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Discrimination of Intact and Injured *Listeria monocytogenes* by Fourier Transform Infrared Spectroscopy and Principal Component Analysis

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Fourier transform infrared spectroscopy (FT-IR, 4000−600 cm^{-1}) was used to discriminate between intact and sonication-injured *Listeria monocytogenes* ATCC 19114 and to distinguish this strain from other selected *Listeria* strains (*L. innocua* ATCC 51742, *L. innocua* ATCC 33090, and *L. monocytogenes* ATCC 7644). FT-IR vibrational overtone and combination bands from mid-IR active components of intact and injured bacterial cells produced distinctive "fingerprints" at wavenumbers between 1500 and 800 cm^{-1}. Spectral data were analyzed by principal component analysis. Clear segregations of different intact and injured strains of *Listeria* were observed, suggesting that FT-IR can detect biochemical differences between intact and injured bacterial cells. This technique may provide a tool for the rapid assessment of cell viability and thereby the control of foodborne pathogens.

KEYWORDS: FT-IR; spectroscopy; *Listeria*; injured cells; principal component analysis

INTRODUCTION

Listeria monocytogenes is recognized as a serious human pathogen. It is ubiquitous and can cause life-threatening illness (*1*, *2*). This pathogen is the most thermally resistant vegetative cell commonly considered when food pasteurization processes are developed. It can survive at low pH and at high salt concentration (>10%). Furthermore, this microbe can grow at refrigeration conditions, often with no signs of food spoilage (*3*, *4*).

There is a need to rapidly determine whether food products are contaminated with *L. monocytogenes* and other pathogens, preferably using a noninvasive method. Genetic methods such as real-time Polymerase Chain Reaction (PCR) are destructive and require a minimum of 2 h. A further limitation is that genetically based methods cannot differentiate between viable and dead or injured cells. Spectroscopic methods offer options for rapid detection of pathogens and can both detect and discriminate between pathogenic strains. Methods based upon vibrational spectroscopic techniques, such as Fourier transform infrared (FT-IR, 4000−600 cm^{-1}) spectroscopy are rapid, require little or no sample pretreatment, and permit the users to collect full spectra in less than a few seconds. FT-IR measures molecular vibrations of biochemical composition and structure, which provides characteristic biochemical "fingerprints" (*5*−*7*) and can easily distinguish structural features of bacteria (*7*, *8*).

FT-IR has been used to monitor *Bradyrhizobium japonicum* growth and its structural changes during growth (*8*). Furthermore, FT-IR has been used to identify and classify *Bacillus cereus* (*9*), *Listeria* spp. (*10*, *11*), *Staphylococcus* spp., *Clostridium* spp., and *Escherichia coli* (*12*), and to investigate microbial colony heterogeneity (*5*). IR methods can also be used to monitor microbial growth and quantify microbes responsible for spoilage in chicken muscles (*6*, *13*).

Although FT-IR can discriminate between different bacteria, the feasibility of this technique to study cell injury has not been reported. Current methods for detecting and enumerating stressed or injured cells can be problematic. For example, *L. monocytogenes* can be injured by many factors, including heating, freezing, drying, sonication, exposure to acids, antibiotics, and sanitizing agents, etc. However, it is very important to be able to detect injured *L. monocytogenes* because sublethally injured cells may recover in food during storage and then grow. Current microbiological methods for recovering injured cells usually involve highly selective media and are time-consuming and labor intensive (*3*, *14*). FT-IR spectroscopy could possibly provide a method to detect injured cells present in foods.

The goal of this study was to develop a rapid, spectroscopic (FT-IR, 4000−600 cm^{-1}) method to detect and discriminate *L. monocytogenes* from other selected *Listeria* strains and to

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investigate how spectral properties of *L. monocytogenes* are affected by sonication injury.

MATERIALS AND METHODS

Preparation of Bacterial Cultures. The bacterial strains used in this study were obtained from the culture collection of the Department of Food Science and Human Nutrition, Washington State University. Selected *Listeria* strains (*L. monocytogenes* ATCC 19114, *L. monocytogenes* ATCC 7644, *L. innocua* ATCC 51742, and *L. innocua* ATCC 33090) from refrigerated slants were activated by streaking onto tryptic soy agar (TSA) (Difco, Detroit, MI) at 37 °C for 24 h. A representative colony was then inoculated into 50 mL of brain heart infusion (BHI) (Difco Laboratories) broth at 37 °C for a 24-h incubation period. At this point, the cells ($\sim 10^9$ cfu/mL) were in the stationary growth phase and were ready for further use. Injury of bacterial cells was accomplished by sonicating (Bransonic Co., Banbury, CT) *L. monocytogenes* ATCC 19114 suspensions at room temperature for 5 min. The sonicated suspensions were cooled frequently in a crushed ice bath to reduce the risk of heat injury to the cells.

Broth (15 mL) of each strain was centrifuged in an RC-S superspeed centrifuge (DuPont Instruments, Newtown, CT) at 6000 rpm for 5 min, the supernatant was discarded, and the precipitate (wet pellet) was resuspended and vortexed in 10 mL of sterile saline (0.9% NaCl) solution. This procedure was repeated three times to remove medium components and harvest pure cells. Then the bacterial/saline suspension (0.5 mL) was dispensed onto an aluminum oxide membrane filter (0.2 μm pore size, 25 mm o.d.) (Anodisc, Whatman Inc., Clifton, NJ). The Anodisc filters were then air-dried under laminar flow at room temperature for 30 min to allow the formation of a homogeneous dried film of bacterial cells.

Bacterial cells were enumerated using a standard spread plating method on TSA. The plates were incubated at 37 °C for 48 h. Cell counts were expressed as colony forming unit (cfu) per milliliter.

FT-IR Spectroscopy. A Thermo Nicolet Avatar 360 FT-IR spectrometer (Thermo Electron Inc., San Jose, CA) was used to collect FT-IR spectra. The Anodisc membrane filters with bacteria were placed in direct contact with an attenuated total reflection (ATR) zinc selenide crystal. This arrangement is widely used to study the chemical composition of smooth surfaces such as biofilms in a relatively undisturbed state (*7*). Spectra of samples ($N = 20$) were acquired at room temperature.

Data Analysis. Data analysis was performed using OMNIC (Thermo Electron Inc., San Jose, CA) and Delight version 3.2.1 (Textron Systems, Wilmington, MA) software. FT-IR spectral features are often overlapped. Therefore, some data preprocessing algorithms were employed to analyze the data, such as binning, smoothing, and second-derivative transformation (*15*, *16*). Binning reduces the number of data points in a spectrum by averaging *n* points into one. Smoothing eliminates high-frequency instrument noise by averaging neighboring data points. Second-derivative transformation separates overlapping absorption bands and removes baseline offsets. First, spectral data were binned by 2 nm and smoothed with a Gaussian function over 12 nm. Then a second-derivative transformation with a 12 nm gap was calculated (*17*, *18*).

In this study, the multivariate statistical analysis technique of principal component analysis (PCA) was used. PCA has been widely used in the interpretation of infrared spectra in medicine, biology, agricultural, and food sciences. It reduces a multidimensional data set to its most dominant features, removes the random variation (noise), and retains the principal components (PC) that capture the related variation (*19*). The multidimensional data are processed by least-squares techniques to a series of orthogonal eigenvectors of the sample covariance matrix. PCA shows whether there are natural clusters in the data and describes similarities or differences from multivariate data sets (*20*). The first PC conveys the largest amount of information, followed by the second PC, and so forth. At a certain point, the variation modeled by any new PC is mostly noise (*21*). The scores for the chemometric model are composed of the weightings for each PC creating the best-fit vector for each sample.

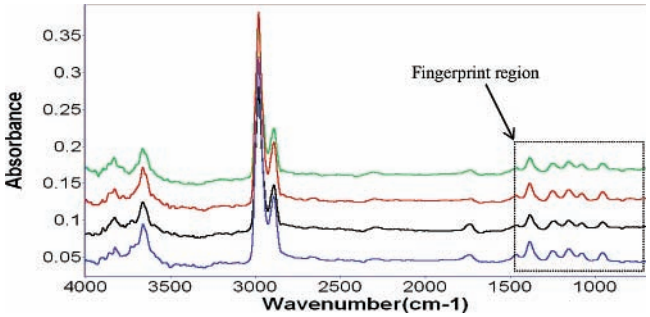

Figure 1. Representative FT-IR spectra for four *Listeria* strains (green, *L. monocytogenes* ATCC 19114; red, *L. monocytogenes* ATCC 7644; black: *L innocua* ATCC 33090; blue, *L. innocua* ATCC 51742).

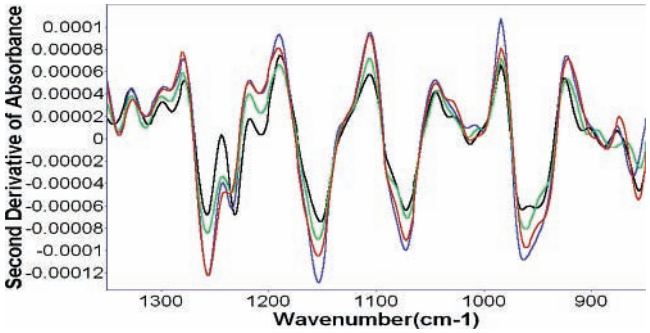

Figure 2. Representative second-derivative transformed FT-IR spectra at the fingerprint region of four *Listeria* strains (green, *L. monocytogenes* ATCC 19114; red, *L. monocytogenes* ATCC 7644; black, *L innocua* ATCC 33090; blue, *L. innocua* ATCC 51742).

RESULTS AND DISCUSSION

Representative FT-IR spectra of four *Listeria* strains (*L. monocytogenes* ATCC 19114, *L. monocytogenes* ATCC 7644, *L. innocua* ATCC 33090, and *L. innocua* ATCC 51742) are shown in **Figure 1**. Each strain exhibited characteristic absorbances at wavenumbers between 4000 and 1500 cm^{-1}. The prominent absorption peaks around 3000 cm^{-1} are mainly due to a CH_2 and CH_3 asymmetric stretch of fatty acids from the bacterial cell wall; the absorption peaks around 1745−1735 cm^{-1} are likely from the >C═O stretch of esters or fatty acids (*7*).

Strain-specific absorbance peaks can also be observed between 1500 and 800 cm^{-1}. Usually, the range below 1500 cm^{-1} is significant for deformation, bending, and ring vibrations and is often referred to as the "fingerprint region" of a FT-IR spectrum (*7*, *22*). The fingerprint region is shown in **Figure 1**. For instance, the peaks at 1455 cm^{-1} are mainly from an asymmetric CH_3 bending mode of the methyl groups of proteins (*8*). These vibrational bands can be correlated to single molecular bonds or functional groups and are important for the identification of biochemical compounds.

A second-derivative transformation makes unique spectral features of the different bacterial strains more prominent. **Figure 2** shows representative second-derivative transformed spectra of the fingerprint region collected from the four bacterial strains in **Figure 1**. Second-derivative transformation is often used to process spectral data because it separates overlapping absorption bands, removes baseline shifts, and increases apparent spectral resolution. In **Figure 2**, the differences in the spectral features between the bacterial strains are distinct. Absorption peaks around 1303 cm^{-1} are from the C−N functional group of the protein; those around 1250 cm^{-1} are from the asymmetric stretching mode of the P═O in phosphate. The peaks between

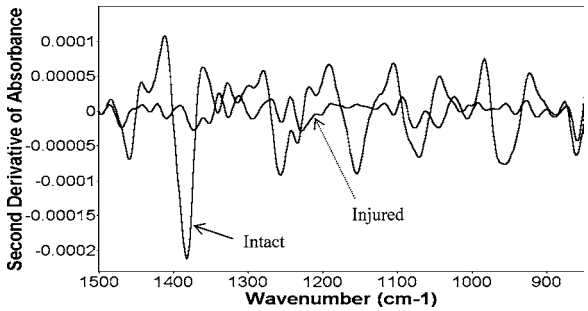

Figure 3. Second-derivative transformed spectrum of intact and sonication-injured *L. monocytogenes* ATCC 19114 cells in the fingerprint region.

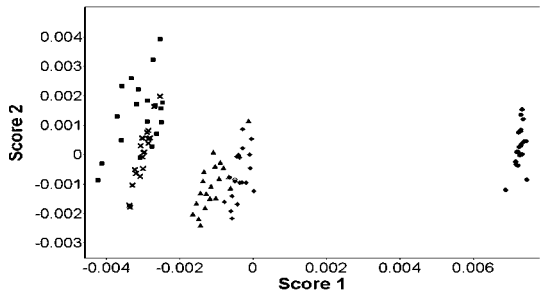

Figure 4. PCA results for intact and sonication-injured *Listeria* strains: (●) sonication-injured *L. monocytogenes* ATCC 19114; (▲) intact *L. monocytogenes* ATCC 19114; (■) intact *L. innocua* ATCC 51742; (×) intact *L. monocytogenes* ATCC 7644; (◆) intact *L. innocua* ATCC 33090.

1200 and 900 cm^{-1} are believed to be from stretching vibrations of the phosphate and the vibrations of polysaccharide moieties (*7*, *8*, *22*).

Spectral measurements may provide insight regarding the condition of injured microbes and how certain types of injury could occur. In this study, sonication was used to damage bacterial cells. **Figure 3** shows typical second-derivative transformed spectra of intact and injured *L. monocytogenes* ATCC 19114 cells. Distinct differences can be observed between these spectra; for example, a band at 1398 cm^{-1} arises primarily from symmetric vibration of protein methyl groups (*8*). The changes to the cells during sonication may have resulted from macromolecular shearing and subsequent redistribution of cell wall components along with possible denaturation of intracellular proteins.

Environmental stresses such as heat and sonication damage bacterial cell walls, protein, and nucleic acid moieties, leading to injury. Some injuries may trigger a physiological response in bacterial cells leading to the production of specific compounds, for example, heat-shock proteins (*23*). Theoretically, these protein compounds have unique absorptions in the FT-IR region providing a unique signature for specific types of bacterial injury. Injury resulting in protein denaturation and rupture of cell walls or cell membranes may also emerge as unique spectral features, although further study is clearly necessary.

PCA was employed to differentiate *Listeria* strains on the basis of differences in their spectral features in the fingerprint region. **Figure 4** shows the PCA results for selected *Listeria* strains (intact and sonication-injured *L. monocytogenes* ATCC 19114; *L. monocytogenes* ATCC 7644; *L. innocua* ATCC 51742; and *L. innocua* ATCC 33090). Clear segregations with distinct sample clusters were observed between all intact strains and the injured *L. monocytogenes* ATCC 19114. This indicates that FT-IR can discriminate between different strains in the genus *Listeria* and between intact and injured cells of this genus. Unique biochemical features of bacteria are discernible within this spectral range. The quantity and distribution of different components in cell wall, the membrane, and cytoplasm, such as proteins and peptides, polysaccharides, phospholipids, peptidoglycan (murein), and nucleic acids, vary among microbial strains, making it possible to use FT-IR to detect and discriminate between foodborne pathogens (*7*).

FT-IR spectra are affected by various factors, including incubation temperature and time, the optical properties of filter membrane used for sample preparation, etc. (*8*, *22*, *24*). In this study, sample preparation procedures were standardized to minimize these influences, generate consistent FT-IR spectral patterns, and obtain reproducible data. The average cell concentration of bacterial strains was consistently 1×10^9 cfu/mL, which provided strong FT-IR signals. Because absorption of water significantly affects FT-IR spectra (*8*), all samples in this study were dried to the same extent before spectral readings were obtained. In addition, data from the fingerprint region (1500−800 cm^{-1}) utilized in the PCA modeling excluded the main water absorption peak at 3400 cm^{-1} due to the O−H stretch of water. Other interfering compounds, such as BHI medium components, were removed by filtration before FT-IR measurements to limit interference from these sample components.

In conclusion, this preliminary study showed that FT-IR could differentiate between *Listeria* strains and between healthy and sonication-injured bacterial cells. However, more work is needed to evaluate the potential of FT-IR to detect, classify, and quantify important foodborne pathogens in complex food systems. Further studies are necessary to investigate how spectral properties of bacteria differ with growth phase, and as a result of injury due to various environmental stresses, including pH, salt, temperature, and other treatments (e.g., addition of antibiotics), and to determine the selectivity and sensitivity of FT-IR methods for the analysis of microbes in various food systems. FT-IR may also be a suitable method for examining bacterial biofilms and as an alternative to traditional microscopic observations.

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