Fourier tansform infrared (FT-IR) spectroscopy: A rapid tool for detection and analysis of foodborne pathogenic bacteria

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Fourier transform infrared (FT-IR) spectroscopy is a physico-chemical method based on measurement of vibration of a molecule excited by IR radiation at a specific wavelength range. FT-IR spectra of bacterial cells can be used to analyze their total composition, including proteins, fatty acids, carbohydrates, nucleic acids, and lipopolysacharides. FT-IR techniques coupled with different chemometrics analyses of the spectra offer a wide range of applications for food microbiology, including detection, differentiation, quantification, and taxonomic level classification of bacteria from culture broth or food matrices. FT-IR spectroscopy is a reliable, rapid, and economic technique which could be explored as a routine diagnostic tool for bacterial analysis by the food industry, diagnostic laboratories, and public health authorities. This chapter highlights the principles of FT-IR spectroscopic analysis of bacteria, the advantages and disadvantages of FT-IR applied to bacterial analysis, various sampling techniques, spectral manipulation, statistical analysis of spectra, and applications in pathogen detection.

Keywords Fourier transform infrared spectroscopy; Pathogen detection; Chemometrics; Bacterial classification

1. Introduction

Rapid and specific detection of foodborne pathogens is an increasingly important task in microbiology and food safety. Bacterial detection, identification, and classification are generally carried out using traditional methods based on biochemical or serological tests and the molecular methods based on DNA or RNA fingerprints. Molecular spectroscopy was introduced as a possible identification approach in the 1950s with limited success [1-3]. The routine use of vibrational spectroscopy for bacterial identification at that time was impractical due to instrument limitations and lack of integrated computational analysis. With the advent of Fourier transform infrared spectroscopy (FT-IR) and computational analysis in the late 1980s and 1990s, Naumann and co-workers reintroduced FT-IR methods for *in-situ* analysis of bacterial cells and complex spectral analysis to identify, differentiate, and classify bacteria [4-8]. Since then, FT-IR spectroscopy has been successfully applied for detection, discrimination, identification, and classification of bacteria belonging to different species, particularly foodborne pathogens such as *Listeria* [9,10], *Escherichia coli* [11,12], *Salmonella* [12,13], *Staphylococcus* [14,15], *Yersinia* [16], and *Bacillus* [17]. FT-IR spectroscopy is not only used as a bacterial identification method, but also provides information about bacterial metabolism [18], growth phase [19], and antibiotic resistance [15]. There is a growing demand for FT-IR spectroscopy in the area of food microbiology due to its technical advancement, simplicity of sample preparation, and speed of analysis.

2. Principles of FT-IR spectroscopy

The infrared region (10-14000 cm^{-1}) of the electromagnetic spectrum is divided into three regions: the near-, mid-, and far-IR. The mid-IR (400-4000 cm^{-1}) is the most commonly used region for analysis as all molecules possess characteristic absorbance frequencies and primary molecular vibrations in this range. Mid-infrared spectroscopy methods are based on studying the interaction of infrared radiation with samples. As IR radiation is passed through a sample, specific wavelengths are absorbed causing the chemical bonds in the material to undergo vibrations such as stretching, contracting, and bending. Functional groups present in a molecule tend to absorb IR radiation in the same wavenumber range regardless of other structures in the molecule, and spectral peaks are derived from the absorption of bond vibrational energy changes in the IR region [20]. Thus there is a correlation between IR band positions and chemical structures in the molecule. In addition to providing qualitative information about functional groups, IR spectra can provide quantitative information, such as the concentration of bacteria in a growth medium.

An IR spectrum is measured by calculating the intensity of the IR radiation before and after it passes through a sample, and the spectrum is traditionally plotted with Y-axis units as absorbance or transmittance and the X- axis as wavenumber units. For quantitative purposes it is necessary to plot the spectrum in absorbance units [21]. FT-IR absorbance spectra follow Beer's law, which relates concentration to absorbance as in Eq. (1)

$$A_\lambda = l\, \varepsilon_\lambda\, c \qquad (1)$$

Where A_λ = Absorbance, L = Pathlength, ε_λ = Absorptivity, c = Concentration

Transmittance is not directly proportional to the concentration and is defined in Eq. (2)

$$\%T = \frac{I_S}{I_R} \qquad (2)$$

Where I_S = Intensity of IR beam after passing through the sample, I_R = Intensity of IR beam before passing through the sample,T= Transmittance

A general schematic of an FT-IR spectrometer is presented in Fig. 1. The IR source emits radiation that is passed through an interferometer, usually a Michelson interferometer with a beamsplitter (a semi-reflecting film usually made of KBr), a fixed mirror, and a moving mirror. The interferometer uses interference patterns to make accurate measurements of the wavelength of light. When IR radiation is passed through a sample, some radiation is absorbed and the rest is transmitted to the detector. The detector measures the total interferogram from all the different IR wavelengths. A mathematical function called Fourier transform converts the interferogram (an intensity versus time spectrum) to an IR spectrum (an intensity versus frequency spectrum). Most mid-IR analyses are performed with a DTGS (deuterated triglycine sulfate) detector due to its ease of use and high sensitivity. When sample measurements must be made at high speed, the mercury cadmium telluride (MCT) detector is used which is 4-10 times more sensitive than the DTGS detector [20].

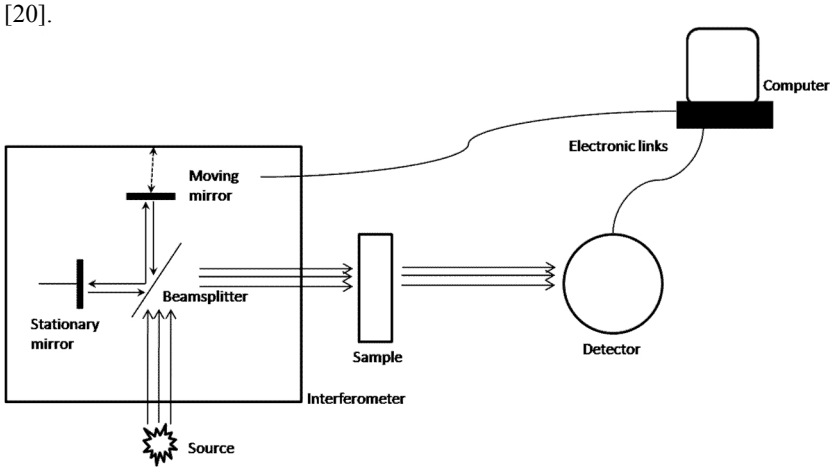

Figure 1. Basic components of an FT-IR spectrometer.

3. Advantages and Disadvantages of FT-IR Spectroscopy

Some of the advantages and disadvantages of using FT-IR spectroscopy methods for analyzing microorganisms are as follows [22-25]:

3.1 Advantages

1.Relatively fast and simple to use: Little or no sample preparation required for spectral acquisition.
2.Sensitive method that requires very little sample: ng-μg.
3.Nondestructive: The bacterial cell remains intact during analysis.
4.Universal method: The instrument and software are readily available and can be used for routine analysis.
5.Qualitative as well as quantitative analysis: Spectra provide information about bacterial cell composition and quantify the number of bacteria or amount of functional groups present in a sample.
6.Multiple sample environment: Samples in the form of liquid, gas, powder, solid, or film can be tested.
7.Identification and discrimination of bacteria: Bacteria can be discriminated based on their physiological state such as live, dead, injured, and treated.
8.Relatively less expensive for bacterial identification compared to several commonly used methods.

3.2 Disadvantages

1.Environmental conditions around the FT-IR instrument can cause variations in the spectra, hence background scans and multiple scans of the same sample are required.
2.Complex samples like mixtures of bacteria produce overlapping spectra, which may lead to misinterpretation of results. Hence a bacterial separation or purification step is required in some instances.
3.A complete library of spectra for each type of bacteria is recommended to facilitate detection.
4.May require standardization, rigorous data collection, and expertise in the chemometric analysis of spectra.

Culture medium, growth time, and growth temperature may cause variations in spectra. Presence of water in a sample may influence the bands at certain specific wavenumber.

4. IR spectra of Bacteria

Each bacterial species has a complex cell wall/membrane composition which gives a unique IR fingerprint (Fig. 2), due to the stretching and bending vibrations of molecular bonds or functional groups present in its proteins, nucleic acids, lipids, sugars, and lipopolysaccharides (LPS). The molecular composition varies from species to species and even at strain levels. Therefore each bacterium will have a unique and characteristic spectrum, and single microorganisms could be identified from an FT-IR spectrum.

To understand FT-IR spectra, some fundamental knowledge of cell surface characteristics and cell composition of bacteria is a prerequisite. The cell surface characteristics vary between Gram-positive and Gram-negative bacteria.The Gram-positive bacteria have a thicker and rigid layer of peptidoglycan (PG, 40-80% by weight of the cell wall) than Gram-negative bacteria (10% by weight of the cell wall). The primary structure consists of parallel polysaccharide chains of alternating *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) residues joined by β(1→4)glycosidic bonds. The parallel chains are linked by penta or tetrapeptides. The amino acid composition of penta or tetra peptide chains varies in different species of bacteria. For example, in the Gram-negative bacterium *E. coli*, the tetrapeptide consists of D-alanine, D-glutamic acid, and *meso*-diaminopimelic acid, on the other hand in the Gram-positive *Staphylococcu*s, it consists of L-alanine, D-glutamine, L-lysine, and D-alanine [26]. Gram-positive cell walls also contain teichoic acids that are covalently bound to the PG, where as Gram- negative cells do not contain teichoic acids, and have lipoproteins covalently bound to the PG in the cell walls. Gram-negative bacteria have an outermembrane (OM) outside of the PG layer which contains phospholipids in the inner layer and LPS in the outer layer [26]. LPS consist of three basic regions: the O-specific side chain (a hetero-polysaccharide which is responsible for antigenic properties), the inner and outer core oligosaccharides, and a lipid anchor called lipid A [27]. O antigens are usually used for typing of bacteria. For example, 76 O-factors have been identified so far in *Yersinia enterocolitica* and *Y. enterocolitica*-like organisms [28]. In addition to the O-antigen, Gram-negative bacteria also contain other antigens like the K-antigen which is associated with the capsule and H-antigens associated with flagella [26]. There appear to be significant differences in the type of sugars and organization of LPS and other antigenic structures of bacteria, and these differences are beneficial in FT-IR and chemometric identification of bacteria.

IR spectra measured for intact cells of bacteria are usually complex and the peaks are broad due to superposition of contributions from all the biomolecules present in a bacterial cell (Fig. 2). In the absence of water, Naumann [4, 29] recommend that five major absorbance regions in IR spectra should be analyzed for the identification of bacteria: the 3000–2800 cm^{-1} spectral region is the fatty acid region (region I); 1700-1500 cm^{-1} contains the amide I and II bands of proteins and peptides (region II); 1500-1200 cm^{-1} is a mixed region of fatty acid bending vibrations, proteins, and phosphate-carrying compounds (region III); 1200-900 cm^{-1} contains absorption bands of the carbohydrates in microbial cell walls (region IV); and 900-700 cm^{-1} is the 'fingerprint region' that contains weak but very unique absorbances that are characteristic to specific bacteria (region V). Regions I and II are the most useful for routine bacterial identification; however, the other regions may be used to better understand minor variations in structure and composition of the bacteria. The fingerprint region is significant for the discrimination of microorganisms at the strain level.

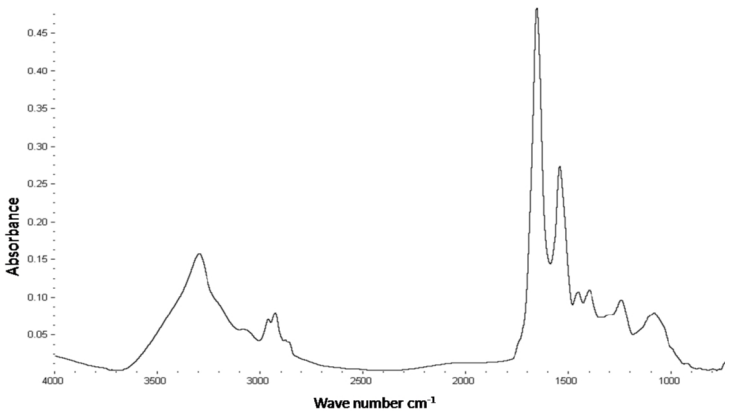

Figure 2. A representative FT-IR absorbance spectrum (400-800 cm^{-1}) of a Gram-negative bacterium, *Salmonella enterica.*

The interpretation of spectra and peak assignments are key steps in the FT-IR analysis of any biological sample. Although most bacterial spectra look very similar on simple visual examination, subtle quantitative differences can be observed on closer inspection. The wave number positions of absorbance peaks, peak intensities, and peak widths are useful for functional group, cell component, and sample identification [24]. There are several interesting peaks that appear on an IR spectrum of bacteria, and most of them represent functional group vibrations in the main biomolecular

constituents like protein, fatty acids, nucleic acid, and carbohydrates. Some tentative band assignments used by Naumann and other researchers [4-7, 24, 29-32] for bacterial identification are given in Table 1. Specific identification can be achieved from spectra by focusing on specific absorbance regions related to those compounds that are diagnostic to a specific pathogen.

Table 1 Assignment of functional groups associated with major vibration bands in mid IR spectra of bacteria [4-7, 24, 29-32].

Wave number (cm^{-1})	Molecular vibrations of functional groups and the biomolecule contributor
3200	N-H stretching of amide A in proteins
2955	C-H asymmetric stretching of $-CH_3$ in fatty acids
2930	C-H asymmetric stretching of $>CH_2$ in fatty acids
2898	C-H stretching of ≥C-H of aminoacids
2870	C-H symmetric stretching of $-CH_3$ in fatty acids
2850	C-H symmetric stretching of $>CH_2$ in fatty acids
1740	>C=O stretching of lipid esters
1715	>C=O stretching of ester, in nucleic acids and carbonic acids
1695-1675	Amide I band components of proteins
1655	Amide I of α-helical structures of proteins
1637	Amide I of β-pleated sheet structures of proteins
1550-1520	Amide II band of proteins
1515	Tyrosine band
1468	C-H deformation of $>CH_2$ in lipids proteins
1415	C-O-H in-plane bending in Carbohydrates, DNA/RNA backbone, proteins
1400	C=O symmetric stretching of COO- group in aminoacids, fatty acids
1310-1240	Amide III band components of proteins
1240	P=O asymmetric stretching of phosphodiesters in phospholipids
1200-900	C-O-C, C-O dominated by ring vibrations in various polysaccharides
1085	P=O symmetric stretching in DNA, RNA and phospholipids
720	C-H rocking of $>CH_2$ in fatty acids, proteins
900-600	"Fingerprint region"

5. Sampling methods and Spectra recording

The most common sampling techniques used for microbial characterization are transmittance, diffuse reflectance, attenuated total reflectance, and microspectroscopy. In addition, techniques such as photoacoustic spectroscopy and specular reflectance are used occasionally.

5.1 Transmittance

In transmission FT-IR spectrsocopy, the sample is placed on one or in between two infrared-transparent plates. The infrared beam then impinges on the sample after passing through the plates and is sensitive to the pathlength (1-20 μm). The advantages of transmission FT-IR are its high signal-to-noise (SNR) ratios, it is a universal method, and it has a comparatively inexpensive sample preparation [20]. The disadvantages of this accessory are the variability of IR absorption by the sample due to differences in thickness and time of sample preparation. In most of the pioneering studies for microbial characterization using FT-IR [4, 5], the transmittance method was used effectively. For transmittance measurements, approximately 10-60 μg of bacetrial cells are suspended in sterile water or saline and dried at 50-60 °C or under vacuum at room temperature after transferring to ZnSe optical plates [4, 5, 29, 33] or polyethylene disposable optical films [34].

5.2 Diffuse reflectance

Diffuse reflectance IR spectroscopy (DRIFTS) is used to analyze solid and powder samples, including freeze-dried biological specimens [29]. The sample is often ground and diluted to 1-10% with potassium bromide (KBr) prior to analysis. KBr is transparent in the IR range. The DRIFT cell reflects radiation to the sample and collects the energy reflected back over a large angle. The advantages of using DRIFTS are the ease of sample preparation and the requirement of a very small sample mass. The disadvantages include the cost of the accessory and limitation to solid sample analysis [20]. Diffuse reflectance is widely used [35,36] for routine analysis where in aliquots of microbial cultures are transferred into wells in sandblasted aluminum plates and dried. Later the plates are mounted on a motorized stage to collect the spectra using DRIFTS.

5.3 Attenuated total reflectance

Attenuated total reflectance (ATR) spectroscopy is based on the phenomenon of total internal reflection, and it gives rise to the biochemical profile of the surface chemistry of a bacterial sample. The ATR crystal can be diamond, zinc selenide, or germanium. The refractive index of the crystal should be higher than that of the sample. The average thickness of a bacterial cell wall ranges from 20-50 nm [37], and the refractive index of a bacterial cell is estimated to be 1.39 [38]. The IR beam can penetrate approximately 300 nm into the bacterial cell. Horizontal ATR (HATR) with a ZnSe crystal is commonly used for microbiological studies. HATR can handle either liquid, solid, film, or powder samples. Sample preparation procedures for ATR include direct transfer of bacterial colonies into small suspensions (20-200 μl), followed by drying onto optical surfaces as thin films; stamping techniques where optical plates were lightly pressed against bacterial colonies on agar plates; and direct analysis of bacterial suspensions by ATR-FT-IR [39]. Samples can be analyzed even without a drying step [12, 39, 40]. Recently studies have reported the use of filter membranes such as polyethylene [41], anodisc [42], aluminium oxide [43], and Metricel^{TM} [12, 40] to capture bacteria from liquid samples before ATR analysis. External pressure needs to be applied for most ATR analyses to ensure good contact of the cells with the ATR crystal.

5.4 FT-IR microscope

An FT-IR microscope combines a light microscope with an FT-IR spectrometer to provide versatility for rapid analysis and visualization of samples [20]. The IR microscope is equipped with a Cassegrain objective, which condenses the incident radiation on the sample and collects the transmitted radiation onto the detector. The FT-IR microscope may be used to analyze biological samples mounted on infrared transparent surfaces by transmission microscopy, or by reflectance microscopy of a sample on a reflecting gold or silver coated disk or reflective metal surface such as polished stainless steel. Direct spectral analysis of microcolonies ($<10^3$ cells per colony spot) can be obtained by a replica stamping technique after dilution plating which reduces the identification time to 6-10 h. Using this method, colony information can be gained through imaging, and cell composition and structural data can be obtained from IR spectra [29, 32]. Thus identification of mixed cultures of bacteria down to the subspecies level can be achieved by micro sampling if the colonies are growing separately on a solid agar [29, 32, 44].

6. Spectral preprocessing

Various manipulations of the spectra can be performed to enhance the appearance and improve the spectral features to facilitate spectral interpretation and analysis [20]. Spectral preprocessing is generally performed with bacterial spectra since most bacterial spectra look similar due to relatively minor compositional differences. The preprocessing of the spectra is usually performed using the software that comes with an FT-IR spectrometer. Some of the common preprocessing methods used for bacterial spectra are described below:

6.1 Baseline correction and smoothing

Baseline correction eliminates the dissimilarities between spectra due to shifts in baseline, and smoothing reduces the high frequency instrumental noise and enhances the information content of a spectrum [20]. It is generally recommended to use baseline correction, smoothing, and binning of bacterial spectra before spectral comparison using chemometric approaches [45, 46].

6.2 Derivatives

The first and second derivative transformations also reduce replicate variability, correct baseline shift, resolve overlapping peaks, and amplify spectral variations [19, 20, 46, 47]. First derivative transformation is generally used for the taxonomic classification of bacteria [9, 10, 44]. Broad overlapping bands of raw spectra become better resolved in a first derivative spectrum as seen in Fig. 3. Spectra of 4 different PCR serotypes (4a, 4c, 1/2b, 1/2c) of *L. monocytogenes* look quite similar (Fig. 3A) in contrast to the first derivatives of the spectra that show clear variations (Fig. 3B). Second derivative spectra increase the number of discriminative features (Fig. 3C) associated with bacterial spectra and improve the clarity of bacterial spectra with a net effect of an increase in spectral resolution [20]. Second derivative spectra are commonly used for bacterial classification [48], studying the growth phase of bacteria, and detecting sublethal injury of bacteria due to heat, chlorine, radicals, and sonication [46, 47, 49, 50].

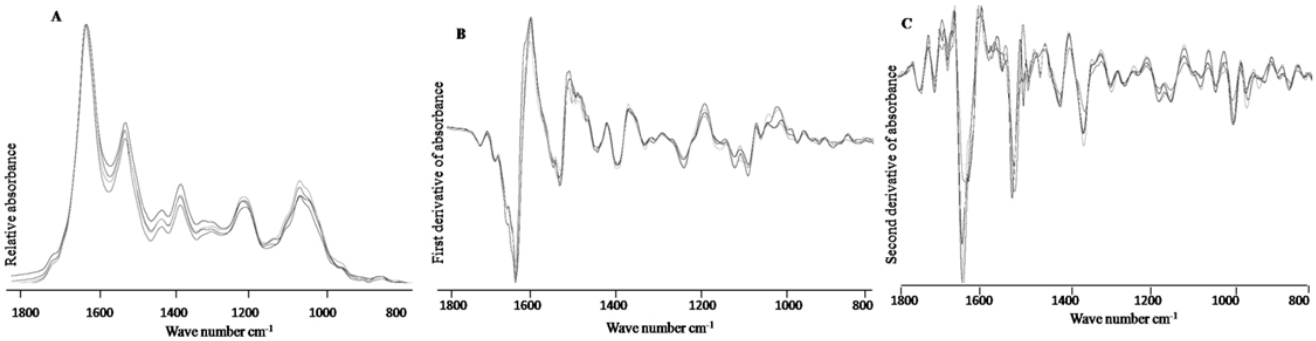

Figure 3. FT-IR spectra (1800-800 cm^{-1}) of four different PCR serotypes (4a, 4c, 1/2b, 1/2c) of *L. monocytogenes*. 3A: Original spectra. 3B: First derivative spectra. 3C: Second derivative spectra

6.3 Normalization

Normalization of spectra eliminates the path length variation and also reduces the differences between each single measurement of the same sample. The spectra are normalized to the most intense band or at the same integrated intensity in a given spectral region. Usually the amide I band is used as an internal standard for normalization [31]. Normalization is usually applied to study the spectra of bacteria at different growth phases. Normalization is also a prerequisite for advanced statistical analysis of bacterial spectra [19, 51].

7. Statistical Analysis/Chemometrics

Various software packages are used to extrapolate the fine data from complex spectra for bacterial characterization using multivariate statistical approaches. Multivariate statistical analysis of FT-IR spectra (chemometrics) can be divided into two types: supervised methods and unsupervised methods. The objective of unsupervised methods is to extrapolate the spectral data without a prior knowledge about the bacteria studied. Principal component analysis (PCA) and Hierarchical cluster analysis (HCA) are examples of unsupervised methods. Supervised methods, on the other hand, require prior knowledge of the sample identity. With a set of well-characterized samples, a model can be trained so that it can predict the identity of unknown samples. Discriminant analysis (DA) and artificial neural network (ANN) analysis are examples of supervised methods.

7.1 Principal component analysis (PCA)

PCA is used to reduce the multidimensionality of the data set into its most dominant components or scores while maintaining the relevant variation between the data points. PCA identifies the natural clusters in the data set with the first principal component (PC) expressing the largest amount of variation, followed by the second PC which conveys the second most important factor of the remaining analysis [42, 45-47], and so forth. Score plots can be used to interpret the similarities and differences between bacteria. The closer the samples are within a score plot, the more similar they are with respect to the principal component score evaluated [43].

7.2 Hierarchical cluster analysis (HCA)

Cluster analysis helps identify similarities between the spectra of microorganisms using the distances between spectra and aggregation algorithms. The distances frequently used are the Pearson product moment correlation coefficient and the Euclidian distance. Ward's algorithm and the average linkage algorithm (also called UPGMA, the unweighted pair group method with arithmetic mean) are the commonly used cluster analysis algorithms for microbial identification [37]. Factorization is also applied to the spectra to break apart the spectral data into the most common spectral variations (factors, loadings, principal components) and the corresponding scores [29,52]. The advantages of factorization are data compression and noise suppression. A dendrogram is a tree diagram frequently used to illustrate the arrangement of the clusters produced by a clustering algorithm (Fig. 4). The left vertical axis of a dendrogram depicts the increasing variance or heterogeneity. The magnitude of this heterogeneity depends on the number of spectra in a cluster and the similarities between them [6]. HCA and dendrograms are used to show the similarities between spectra of bacteria representing the same species, genus, serotypes, or haplotypes [5, 6, 9]. In addition to finding the similarities between bacteria, an unknown bacterium can be identified by calculating the spectral distance between it and known bacteria or by introducing it in the HCA. Figure 4 illustrates the cluster analysis of six MLGT (multilocus genotyping) haplotypes of *L. monocytogenes* belonging to three PCR serotypes (4b, 1/2b, 1/2a). The three main clusters

in this dendrogram correspond to the three PCR serotypes of *L. monocytogenes*. The subclusters within the 4b and 1/2b main clusters correspond to different MLGT haplotypes of *L. monocytogenes.*

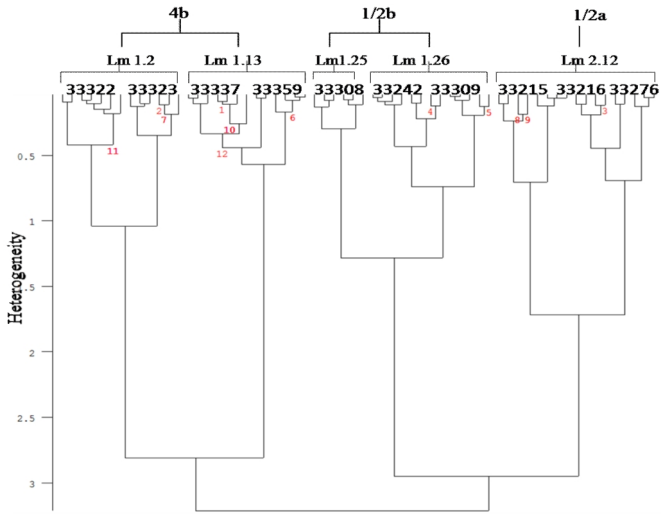

Figure 4. Dendrogram of a Hierarchical Cluster Analysis performed with FT-IR spectra of different PCR serotypes (4b, 1/2b, 1/2a) of *L. monocytogenes* collected by FT-IR microspectroscopy. Cluster analysis was performed using vector normalized first derivative spectra using Wald's algorithm and squared Euclidian distance measurements. A total of 60 spectra collected from 10 strains (designated by five digit numbers) of *L. monocytogenes* belonging to 6 different MLGT haplotypes (designated by Lm) were analyzed.

7.3 Canonical variate analysis (CVA) and Discriminat analysis

CVA is a common chemometric analysis used for differentiating sample types and is an extension of PCA. CVA is usually conducted on reduced data [31] and groups data by estimating the canonical variates, which are linear combinations of the original variable. Canonical variate analysis gives a graphical representation of the similarities and differences between spectra as shown in Fig. 5B. Discriminant analysis is another multivariate statistical measure for the classification of objects into groups or clusters by determining the similarity of a set of values from an unknown sample to a set of values measured from a set of known samples [53]. The extent of difference is calculated by the Mahalanobis distance (D_M) which gives a statistical measure, in terms of standard deviations, of how well the spectrum of the unknown sample matches the spectra of the known samples [54]. The Mahalanobis distance (D_M) is mathematically defined as:

$$D_M = ((X_1 - X_2)W^{-1}(X_1 - X_2)^T)^{0.5} \quad (3)$$

Where, W = pooled estimate of the within group covariance matrix, X_1 and X_2 = mean vectors for the two groups, and
T= transpose matrix

Mahalanobis distances were used for classifying *Listeria* strains [51], differentiating *Salmonella* serovars and *E. coli* based on their LPS spectra [11, 55], discriminating *Salmonella* based on outer membrane protein spectra [56], discriminating *S. aureus*[14], and detection and differentiation of bacteria from food [40] and mixed cultures [57,58].

7.4 Soft Independent Modelling by Class Analogy (SIMCA) and Artificial Neural Network (ANN)

SIMCA is used for supervised classification of spectral data. The method requires a training data set consisting of samples with a set of attributes and their class membership (such as type of bacteria). The term soft refers to the fact the classifier can identify samples as belonging to multiple classes and not necessarily producing a classification of samples into non-overlapping classes. Before SIMCA modeling, the samples belonging to each class need to be analyzed using PCA; only the significant components are retained and further analyzed. SIMCA models were used to classify bacteria [59, 60], discriminate spectra of bacteria at different growth phases [19], and also to differentiate between viable and sub-lethally injured bacteria [46]. ANN is a supervised and self-training system. This method also requires a training data set and validation data set. ANN-based analysis was reported for the classification of *Listeria* to the species level and *L. monocytogenes* to serogroups and serovar levels [9,10]. For the ANN-based classification, HCA was used as a validation method. ANN is an emerging method and according to Goodacre et al [36] and Rebuffo et al [9, 10] it is superior to PCA and HCA for discrimination of bacteria.

7.5 Partial Least Squares Regression (PLSR)

The partial least squares method is used to bulid predictive models for qualitative as well as quantitative anlysis of bacteria with no restriction on the wavenumber range that can be selected for calibration. PLSR models extract the maximum amount of information from the spectra based on the latent variables or factors which are used in the calibration and prediction steps. In PLSR, PLS components are extracted from a data matrix X in order to explain as much of the variation in a reference matrix Y as possible while at the same time accounting for the variation in the data matrix X [59, 60]. When PLSR is used for discrimination, it is called discriminant PLSR. In order to build a PLSR model, a calibration data set (known sample), validation data set, and test sample are needed. The optimal number of PLS components is found by calculating the minimum root mean square error (RMSE) for each PLS component and controlling subsequently if the change in RMSE for the preceding components is substantial. The RMSE is calculated by cross-validation. The root mean square error of prediction (RMSEP), which represents an objective assessment of the overall error between modeled and reference values [61], should also be calculated to evaluate if the model can be used for analyzing new spectra. Discriminant PLSR models were used to classify and identify different species of pathogenic bacteria [13, 60].

8. Applications of FT-IR in Food Microbiology

Rapid and simple identification of microorganisms on food products is an essential task for the food industry. Traditional methods such as conventional plating, biochemical tests, and immunological methods have several steps and may take a long time to get confirmatory results. FT-IR methods can provide biochemical fingerprints of bacteria within a short time frame (some analyses take only minutes) in a simple and economical way. For this reason, FT-IR is increasingly gaining importance in the field of food microbiology for the applications listed below:

8.1 Detection and quantification of bacteria from culture and food

Detecting pathogenic bacteria in a sample using FT-IR is based on identifying a spectral change or increase in peak intensity from the baseline spectra of an uncontaminated sample (food, growth media). The results should be verified through the use of a spectral library search of the known bacteria [24]. Detecting pathogenic bacteria in a sample using FT-IR spectra can be done by either a direct method or an indirect method. In the direct method, IR spectra of culture or contaminated food are collected directly from the sample. For example, studies have differentiated and quantified different species of bacteria from an apple juice matrix using an ATR method and from an apple surface using FT-Raman spectroscopy [62, 63]. In most cases the spectra collected may contain noise due to food particles or culture medium components. Indirect detection eliminates the noise due to media components by using a bacterial separation step such as filtration [40, 43, 64, 65] or immunomagnetic separation [40, 65] prior to spectral acquisition. The detection limit for pathogens from culture broth or food varies between 10^3-10^6 cfu/ml depending on the type of food and method used for detection. If the level of bacteria in a food is lower than the detection limit, selective enrichment is required for the cell number to increase [40, 65]. There are a few reports on the FT-IR detection of foodborne pathogens from solid foods. In order to discriminate *Salmonella*-contaminated beef, a suitable headspace sampling system was designed and used to collect the headspace volatiles from the packed meat to an FT-IR gas cell [66]. Numerous studies [40, 42 ,55, 63, 67, 68] have reported the detection and identification of single types of bacteria, a few studies reported the detection of mixed cultures [45, 69, 70], and a mathematical model for identifying a particular pathogen in a mixed culture was developed [69]. FT-IR detection of bacteria from food could be used routinely only if the spectral database for most pathogens is established.

8.2 Discrimination of viable, injured, and dead bacteria

Bacterial viability determination is one of the major concerns in the food industry because injured bacteria cause a significant health threat if they revive during food distribution and storage and it is important to examine the efficacy of various intervention treatments used in food processing. Conventional microbiology methods cannot give an accurate measure of both live and dead cells. The fluorescent dye techniques and quantitative PCR methods, developed to overcome this problem, are prone to variations depending on physiological and biochemical heterogeneity of the target bacteria and sample matrix [71]. FT-IR methods, on the other hand, are based on the biochemical composition of cells, are less prone to variation, and hence are suitable for differentiation of live and dead cells. FT-IR methods have been used to study sonication-injured *L. monocytogenes* [47], heat-injured *S. typhimurium* and *L. monocytogenes* [46], chlorine-injured *Pseudomonas aeruginosa* and *E. coli* in water [50], radical (ascorbic acid, hydrogen peroxide) induced damage of *Micrococcus luteus* [49], and heat-killed *E. coli* O157:H7 in ground beef [65]. In most of these experiments, spectral differences between live and injured bacteria were barely discernable due to the minor compositional differences. Hence second derivative (Fig. 5A) pre-processing was carried out to increase the number of discriminative features and principal component analysis (Fig. 5B) and SIMCA models were used to correctly classify live and injured cells. Second derivative spectra of uninoculated ground beef and live and heat-killed *E. coli* O157:H7 acquired from inoculated ground beef using a filtration-based FT-IR approach [65] are shown in Fig. 5A. The major differences

between the spectra of live and dead cells were observed in the amide and nucleic acid regions due to heat-induced denaturation of these biomolecules. A Cooman plot shows the clear separation of spectra of uninoculated sample (control) and spectra of live and dead cells of *E. coli* O157:H7 acquired from inoculated ground beef (Fig. 5B).

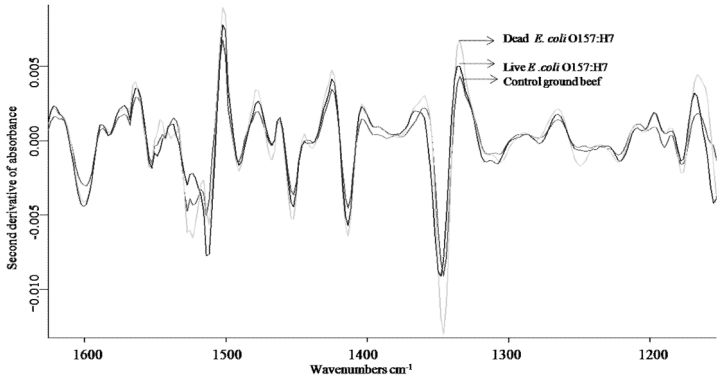

Figure 5A. Second derivative FT-IR spectra acquired from ground beef and ground beef inoculated with live and heat-treated (dead) *E. coli* O157:H7

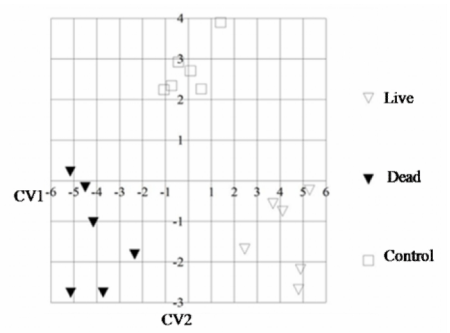

Figure 5B. Cooman Plot showing the separartion of FT-IR spectra of live and heat-treated *E. coli* O157:H7 acquired from inoculated ground beef and control ground beef

8.3 Analysis of structural components of bacteria

FT-IR spectra of intact bacterial cells cannot provide complete information of specific cellular components due to overlapping absorbance bands. The specta of bacteria may also change depending on the growth medium and culture conditions. FT-IR techniques were used to gain insight of bacterial cell structures and surface components before the pioneering study of FT-IR characterization of bacteria [72], and later studies reported the discrimination of bacteria based on cellular components. Species and strain level identification of 14 Gram-positive and Gram-negative foodborne pathogens was reported by Whittacker et al [73] using the spectra of fatty acid methyl esters isolated from these bacteria. IR spectra of outer membrane proteins of *Salmonella* were used to differentiate between six *Salmonella enterica* serovars [56] and also for taxonomical classification of phage types of *S. Enterica* Enteritidis [13]. Spectra of crude LPS extracts from *E. coli* and *S. enterica* were used for serotype level differentiation with 95-100% correct classification [11, 55].

8.4Taxonomic classification of bacteria

Taxonomic level classification of foodborne pathogens is important for epidemiological investigation, outbreak detection and trace-back, source tracking, and ultimately for pathogen control programs. Classical methods used in microbial taxonomy are microscopy, staining techniques, biochemical assays, and serological tests which are time consuming and laborious. At present various molecular subtyping methods such as MLVA, PFGE, MLGT, and MLST are carried out to establish the relatedness of different outbreak strains of foodborne pathogens. Although very effective, these methods are time consuming, expensive, and require trained personnel. FT-IR methods have been successfully applied for the discrimination and classification of a variety of microorganisms at genus, species, and sub-species levels since the pioneering study by Naumann [4,6], and these were thoroughly reviewed elsewhere [23, 25]. FT-IR spectra represent phenotypic and genetic fingerprints of bacteria thereby allowing the differentiation of bacteria at different taxonomic levels even down to haplotype levels. Recent studies related to FT-IR classification of foodborne pathogens are summarized in Table 2. The different spectroscopic and chemometric methods used are tabulated along with the specificity of the methods. Microbial spectra are very complex and contain superimposed bands; therefore, different preprocessing and pattern recognition techniques (described in section 6 and 7 of this chapter) have to be used in order to extract the data, and the chemometric approaches used in the studies are also summarized in Table 2.

Table.2: Review of recently published studies on taxonomic classification and differentiation of foodborne pathogenic bacteria

Bacteria and number of isolates used	**IR Technique used**	**Percentage correct identification and taxonomic level**	**Chemometric approach**	**Ref**
S. enterica Enteritidis 45 isolates	Transmit	98-100% at phage type level using outer membrane protein (OMP) extract	PLSR-DA	[13]
Yersinia enterocolitica 123 strains	Transmit	98.3 % at biotype level, and 92.5% at serotype level	ANN	[16]
Listeria 5 species 25 stains	Transmit	92.8% at species level	ANN	[44]
	Transmit Microspect	79.2% at species level		
Salmonella enterica 20 strains	ATR	100% at serovar level and 90% at strain level from food matrix 96% at serovar level and 85% at strain level of unknown (blind set)	SIMCA and MD	[74]
	Microspec			
Listeria 5species, 30 strains	Microspec	93% at species level 80% at strain level	Canonical DA and PLSR-DA	[75]
	Macro/ Transmit	100 % at species and strain level		
L. monocytogenes 272 strains	Transmit	98.8% at serovar level (O antigen serogroup) 91.6% at serovar level (H antigen)	ANN and HCA	[9]
L. monocytogenes and other *Listeria* species 520 strains	Transmit	96% at species level 85-93% at strain level	SD, ANN, and HCA	[10]
L. monocytogenes 89 strains	Transmit	100% based on susceptibilities towards sakacin using PCA. PLSR model had a correlation coeffiient (R) of 0.98	PCA and PLSR	[60]
Bacillus, *E. coli*, *Salmonella, Listeria* 8 strains	ATR	94% at strain level	PCA and SIMCA	[48]
S. aureus and other staph species 39 species and subspecies	Transmit	*S. aureus* was identified correctly (100%) and discriminated from all other species. In a validation analysis 97% *S. aureus* were correctly classified	PCA, FDA CVA, and MD	[14]
Salmonella enterica 6 serotypes	Reflectance Microspec	100% at serotype level using OMP extract	CVA and DA	[56]
E. coli 5 strains	Reflectance Microspec	80% using intact cells and 95% using OMP extract at serotype level	CVA and DA	[11]
Campylobacter coli and *Campylobacter jejuni* 17 isolates		98.72% correct typing of ERIC (Enterobacterial repetitive intergenic consensus) PCR types	CVA and HCA	[76]
Campylobacter coli and *Campylobacter jejuni* 26 isolates	Transmit	100% of training set and 99.16% of test set at subspecies level	ANN, MLP, and PNN	[77]
Bacillus and *Clostrdium* endospores 5 species	Reflectance Microspec	57% and 75% correct speciation of spores using untreated spores and autoclaved spores respectively	PCA, HCA, and SIMCA	[78]
B. subtilis and *B. cereus* 10 strains	ATR	100% at species level. Strain differentiation was better with ATR than diffuse reflectance	PCA, DFA, and HCA	[61]
	Diffuse reflectance			

ATR: Attenuated total reflectance, Transmit: Transmittance, Microspec: Microspectroscopy, PLSR: Partial least squares regression, DA: Discriminant analysis, ANN: Artificial neural network, SIMCA: Soft independent modelling by class analogy, MD: Mahalanobis distance, HCA: Hierarchical cluster analysis, SD: Spectral distance, FDA: Factorial discriminant analysis, MLP: Multilayer perceptron, PNN: Probabilistic neural network, DFA: Discriminant function analysis.

Conclusion

The most attractive features of FT-IR spectroscopy methods for food microbiology applications are their extreme rapidity, simplicity, and uniform applicability to any group of microorganisms. FT-IR methods are highly specific and sensitive and enable identification of bacteria even down to strain levels. IR fingerprinting methods have greatly improved, commercially manufactured IR instruments for microbiology are available, and spectral databases for different bacteria have been created and validated. Fully automated FT-IR systems for detection, quantification, and differentiation of microorganisms are already being introduced. With the advancements in IR technology and computational analysis, FT-IR methods could be promising diagnostic tools for routine epidemiological investigation of foodborne bacteria by various public health authorities.

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