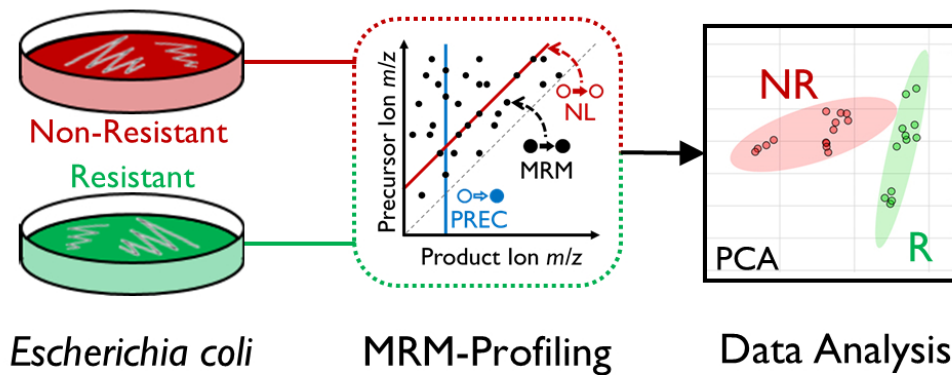


Figure 3. Distribution of transitions selected from t-test between controls and sample treated with amoxicillin/clavulanate at half MIC. “Saturated Chain” refers to the saturated fatty acyl chain residues. “Unsaturated Chain” refers to fatty acyl chain residues with degree of unsaturation ranging from 1 to 5.

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24 Bacterial strains are classified as resistant or non-resistant using MRM profiling and multivariate analysis

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