Nanospray desorption electrospray ionization mass spectrometry of untreated and treated probiotic Lactobacillus reuteri cells

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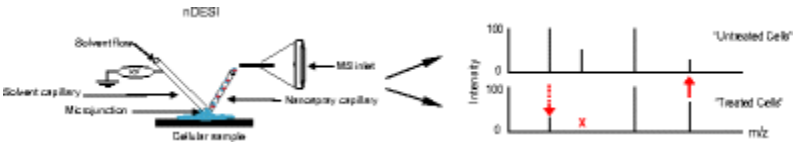
Abstract:

Mass spectrometry has proven to be a useful technique for rapid identification of bacterial cells. Among various ionization techniques in mass spectrometry, matrix-assisted laser desorption/ionization (MALDI) has been commonly used for the identification of bacterial cells. Recently, MALDI mass spectrometry has also been utilized to distinguish cellular responses. Ambient ionization techniques do support whole bacterial cell analysis, which include desorption electrospray ionization (DESI). Nanospray DESI (nDESI) is a new variant of DESI, and its application to whole-cell mass spectrometry is limited. In this project, the use of nDESI mass spectrometry to measure probiotic *Lactobacillus reuteri* (LR) cells is explored. A unique and reproducible mass spectral pattern of untreated LR cells was obtained by using 50% methanol/water as nDESI solvent. The use of nDESI mass spectrometry is further extended to distinguish untreated LR cells from treated LR cells that have been exposed to low pH. These findings demonstrate the feasibility of using nDESI in whole-cell mass spectrometry.



Keywords: Whole-cell mass spectrometry | Bacterial cells | Nanospray DESI

Article:

Introduction

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is used widely in clinical laboratories as a means of rapidly identifying bacterial strains [1,2,3]. This is

accomplished by mixing the bacterial culture with a matrix compound and spotting onto a MALDI sample plate prior to the analysis. A schematic diagram of the MALDI process is shown in Fig. 1A. This direct approach to identify bacterial cells is often referred as whole-cell mass spectrometry or intact cell mass spectrometry, because intact bacterial cells are mixed with MALDI matrix prior to the measurements. Bacterial strains are differentiated by detecting specific molecular ions from the cellular samples, and the intensity of their corresponding peaks in the mass spectra can also be taken into consideration. The mass spectral patterns obtained from a variety of known bacterial cells form a spectral library or database that can be referenced when bacterial cells in a particular sample are being identified [4,5,6].

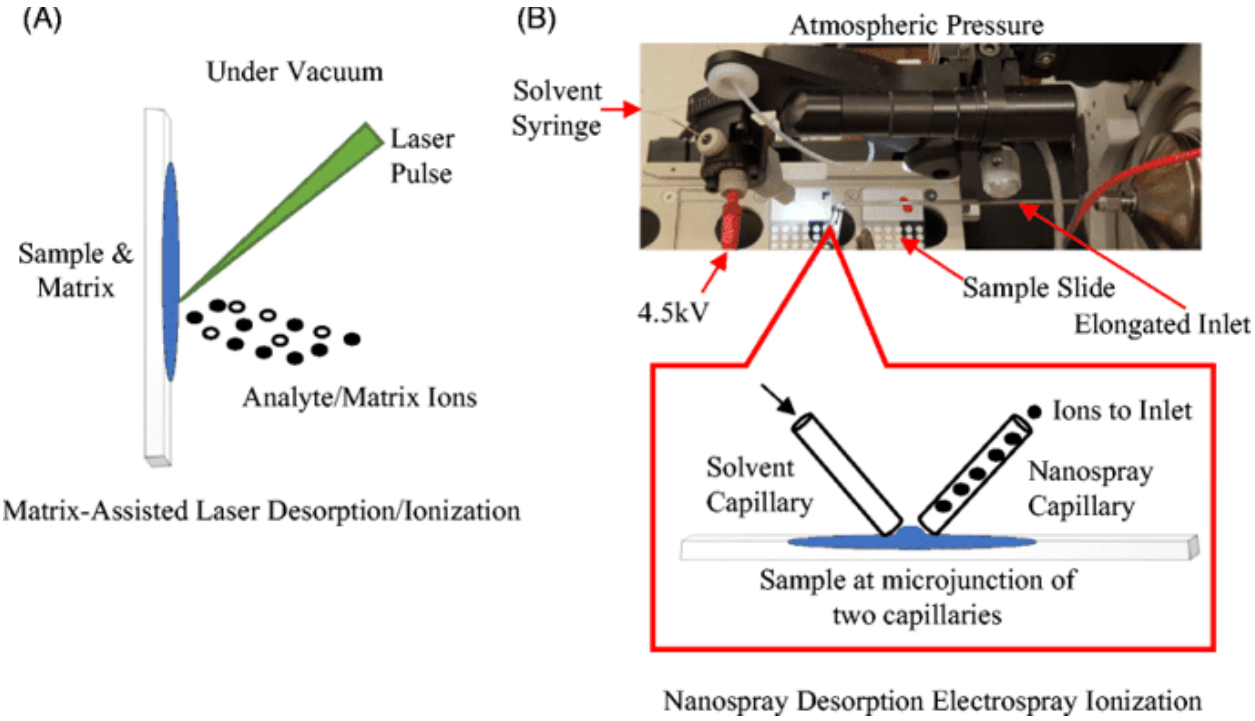


Fig. 1. Schematic diagram of (**A**) matrix-assisted laser desorption/ionization (MALDI) and (**B**) nanospray desorption electrospray ionization. A 2–4 µL sample was air-dried on a glass slide. Fused silica capillary with 49 µm internal diameter was ~ 1–2 mm above the sample, and the flow rate of solvent was 3.5 µL/min

Whole-cell mass spectrometry has also been used to monitor the responses from cells that have been exposed to a variety of conditions. This includes detecting bacterial responses to different environmental conditions by measuring the cells directly using MALDI mass spectrometry [7, 8]. Among other applications of whole-cell mass spectrometry, it has been used to detect and monitor the responses from mammalian cells. Using MALDI mass spectrometry, the response of mammalian cells which had been exposed to known toxic chemicals was detected by comparing the mass spectral patterns [9]. Other studies have also shown the capability of MALDI mass spectrometry to detect cellular responses to various toxicants [10].

Mass spectrometric ambient ionization technique enables samples to be ionized outside the mass spectrometer in an open laboratory environment or in their original condition [11,12,13]. The ability of samples to be ionized outside the mass spectrometer reduces the workload and simplifies the procedure for preparing the samples before the mass spectrometric analysis can be carried out [14, 15]. Bacterial samples have been studied by using ambient ionization techniques.

It has been utilized particularly for identifying bacteria and also for examining colonies of bacteria directly from agar plates [14, 16, 17]. Desorption electrospray ionization (DESI) mass spectrometry has been used to rapidly distinguish different types of bacteria [18]. Unlike MALDI mass spectrometry, DESI and other ambient ionization techniques are more suited to studying compounds in low mass ranges [19,20,21].

Nanospray desorption electrospray ionization (nDESI) mass spectrometry is a new variant of DESI source that makes use of a liquid junction to deliver charged analyte ions into the mass spectrometer (Fig. 1B). Instead of using a flow of gas to direct a charged solvent in the conventional DESI, a solvent capillary, which is also known as the primary capillary, is utilized to deliver the solvent in small charged droplets to the sample surface, and a secondary capillary (or nanospray capillary) is used to transfer the charged analyte ions into the mass spectrometer [22]. The droplets that form on the sample surface bridge the gap between the two capillaries and enable the transfer of sample into the mass spectrometer [23, 24]. Ambient ionization sources like nDESI enable samples to be ionized without any MALDI matrix compound, thus simplifying the protocols for sample preparation. In this study, the use of nDESI mass spectrometry to measure a specific bacterial cell culture was first explored. This was followed by exploring the use of nDESI mass spectrometry to distinguish untreated bacterial cells from the same cell type but treated with low pH, which is a critical factor for the survival of bacterial cells.

Materials and methods

Preparation of bacterial samples

Lactobacillus reuteri (ATCC 23272) samples were prepared freshly by inoculating 0.5–1 μL of a stock culture into 5 mL of de Man-Rogosa-Sharpe (MRS) medium and incubated under 5% of CO_2 gas at 37 °C in the darkness until OD_{600} reading of ~ 1.8 was attained, which corresponded to the mid-log phase of *Lactobacillus reuteri* (LR) cells. The blank for the OD_{600} readings was MRS medium without any cells.

Harvesting and washing bacterial cells

To harvest the bacterial cells, the culture was transferred into a 15-mL centrifuge tube and centrifuged at 3000*g* for 3 min at 4 °C. The supernatant was discarded. The cell pellet was re-suspended in 1 mL of fresh ice-cold MRS medium and transferred into a microcentrifuge tube. After spinning down the cells, the cell pellet was washed two times with 1 mL of autoclaved ice-cold deionized water. Following the last wash, the pellet was re-suspended in about 200–500 μL of autoclaved ice-cold deionized water.

Treatment of LR cells at low pH

The *Lactobacillus reuteri* cells were grown under normal conditions, and OD_{600} of ~ 1.8 was attained before being harvested and re-suspended in an MRS medium that had been adjusted to pH 4 by using hydrochloric acid. After a 1-h incubation inside the incubator, the bacterial cells were transferred, washed, and re-suspended in autoclaved ice-cold deionized water as described

above. In order to have a control sample for comparison, a separated and equal volume of cell culture was harvested and re-suspended in fresh MRS medium at the normal pH and handled in the same way as the treated cells.

Nanospray DESI MS analysis

The 2–4 μL samples that contained whole LR bacterial cells were dried under room temperature on a glass slide. Mass spectrometric measurements were performed by using a Thermo LTQ Orbitrap equipped with a DESI ionization source from Prosolia. Modification of this source was accomplished by extending the length of existing spray capillary, thus allowing droplets of solvent to be delivered onto the sample surface. The length of nanospray capillary was measured and cut to an appropriate length for lining it up with the mass spectrometry inlet and the solvent capillary as shown in Fig. 1. The alignment of the nanospray capillary was further adjusted while a control was being measured. The rhodamine dye (443 *m*/*z*) in a red Sharpie™ was used as a control in this study. Droplets of 50% methanol/water solvent were delivered to the sample by using a 250-μL syringe with a flow rate of ~ 3.5 μL/min. The spray voltage was set to 4.5 kV and the capillary voltage at 55 kV, which were default settings for the Prosolia DESI ionization source. The angle of the solvent capillary also remained at the default position as this is less critical for nDESI experiments, because a stable signal can be obtained when the solvent capillary and the nanospray capillary form a liquid junction. Each mass spectrum was acquired in positive-ion full-scan mode with a mass range of 125–1000 *m*/*z* for ~ 3 min which resulted in about 325 scans. Negative controls were acquired by obtaining spectra from 50% methanol/water and MRS medium. In order to maintain the signal intensity over the acquisition time, the position of the cellular sample was adjusted via the *xy*-stage on the nDESI ion source, such that different parts of the same sample were aligned with the nanospray capillary.

Results and discussion

Acquisition of unique mass spectral pattern from untreated LR

To achieve rapid identification of bacterial samples, one important factor is minimal sample preparation. For bacterial cells that are isolated from either liquid cultures or biological samples, the cells ought to be washed in order to reduce the background noise from sample matrix [20, 25]. Hence, the washing step is required in the protocols for preparing the cellular samples that are directly detected by using mass spectrometry. In comparison to the protocol for preparing MALDI cellular samples, which requires the coexistence of a specific MALDI matrix compound in the sample, the preparation of nDESI samples including the bacterial samples in this study is relatively simple [9]. As shown in the “Materials and methods” section, the bacterial samples are ready to be measured right after the washing step is completed. Since nDESI operates under the ambient air pressure, versus loading the samples into a high-vacuum MALDI ion source, the nDESI method would save time and is more compatible to high-throughput settings.

Different solvents that would support the DESI process had been previously reported by other research groups [15]. By comparing the signals and their signal intensities that could be acquired from LR cells when different solvents were used, the optimum solvent for supporting nDESI was

identified to be 50% methanol/water. The optimization of the solvent flow rate as well as the angle between the nanospray capillary and the sample surface did not yield any significant improvement on the signals being acquired from the LR cellular sample. In comparison to the flow rate in a standard nano-ESI ion source, 3.5 µL/min flow rate of the selected solvent to support nDESI is relatively high. However, the actual flow rate of the solvent in the nanospray capillary after making contact with the sample in an open space is expected to be lower than 3.5 µL/min (Fig. 1B).

In order to ensure that the mass spectral pattern obtained with nDESI was unique to LR cells, spectral patterns of 50% methanol/water and the MRS cell culturing medium were obtained as negative controls. A mass spectral pattern is defined as all the *m*/*z* ratio of each detectable peak, i.e., signal-to-noise ratio ≥ 2, within the predetermined mass range and their corresponding peak intensity. Figure 2A shows the spectral pattern acquired from whole LR cells, which is different from those spectral patterns obtained from the negative controls as shown in Fig. 2B, C. Hence, the LR spectral pattern is unique and corresponds to untreated LR cells. If the LR cells were not washed as described in the "Materials and methods" section, the spectral pattern looked similar to the one obtained from the MRS medium (Fig. 2B). After washing the cells, higher signals could be obtained if the cells were air-dried.

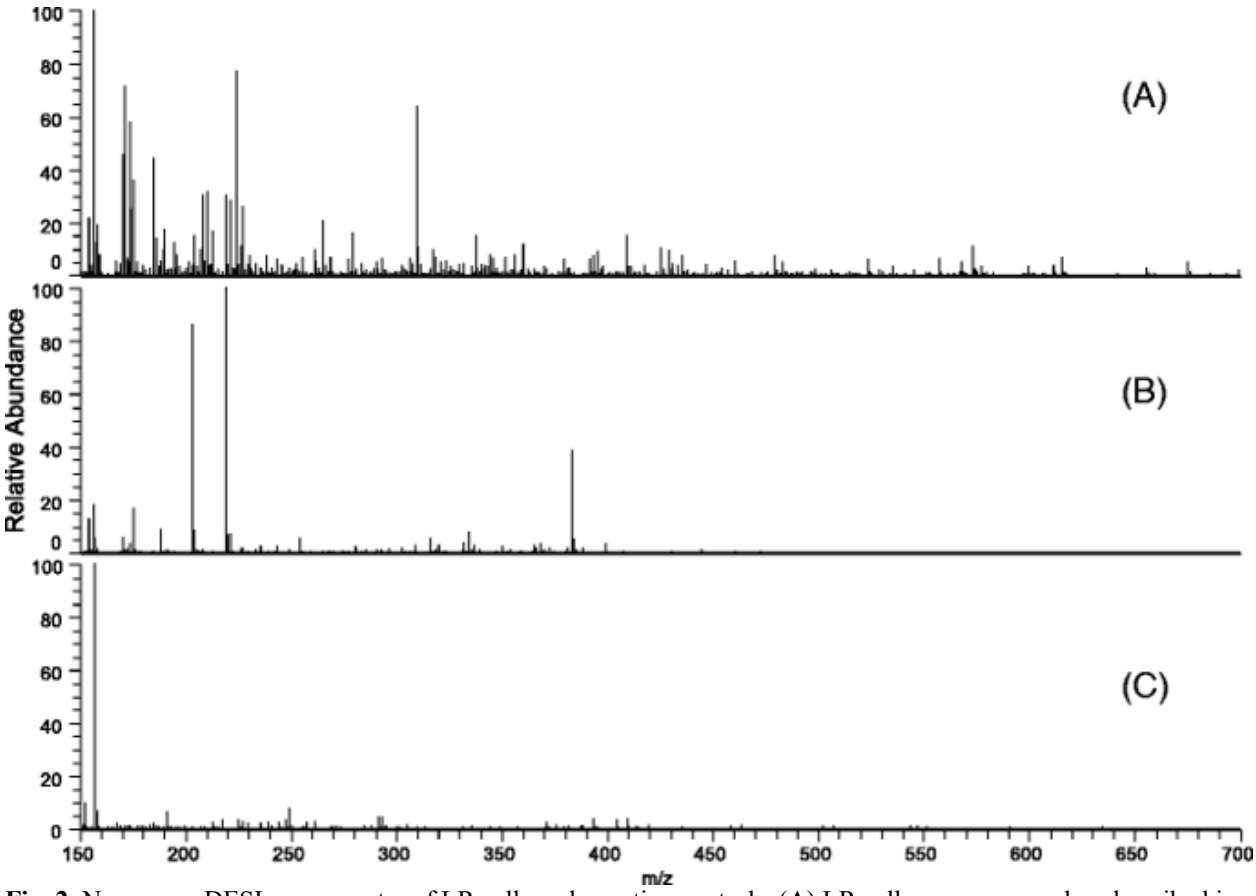
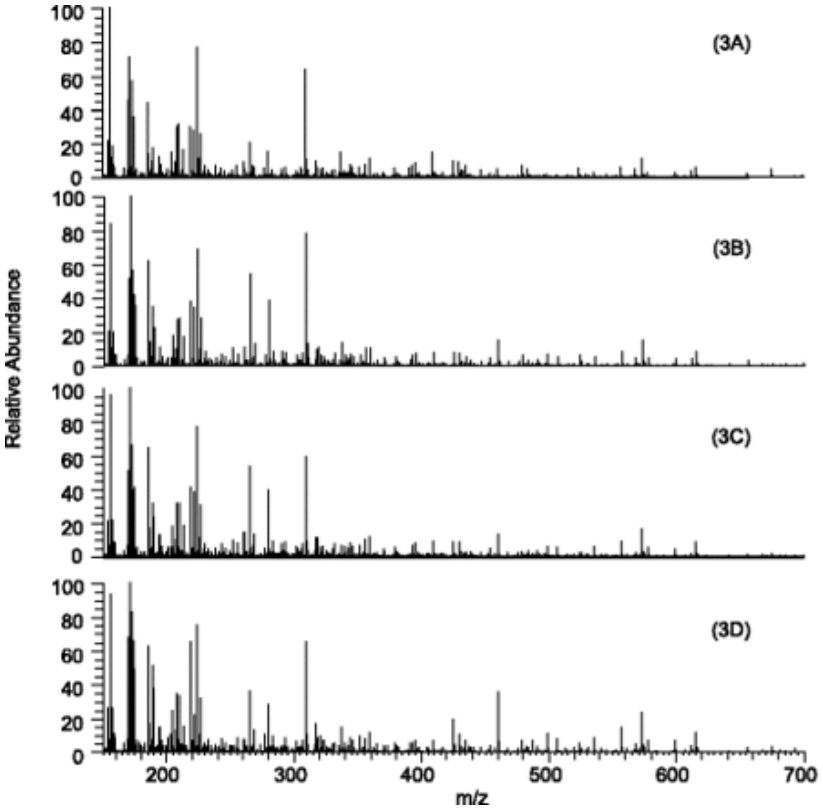


Fig. 2. Nanospray DESI mass spectra of LR cells and negative controls. (**A**) LR cells were prepared as described in the method. (**B**) Dried MRS cell culturing medium. (**C**) 50% methanol/water solvent

Reproducibility of LR mass spectral pattern

To ensure nDESI is suitable for whole-cell mass spectrometry, the ability to acquire the same mass spectral pattern from the selected bacterial model of *Lactobacillus reuteri* is critical. To test the reproducibility of nDESI measurements, LR cells were cultured, washed, and analyzed on separated days. The results shown in Fig. 3 indicate the spectral pattern of LR cells is distinct and can be obtained reproducibly. The coefficient of variation of intensities of major peaks among the four spectra in Fig. 3 was calculated, and the average was equal to 11.556%. The spectral patterns display small variations in the overall peak intensity, which could be attributed to different amounts of LR cells being measured and/or other factors such as minor changes in the concentration of growth media components that could be caused by autoclaving [18].



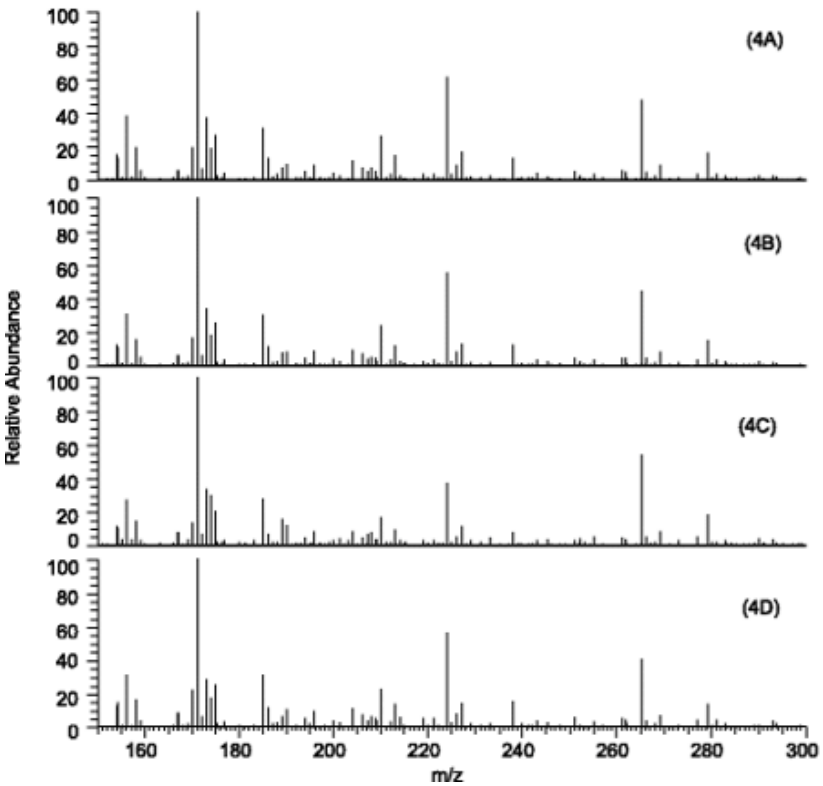
m/z	CV (%)
154.0265	12.010
156.0422	18.808
158.0215	5.9961
170.0579	9.7151
171.0168	4.0602
171.0532	3.7573
173.0576	7.7553
174.0178	14.731
174.8961	19.063
185.0689	5.2952
190.0128	10.278
207.9986	11.045
219.0269	26.427
221.0425	4.4502
223.9725	16.196
227.0796	4.3680
265.1875	19.144
309.1832	14.899
	11.556 Average

Fig. 3. Reproducibility of mass spectral pattern of untreated LR cells. 3A–3D are nDESI mass spectra acquired on different days. Coefficient of variation (CV) of peak intensity at selected *m/z* ratios that are > 20% relative abundance is calculated with ≥ 3 replicates, except those values with an underline. Outliers are identified by comparing to ± 90% confidence interval

Different spectral pattern obtained from treated LR cells

As demonstrated in an earlier report from our research group, cellular response to stress conditions could be detected via the changes in MALDI mass spectra obtained from cells that had been exposed to stress conditions [9]. Using the spectral pattern of untreated LR cells as a reference, variations in the nDESI mass spectrum of LR cells that had been exposed to a low pH condition could be detected. To ensure the spectral pattern of treated LR cells was reproducible, the same experiment of treating the LR cells was repeated. As shown in Fig. 4, the results were

reproducible. The coefficient of variation of intensities of major peaks among the four spectra in Fig. 4 was calculated, and the average was equal to 13.181%.



m/z	CV (%)
156.0422	19.671
158.0215	12.223
170.0580	7.9261
171.0169	4.0792
171.0532	16.146
173.0576	15.389
174.0178	16.873
174.8961	4.0851
185.0689	14.567
209.9569	17.067
223.9726	15.663
227.0796	8.3832
265.1875	17.028
279.2032	15.428
	13.181 Average

Fig. 4. Reproducibility of mass spectral pattern of 1-h treated LR cells. 4A–4D are nDESI mass spectra acquired on different days. Coefficient of variation (CV) of peak intensity at selected *m*/*z* ratios that are > 20% relative abundance is calculated with ≥ 3 replicates, except the value with an underline. Outliers are identified by comparing to ± 90% confidence interval

In order to determine whether the spectral patterns obtained from treated LR cells are correlated to each other, the experimental data was analyzed by using the principal component analysis (PCA). For the PCA calculation, all the *m*/*z* values of each detectable peak with ≥ 10% peak intensity were considered as observable and their corresponding peak intensity was considered as variable. As shown in the lower right-hand side of Fig. 5A, the Pearson correlation coefficient of the four repeated mass spectrometric measurements (4A–4D) ranges from 0.933 to 0.960, which indicates the spectral pattern of treated LR cells is statistically reproducible. Also, as shown in the upper right-hand side of Fig. 5A, similar results on the correlation of spectral patterns that corresponded to the repeated measurements of untreated LR cells (3A–3D) are achieved. Equally important, the PCA plot of correlation in Fig. 5A shows that the reproducible spectral patterns of untreated LR cells (3A–3D) are grouped together but segregated in a different segment in comparison to those corresponding to treated LR cells (4A–4D). This represents the spectral patterns of untreated LR cells, and treated LR cells are different.

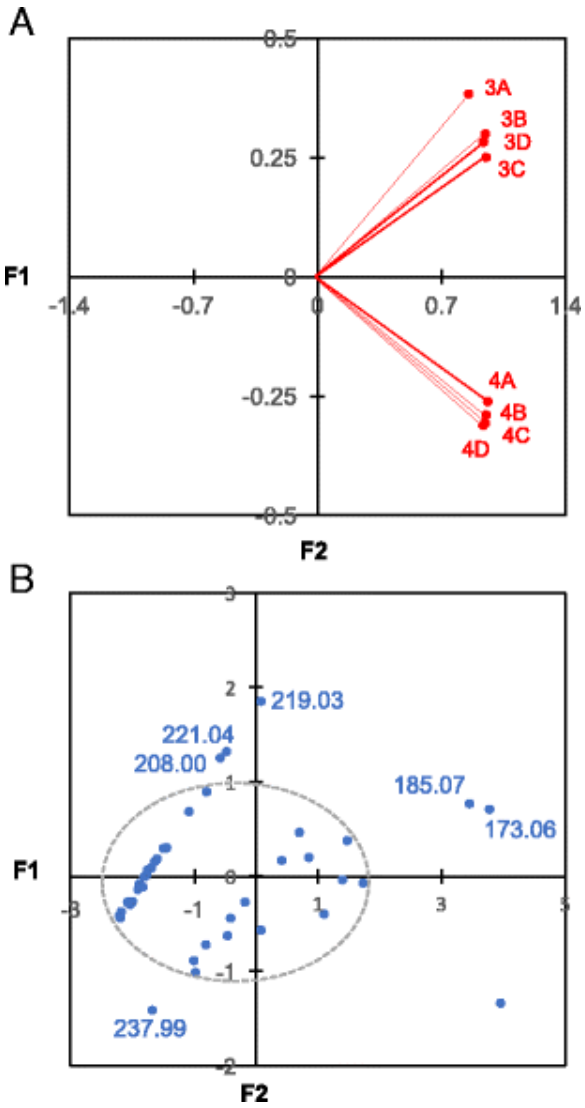


Fig. 5. Results from principal component analysis (PCA). (**A**) Plot of correlation. The Pearson correlation coefficient of factor 1 (F1) is plotted against factor 2 (F2). 3A–3D represent the replicates of spectral pattern obtained from control untreated LR cells (Fig. 3), and 4A–4D represent the replicates of spectral pattern obtained from LR cells treated for 1 h (Fig. 4). (**B**) Plot of PCA scores of each selected *m*/*z* peak. The labeled data points outside the highlighted area (dotted line) correspond to *m*/*z* values that can be used to distinguish between the spectral patterns of untreated and treated LR cells

To investigate the differences in the spectral patterns between the untreated and treated LR cells, the PCA scores of each observable (i.e., *m*/*z* value) are plotted in Fig. 5B, and the observables with a relatively high or low score are labeled. By comparing the spectral patterns between untreated and treated LR cells, an extra peak is detected at 237.99 *m*/*z* which has the lowest PCA score in the lower left-hand side of Fig. 5B. In another segment on the upper left-hand side of Fig. 5B, the data points with higher PCA score which correspond to the peaks at 208.00, 221.04, and 219.03 *m*/*z* are missing or undetectable in the treated spectral pattern. The remaining labeled data points with higher PCA score in the upper right-hand side of Fig. 5B corresponding to the peaks at 185.07 and 173.06 *m*/*z* have lower intensity in the treated spectral pattern in comparison to that in the spectral pattern of untreated LR cells.

In order to visualize the differences in the spectral patterns of untreated and treated LR cells, the representative spectra are stacked in Fig. 6. The changes in the spectral pattern of treated LR cells may include the presence of additional peak(s) and/or the absence of detectable peak(s), plus the changes in peak intensity, both increasing and decreasing peak intensity. Some of these changes in the treated spectral pattern are highlighted in Fig. 6B. The prolonged exposure of LR cells to the same pH condition results in more pronounced changes in the mass spectral pattern (Fig. 6C). The unique mass spectral pattern of LR cells and its variation resulting from a specific treatment under the low pH do comply with the results in an earlier report, in which changes in the protein level were detected by comparing the gel images of lysates extracted from the same LR cells with or without any low pH treatment [26]. Additional differential peaks with their *m*/*z* ratios and peak intensities are summarized in Fig. 7. In Fig. 7A, it shows the spectral patterns of LR cells (untreated or treated) are free from any signal that corresponds to the components used in the culturing medium (see Electronic Supplementary Material (ESM) Fig. S1). Whereas, in Fig. 7B, it shows the list of differential peaks with either increasing or decreasing peak intensity as the results of treating the LR cells under low pH (see ESM Fig. S2). These peaks further allow the differentiation of spectral patterns in Fig. 6, plus supporting a possibility on identifying new biomarkers for differentiating bacterial cellular responses in the future by using this experimental approach.

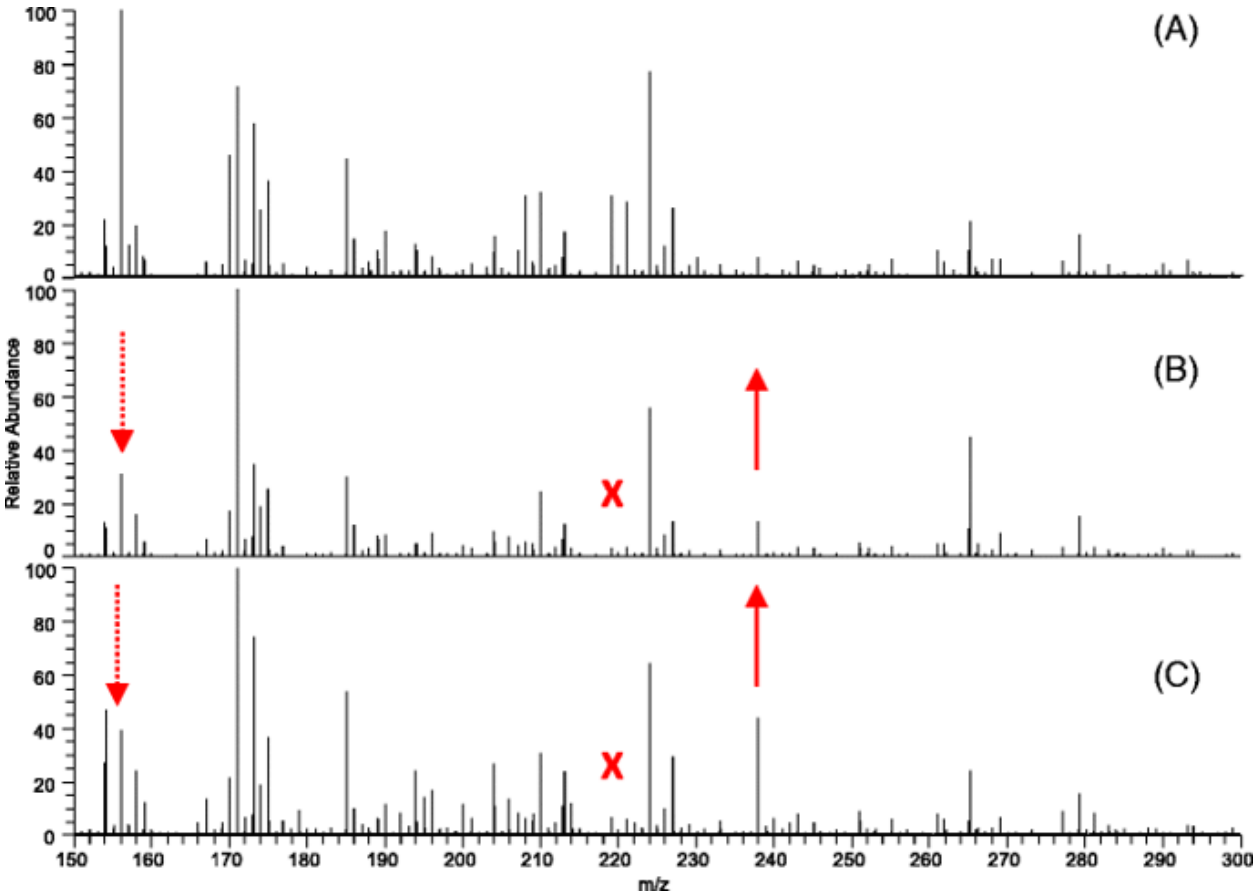


Fig. 6. Comparing the differences in spectral pattern obtained from untreated LR cells (**A**) and LR cells treated for either 1 h (**B**) or 4 h (**C**) at low pH. One of the peaks with lower intensity as a result of treating the cells is labeled with a dotted downward arrow. The missing peak and extra peak as a result of treating the cells are labeled with a cross and solid upward arrow, respectively

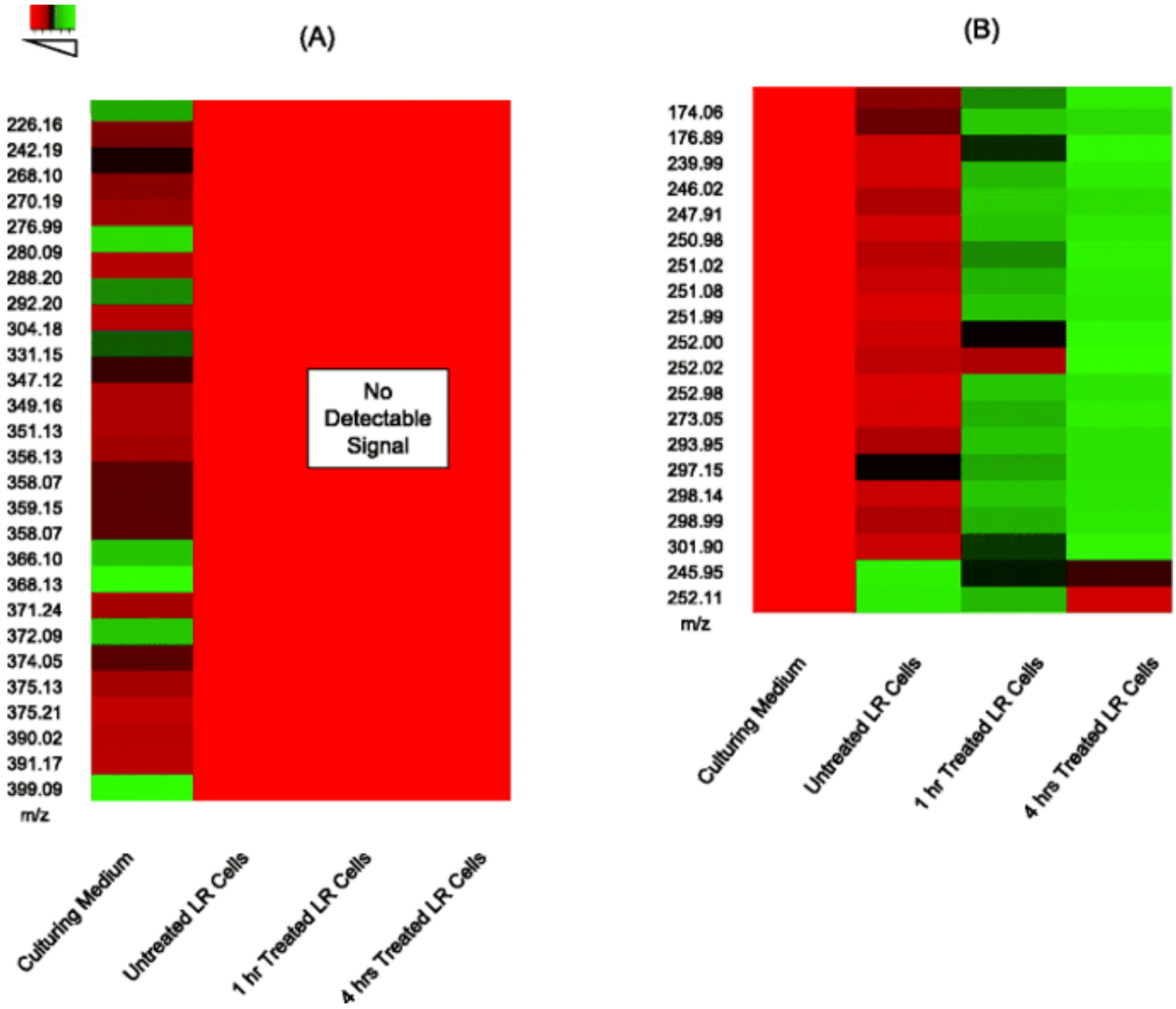


Fig. 7. Heat maps of peak intensities at selected *m/z* ratios in the mass spectrum of culturing medium, untreated LR cells, 1 h treated LR cells, and 4 h treated LR cells. (**A**) Signals only detected from culturing medium. (**B**) Differential signals were detected from untreated and treated LR cells, but no signal was detected from culturing medium. Maps were created by using Heatmapper (www2.heatmapper.ca)

Conclusions

Based on the initial results of this pilot study, nDESI ion source does support the reproducible acquisition of a unique mass spectrum from whole LR bacterial cells directly. Supported by the results from an orthogonal PCA, the cellular response from LR cells to a treatment at low pH could be detectable by using the nDESI method. This represents the first proof of concept on using nDESI mass spectrometry to distinguish a sample of treated bacterial cells from those that contain untreated cells. The nDESI method is complementary to the current MALDI method in which cellular samples are also measured directly by mass spectrometry. This is because the two ionization techniques are based on entirely different principles; thus, the efficiency on ionizing various compounds in the same sample are expected to be different. Also, without the use of any MALDI matrix, the nDESI signals are free from any MALDI matrix interference. These explain

why the optimal nDESI signals were acquired in the lower mass range and may not overlap significantly with the MALDI signals. Hence, if both nDESI and MALDI are used to measure the same cellular sample, the two methods do complement with each other and provide a more comprehensive coverage on the sample contents being investigated. Similar to the MALDI method, which has been proven to be useful for identifying bacterial cells in the area of clinical microbiology, the information on the identity of each detectable peak in the nDESI method may not be required. This is simply due to the comparative nature of the nDESI method, which relies on identifying the variations in the mass spectral pattern of a sample of interest in comparison to the spectral pattern obtained from a reference. While being a simple comparative method, the nDESI method does provide high specificity and accuracy. This is because more than one particular signal in a single mass spectrum are being used to determine whether there is any variation in the spectral patterns. Similar to the conventional DESI methods, the nDESI method is theoretically applicable to the measurements of other cell lines with or without any treatment. With the ease on carrying out bacterial cell culturing in the laboratories and the extensive knowledge on the cellular processes in bacterial cells, the readily available bacterial strains have often been the choice for cellular models in many different research areas. To confirm the identity of a bacterial cell culture and/or determine whether the culture has been contaminated by another type of cells, the direct MALDI MS measurements of bacterial cells have been demonstrated to be a viable approach. By replacing the conventional MALDI ion source with nDESI or other ambient ionization techniques, it would simplify the sample preparation process before the MS measurements. This technological development is expected to pave the way for using direct MS measurements of bacterial cells in the ongoing studies of various microbiomes. Similar to the MALDI MS method, the nDESI approach holds the potentials to speed up the identification of bacterial cell cultures as well as investigating the possible responses from bacterial cells after exposing the cells to specific conditions. For the food or pharmaceutical industries, the nDESI approach may represent a way to faster or more frequent quality control testing, which, in turn, further improves the quality of our consumable products.

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Conflict of interest. The authors declare that they have no competing interests.