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Mass spectrometry has proven to be a useful tool in microbiology research, especially for rapid identification of bacterial strains based on their unique mass spectral pattern. Traditionally, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is used for the analysis of intact bacterial cells. Studies have demonstrated the capability of MALDI mass spectrometry as a tool in distinguishing cellular response to various stressing conditions based on their mass spectral pattern.

Ambient ionization techniques allow intact bacterial cells to be analysed in their native state without the use of a matrix for ionization. Several ambient ionization techniques have been utilized as a means of analysing whole cells without any specific reagents or extraction procedures. One of these techniques, called nanospray desorption electrospray ionization (nanospray DESI), is a modification of DESI mass spectrometry that has been used to analyse bacteria directly on the agar plate. In this project, nanospray DESI mass spectrometry will be explored as a tool for rapidly distinguishing the response of bacterial cells to low pH conditions. Probiotic Gram-positive *Lactobacillus reuteri* cells were utilized as a model cell culture because of their importance in maintaining the health of the human digestive tract. After culturing the *L. reuteri* cells under 5% CO_2 in the darkness, the cells were harvested and washed prior to the nanospray DESI measurements. A clear, reproducible spectral pattern of the untreated cells was obtained using a solvent of 50% methanol and water. Adjustments of the methods revealed that at

an OD_{600} of 1.8, and when washed with water only, the spectral pattern was visible and reproducible.

Exposure to low pH conditions was conducted by incubating the *L. reuteri* cells in pH adjusted media for one hour and four hours. By setting the mass spectral pattern of the untreated bacterial cells as a reference, our results show that the spectra of the bacterial cells exposed to the low pH media differs from the untreated cells. These findings demonstrate the capability of nanospray DESI mass spectrometry as a tool for rapidly distinguishing the bacterial response to stress. Using these protocols, this method can be expanded to study the cellular response of bacteria to different types of cellular stress.

DETECTING THE CELLULAR RESPONSE OF *LACTOBACILLUS REUTERI* WITH NANOSPRAY DESORPTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY

by

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CHAPTER I

INTRODUCTION

1.1 Mass Spectrometry Overview

Mass spectrometry is an instrumentation method that allows identification and analysis of analytes based on their mass. This instrument makes use of three key components in order to detect the compounds: the ion source, mass analyzer and detector. In order to charge the compounds, an ion source is utilized. This part of the instrument can vary depending on the composition of the analyte, and there are a vast variety of ionization sources that can be selected to conduct research on many compounds and substances[1]. This is very important in the mass spectrometry process because the transfer of the analyte through the mass spectrometer relies on differences in potential, and the charge on the compounds is also used to facilitate the analysis of the compound. The mass analyzer is used as a selection or a filtering tool in the mass spectrometer. This process allows researchers to isolate known compounds for analysis based on the mass, which can be accomplished using different types of analyzers like the quadrupole mass analyzer or the time-of-flight mass analyzer. The many variations of ionization sources and mass analyzers gives this instrument versatility to be used as a tool for analysis of different sample compositions and in various fields of research[1, 2]. Some research areas of interest include clinical and microbiological applications which utilize mass

spectrometry. One of these is the ability of mass spectrometry to analyze whole/ intact cells with minimal sample preparation.

1.2 Bacterial Identification with Mass Spectrometry

The use of mass spectrometry to characterize bacteria was first explored by Anhalt and Fenselau in 1975. Anhalt et al analyzed several species of bacteria with pyrolysis mass spectrometry, a type of gas-liquid chromatography method that uses thermal heat to vaporize and separate analytes for analysis. This paper demonstrated that by conducting the experiments using lower temperatures and using bacterial extracts from lyophilized bacteria samples, the researchers were able to distinguish several types of bacterial species[3]. These earlier methods of characterizing bacteria using mass spectrometry required several extraction steps, in order to prepare the samples for mass spectrometry analysis. Identification of bacteria in clinical and microbiological laboratories is often linked closely to diagnosis of infections. Therefore, methods that are used to identify the species must be rapid, in order to facilitate treatments.

1.2.1 Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

Current methods that use mass spectrometry to conduct bacterial identification are performed using ionization sources like matrix assisted laser desorption ionization (MALDI), because it is well suited for analyzing large compounds like lipids and peptides that can be found on bacterial cells[4, 5]. This method makes use of a matrix compound to aid in ionizing large molecules and has some key characteristics, such as a

low molecular weight, ability to absorb UV and IR light, and the presence of a chromophore. Samples are prepared for analysis by first being mixed with the matrix and dried onto the MALDI plate. During the instrument analysis, a pulsed laser is used to oblate the sample, resulting in the desorbed and protonated analytes being sent into the mass analyzer for analysis.[2] The use of the matrix in MALDI mass spectrometry can allow singly charged ions to be detected, which enables it to be applied as a means of analyzing and identifying large proteins and other molecules. This mass spectrometry ionization source is utilized in clinical research laboratory settings to identify protein biomarkers and recently in microbiological settings as a means of identifying and distinguishing bacterial strains[6].

1.2.2 How Are Bacteria Identified with MALDI-MS?

MALDI mass spectrometry simplified the process of identifying bacteria because it enables bacterial cells to be analyzed without any additional sample preparation steps. Bacteria can be grown on agar, applied directly to the MALDI target plate followed by application of the matrix and analysis.[7] This method can also be used to analyze bacteria that are grown in liquid media and the process is also rapid. Isolation of bacteria grown in liquid media is usually followed by a washing step, before the matrix can be mixed with the sample and analyzed.[8] This direct approach to identifying bacteria has been given the terms ‘whole cell mass spectrometry ‘and ‘intact cell mass spectrometry’ in the literature, because the bacterial cells are whole/intact until they are mixed with matrix for instrument analysis[9]. MALDI mass spectrometry differentiates between bacterial species based on the mass spectra that is obtained from the analysis. The masses that are detected

by the instrument and their intensities form what is known as a spectral fingerprint, which will vary depending on the type of bacteria analyzed. These patterns in the mass spectra are then used to form a library or database that can be referenced when analyzing bacteria originating from various types of biological samples in clinical laboratories. These databases are utilized in clinical laboratories as a means of rapidly differentiating between many strains of bacteria[10, 11].

1.2.3 Analyzing Cell Response with Mass Spectrometry

Whole cell mass spectrometry distinguishes the bacteria based on the patterns obtained in instrument analysis and this method can be used as a way to monitor bacterial growth. Arnold et al[12] demonstrated this capability of whole cell mass spectrometry by analyzing *Escherichia coli* cells during different points in the growth cycle. The researchers showed that the intensities of the peaks seen in the spectral fingerprint of the bacterial cells changed during the growth cycle. These peaks were correlated by Arnold et al as ribosomal proteins and they noted that the changes in the environment of the culture media due to the growth of the bacteria demonstrates the need to control growth times when conducting whole cell mass spectrometry.[12, 13] The ability of this method to show how proteins in the bacteria can change depending on the growth conditions can be applied further to assess how bacteria respond to certain environments.

The assessment of bacterial response to environmental conditions using whole cell mass spectrometry was also demonstrated by Marvin-Guy-et al[14]. The researchers used MALDI mass spectrometry to look at the spectral fingerprint of bacterial cells that

were stressed, in order to compare this spectra to the fingerprint of a control or an un-stressed bacterial sample. The researchers stressed the bacteria by exposing them to different concentrations of bile salts, followed by instrument analysis. The spectral fingerprint of the control sample differed from the treated samples, and the researchers identified new peaks in the spectral fingerprint that could be seen in the samples exposed to bile salts.[14] This variation also could be seen in the three concentrations used to treat the bacteria, demonstrating that it is possible to analyze the bacterial response to conditions of stress. Further applications of utilizing MALDI mass spectrometry to detect and monitor cell response involved the use of mammalian cells. In this study, whole cell mass spectrometry was used to distinguish the in vitro cellular response of mammalian cells that were exposed to toxic chemicals. The researchers found that the mass spectral fingerprint of the mammalian cell changed after exposure to the toxic chemicals. These changes were able to be distinguished based on the type of toxin.[15] This whole cell mass spectrometry method has also been utilized as a means of detecting cellular conditions such as apoptosis and necrosis, two different types of cell death. In this study, the researchers found that using MALDI mass spectrometry, differences in the spectral pattern could be distinguished in cells exposed to compounds capable of inducing apoptosis and cell death caused by necrosis[16].

The ability to rapidly distinguish between these two types of cell death demonstrate that whole cell mass spectrometry can be utilized as a prescreening tool for cytotoxicity studies.[10] This method also has applications in studies that test environmental compounds for potential toxicity.[16, 17] These studies demonstrate the use of

mass spectrometry as a tool for rapidly examining the cellular response to toxic compounds or environments and can be utilized in the form of quick, untargeted analysis to learn more about the different types of responses that cells have to toxins, and aid in ascertaining the methods in which the toxins affect the cells.

1.3 Ambient Ionization Techniques

Ambient ionization is a technique that enables samples to be ionized outside of the mass spectrometer in the open laboratory environment, or in their original condition.[18] Ionization of the samples for mass spectrometry analysis usually takes place under vacuum conditions. To perform ionization techniques in the open atmosphere, desorption and extraction of the charged analytes typically happen at the same time and can be performed with ionization sources that involve spraying, heating and laser techniques to charge the analyte. These ions are then directed into the mass spectrometer through an external mass spectrometer inlet.[19] The ability of samples to be ionized outside the mass spectrometer reduces the amount of sample preparation that is typically conducted for mass spectrometry analysis, making it applicable for direct analysis of many different sample compositions. There have been a wide variety of ambient ionization sources developed that all have very different methods of generating the charged analytes for analysis[20]. These techniques have been applied in imaging experiments to identify compounds in tissue samples. Imaging techniques utilizing ambient ionization allow researchers the ability to directly study components in complex samples without undergoing long sample preparation techniques. This is also an advantage of using

ambient ionization techniques in forensic analysis, because samples can be examined undisturbed and in their natural state[21, 22]. Bacterial samples have also been studied with ambient ionization and it has been utilized particularly for identifying bacteria and also for examining colonies of bacteria directly on agar plates[20, 23, 24]. Two ambient ionization techniques that have been applied towards bacterial profiling are discussed below.

1.3.1 Desorption Electrospray Ionization

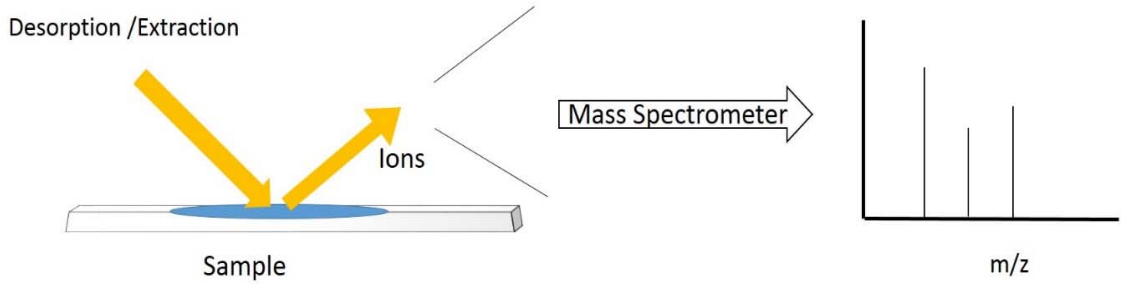
One ambient ionization source that has been utilized to study whole bacterial cells is desorption electrospray ionization (DESI) mass spectrometry. This ionization source employs the use of an electrospray source in order to spray charged solvent onto the sample. The charged analyte is then carried through the air into the mass spectrometer for analysis. This solvent is usually directed to the sample with nitrogen gas, and this gas is utilized to direct the samples into the mass spectrometer inlet[25]. Performance of DESI requires the angle (known as the incident angle) of the solvent spray to line up with the mass spectrometer in order to get good signal of the compounds being tested, which is determined by high signal-to-noise ratios.[18] This ionization method has been used to analyze several strains of bacteria[26]. The samples were prepared only with one washing step and the researchers noted that washing the bacteria is not required when using solid agar media. The bacteria studied by Meetani et al [26] were identified by peaks in the 50-500 Dalton range. The DESI ionization source is more suited to studying compounds in lower mass ranges; research has been published by Song et al [27, 28] studying intact bacterial cells with the DESI ionization source at a range of 500-2000 Daltons. This is

typical of ionization sources that utilize an electrospray mechanism to desorb and transfer ions to the mass spectrometer.

1.3.2 Nanospray Desorption Electrospray Ionization

Nanospray desorption electrospray ionization (nanospray DESI) mass spectrometry is a modification of the DESI source that makes use of a liquid junction system to deliver the charged analyte into the mass spectrometer. Instead of using gas to direct the charged solvent, a solvent capillary (known as the primary capillary) is utilized to deliver the solvent in small charged droplets to the sample, and a secondary capillary (known as the nanospray capillary) is used to transfer the charged analytes into the mass spectrometer.[29] The droplets that form on the sample surface bridge the gap between the two capillaries and enables the transfer of the sample into the instrument for analysis. **(Fig 1)** Because of this 'liquid junction' that is formed on the sample surface, nanospray DESI is especially suited for surface sampling experiments, imaging of small molecules in tissue samples and it has also been utilized to analyze colonies of bacteria directly on the agar plate. These types of experiments have been conducted using nanospray DESI as a means of understanding more about interactions between bacteria that form biofilms[29-31]. The formation of the liquid bridge is believed to improve the transfer of ions into the mass spectrometer and may help in obtaining high signal-to-noise ratios.

Figure 1. Schematic Diagram of Nanospray DESI.



Through a primary capillary on the left hand side, solvent that has been charged is delivered and forms a droplet on top of the sample. This serves as a liquid junction between the nanospray capillary on the right hand side, which then creates the ions from the sample.

1.4 Specific Aims

1.4.1 Acquisition of Mass Spectral Pattern from Gram Positive Probiotic Bacterial Cells

Nanospray DESI has been utilized for experiments involving bacteria grown in agar plates because the liquid junction created by the solvent capillary and the secondary capillary allow movement across the surface of the sample. In this project, the use of nanospray DESI will be explored utilizing bacteria cultured in liquid media, which has not been reported in the literature. The goal, therefore will be to first acquire a reproducible spectral pattern (or fingerprint) of a bacteria species. The bacteria that will be used in these experiments is *Lactobacillus reuteri*, a Gram positive bacterial strain typically found in the human gut microbiome. The spectrum obtained from these experiments will be used as a control or reference spectrum in subsequent studies.

1.4.2 Use of *Lactobacillus reuteri* as a Gram Positive Probiotic Bacterial Cell Model

Lactobacillus reuteri is a type of probiotic bacteria which is known to aid in maintenance of gastrointestinal health. These probiotic species are also reported to benefit the immune system by preventing harmful pathogens from populating the gut. Several species of probiotic bacteria have been shown to have the capability of producing compounds that have antimicrobial activity[32, 33]. Studies of these probiotic bacteria have been conducted in order to ascertain how these microbes aid in preventing harmful pathogens from populating the gut[32]. Studying *Lactobacillus reuteri* using nanospray DESI will allow a fast method of studying the response of this microbe to different stressed environments, and can demonstrate how this method can be used to rapidly differentiate the cellular response of other bacterial species.

1.4.3 Detection of the Cellular Response to Stress Conditions

Nanospray DESI will be used to study intact bacterial cells that have been grown in liquid media which has not been reported in the literature. The goal will be to explore the use of nanospray desorption electrospray mass spectrometry as a tool for monitoring the response of bacteria to conditions of stress. This will be accomplished by using the spectra of untreated cells as a reference and identifying peaks that correspond to the response of bacterial cells exposed to the specific stress condition. By quantifying the cellular response, this experiment will further demonstrate the capability of this novel method to monitor the responses from bacterial cells. The stress condition utilized in this experiment is low pH exposure. Altering growth conditions of bacteria, such as pH,

changes the protein expression of the bacteria as they adapt to the environmental stress. *Lactobacillus reuteri* have been shown to adapt the expression of proteins when exposed to low pH conditions. This adaptation is done to ensure their survival in low pH environments such as the human digestive tract.[34] These protein changes can potentially lead to differences in the cell membranes of the bacterial cells and the goal will be to detect the cellular response using the spectral pattern obtained with nanospray DESI mass spectrometry. This will demonstrate that nanospray DESI can be used to detect the response of cells to stress conditions and can also expanded as a means of potentially quantifying the response of cells to toxic compounds.

CHAPTER II

ACQUISITION OF THE MASS SPECTRAL PATTERN OF GRAM POSITIVE BACTERIAL CELLS

2.1 Introduction

Identification of bacteria with mass spectrometry relies on obtaining a reproducible spectrum of the bacteria.[4] This is usually accomplished by controlling the growth conditions of the bacterial strain. Analysis of different strains of bacteria with MALDI or even DESI have shown that bacteria can be distinguished rapidly using these mass spectral profiles when conditions like culture media and growth times are the same.[35] Controlling when the *Lactobacillus reuteri* cells are harvested will ensure that the spectra obtained with nanospray DESI mass spectrometry will be reproducible. Studies have also shown that the amount of bacterial cells present in the culture tube is a significant factor in obtaining spectra of the bacteria.[36] Taking into account these factors will aid in obtaining a reproducible spectral pattern of the bacteria. Reproducibility of the spectral pattern allows whole cell mass spectrometry to be useful in the rapid identification and differentiation of various bacterial strains. Databases can then be created using reference spectra that can be utilized to accelerate the process of identifying and distinguishing strains of bacteria.[37] Therefore our initial step in this project is to use the nanospray DESI ionization source and obtain a spectral pattern of the *Lactobacillus reuteri* cells that can be utilized as a reference.

2.2 Methods

2.2.1 Preparation of Bacterial Samples

The *Lactobacillus reuteri* cells were cultured by inoculating 0.5-1 µL of a stock culture of *L. reuteri* (ATCC 23272) into 5 mL of de Man Rogosa and Sharpe (MRS) media and incubating with 5 percent CO_2 at 37°C overnight to reach an approximate OD_{600} reading of 1.6-1.8. This was determined using optical density readings from a Thermo Spectronic UV spectrophotometer set to measure absorbance readings at 600 nm. Harvesting the bacterial cells around this OD_{600} reading ensured that there was enough bacterial cells for the nanospray DESI analysis.

2.2.2 Washing of Bacterial Pellet

To isolate the bacterial pellet, the entire tube of bacteria was transferred into a 15 mL centrifuge tube and centrifuged at 3000_g for 3-4 min at 4°C. The supernatant was discarded and the cells transferred into an Eppendorf tube. The pellet was washed two times with 1X Phosphate Buffered Saline (PBS) buffer and a third time with autoclaved deionized water; these washing steps were conducted to remove the salts found in the MRS media. The bacterial pellet was re-suspended in enough autoclaved deionized water to make a very cloudy suspension (about 200-500 µL) for mass spectrometry analysis.

2.2.3 Instrument Analysis

The bacterial samples were analyzed as 2-4 µL aliquots of the suspension dried onto glass slides. Mass spectrometry was performed using an LTQ Orbitrap equipped

with a DESI ionization source. This source was modified to extend the existing spray capillary length to allow functionality as a primary capillary. The nanospray capillary was measured to an appropriate height that enabled it to be adjusted and line up with the mass spectrometry inlet and the solvent capillary. To ensure that the nanospray capillary is properly aligned with the mass spectrometer inlet, rhodamine dye was used to test the alignment of the set up. The 250 µL solvent syringe was set to flow at a flow rate of 2-5 µL/min. The spectra was acquired in positive mode using a mass range of 125-1000 m/z for 3 minutes. Negative controls were acquired by obtaining spectra of the solvent, MRS media and the PBS buffer used to wash the bacterial cells.

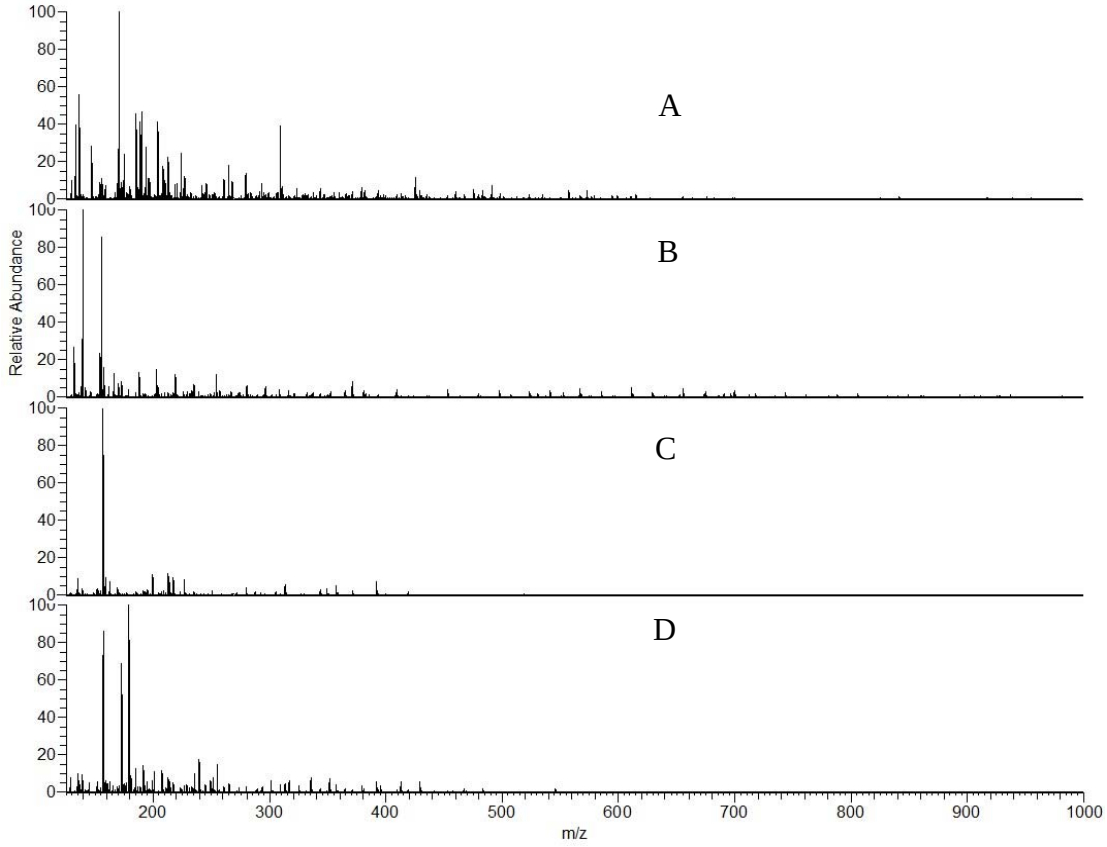
2.3 Results and Discussion

2.3.1 Spectral Pattern of *Lactobacillus reuteri*

The goal of this experiment was to obtain reproducible spectra of our model bacteria strain, *Lactobacillus reuteri*. Analysis of the bacteria using the mass spectrometer was performed initially using two different solvents, 50% methanol-water and 50% acetonitrile-water. This was conducted in order to determine which would yield clear, reproducible spectra. The 50% methanol-water solvent is generally used in DESI experiments and in this case, resulted in a higher signal when analyzing the bacterial cell samples. This can be seen in a comparison of the two spectra that is depicted below **(Fig.3)** Because of this, it was determined that the 50% methanol-water solvent would yield clear reproducible spectra and therefore be ideal in conducting these experiments. In order to ensure that the spectra obtained with the nanospray DESI instrument was

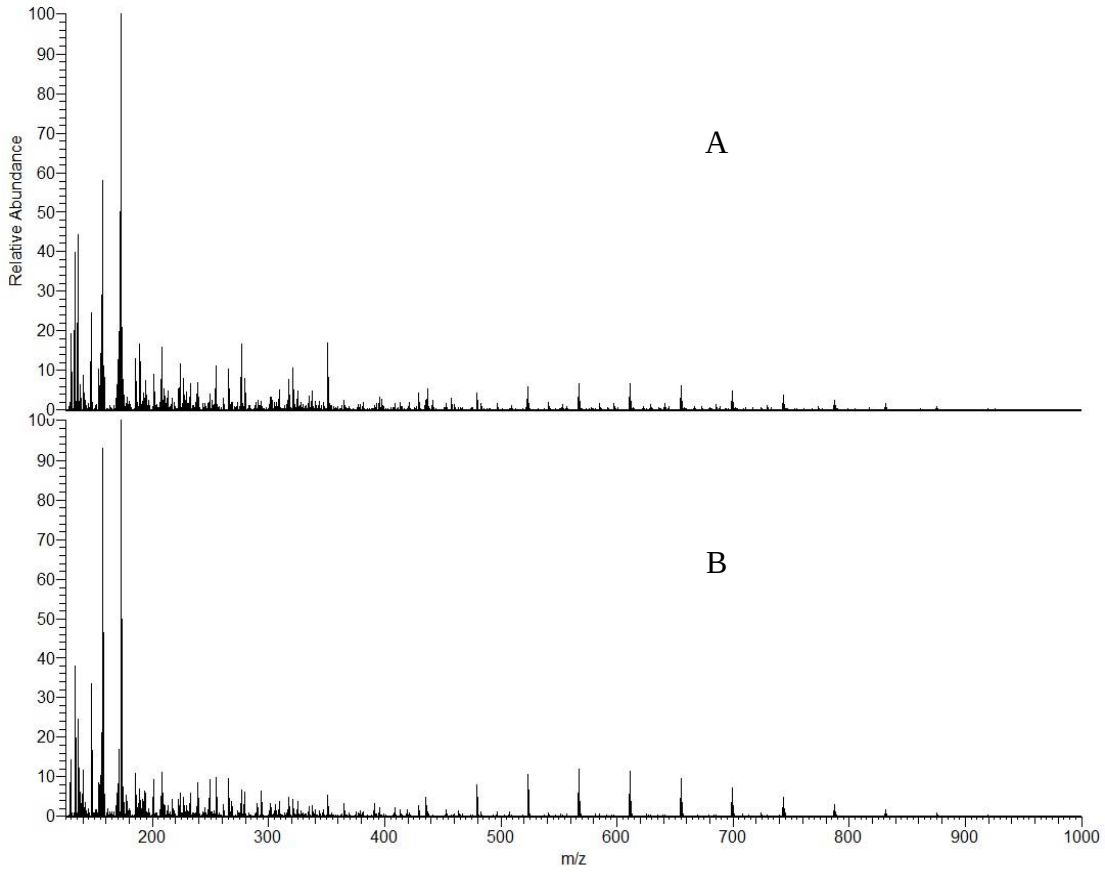
unique to the bacterial cell samples, spectra of the methanol-water solvent, the PBS buffer and the MRS media were also obtained as negative controls. As shown in the figures below, the spectral pattern shown below is unique to the *Lactobacillus reuteri* cells sample **(Fig 2)**.

Figure 2. Nanospray DESI Mass Spectra of *L. reuteri* and Controls.



Results of nanospray DESI analysis of (A) *L. reuteri* cells (B) MRS media (C) 50% Methanol Solvent (D) PBS buffer. Because the spectral pattern shown in (A) is not present in the negative controls, we can conclude that it is unique to the bacteria sample.

Figure 3. Comparison of Using Methanol or Acetonitrile Solvents for Nanospray DESI.



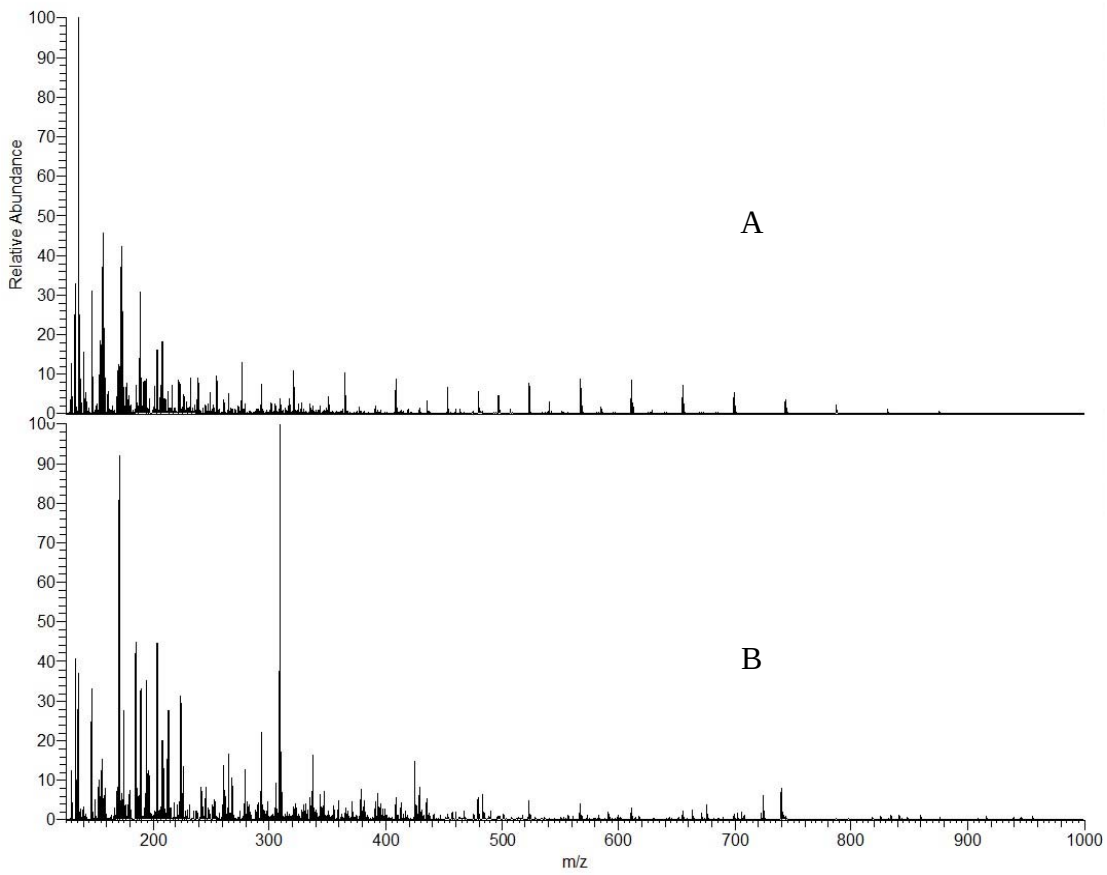
Comparison of the *L. reuteri* spectral pattern using 50% acetonitrile-water (A) and 50% methanol-water solvent (B). Higher signal-to-noise ratios were obtained when performing instrument analysis with the methanol-water solvent.

2.3.2 Use of PBS Buffer in the Washing Step

The use of PBS buffer to wash the bacterial cells was initially conducted as a means of ensuring that the bacterial cells remained intact during the washing process. This is due to the effect of cell wall lysis that could occur because of the osmotic differences in the water solvent and the contents of the bacterial cell. Using PBS buffer to

suspend cells for analysis was previously performed in our laboratory's past experiments when analyzing mammalian cells using MALDI mass spectrometry[15]. Our initial experiments were conducted using PBS buffer, however the use of PBS buffer to wash bacterial cells is not reported in literature referencing analysis of bacterial cells using whole cell mass spectrometry techniques. The protocol in preparing bacterial samples for analysis only utilizes water to wash and/or suspend the bacterial cells, which is also the case for experiments utilizing DESI mass spectrometry [8, 12, 14, 27]. For our next experiments, we modified the methods detailed above and analyzed the *Lactobacillus reuteri* cells after washing them twice with water. Shown below are the results. A comparison of these results shown below **(Fig.4)** with that of the bacterial cells washed with PBS buffer reveal that there is a much more distinct mass spectral pattern.

Figure 4. Results from Using a Revised Method for Washing the Cells.



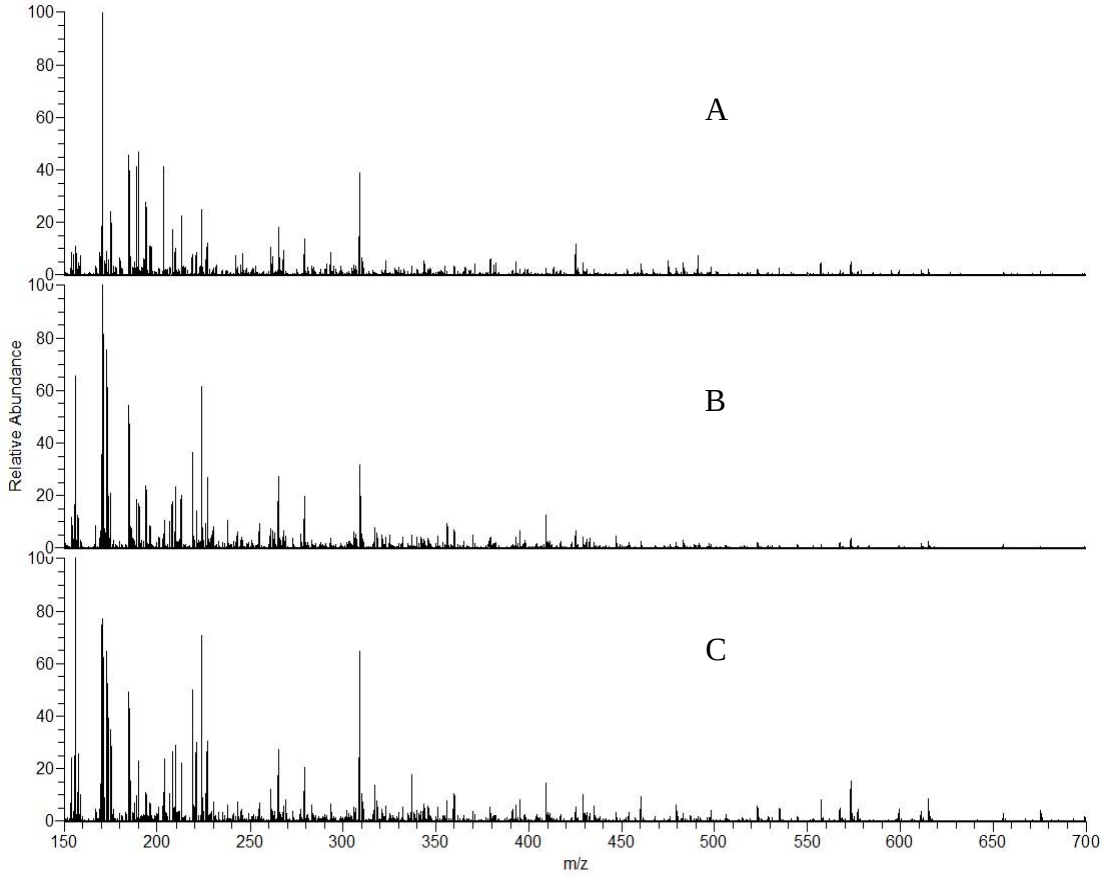
(A) Spectral pattern of *L. reuteri* washed two times with PBS buffer. (B) Spectral pattern of *L. reuteri* cells washed twice only using water. Omitting the PBS buffer in the washing step yielded clearer pattern of the bacterial cells.

2.3.3 Reproducibility of Spectra

An important part of obtaining reference spectra of the bacterial samples is being able to reproduce the results. Achieving the same spectral pattern of the *Lactobacillus reuteri* cells, ensures that the nanospray DESI method is capable of being used as a tool for rapid bacterial identification. Reproducibility was also conducted by carrying out the

experiment on separate days, in order to ascertain that the pattern shown is reproducible. This can also be seen in the figure shown below **(Fig. 5)**. These results were obtained after the methods were modified to omit the PBS buffer as a washing agent. As noted previously, the mass spectral pattern of the *L. reuteri* cells is distinct and clear.

Figure 5. Reproducibility of Mass Spectral Pattern from Nanospray DESI Analysis.



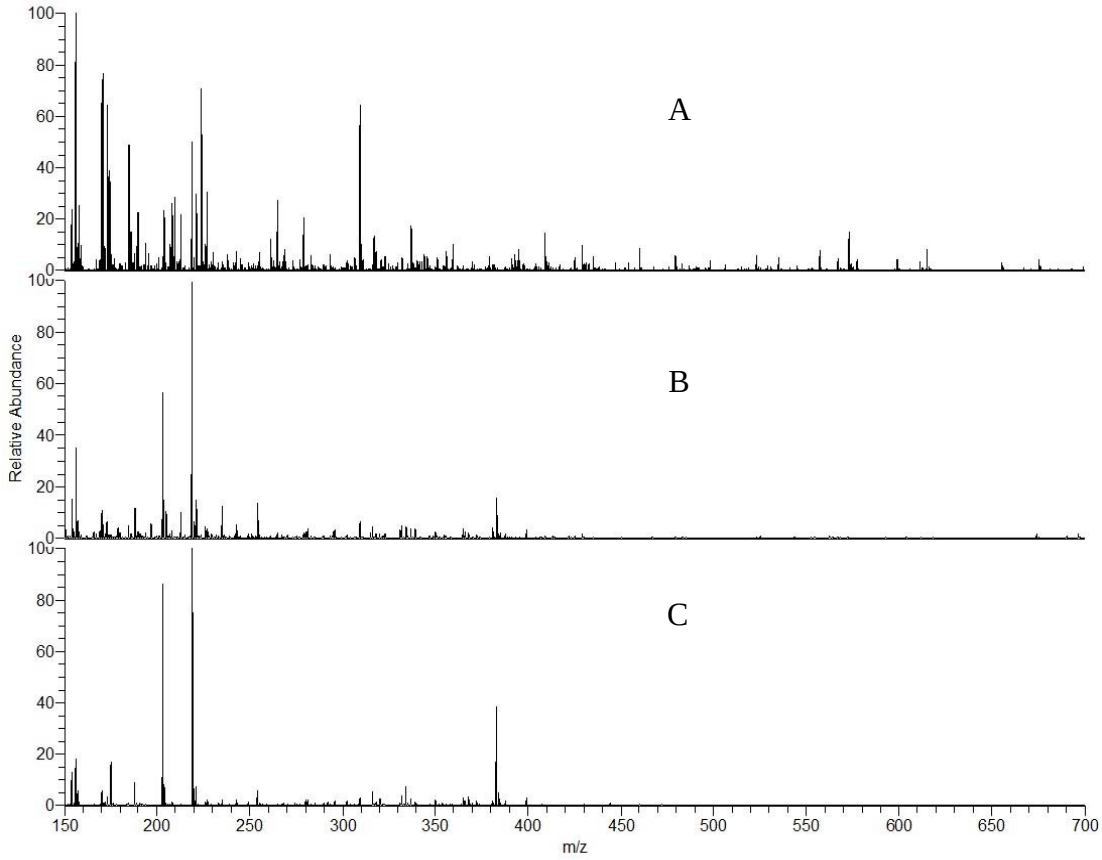
Spectral pattern of *L. reuteri* obtained during three separate trials which occurred on different days. The spectral pattern obtained after the (A) first analysis, (B) second analysis, and (C) third analysis are reproducible.

2.3.4 Necessity of Washing the Bacterial Pellet

Rapid identification of bacterial samples relies on minimal sample preparation. This is an important aspect in clinical research that uses mass spectrometry to identify bacterial species before commencing treatment. Minimal sample preparation is also important when using nanospray DESI to obtain the spectra of bacteria. In the method detailed above **(Section 2.2)**, there is a washing step included as a means of removing

any components from the MRS media used to incubate the bacterial cells. A set of experiments were conducted to ascertain the necessity of the washing step. These analyses showed that components from the MRS media were very prominent in the spectra of the *L. reuteri* cells that were not washed. This comparison is shown in the figure below **(Fig 6)**. These components from the MRS media can potentially interfere with our goal of attempting to distinguish the cell response using the spectral pattern of the bacterial cells. Therefore, it was determined that washing the bacteria with water was necessary and should be included as an important step in these experiments.

Figure 6. Comparison of Mass Spectral Pattern-Washing the Bacterial Pellet.



Nanospray DESI mass spectrum of (A) L. *reuteri* cells prepared with the modified washing step, (B) without the modified washing step and (C) the MRS media. Components from the MRS media (C) are prominent in the spectra of the unwashed bacterial cells (B). The washing step is necessary to prevent the interference of these MRS media components in our further experiments.

2.3.5 Further Applications of Nanospray DESI Method

The identity of the peaks shown in the spectra above most likely correspond to parts of the bacterial cell such as proteins, lipids, and components of the cell membrane such as ribosomes.[35, 38] Whole cell mass spectrometry techniques using MALDI and nanospray DESI are capable of identifying these components unique to specific strains.

This enables these techniques to be applicable for a wide variety of experiments. Because ambient ionization techniques like DESI and nanospray DESI are usually applied to study compounds with a lower mass range, (100-1000 m/z), the peaks shown in the spectra above are likely to be fragments of cell membrane components and lipids or proteins with smaller molecular weights. Rapid identification of peaks specific to certain strains can be used as a pre-screening tool in order to ascertain potential targets for in-depth studies. The spectral pattern of *Lactobacillus reuteri* shown in the figures above, are capable of being used for such work, due to the capabilities of the Orbitrap instrument in identifying compounds.

2.4 Conclusion

Nanospray DESI has been documented to study bacteria that are cultured in agar plates, and these results show that this instrumentation technique is also capable of analyzing bacterial cells that are cultured in liquid media. Obtaining spectra of the bacterial cells directly from the culture tube reduces the sample preparation time and further demonstrates the capability of the nanospray DESI instrument in directly analyzing bacterial cells. This can then be further expanded to identify the spectral pattern of other types of probiotic bacteria and even other strains. By acquiring the reference pattern of *Lactobacillus reuteri,* further study of the instrumentation technique can be utilized. One application, in particular, is testing the capability of nanospray DESI in detecting the cellular response of the *Lactobacillus reuteri* to a stress condition.

CHAPTER III

DETECTING THE CELLULAR RESPONSE TO A STRESS CONDITION

3.1 Introduction

Whole cell mass spectrometry has been shown to distinguish between cells that have been cultured or exposed to different environmental conditions. Using MALDI mass spectrometry, Arnold et al[12] demonstrated that the mass spectra of bacteria changes during their growth cycle. This is due to the variations in protein expression that occurs during the growth cycle. Changes in the protein expression occur as the bacteria adapt to different environments, such as different culture media and also during exposure to toxins, or other conditions that are likely to stress them. Exposure of bacterial cells to stress was conducted by Marvin Guy et al[14] and using MALDI mass spectrometry peaks in the mass spectra were identified as resulting from the stress condition.

The ability to rapidly distinguish between cells that are cultured in different environments has the capability of being utilized in toxicity studies. Experiments have been conducted utilizing mammalian cells and detection of peaks associated with apoptosis were identified using MALDI mass spectrometry of whole cells.[16] Other studies have demonstrated that cellular responses to different types of toxic compounds can be differentiated using this mass spectrometry method. This has been conducted by researchers using mammalian cells and also with fish cell lines.[15, 17]

Utilizing nanospray DESI, our next goal is to expose the *Lactobacillus reuteri* cells to a stress condition and compare the resulting spectra to that of the control. This will enable us to determine the capabilities of the nanospray DESI instrumentation method as a tool for rapidly distinguishing cellular response to stress.

3.2 Methods

3.2.1 Preparation of Bacterial Samples

Two culture tubes of *Lactobacillus reuteri* bacteria cells were prepared by inoculating 0.5-1 µL of a stock culture of *L. reuteri* (ATCC 23272) into 5 mL of de Man Rogosa and Sharpe (MRS) media and incubated with 5 percent CO_2 at 37°C to an OD_{600} reading of 1.6-1.8. This was determined using optical density readings from a Thermo Spectronic UV spectrophotometer set to measure absorbance readings at 600 nm.

3.2.2 Washing of Bacterial Pellet

To isolate the bacterial pellet, the entire tube of bacteria was transferred into a 15 mL centrifuge tube and centrifuged at 3000_g for 3-4 min at 4°C. The supernatant was discarded and the cells transferred into an Eppendorf tube. The pellet was washed two times with autoclaved deionized water; these washing steps were conducted to remove the salts found in the MRS media. The bacterial pellet was re-suspended in enough autoclaved deionized water to make a very cloudy suspension (about 200 µL) for mass spectrometry analysis.

3.2.3 Exposure to Low pH

To obtain bacterial samples of stressed cells, the *Lactobacillus reuteri* samples were prepared by incubating the bacteria in the MRS media and harvesting at an OD_{600} of 1.8. The cells were then centrifuged and re-suspended in MRS media adjusted to a pH of 4 using hydrochloric acid. After incubation for an hour, the bacteria pellet was transferred into a 15 mL centrifuge tube and centrifuged at 3000_g for 3-4 min at 4°C. The supernatant was discarded and the cells transferred into an Eppendorf tube. The pellet was then washed and re-suspended in the autoclaved deionized water for analysis. In order to have a control sample for comparison, two sets of bacteria were cultured and the untreated cells were re-suspended in fresh MRS media and incubated for the extra hour in the normal conditions. This was done to ensure that there was a same-day comparison of the resulting mass spectra of the cells.

3.2.4 Instrument Analysis

The bacterial samples were analyzed as 2-4 µL aliquots of the suspension dried onto glass slides. Mass spectrometry was performed using an LTQ Orbitrap equipped with a DESI ionization source. This source was modified to extend the spray capillary and include a primary solvent capillary. The nanospray capillary was adjusted to line up with the mass spectrometry inlet and the solvent capillary and the instrument was calibrated with rhodamine. This calibration was done to ensure that the nanospray capillary was aligned to the mass spectrometry inlet. The 250 µL solvent syringe was set to flow at a flow rate of 2-5 µL/min. The spectra was acquired in positive mode using a

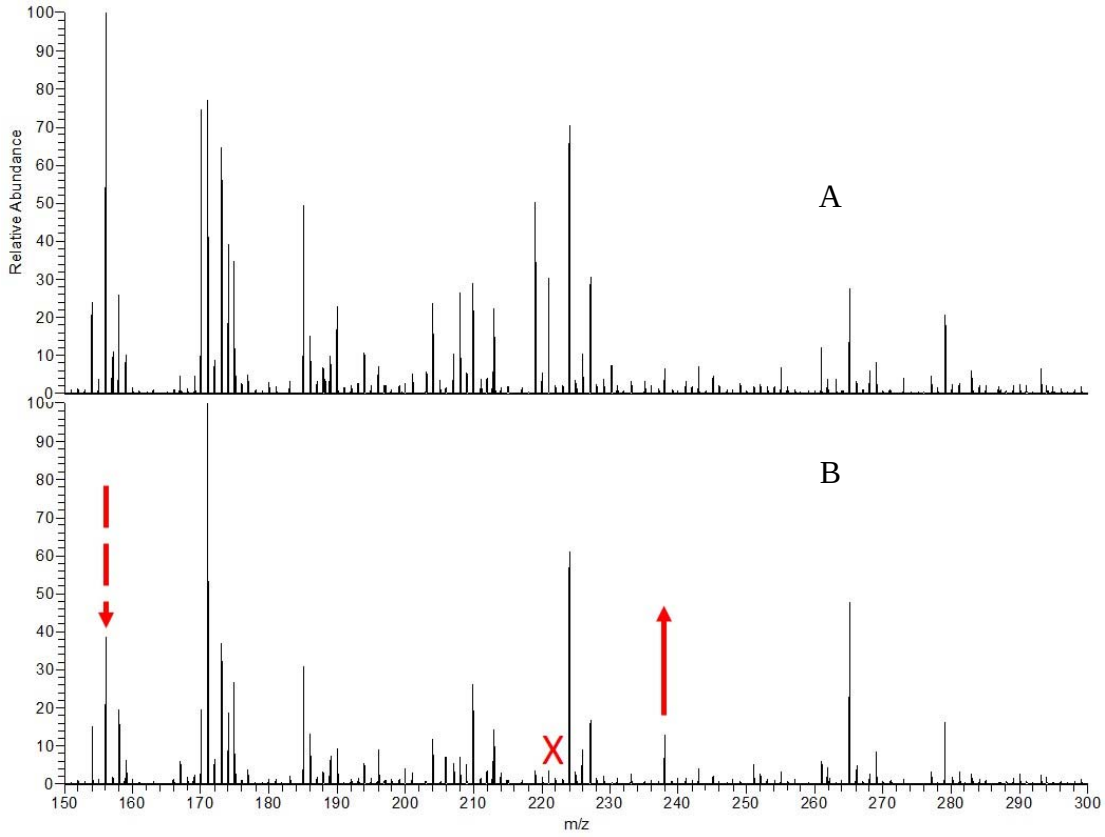
mass range of 125-1000 m/z for 3 min. Negative controls were acquired by obtaining spectra of the solvent and the MRS media used to grow the bacterial cells. The nanospray capillary was changed after analysis of the control and cells exposed to the low pH condition. This was done to prevent any contamination and to ensure that the spectra of the treated cells and the control cells are distinguishable.

3.3 Results and Discussion

The goal of these experiments was to determine the response of the *Lactobacillus reuteri* bacteria to a stress condition. Using the same growth conditions detailed in the methods section, the bacteria cells were cultured. An additional step was performed of exposing one culture tube to MRS media that had been adjusted to a low pH of 4 for an hour. The results shown below **(Fig.7)** were obtained after the cells were washed according to the methods detailed above. Differences in the spectra were observed at the lower part of the mass range spectrum from 150-500 m/z. Distinguishing the cellular responses of cells using mass spectrometry relies on the ability to identify changes in the mass spectral pattern. Using the spectra of the untreated *Lactobacillus reuteri* cells as a reference, variations in the spectra of the cells exposed to the low pH media were identified. These changes in the spectra were noted if the peak intensity was lowered, increased, or if peaks present in the untreated cell spectral pattern were absent in the spectra of the treated cells. In the figure below, the dotted arrow depicts the place in the spectra where the intensity of the peaks were lowered in the exposed cells. The (X)

denotes peaks in the spectra that are absent, while the straight arrow shows an increase in the intensity of the peaks.

Figure 7. Unique Mass Spectral Pattern from Treated and Untreated Cells.



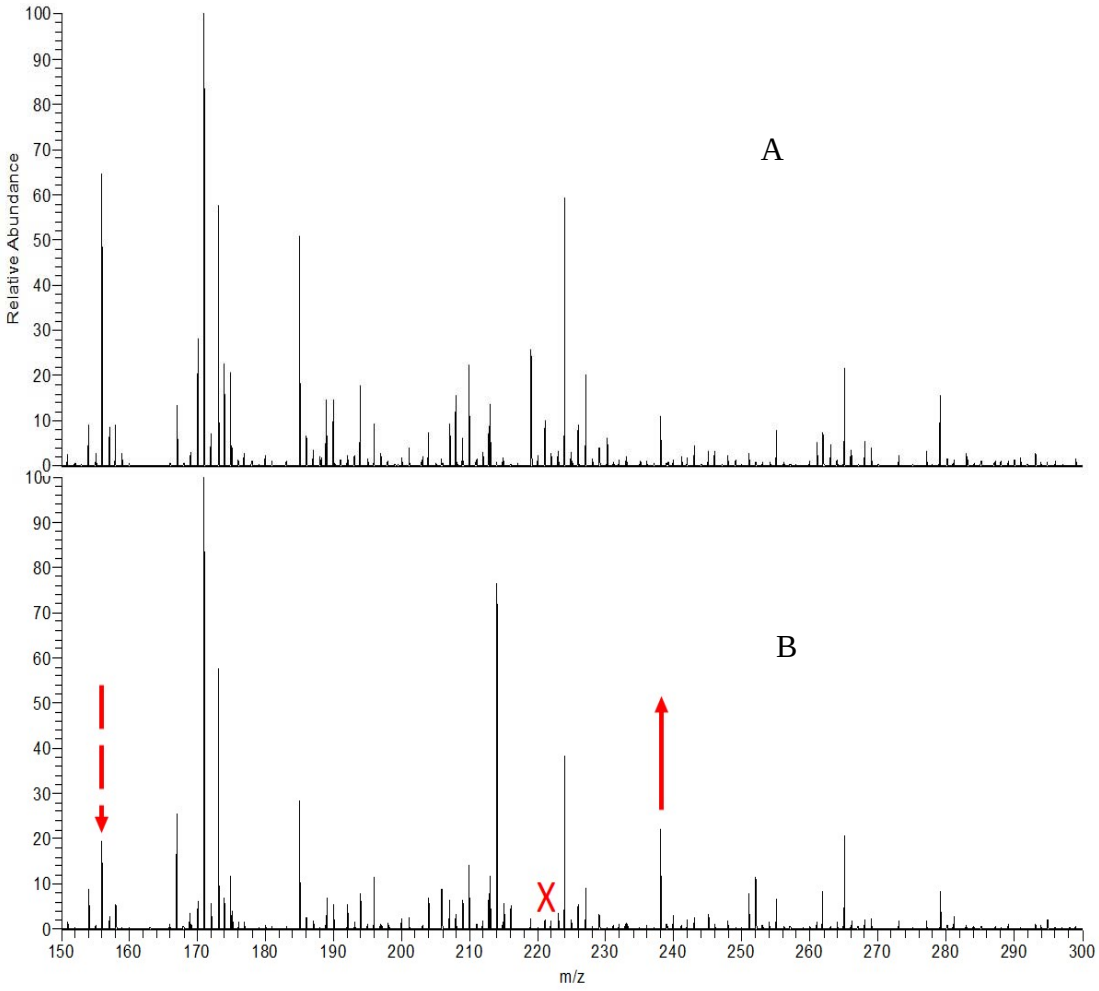
Comparison of (A) untreated *L. reuteri* cells and (B) *L. reuteri* cell exposed to the low pH condition. Changes in the spectral pattern, marked in red, can be observed in the treated cells.

3.3.1 Reproducibility of the Cell Response Variations

An important aspect of using mass spectrometry to detect the cellular response is to ensure that the differences shown in the untreated versus the treated cells are reproducible. Determining this will ensure that the nanospray DESI method is reliable

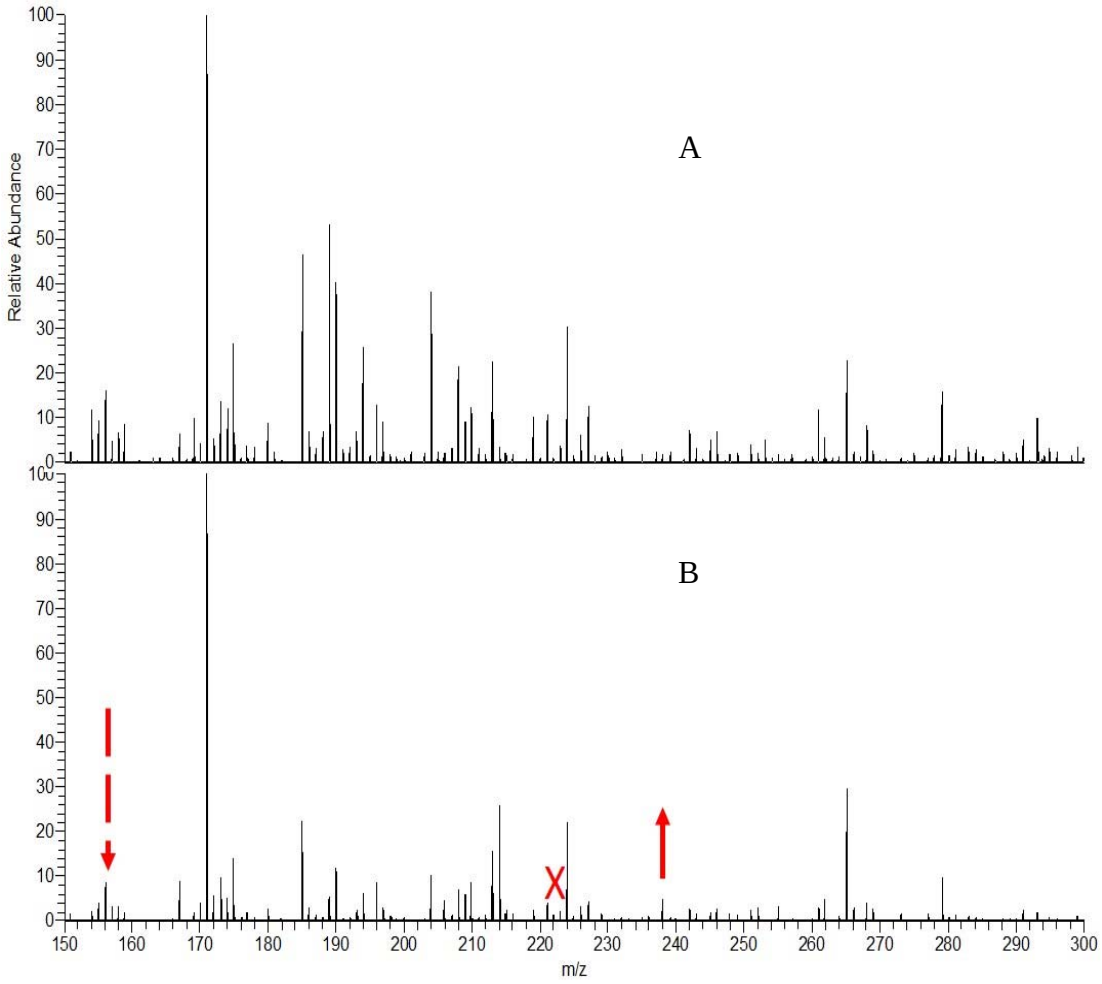
and capable of distinguishing the changes resulting from the cells being exposed to stress conditions. Experiments were conducted over several days to ensure that these results were consistent. Shown in the figures below **(Fig.8-9)** are the results of these studies. Fluctuations in the signal intensity of the peaks is a phenomenon that is typical when analyzing biological compounds using mass spectrometry. This is most likely due to small changes in the MRS media nutrients due to factors such as autoclaving, measuring.[26] These small fluctuations can also be seen in the spectra of the control, even though the spectra of the untreated *L. reuteri* cells are reproducible.

Figure 8. Reproducibility of Mass Spectral Pattern from Treated and Untreated Cells.



Comparison of (A) untreated *L. reuteri* cells and (B) cells exposed to low pH conditions. The changes marked in red are reproducible when compared with the figure above. (**Fig. 7**)

Figure 9. Second Trial of Reproducibility - Treated versus Untreated.



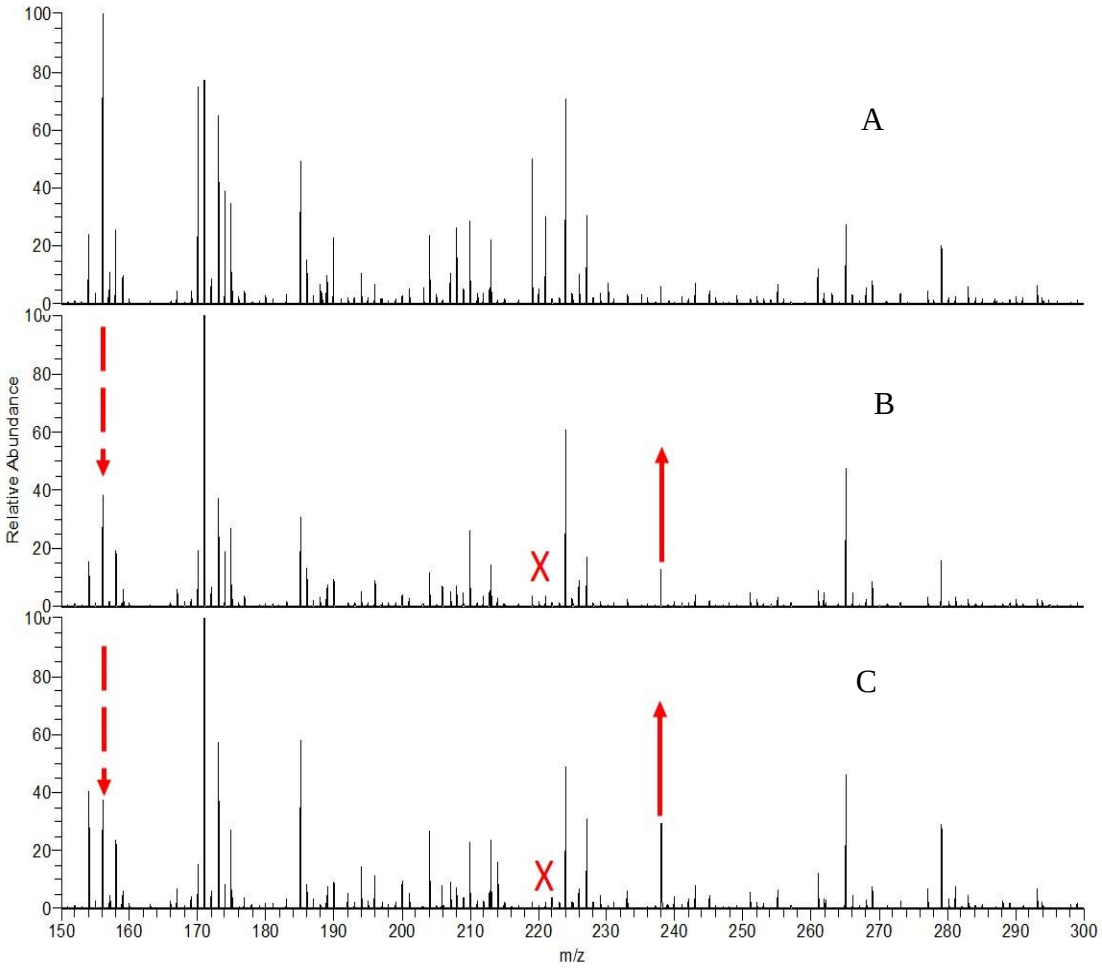
Nanospray DESI comparison of (A) untreated *L. reuteri* cells and (B) cells exposed to low pH conditions. The changes marked in red are reproducible when compared with the figure above. (**Fig. 8**)

3.3.2 Quantitative Measurement on Extended Treatment

The duration of the exposure to the toxic environment can also affect how bacteria respond to toxic environments. The responses of the bacteria to the toxic condition are likely to become more pronounced. This was noted by researchers when

using MALDI mass spectrometry to detect the response of a bacterial strain to varying conditions of salts. The intensity of certain peaks increased as the stress condition became more pronounced. [14] To test this phenomenon, the *Lactobacillus reuteri* cells were exposed for four hours to the low pH MRS media, and a comparison was made using the spectra of the control as well as the spectra of the cell exposed for one hour. The results of this experiment are shown below. **(Fig. 10)** When the exposure time increased, the cellular response was more pronounced in certain parts of the spectra. These changes are indicated in the figure. As the bacterial cells are exposed to the stress condition for longer periods of time, the response of the cells are likely to become more pronounced, as they adapt to the differences in their environment. These differences could potentially be quantified using the intensity of the peaks, and be utilized to further examine the effect of the toxic condition on the bacterial cells.

Figure 10. Quantitative Measurements of Extended Treatment.



Comparison of (A) untreated *L. reuteri* cells, (B) cells exposed to low pH for 1 hour and (C) cells exposed to low pH for 4 hours. An increase in the peak intensity (Straight arrow) corresponds to the response of the prolonged exposure to the stress condition.

3.3.3 Implications of Results

The growth of bacteria is very dependent on environmental conditions. Many different bacterial strains adapt to thrive in a wide variety of environments, and *Lactobacillus reuteri*, is a strain that is well adapted to residing in the human gut

microbiome. Because these conditions are fairly acidic, this strain of bacteria can adapt to acidic stress conditions by expressing different proteins. When the *Lactobacillus reuteri* cells are exposed to acidic conditions, proteins associated with stress-response, metabolism and regulation of transcription and translation processes are upregulated.[34] These changes can then be detected using whole cell mass spectrometry methods like the nanospray DESI instrumentation utilized in these experiments. The information obtained with the Orbitrap mass spectrometer can be used as a means of understanding more about the cellular responses of probiotic bacteria like *Lactobacillus reuteri* in different types of stress conditions that may be found in the human gut microbiome.

3.4 Conclusion

When bacterial cells are exposed to environments different from their normal conditions, they are capable of adapting very quickly to these new changes. This ability to rapidly adapt to new conditions enable bacteria to survive in toxic conditions. Detecting these changes rapidly, demonstrates the capability of nanospray DESI in distinguishing cellular response to these changes. The ability to carry out these comparisons quickly can allow researchers to rapidly detect the response of cells to toxic compounds and environments. These results can also be quantified, due to the ability of the nanospray DESI to detect changes as the exposure to the stress condition is extended. Quantification of the cellular response would enable researchers to determine the effect of the stress condition on the bacterial cells. This information would be of potential importance in the field of toxicology, and this nanospray DESI method could be utilized

as a form of preliminary toxicity screening to rapidly identify the response to toxic compounds and environments.[15, 17]Because the Orbitrap mass spectrometer has strong mass identification capabilities, determination of the identity of those peaks present and absent in the treated cells can be identified and potentially aid in studies of bacterial stress response mechanisms.

CHAPTER IV

CONCLUSION

4.1 Conclusion

Nanospray DESI has been documented to study bacteria that are cultured in agar plates, and these results show that this instrumentation technique is also capable of analyzing bacterial cells that are cultured in liquid media. These results also demonstrate the importance of washing the bacterial cells which ensures that the spectra obtained will be clear and reproducible. Further applications of this nanospray DESI instrumentation include analysis of different probiotic bacteria as well as other strains. These experiments also explored the potential of this method as a tool for rapidly detecting the cellular response to stress conditions. Our results show that this instrumentation method is capable of detecting the cellular response in a way that is reproducible. Quantification of the cellular response is also possible using nanospray DESI mass spectrometry. By obtaining the spectral pattern of cells exposed to various concentrations of toxic compounds or exposure times, differences in the intensity can be used as a means of quantifying how much the cells are affected by the stress environments.

4.2. Potential Applications of Nanospray DESI

Using nanospray DESI to conduct whole cell mass spectrometry analysis of bacterial samples is applicable to many different fields of research such as clinical and toxicological studies. In the clinical laboratories, nanospray DESI instrumentation

methods can be applied towards the identification of bacteria that are difficult to culture and therefore aid in reducing sample preparation steps. Rapidly detecting the cellular response of bacteria with this method can be applied as a pre-screening tool for rapidly testing the toxicity of compounds. Information generated from this preliminary screening can be explored further due to the high resolution capabilities of many mass spectrometers.

Because nanospray DESI mass spectrometry is capable of detecting the cell response to stress conditions, it can be applied towards monitoring the growth of bacteria, which can be useful for quality control assessment in the food industry. Nanospray DESI mass spectrometry methods could be applied towards monitoring the growth of bacteria such as lactic acid bacteria cultures that are used in dairy products. This instrumentation method could also be relevant for assessing the growth of other probiotic bacteria that are utilized in dairy products and supplements.

Nanospray DESI mass spectrometry has the potential to be utilized as quick method of studying the bacterial cell response to various conditions, and therefore aid in understanding more about these stress response mechanisms. Standardization of the nanospray DESI instrument set up, will allow this method to be applied towards other fields of research that can take advantage of its capability to aid in quickly obtaining data from complex samples.

REFERENCES

1. El-Aneed, A.; Cohen, A.; Banoub, J., Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. *Applied Spectroscopy Reviews* **2009,** *44* (3), 210-230.

2. Awad, H.; Khamis, M. M.; El-Aneed, A., Mass Spectrometry, Review of the Basics: Ionization. *Applied Spectroscopy Reviews* **2015,** *50* (2), 158-175.

3. Anhalt, J. P.; Fenselau, C., Identification of bacteria using mass spectrometry. *Analytical Chemistry* **1975,** *47* (2), 219-225.

4. Fenselau, C.; Demirev, P. A., Characterization of intact microorganisms by MALDI mass spectrometry. *Mass Spectrometry Reviews* **2001,** *20* (4), 157-171.

5. Holland, R. D.; Duffy, C. R.; Rafii, F.; Sutherland, J. B.; Heinze, T. M.; Holder, C. L.; Voorhees, K. J.; Lay Jr, J. O., Identification of Bacterial Proteins Observed in MALDI TOF Mass Spectra from Whole Cells. *Analytical Chemistry* **1999,** *71* (15), 3226-3230.

6. Singhal, N.; Kumar, M.; Kanaujia, P. K.; Virdi, J. S., MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Frontiers in Microbiology* **2015,** *6*, 791.

7. Braga, P. A. C.; Tata, A.; Goncalves dos Santos, V.; Barreiro, J. R.; Schwab, N. V.; Veiga dos Santos, M.; Eberlin, M. N.; Ferreira, C. R., Bacterial identification: from the agar plate to the mass spectrometer. *RSC Advances* **2013,** *3* (4), 994-1008.

8. Zhou, N.; Wang, N.; Xu, B.; Wang, J.; Fang, J.; Dong, F.; He, K.; Yang, X., Whole-cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for rapid identification of bacteria cultured in liquid media. *Science China Life Sciences* **2011,** *54* (1), 48-53.

9. Holland, R. D.; Wilkes, J. G.; Rafii, F.; Sutherland, J. B.; Persons, C. C.; Voorhees, K. J.; Lay, J. O., Rapid Identification of Intact Whole Bacteria Based on Spectral Patterns using Matrix-assisted Laser Desorption/Ionization with Time-of-flight Mass Spectrometry. *Rapid Communications in Mass Spectrometry* **1996,** *10* (10), 1227-1232.

10. Bizzini, A.; Durussel, C.; Bille, J.; Greub, G.; Prod'hom, G., Performance of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of Bacterial Strains Routinely Isolated in a Clinical Microbiology Laboratory. *Journal of Clinical Microbiology* **2010,** *48* (5), 1549-1554.

11. Dubois, D., Performances of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid identification of bacteria in routine clinical microbiology. *J Clin Microbiol* **2012,** *50*.

12. Arnold, R. J.; Karty, J. A.; Ellington, A. D.; Reilly, J. P., Monitoring the Growth of a Bacteria Culture by MALDI-MS of Whole Cells. *Analytical Chemistry* **1999,** *71* (10), 1990-1996.

13. Wieme, A. D.; Spitaels, F.; Aerts, M.; De Bruyne, K.; Van Landschoot, A.; Vandamme, P., Effects of Growth Medium on Matrix-Assisted Laser Desorption–Ionization Time of Flight Mass Spectra: a Case Study of Acetic Acid Bacteria. *Applied and Environmental Microbiology* **2014,** *80* (4), 1528-1538.

14. Marvin-Guy, L. F.; Parche, S.; Wagnière, S.; Moulin, J.; Zink, R.; Kussmann, M.; Fay, L. B., Rapid identification of stress-related fingerprint from whole bacterial cells of Bifidobacterium lactis using matrix assisted laser desorption/ionization mass spectrometry. *Journal of the American Society for Mass Spectrometry* **2004,** *15* (8), 1222-1227.

15. Chiu, N. H.; Jia, Z.; Diaz, R.; Wright, P., Rapid differentiation of in vitro cellular responses to toxic chemicals by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Environ Toxicol Chem* **2015,** *34* (1), 161-6.

16. Dong, H.; Shen, W.; Cheung, M. T.; Liang, Y.; Cheung, H. Y.; Allmaier, G.; Kin-Chung Au, O.; Lam, Y. W., Rapid detection of apoptosis in mammalian cells by using intact cell MALDI mass spectrometry. *Analyst* **2011,** *136* (24), 5181-9.

17. Kober, S. L.; Meyer-Alert, H.; Grienitz, D.; Hollert, H.; Frohme, M., Intact cell mass spectrometry as a rapid and specific tool for the differentiation of toxic effects in cell-based ecotoxicological test systems. *Anal Bioanal Chem* **2015,** *407* (25), 7721-31.

18. Cooks, R. G.; Ouyang, Z.; Takats, Z.; Wiseman, J. M., Ambient Mass Spectrometry. *Science* **2006,** *311* (5767), 1566-1570.

19. Badu-Tawiah, A. K.; Eberlin, L. S.; Ouyang, Z.; Cooks, R. G., Chemical Aspects of the Extractive Methods of Ambient Ionization Mass Spectrometry. *Annual Review of Physical Chemistry* **2013,** *64* (1), 481-505.

20. Harris, G. A.; Galhena, A. S.; Fernández, F. M., Ambient Sampling/Ionization Mass Spectrometry: Applications and Current Trends. *Analytical Chemistry* **2011,** *83* (12), 4508-4538.

21. Takáts, Z.; Wiseman, J. M.; Cooks, R. G., Ambient mass spectrometry using desorption electrospray ionization (DESI): instrumentation, mechanisms and applications in forensics, chemistry, and biology. *Journal of Mass Spectrometry* **2005,** *40* (10), 1261-1275.

22. Weston, D. J., Ambient ionization mass spectrometry: current understanding of mechanistic theory; analytical performance and application areas. *Analyst* **2010,** *135* (4), 661-668.

23. Chingin, K.; Liang, J.; Chen, H., Direct analysis of in vitro grown microorganisms and mammalian cells by ambient mass spectrometry. *RSC Advances* **2014,** *4* (11), 5768-5781.

24. Fang, J.; Dorrestein, P. C., Emerging mass spectrometry techniques for the direct analysis of microbial colonies. *Current Opinion in Microbiology* **2014,** *19*, 120-129.

25. Venter, A.; Nefliu, M.; Graham Cooks, R., Ambient desorption ionization mass spectrometry. *TrAC Trends in Analytical Chemistry* **2008,** *27* (4), 284-290.

26. Meetani, M. A.; Shin, Y. S.; Zhang, S.; Mayer, R.; Basile, F., Desorption electrospray ionization mass spectrometry of intact bacteria. *Journal of Mass Spectrometry* **2007,** *42* (9), 1186-1193.

27. Song, Y.; Talaty, N.; Tao, W. A.; Pan, Z.; Cooks, R. G., Rapid ambient mass spectrometric profiling of intact, untreated bacteria using desorption electrospray ionization. *Chemical Communications* **2007,** (1), 61-63.

28. Song, Y.; Talaty, N.; Datsenko, K.; Wanner, B. L.; Cooks, R. G., In vivo recognition of Bacillus subtilis by desorption electrospray ionization mass spectrometry (DESI-MS). *Analyst* **2009,** *134* (5), 838-841.

29. Roach, P. J.; Laskin, J.; Laskin, A., Nanospray desorption electrospray ionization: an ambient method for liquid-extraction surface sampling in mass spectrometry. *Analyst* **2010,** *135* (9), 2233-2236.

30. Watrous, J.; Roach, P.; Alexandrov, T.; Heath, B. S.; Yang, J. Y.; Kersten, R. D.; van der Voort, M.; Pogliano, K.; Gross, H.; Raaijmakers, J. M.; Moore, B. S.; Laskin, J.; Bandeira, N.; Dorrestein, P. C., Mass spectral molecular networking of living microbial colonies. *Proceedings of the National Academy of Sciences of the United States of America* **2012,** *109* (26), E1743-E1752.

31. Lanekoff, I.; Laskin, J., Imaging of lipids and metabolites using nanospray desorption electrospray ionization mass spectrometry. (1940-6029 (Electronic)).

32. Cleusix, V.; Lacroix, C.; Vollenweider, S.; Duboux, M.; Le Blay, G., Inhibitory activity spectrum of reuterin produced by Lactobacillus reuteri against intestinal bacteria. *BMC Microbiology* **2007,** *7*, 101-101.

33. Li, J.; Wang, W.; Xu, S. X.; Magarvey, N. A.; McCormick, J. K., Lactobacillus reuteri-produced cyclic dipeptides quench agr-mediated expression of toxic shock syndrome toxin-1 in staphylococci. *Proceedings of the National Academy of Sciences* **2011,** *108* (8), 3360-3365.

34. Lee, K.; Lee, H.-G.; Pi, K.; Choi, Y.-J., The effect of low pH on protein expression by the probiotic bacterium Lactobacillus reuteri. *PROTEOMICS* **2008,** *8* (8), 1624-1630.

35. Valentine, N.; Wunschel, S.; Wunschel, D.; Petersen, C.; Wahl, K., Effect of Culture Conditions on Microorganism Identification by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Applied and Environmental Microbiology* **2005,** *71* (1), 58-64.

36. Williams, T. L.; Andrzejewski, D.; Lay Jr, J. O.; Musser, S. M., Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells. *Journal of the American Society for Mass Spectrometry* **2003,** *14* (4), 342-351.

37. Seng, P.; Drancourt, M.; Gouriet, F.; La Scola, B.; Fournier, P.-E.; Rolain, J. M.; Raoult, D., Ongoing Revolution in Bacteriology: Routine Identification of Bacteria by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry. *Clinical Infectious Diseases* **2009,** *49* (4), 543-551.

38. Easterling, M. L.; Colangelo, C. M.; Scott, R. A.; Amster, I. J., Monitoring Protein Expression in Whole Bacterial Cells with MALDI Time-of-Flight Mass Spectrometry. *Analytical Chemistry* **1998,** *70* (13), 2704-2709.