

Automated species and strain identification of bacteria in complex matrices using FTIR spectroscopy

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ABSTRACT

Fourier Transform Infrared (FTIR) spectroscopy provides a highly selective and reproducible means for the chemically-based discrimination of intact microbial cells which make the method valuable for large-scale screening of foods. The goals of the present study were to assess the effect of chemical interferents, such as food matrices, different sanitizing compounds and growth media, on the ability of the method to accurately identify and classify *L. innocua*, *L. welshimeri*, *E. coli*, *S. choleraesuis*, *S. subterranea*, *E. sakazakii*, and *E. aerogenes*. Moreover, the potential of FTIR spectroscopy for discrimination of *L. innocua* and *L. welshimeri* of different genotypes and the effect of growth phase on identification accuracy of *L. innocua* and *L. welshimeri* were tested. FTIR spectra were collected using two different sample presentation techniques – transmission and attenuated total reflection (ATR), and then analyzed using multivariate discriminant analysis based on the first derivative of the FTIR spectra with the unknown spectra assigned to the species group with the shortest Mahalanobis distance. The results of the study demonstrated 100% correct identification and differentiation of all bacterial strains used in this study in the presence of chemical interferents or food matrices, better than 99% identification rate in presence of media matrices, and 100% correct detection for specific bacteria in mixed flora species. Additionally, FTIR spectroscopy proved to be 100% accurate when differentiating between genotypes of *L. innocua* and *L. welshimeri*, with the classification accuracy unaffected by the growth stage. These results suggest that FTIR spectroscopy can be used as a valuable tool for identifying pathogenic bacteria in food and environmental samples.

Keywords: FTIR spectroscopy, pathogen detection, Mahalanobis distance

1. INTRODUCTION

The lack of a rapid, economical and simple detection and identification method for foodborne pathogens continues to pose an impediment to providing real-time assessments for managing foodborne risks. Conventional cultural procedures for bacterial identification include multiple subcultures and biotype- or serotype- identification steps and require up to a week to obtain confirmed results¹⁻³. Moreover, these methods are often misleading and provide only retrospective information about a food product sample⁴. Recently, much effort has been focused on development of new molecular and immunological techniques; however their use for routine bacterial detection and identification is still limited due to high costs and the requirement for trained personnel³. In addition, reliability of such nucleic acid amplification methods can be decreased due to the presence of residual matrix components that inhibit enzymatic reactions⁵.

Fourier transform infrared (FTIR) spectroscopy offers options for rapid discrimination, classification and identification of intact microbial cells⁶. Agent identification with FTIR spectroscopy involves the observation of vibration properties of chemical bonds when excited by an infrared beam. An infrared spectrum provides information about the biochemical composition of the cell. Each bacterial species produces a unique fingerprint-like FTIR spectra⁷⁻⁸. Unlike existing biological technologies that are both labor intensive and expensive due to the high volume of reagents and supplies required for strain identification, FTIR spectroscopy has great potential in the food industry as a rapid and cost-effective pathogen identification method.

Use of FTIR spectroscopy in analysis of food-borne bacteria has been investigated since the 1980s, when its application in diagnostic microbiology was reported⁹. Since then, FTIR spectroscopy was successfully applied for identification and classification of food-borne bacterial strains and species from a variety of different genera, including *Listeria*^{3,10}, *Bacillus*^{11,12}, *Staphylococcus*, *Clostridium*, *Escherichia coli*^{11,13}, and *Lactobacillus*¹⁴, as well as for identification of food-borne, fermentative yeasts⁷. Recently, FTIR spectroscopy was used to differentiate between intact and sonication-injured *L. monocytogenes*⁶ and to detect microbial spoilage of meat. The effects of components of growth media and sampling procedure on the expression of FTIR spectra of *B. cereus* and *L. monocytogenes* were also examined in the past¹. However, it remains unclear whether FTIR spectroscopy is able to accurately identify and differentiate bacteria in the presence of a variety of chemical interferences such as selective media ingredients, chemical sanitizers or food matrices or if various growth phases have an effect on bacterial identification.

In this paper we describe the results from FTIR spectroscopy applied to a diverse collection of pathogen simulants, including *Listeria innocua*, *Listeria welshimeri*, *Escherichia coli* K12, *Escherichia coli* ATCC 29181, *Salmonella choleraesuis*, *Salmonella subterranea*, *Enterobacter sakazakii*, and *Enterobacter aerogenes*, in order to evaluate the ability of the method to accurately identify foodborne microorganisms. We apply multivariate discriminant analysis for agents' identification based on the first derivative of the FTIR spectra with the unknown spectra assigned to the species/strain group with the shortest Mahalanobis distance. The measurements and results are subdivided into three categories of challenges: (1) pathogen target identification, (2) pathogen target identification in various sanitizing compounds, growth media, and food matrices, and (3) pathogen target identification in mixed flora samples. The first two objectives were intended to demonstrate the capability of FTIR to correctly identify pathogen targets in non-ideal sample conditions. The third objective was intended to demonstrate the capability of FTIR to correctly identify and discriminate pathogen targets in complex backgrounds containing additional bacterial populations. Finally, we describe experiments which demonstrate the method's insensitivity to growth phase and its ability to differentiate bacteria within the same genotype.

2. SAMPLE PREPARATION AND METHODOLOGY

2.1 Strains and growth conditions

The selected strains (*Listeria innocua* CWD 142, *Listeria welshimeri* FSL H6-O17, *Escherichia coli* K12 HB 101, *Escherichia coli* (ATCC 29181), *Salmonella choleraesuis* (ATCC 13314), *Salmonella subterranea* (ATCC BAA-836), *Enterobacter sakazakii* (ATCC 29544), and *Enterobacter aerogenes* (ATCC 13048)) were chosen as bio safety level one (BSL-1) simulants and controls for selected food borne pathogens *L. monocytogenes*, *E. coli* O157:H7, *Salmonella typhimurium*, and *E. sakazakii*. These bacterial cultures represent well known contaminants of food processing environments and present major public health implications to food processors. The selected bacterial strains were obtained from the American Type Culture Collection (ATCC), Manassas, Virginia and from the strain collection of the Department of Nutrition and Food Sciences, University of Vermont.

2.2 Sample preparation

Each culture was grown in ten 9-ml volumes of TSB for 18h at 37C° to take into account the fluctuations of cultures grown on the same day. Five ml of freshly grown bacterial cultures from each tube were pelleted by centrifugation at 5,000 X g for 15 min. and washed three times to remove media components. The bacterial suspension (100µl) was then deposited in concentration of 10⁸ – 10⁹ cells per ml on sterile non-breakable microporous polyethylene cards and dried at ambient temperature for 3-4 h in a laminar flow cabinet to form a homogeneous dried film of bacterial cells. Bacterial cells were enumerated on nonselective 3M™ Petrifilm™ Plates for each culture tube to ensure consistent numbers of bacterial cells.

2.3 Effect of growth media

To test the influence of growth media on the FTIR reflection spectra, selected strains were grown in various selective media. Each bacterial culture was inoculated in ten 9-ml tubes of respective selective broth and grown at 37C° for 18h. Thereafter, 5ml of each strain from each tube was centrifuged at 6000 rpm for 15min. The supernatant was then

discarded, and the pellet was re-suspended and vortexed in 5ml of sterile Butterfield's phosphate buffer. This procedure was repeated three times to remove media components and to harvest pure cultures. Two sampling replications for each bacterial suspension were deposited on the membrane cards at levels of $10^8 - 10^9$ CFU/ml. Then the filters were dried under laminar flow at room temperature for 3-4 h.

2.4 Effect of growth phase

L. innocua CDW 142 and *L. welshimeri* FSL H6-017 were used for growth experiments. These bacterial cultures were inoculated in TSB media at an initial level of 100 CFU/ml and grown at 37°C with agitation to the stationary phase. Optical density at 600 nm (OD600) was determined every hour and bacterial counts were done at each time point by plating preparations in duplicates onto 3M™ Petrifilm™ Plates. Growth curves were generated by using absorbance values. From these curves the approximate mid-exponential- and stationary-phase points were determined for each culture and used as reference times to perform the FTIR spectra experiments. Next, ten 9ml sterile TSB media were inoculated with *L. innocua* CDW 142 and *L. welshimeri* FSL H6-017 cultures and grown at 37°C to reach logarithmic or stationary phase. Five ml aliquots of cultures were removed from each tube, centrifuged and washed as described earlier, and placed on the membrane filters in duplicates. The filters were dried under laminar flow at room temperature for 3-4 h before the FTIR spectra analysis.

2.5 Effect of genotypes

L. innocua Ribotype 1-1910-7, *L. innocua* Ribotype 5-415-5, *L. welshimeri* Ribotype 1-1921-4, *L. welshimeri* Ribotype 2-864-3 were used in these experiments. These cultures were grown in TSB media at 37°C.

2.6 Effect of food matrices

Retail commercial samples of nonfat dry skim milk powder and powdered infant formula were purchased for study. Overnight cultures of *E. sakazakii* ATCC 29544 and *E. aerogenes* ATCC 13048 were inoculated into samples (10 sample tubes per culture) at 37°C for an 18h incubation period.

2.7 Effect of sanitizing chemicals

Commonly used food processing plant environmental sanitizers including sodium hypochlorite, quaternary ammonium compound, iodine based sanitizer, and preservative Ethylene Diaminetetraacetic Acid (EDTA) were evaluated as chemical interferents during bacterial spectral signature analysis. One ml of 18 h cultures of *L. innocua* CWD 142 and *L. welshimeri* FSL H6-017 were grown in TSB at 37°C and then exposed for 2 and 3 min to 50 and 200ppm of sodium hypochlorite and quaternary ammonium compound; 6 and 25ppm of iodine based sanitizer; 50 and 300ppm of EDTA to reflect concentrations commonly used in food plant environments and food preservation. Samples were then centrifuged, washed and analyzed.

2.8 Effect of mixed species samples

Strains of *Listeria innocua* CWD 142, *Listeria welshimeri*, *Pseudomonas fluorescens* ATCC 11150 and *Lactobacillus plantarum* ATCC 8014 were used for this study. Each selected strain was grown in twenty 9 ml volumes of TSB for 18 h at 37°C. Equal parts (1 ml) of *L. innocua*, *P. fluorescens*, and *L. plantarum* from freshly grown bacterial cultures from each tube were mixed to achieve a total of 3 ml suspension that was then pelleted by centrifugation at 5,000 X g for 15 min (Centrifuge model HSC10K, Savant Instrument Inc., Farmingdale, NY) and washed three times to remove media components. The mixed bacterial suspension (100 µl) was then deposited on sterile non-breakable microporous polyethylene cards (Thermo Electron Corp, Madison, WI), which are compatible with qualitative mid-range (4000 cm^{-1} to 400 cm^{-1}) infrared spectroscopy, and dried at ambient temperature for 3-4 h in a laminar flow cabinet to form a homogeneous dried film of bacterial cells. Six sampling replications were done for each of the pelleted suspension to verify repeatability of the method, thus resulting in a total of 120 replicates. Similarly, 120 replicates with equal parts *L. welshimeri*, *P. fluorescens*, and *L. plantarum* were prepared. Concentration of the freshly grown bacterial cultures was enumerated on nonselective 3M Petrifilm aerobic count plates (3M Co., St. Paul, MN) to ensure consistent numbers of bacterial cells.

3. MEASUREMENTS AND ANALYSIS METHODOLOGY

3.1 FTIR spectroscopy

Two different spectral sampling methods were investigated in this study - Transmission and Attenuated Total Reflection (ATR). Both the transmission spectra and the ATR FTIR spectra were measured for each bacterial sample with a Thermo Nicolet IR200 FTIR spectrometer (Thermo Electron Inc., San Jose, CA), equipped with a KBr beam splitter and a DTGS detector. Preprocessing and processing of FTIR spectral data were performed using EZ OMNIC software. For the reflection analysis, the membrane filters with bacteria were placed in direct contact with an ATR ZnSe crystal. Spectra of samples were collected at room temperature at spectral range from 399.1926 cm^{-1} to 3999.64 cm^{-1} at a spectral resolution of 2 cm^{-1} . Each spectrum was composed of an average of 16 separate scans. A background spectrum was recorded with a blank filter membrane and then each spectrum was obtained by subtracting the single-beam spectrum against that of the blank filter membrane. To minimize problems from unavoidable baseline shifts and to enhance the resolution of the bands, base line shifts were removed by taking the first derivative of the spectral data which was then used for further analysis.

3.2 Bacterial identification testing

Identification algorithms were developed specifically for this study by QuantaSpec Inc. The algorithms were tested using a Cross Validation approach, where one spectral sample is removed from the data set and is used as an “unknown” to test the identification algorithm, which is generated from the remaining data set referred to as a training set. Next, a different spectral sample is removed from the data set and is used as an “unknown” to test the algorithm generated by the remaining training set. This process was repeated until each spectral sample had been used as an unknown. Accuracy of the approach was determined by comparing the algorithm predicted identity of the “unknown” to the true identity of the sample.

3.3 Data analysis

Data analysis was performed using multivariate discriminant analysis of the first derivative of the FTIR spectral data. ATR and transmission data were analyzed separately. Multivariate discriminant analysis is closely related to principal components analysis which has been widely used to discriminate FTIR spectra of bacteria^{1,8,10,12}. Here, linear combinations of the spectra, or canonical variates, are formed to maximize the distance between two or more predefined group means and to provide the best discrimination of the groups. The groups were scaled so that the distances between them represent the Mahalanobis distances, M_{ij} , which were calculated as follows:

$$M_{ij} = (x_i - x_j) S^{-1} (x_i - x_j),$$

where, S is the pooled estimate of the within-group covariance matrix and x_i and x_j are mean vectors for the two groups. Thus, M_{ij} is the distance between groups in within-group standard deviation units. Canonical scores for the two most significant variates were used to create a graphical two-dimensional representation of the data.

4. RESULTS

4.1 Spectral identification of pathogen targets

Discrimination of *L. innocua*, *L. welshimeri*, *E. coli* ATCC 29181, *E. coli* K12, *S. choleraesuis*, *S. subterranea*, *E. sakazakii*, and *E. aerogenes* was successfully accomplished using ATR and transmission analysis. Transmission FTIR spectra and ATR spectra are shown in Fig. 1 and Fig. 2, respectively, for various strains.

Our multivariate discriminant analysis algorithm showed successful differentiation, resulting in 100% discrimination among these bacterial cultures. One hundred fifty nine spectra collected using the ATR approach, were correctly assigned to their species/strain group, and 157 spectra, collected with transmission approach, were correctly assigned to their species/strain group. These results were obtained when the full spectral region between 399.1926 and 3999.64 cm^{-1} was included. We found that the accuracy of the method dropped from 100% to 98% when a narrower spectral region between 400 and 2000 cm^{-1} was used for the ATR data.

The multivariate approach demonstrated the ability of infrared spectroscopy to successfully discriminate among cultures of all tested bacterial species. Differentiation of the spectra is shown in Fig. 3 for Transmission and ATR spectra, where the bacterial species are separated into eight distinct clusters. The method demonstrated a very good reproducibility by the tight clustering of specific bacterial samples grown in separated tubes on the same day and when repeated on a subsequent day. Interestingly, the ATR data analysis resulted in gram-negative *E. coli* K12, *E. coli* ATCC 29181, *S. cholerasuis*, *S. subterranean*, *E. sakazakii*, and *E. aerogenes* bacterial clusters grouped closely together, which may be attributed to genetic similarities shared by members of the same genera *Enterobacteriaceae*.

4.2 Pathogen identification in growth media, sanitizing compounds, and food matrices

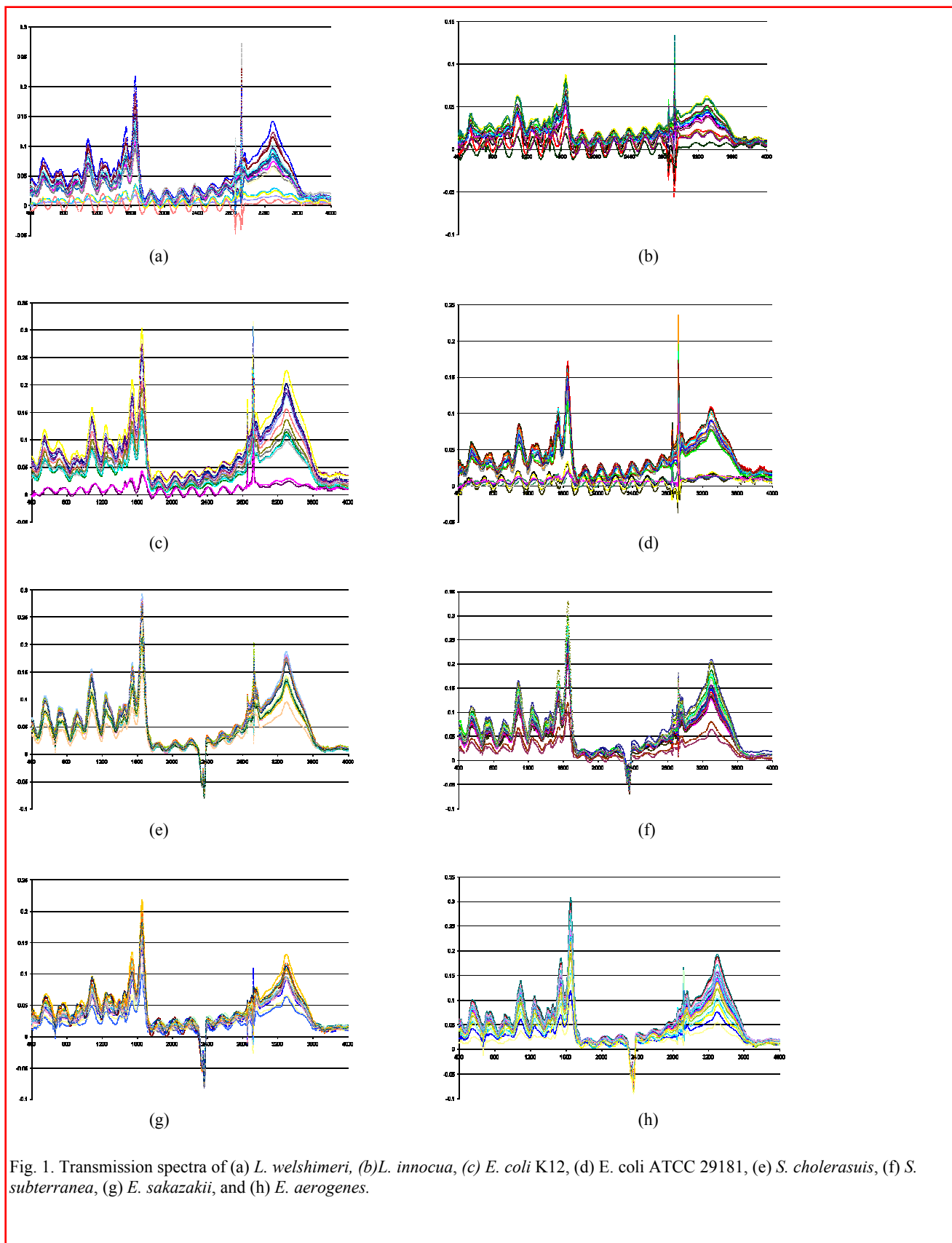
The robustness of the method was further demonstrated by the cross-validation approach that showed that the separation of bacterial species was insignificantly affected by variations in broth medium and sanitizer matrices. The selective enrichment media that were used in this research represent broths that are commonly used for bacterial detection from environmental and food sources. Using cultures grown under various conditions, it was discovered that in most cases the cells grown in selective media exhibited the same spectra as the cells grown in non-selective enrichment broths. When the statistical analysis was performed to evaluate the effect of media on classification, some of the samples were incorrectly classified; in all cases such incorrect identification occurred for bacteria that belong to the same genus. For the transmission data, the method identified *E. coli* K12 grown in TSB media as *E. coli* ATCC 29181 also cultured in TSB, and *L. welshimeri* FSL H6-O17 grown in TSB as *L. innocua* CWD 142 also grown in TSB. When the ATR data was analyzed, 3 analytic mistakes were noted: *S. subterranea* ATCC BAA-836 grown in TSB was identified as *S. cholerasuis* ATCC 13314 also cultured in TSB, *S. subterranea* ATCC BAA-836 cultured in SCB was identified as *S. cholerasuis* ATCC 13314 also cultured in SCB, and *E. coli* K12 grown in TSB media was selected as TSB cultured *E. coli* ATCC 29181. Nonetheless, the identification rate for all species was above 99%. Of 319 samples collected with ATR approach, 316 were correctly identified (99.1%); 317 out of 319 (99.4%) collected with transmission technique samples were correctly assigned to their species/strain group. This demonstrated reliability of the method in distinguishing bacteria in presence of media matrices. The results for all measurements in this section are summarized in Table 1.

FTIR spectroscopy was proven to be 100% specific in bacterial differentiation in the presence of commonly used sanitizers and preservative. When the cross validation approach was applied to the transmission and ATR data, 100% of spectra representing *L. innocua* CWD 142 and *L. welshimeri* FSL H6-O17 cultures treated with iodophor, quaternary ammonia and chlorine sanitizers were correctly identified. Moreover EDTA was proven to have no effect on identification as 100% of *L. innocua* CWD 142 and *L. welshimeri* FSL H6-O17 spectra were successfully assigned to their species group, as depicted in Table 1.

Finally, *E. sakazakii* ATCC 29544 and *E. aerogenes* ATCC 13048 were used as simulants of pathogenic *E. sakazakii* for investigation of the impact of food matrices on FTIR detection. Samples were prepared by using overnight bacterial cultures grown in reconstituted nonfat dry skim milk powder (NDSMP) and powdered infant formula. Table 1 depicts a 100% (79/79) successful rate of differentiation of the ATR spectra and 100% (80/80) rate for the transmission spectra.

4.3 Pathogen target identification in mixed flora samples

Cross validation testing showed that the presence of mixed bacterial species in samples did not affect the ability to discriminate between samples with *L. welshimeri* and *L. innocua* as all samples were correctly identified with either transmission (240/240) or ATR (239/239) spectral data. These results indicate that it is possible to detect a pathogen in a sample of multi-species bacterial flora and discriminate amongst closely related interferents. These findings suggest that FTIR could be useful for the automated reagent-less detection and identification of bacteria in environmental samples with low false negative rates.



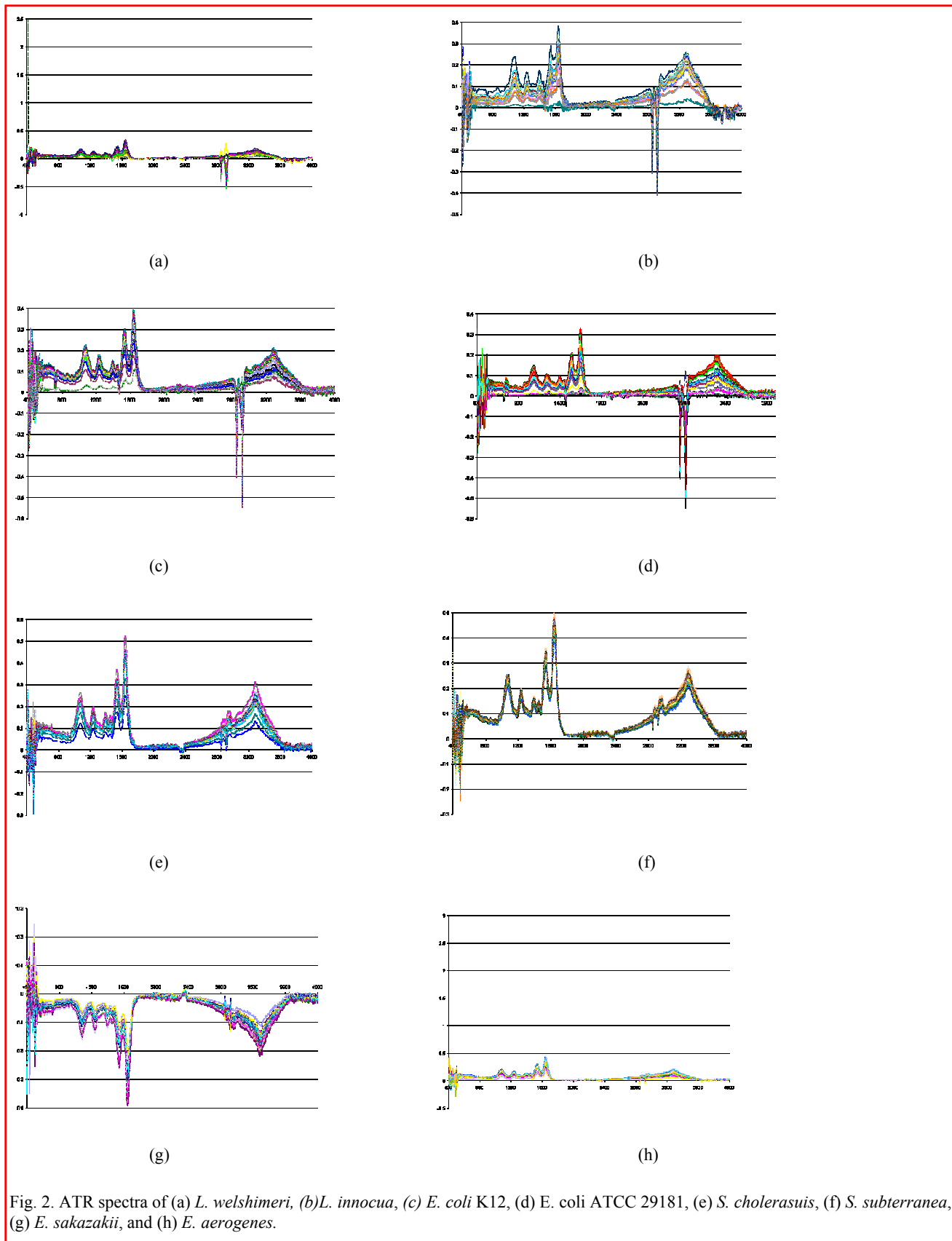


Fig. 2. ATR spectra of (a) *L. welshimeri*, (b) *L. innocua*, (c) *E. coli* K12, (d) *E. coli* ATCC 29181, (e) *S. choleraesuis*, (f) *S. subterranea*, (g) *E. sakazakii*, and (h) *E. aerogenes*.

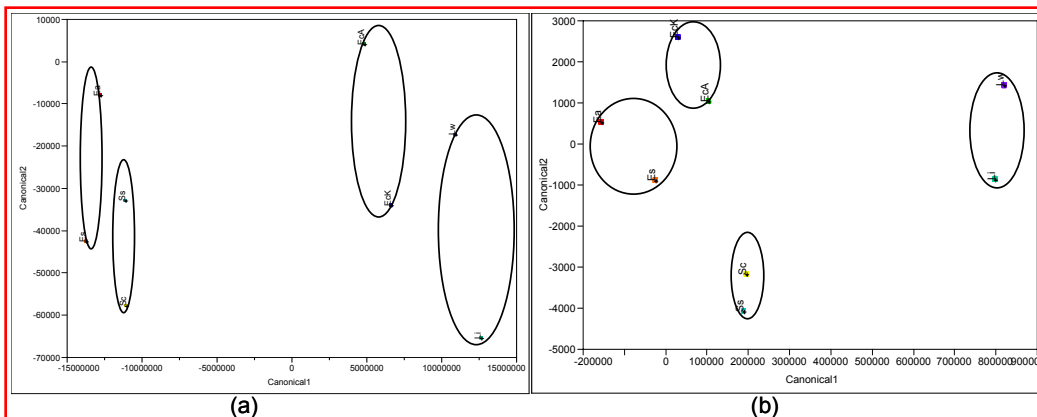


Fig. 3. Discrimination of *L. innocua* (*Li*), *L. welshimeri* (*Lw*), *E. coli* ATCC 29181 (*EcA*), *E. coli* K12 (*EcK*), *S. choleraesuis* (*Sc*), *S. subterranean* (*Ss*), *E. sakazakii* (*Es*), and *E. aerogenes* (*Ea*) using multivariate discriminant analysis for (a) Transmission, and (b) ATR FTIR data.

Table 1. ID rate of pathogen simulants as affected by growth phase, genotype, growth media and chemical interferents.

Aim of the study	Strains used (replicates ATR/Transmission)	ATR [correct/total]	Transmission [correct/total]
Differentiation of Bacteria	<i>L. innocua</i> CWD 142 (20/20), <i>L. welshimeri</i> FSL H6-O17 (19/20) <i>E. coli</i> K12 HB 101 (20/20) <i>E. coli</i> ATCC 29181 (20/20) <i>S. choleraesuis</i> ATCC 13314 (20/17) <i>S. subterranea</i> ATCC BAA-836 (20/20) <i>E. sakazakii</i> ATCC 29544 (20/20) <i>E. aerogenes</i> ATCC 13048 (20/20)	159/159 (100%)	157/157 (100%)
Effect of Growth Phase	<i>L. innocua</i> CWD 142, log phase (20/20) stationary phase (20/20) <i>L. welshimeri</i> FSL H6-O17 log phase(20/20) stationary phase (20/20)	80/80 (100%)	80/80 (100%)
Effect of Genotype	<i>L. innocua</i> Ribotype 1-1910-7 (20/20) <i>L. innocua</i> Ribotype 5-415-5 (20/20) <i>L. welshimeri</i> Ribotype 1-1921-4 (20/20) <i>L. welshimeri</i> Ribotype 2-864-3 (20/20)	80/80 (100%)	80/80 (100%)
Effect of Growth Media	<i>L. innocua</i> CWD 142, (40/40) <i>L. welshimeri</i> FSL H6-O17 (39/39) <i>E. coli</i> K12 HB 101 (40/40) <i>E. coli</i> ATCC 29181 (40/40) <i>S. choleraesuis</i> ATCC 13314 (40/40) <i>S. subterranea</i> ATCC BAA-836 (40/40) <i>E. sakazakii</i> ATCC 29544 (40/40) <i>E. aerogenes</i> ATCC 13048 (40/40)	316/319 (99.1%)	317/319 (99.4%)
Effect of Iodophor Sanitizer	<i>L. innocua</i> CWD 142, (40/40) <i>L. welshimeri</i> FSL H6-O17 (40/40)	80/80 (100%)	80/80 (100%)
Effect of Quarternary Ammonia	<i>L. innocua</i> CWD 142, (40/40) <i>L. welshimeri</i> FSL H6-O17 (40/40)	80/80 (100%)	80/80 (100%)
Effect of Chlorine sanitizer	<i>L. innocua</i> CWD 142, (40/40) <i>L. welshimeri</i> FSL H6-O17 (39/40)	79/79 (100%)	80/80 (100%)
Effect of EDTA	<i>L. innocua</i> CWD 142, (40/39) <i>L. welshimeri</i> FSL H6-O17 (40/40)	80/80 (100%)	79/79 (100%)
Effect of Food Matrices	<i>E. sakazakii</i> ATCC 29544 (39/40) <i>E. aerogenes</i> ATCC 13048 (40/40)	79/79 (100%)	80/80 (100%)
Effect of Mixed Species Flora	<i>L. innocua</i> CWD 142 w/ <i>P. flourescens</i> , and <i>L. plantarum</i> , (120/120) <i>L. welshimeri</i> FSL H6-O17 w/ <i>P. flourescens</i> , and <i>L. plantarum</i> (119/120)	239/239 (100%)	240/240 (100%)

5. CONCLUSIONS

FTIR spectroscopy can be used for rapid, accurate, reagent-less identification of micro-organisms in food matrices. In addition, FTIR spectroscopy can differentiate and identify the presence of specific species in multi-species samples. Furthermore, FTIR spectroscopy is not adversely affected by sanitizers or preservatives, an important advantage over PCR and ELISA-based detection methods. These advantages show FTIR spectroscopy's potential for analysis of field samples not just pure cultures. There was little difference in the accuracy of transmission versus ATR-based spectroscopy in the present study. The ATR approach is favored for field applications as it does not require a transparent substrate and thus uses less consumables. There was some indication that identification accuracy is reduced when the range of spectral data is reduced. It is possible that accurate identification may be accomplished with a reduced spectral range if the algorithm is developed with a larger number of replicates in the training set. Identification of the minimal spectral range required for accurate identification is an interesting goal for further research.

6. ACKNOWLEDGEMENTS

This work was funded by the US FDA under a Phase I SBIR grant.

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