REVIEW
Medical Biotechnology
Biotechnology Journal
www.biotechnology-journal.com

An Overview of the Evolution of Infrared Spectroscopy Applied to Bacterial Typing

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The sustained emergence of new declared bacterial species makes typing a continuous challenge for microbiologists. Molecular biology techniques have a very significant role in the context of bacterial typing, but they are often very laborious, time consuming, and eventually fail when dealing with very closely related species. Spectroscopic-based techniques appear in some situations as a viable alternative to molecular methods with advantages in terms of analysis time and cost. Infrared and mass spectrometry are among the most exploited techniques in this context: particularly, infrared spectroscopy emerged as a very promising method with multiple reported successful applications. This article presents a systematic review on infrared spectroscopy applications for bacterial typing, highlighting fundamental aspects of infrared spectroscopy, a detailed literature review (covering different taxonomic levels and bacterial species), advantages, and limitations of the technique over molecular biology methods and a comparison with other competing spectroscopic techniques such as MALDI-TOF MS, Raman, and intrinsic fluorescence. Infrared spectroscopy possesses a high potential for bacterial typing at distinct taxonomic levels and worthy of further developments and systematization. The development of databases appears fundamental toward the establishment of infrared spectroscopy as a viable method for bacterial typing.

1. Introduction

Infrared (IR) radiation was firstly recognized by Sir William Herschel in 1800 by the observation of sunlight decomposition through a prism. In his experiments, Herschel measured the temperature of each rainbow color noticing that the temperature increased from the blue to the red part of the spectrum. He also realized that immediately after the red part of the spectrum, the temperature was even higher and hypothesized that it should be another type of light that could not be seen. These findings were the first recognition of the existence of the IR radiation.[1] However, the interest in the IR radiation re-emerged only about 100 years later with Coblentz[2] works that recorded and analyzed the IR spectra of hundreds of organic and inorganic compounds. These incipient but fundamental works led to the recognition that each compound has a unique spectrum in the IR region that reflects the chemical nature of its functional groups. Almost simultaneously to the Coblentz's work, Michelson conceives the interferometer[3] that would later become an essential tool in IR spectroscopy. The first commercial IR spectrometers appeared in 1957, and in 1966 Joseph Fourier developed the Fourier transform algorithm allowing the development of IR spectrometers as are known today. Nonetheless, it was only with Naumann, Helm, and Holt works in the early 90s that IR spectroscopy was truly explored in the context of bacteria identification.[4–6] According to Naumann and co-workers, to each microbial cell could be associated an IR spectrum with a specific fingerprint signature that could be used for discrimination and/or identification purposes.[6] After these initial works, bacterial typing at different taxonomic levels resourcing to IR spectroscopy has been reported with variable degrees of success.

In this review, we cover fundamental aspects of IR spectroscopy, a literature review on bacterial typing as well as the advantages and limitations of the technique in this context. A comparison with competing spectroscopic techniques, such as matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), RAMAN, and intrinsic fluorescence (IF), as well as with gold standard molecular biology typing methods is elaborated. Perspectives on the evolution of the IR technique in this particular context are discussed.

2. Infrared Spectroscopy Principles

IR spectroscopy is based on the interaction of an IR beam with a sample. The nature of the IR radiation (near, mid, or far infrared) and/or the processing methods define the technique designation. Near infrared spectroscopy (NIRS) refers to the use of near

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DOI: 10.1002/biot.201700449

infrared radiation. Similar designations are used for mid and far IR radiation. Dispersive instruments using some type of wavelength decomposition are intrinsically different from Fourier-transform instruments. The latter make use of an interferometer and the Fourier-transform algorithm. These spectroscopic techniques can be used to characterize solids, liquids, or gaseous samples with the aid of different accessories depending on the physical state. The interaction of the IR beam with the sample might resource to different sampling techniques: transmission and reflection (**Figure 1**).

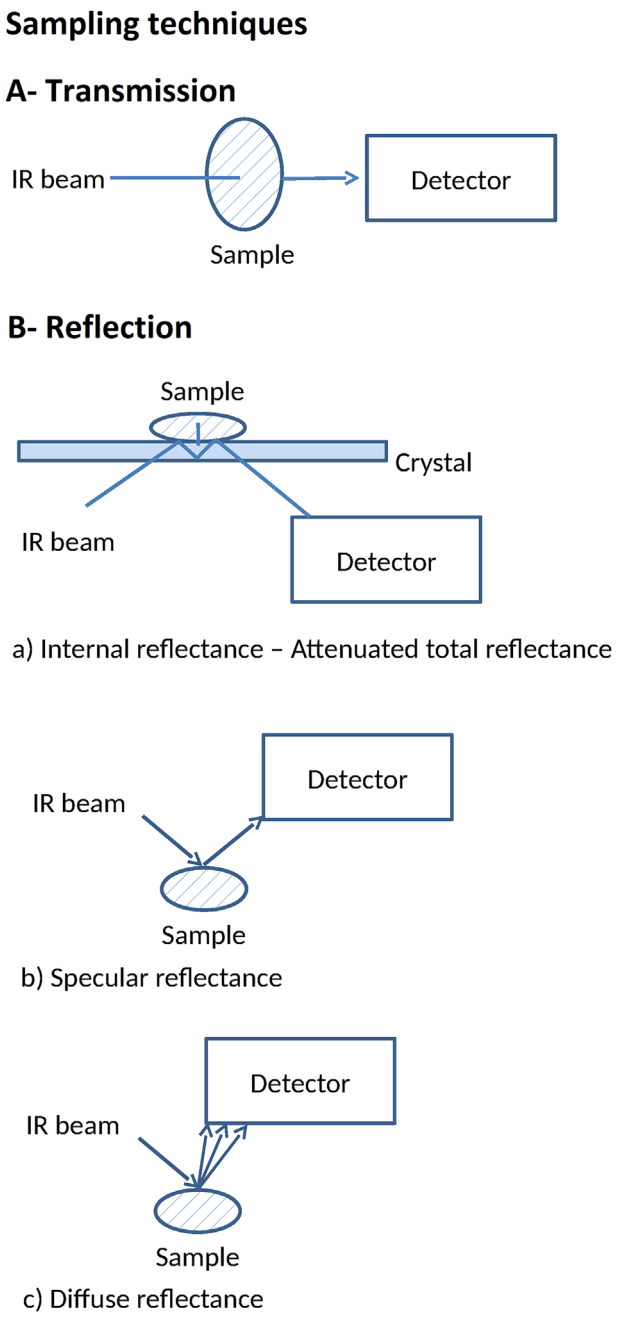

Figure 1. Sampling techniques (A: transmission and B: reflection) commonly used in infrared spectroscopy.

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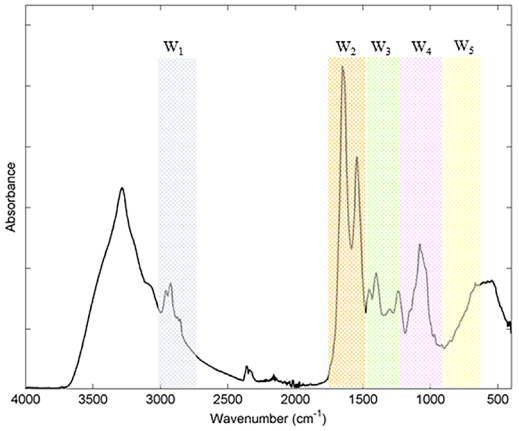
Transmission techniques are more common and are based on the detection of the transmitted IR radiation. Reflection techniques are based on the reflection of the IR beam after contacting with the sample and receive distinct designation depending on reflection process (specular, internal, and diffuse). In attenuated total reflectance (ATR), an IR beam undergoes total internal reflectance in a crystal and the generated evanescent wave interacts with the sample (Figure 1). This is a sampling technique widely used in the context of bacterial typing due to the associated versatility. No sample preparation is required, thus bacterial cells can be placed directly on the ATR crystal (usually made of ZnSe or diamond) surface. IR radiation induces the vibration of covalent bonds between atoms. These vibrations are usually named stretching, bending, rocking, scissoring, and twisting and occur at a specific frequency that is characteristic of each chemical bond. The result is an infrared spectrum that reflects the whole chemical composition of the sample. Typically, an infrared spectrum of a biological material presents characteristic bands due to lipids (3000–2800 cm^{-1}), proteins/amides I and II (1700–1500 cm^{-1}), phospholipids/DNA/RNA (1500–1185 cm^{-1}), polysaccharides (1185–900 cm^{-1}), and the fingerprint region (900–600 cm^{-1}) (**Figure 2**A and B).

3. Infrared Spectroscopy and Bacteria

Since Naumann's work,[6] a growing interest on the application of IR spectroscopic based techniques in the microbiology field has emerged and led to several hundreds of published studies. Noteworthy, bacterial typing at different taxonomic levels, detection of contaminations,[7–9] and antibiotic resistance[10–15] were among the most reported. These applications were mostly selected given the limitations (e.g., cost per analysis) of the gold standard molecular biology techniques for these purposes. In this context, IR spectroscopy emerges as a more cost effective, convenient, and fast alternative. Most of the reported studies (above 90%) aiming at bacterial typing were developed based on the mid infrared radiation, commonly with Fourier-transform infrared spectroscopy (FTIRS) or Fourier-transform infrared spectroscopy with attenuated total reflectance (FTIRS-ATR). From now on, we will use these two acronyms when addressing IR spectroscopy in the mid IR range.

A



B

Wave number (cm^{-1})	Spectral region	Assignment
~3500		O-H stretching of hydroxyl groups
~3200		N–H stretching in resonance with overtone
~2955	W_1	CH_3 asymmetric stretching
~2930	W_1	CH_2 asymmetric stretching
~2918	W_1	CH_2 asymmetric stretching
~2898	W_1	C-H stretching in methylene groups
~2870	W_1	CH_3 symmetric stretching
~2850	W_1	CH_2 symmetric stretching
~1740	W_2	C=O stretching of esters
~1715	W_2	RNA C=O stretching
~1680-1715	W_2	C=O stretching
~1695	W_2	Amide I bands
~1685	W_2	Antiparallel pleated sheets
~1675	W_2	Proteins b-turns vibrations
~1655	W_2	DNA C=O stretching; N–H bending; RNA C=O stretching Amide I of a-helical structures
~1637	W_2	Amide I of b-pleated sheet structures
~1550-1520	W_2	Amide II
~1515	W_2	Tyrosine band
~1485	W_3	$(CH_3)_3N^+$ asymmetric bending
~1400	W_3	C=O symmetric stretching of COO^-
~1310-1240	W_3	Amide III
~1250-1220	W_3	P=O asymmetric stretching of $>PO^-_2$
~1200-900	W_4	C–O, C–C stretching, C–O–H, C–O–C deformation
~1090-1085	W_4	P=O symmetric stretching of $>PO^-_2$
~720	W_5	C-H rocking of $>CH_2$
~900-600	W_5	Fingerprint region

Figure 2. A) Typical bacterial infrared spectrum (Region W_1: fatty acids; Region W_2: proteins; Region W_3: mixed region; Region W_4: polysaccharides; Region W_5: fingerprint region); (B) characteristic infrared absorption bands.

3.1. Bacterial Typing at the Genus Level

Bacterial identification at the genus level is commonly performed through culture-based methods, which rely on the recognition of some specific biochemical characteristics, or in non-culture based methods such as 16S rRNA sequencing. Only a few studies were found in the literature exploiting the potential of FTIRS and/or FTIRS-ATR to discriminate bacteria at the genus level (**Table 1**). This fact is probably due to the relatively simplicity of genus discrimination using even quick and cheap phenotypic methods. In 2010, Boudau and co-workers,[16] used FTIRS to analyze sulphate and thiosulphate reducing bacteria associated with bio-corrosion processes through the construction of a reference spectral library. Hierarchical clustering analysis (HCA) allowed the successful discrimination of sulfur reducing bacterial strains at the genus level. Previously to Boudau's work, an attempt to discriminate these two bacterial groups (sulphate and thiosulphate reducing bacteria) in the context of bio-corrosion processes, did not report better results.[17] More recently, San-Blas and co-workers[18] successfully discriminate three genera of the *Enterobactereaceae* family: *Xenorhabdus*, *Photorhabdus*, and *Escherichia*. These genera are associated with insect lethal septicemia. A combined approach involving FTIRS-ATR and chemometrics showed that substantial spectral differences were visible below 1400 cm^{-1}, which are commonly associated with phosphate and carbohydrates molecules. Principal component analysis (PCA) as well as a HCA revealed three distinct clusters, each containing isolates of one single genus. In 2014, Nagib et al.[19] discriminated bovine mastitis *Trueperella pyogenes* isolates belonging to *Arcanobacterium* and *Actinomyces* genus with an artificial neural network (ANN). More recently, Al-Holy and co-workers[20] discriminated *Bacillus* from *Alicylobacillus* isolates through PCA and soft independent modeling of class analogy (SIMCA).

3.2. Bacterial Typing at the Species Level

Bacterial identification and/or discrimination at the species level remain a challenge in microbiology, particularly in the case of closely related species that share many phenotypic and/or genotypic characteristics. Routine methods for species identification include DNA–DNA hybridization, amplification by polymerase chain reaction, and sequencing of species-specific DNA regions or genes. Multiple commercial phenotypic tests are also available in the market for different bacterial systems. However, these methods are laborious, time consuming, and expensive. Moreover, most of the commercially available phenotypic tests were formulated several years ago and were never updated, though presenting a high rate of miss identifications particularly for recently described species. These issues clearly contributed to the high number of studies that have been published with alternative techniques for bacterial typing at the species level (Table 1). IR-based discrimination of Gram-positive bacteria has been over explored particularly in the case of *Listeria* and *Bacillus* genera with several works exclusively dedicated to species discrimination. The first reports exploring IR spectroscopy for bacterial typing at the species level (after Naumann's work), were performed in the later 90s. Among them, a report on the successful discrimination of *Listeria* species by FTIRS and canonical variate analysis

Table 1. Genus and/or species studies using IR spectroscopy for bacterial typing.

Bacteria	Discrimination level	Infrared technique	Chemometric methods	References
Sulphate and thiosulphate reducing bacteria	Genus	FTIRS, FTIRS-ATR	HCA, PCA	[16,17]
Alicylobacillus	Genus	FTIRS	PCA, SIMCA	[20]
	Strain	FTIRS-HATR	LDA, PCA	[75]
Photorhabdus	Genus	FTIRS-ATR	HCA, PCA	[18]
Xenorhabdus	Genus	FTIRS-ATR	HCA, PCA	[18]
Arcanobacterium	Genus	FTIRS	ANN	[19]
Actinomyces	Genus	FTIRS	ANN	[19]
*Trueperell*a	Genus	FTIRS	ANN	[19]
	Species	FTIRS	ANN	[19]
Bacillus	Genus	FTIRS	PCA, SIMCA	[20]
	Species	FTIRS, FTIRS-ATR, Diffuse Reflectance-FTIRS	PCA, HCA, DFA, CVA, SIMCA	[26–30,49,50,54,55]
	Vegetative/Sporulated	FTIRS	PCA, CART	[83]
Escherichia	Genus	FTIRS-ATR	HCA, PCA	[18]
	Species	FTIRS, FTIRS-ATR	HCA, ANN, PCA, SIMCA, CVA	[49-54,86,92]
Escherichia coli	Sequence type	FTIRS, FTIRS-ATR	PC-DFA, PLSDA, HCA, SIMCA	[67,68]
	Strain	FTIRS	PCA, SIMCA, CVA	[49,50,52,73]
	MLVA profile	FTIRS	HCA, CVA	[72]
Listeria spp.	Species	FTIRS, FTIRS-ATR, FTIR microspectroscopy	CVA, ANN, SCDA, PLSRDA, PCA	[5,21–25,27,49,50]
Lactobacillus spp.	Species	FTIRS	HCA, ANN	[31,32,39,40,53]
Enterococcus spp.	Species	FTIRS, Diffuse Reflectance FTIRS	HCA, ANN	[33,36,39–41,51,92]
Listeria monocytogenes	Serotype	FTIRS	CVA, ANN, HCA	[21,25,56,57]
	Epidemic clones	FTIRS	CVA, LDA	[76]
	Halotype	FTIRS	HCA, CVA	[57]
	Intact/Injured	FTIRS	CVA, LDA, PCA	[76,84]
Enterococcus faecium	PFGE type	FTIRS	PLSDA	[71]
Streptococcus spp.	Species	FTIRS, Diffuse Reflectance FTIRS	ANN, HCA	[4,34,36,39,40]
Streptococcus pneumonia	Serotype	FTIRS		[64]
Staphylococcus spp.	Species	FTIRS, FTIRS-ATR	FDA, HCA, PLS, KPLS, SIMCA	[4,35,39,41,42,50,51]
Staphylococcus aureus	Serotype	FTIRS	ANN, HCA, PCA	[65]
	MRSA strains	FTIRS	PCA, KNN	[74]
Clostridium spp.	Species	FTIRS	HCA	[4,53]
Carnobacterium spp.	Species	FTIRS	PCA, UPGMA, ANN	[37,40]
Lactococcus spp.	Species	FTIRS	HCA, ANN	[39,40]
Leuconostoc spp.	Species	FTIRS	ANN	[40]
Oenococcus oeni	Species	FTIRS	ANN	[40]
Pedicoccus spp.	Species	FTIRS	ANN	[40]
Weissella spp.	Species	FTIRS	ANN	[40]
Corynebacterium spp.	Species	FTIRS	HCA	[38,39,41]
Brevibacterium spp.	Species	FTIRS	HCA	[38,39,41]
Arthrobacter spp.	Species	FTIRS	—	[41]
Kokuria spp.	Species	FTIRS	—	[41]
Microbacterium spp.	Species	FTIRS	—	[41,86]
Brachybacterium spp.	Species	FTIRS	—	[41]

(*Continued*)

Table 1. (*Continued*)

Bacteria	Discrimination level	Infrared technique	Chemometric methods	References
Micrococcus luteus	Species	FTIRS	—	[41]
Rathayibacter tritici	Species	FTIRS	—	[41]
Lactococcus cremoris	Species	FTIRS	PLS, KPLS	[42]
Rhodococcus erythropolis	Species	FTIRS	—	[38]
Acinetobacter spp.	Species	FTIRS, FTIRS-ATR	HCA, PCA, DFA, ANN	[43-45]
Acinetobacter baumannii	Sequence type	FTIRS-ATR	PLSDA	[69,70]
Burkholderia cepacia complex	Species	FTIRS	PLS, PCA, SIMCA, ANN	[45,46]
	Ribopatterns	FTIRS	PCA, PLS-DA	[46]
Pseudomonas spp.	Species	FTIRS	HCA, ANN, PCA, SIMCA	[45,49,53,86,92]
Stenotrophomonas maltophilia	Species	FTIRS	PCA, ANN	[45,55]
Ralstonia pickettii	Species	FTIRS	ANN	[45]
Achromobacter spp	Species	FTIRS	ANN	[45]
Yersinia spp.	Species	FTIRS, FTIRS-ATR	ANN, SIMCA, PCA, CVA	[47,50,52]
Yersinia enterocolitica	Serotype	FTIRS	ANN	[47]
	Biotype	FTIRS	ANN	[47]
Yersinia ruckeri	Biotype	FTIRS	ANN	[80]
Oscillatoria limosa	Species	FTIRS-ATR	PCA, ANN	[48]
Arthrospira platensis	Species	FTIRS-ATR	PCA, ANN	[48]
Phormidium spp.	Species	FTIRS-ATR	PCA, ANN	[48]
Scytonem javanicum	Species	FTIRS-ATR	PCA, ANN	[48]
Nostoc punctiform	Species	FTIRS-ATR	PCA, ANN	[48]
Salmonella spp.	Species	FTIRS-ATR	PCA, SIMCA, CVA	[50,52]
	Live/Dead	FTIRS-ATR	PCA, SIMCA	[63]
Salmonella enterica	Serotype	FTIRS, FTIRS-ATR	PCA, SIMCA, CVA	[68–63]
	Strain	FTIRS	HCA	[77]
	Phage type	FTIRS	PLS-DA	[81]
Shigella spp.	Species	FTIRS-ATR	PCA, SIMCA, CVA	[50,52]
Vibrio spp.	Species	FTIRS-ATR	PCA, SIMCA	[50]
Enterobacter spp.	Species	FTIRS	HCA	[51]
Citrobacter spp.	Species	FTIRS	HCA	[51]
Klebsiella spp.	Species	FTIRS	HCA, ANN	[51,92]
Proteus mirabilis	Species	FTIRS	HCA, ANN	[51,92]
	Lipopolyssacharide structure	FTIRS-ATR	HCA, PCA, RF	[85]
Geobacillus caldoxylosilyticus	Species	FTIRS	HCA	[53]
Thermobrachium celere	Species	FTIRS	HCA	[53]
Thermoanaerobacter ethanolicus	Species	FTIRS	HCA	[53]
Micrococcus luteus	Species	FTIRS	PCA, HCA	[53,54]
Pantoea agglomerans	Species	FTIRS	PCA, HCA	[54]
Legionella pneumophila	Serogroup	FTIRS	-	[66]
Brucella spp.	Biovars	FTIRS	PCA, LDA	[78]
Xanthomonas oryzae	Pathovars	FTIRS	-	[79]
Campilobacter spp.	Genotype	FTIRS	ANN	[82]

(CVA) combined approach.[5,21] Some years later, Rebuffo et al.[22] used a large and diverse collection of 277 isolates from five *Listeria* species and proved that this technique was able to successfully discriminate 96% of the isolates resourcing to an ANN. It was demonstrated the advantage of using macrosamples instead of a microsample approach.[23] Janbu et al.[24] also discriminated five *Listeria* species with FTIRS and FTIR micro-spectroscopy. Obtained discrimination success rates were approximately 93 and 100%, respectively. These results were obtained with step-wise canonical discriminant analysis (SCDA) and partial least squares regression discriminant analysis (PLSRDA). Several years later, Romanolo et al.[25] discriminated isolates from six *Listeria* species resourcing to a previously constructed spectral library by the same authors. Concerning the *Bacillus* genus, the first attempt to species discrimination was performed by Lin et al.[26] and Beattie et al.[27] Lin and collaborators discriminated eight *Bacillus cereus* group species solely based on the comparison of IR peak patterns. Beattie et al.[27] worked with *B. cereus*, *Bacillus mycoides*, *Bacillus thurigiensis*, *Bacillus circulans*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus subtilis* (the method was CVA). Later, Winder and Goodacre[28] concluded that FTIRS-ATR and diffuse-reflectance FTIRS possess the same discriminatory power to discriminate *B. cereus* from *B. subtilis*. In 2013, a more comprehensive study in the context of industrial dairy processing environments and product spoilage[29] also used FTIRS combined with HCA to discriminate several *Bacillus* and *Geobacillus* species. Recently, Branquinho et al.[30] proved that FTIRS combined with chemometric methods was very useful to assist in the identification of a new *Bacillus* species. *Lactobacillus* species were also successfully discriminated by FTIRS and chemometrics by Oust et al.[31] and Bosch et al..[32] Less explored Gram-positive bacteria include *Enterococcus*, *Streptococcus*, and *Staphylococcus* genus for which only a few reports were found in the literature. Kirschner and co-workers[33] performed a comparative study comprising six *Enterococcus* species using phenotypic, genotypic, and IR spectroscopic techniques. They concluded that FTIRS combined with HCA was suitable for species discrimination. In 2014, Schabauer[34] proved that FTIRS could improve the diagnosis of mastitis associated with *Streptococcus* species. *Staphylococcus aureus* was also successfully discriminated[35] from other *Staphylococcus* species by FTIRS and factor discriminant analysis (FDA). Several other works were performed on Gram-positive bacteria considering two or more genus. Helm,[4] one of the pioneers on the utilization of IR radiation for bacterial typing, successfully discriminated *Staphylococcus*, *Streptococcus*, and *Clostridium* species resourcing to cluster analysis. However, in this work, bacterial isolates were growth in different culture media (e.g., Columbia blood agar was used for *Staphylococcus*). As the IR-based approach relies on the phenotype and it is known, from the very beginning, that different culture media will strongly influence the spectra the discrimination was obviously biased by the culture media. In 1996, Goodacre[36] proved the ability of diffuse reflectance-absorbance FTIRS and ANN to discriminate nineteen strains of *Streptococcus* and *Enterococcus* species. Other works proved the ability of FTIRS combined with chemometrics for *Carnobaterium* typing,[37] to discriminate species of *Actinomycetes*,[38] lactic acid bacteria,[39,40] food-borne pathogens commonly found in cheese and milk,[41,42] and pathogens of domestic ruminants and pigs causing mastitis such as *Trueperella* spp.[19] Regarding Gram-negative bacteria, only *Acinetobacter*, *Burkholderia*, and *Yersinia* genus were explored for species discrimination exclusively within the genus itself. *Acinetobacter* genus comprises several genomic and closely related species and their accurate identification remains a challenge for microbiologists. Winder and co-workers[43] developed a discrimination method to differentiate *Acinetobacter* genomic species a from environmental isolates (obtained from activated sludge). Depending on the chemometric method used for grouping the genomic species, this work indicated a variable degree of success regarding species discrimination. Later, Sousa et al.[44] developed a flowchart for species identification within the so-called *Acinetobacter calcoaceticus – Acinetobacter baumannii* complex based on IR spectra (FTIRS-ATR) and HCA with a high success rate. Six species belonging to the complex were discriminated with sensitivities and specificities ranging from 90 to 100%. Regarding the *Burkholderia cepacia* complex two studies were found in the literature addressing the ability of IR spectroscopy has a typing method. Both studies were developed from cystic fibrosis patients isolates. Bosch and co-workers[45] developed a discrimination system consisting on two hierarchical levels able to discriminate *B. cepacia* complex and other Gram negative bacteria that usually colonize cystic fibrosis patients. The first level was able to discriminate *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Ralstonia pickettii*, *Acinetobacter* spp., and *Achromobacter* spp. The second level allowed the discrimination of four *B. cepacia* complex species with 93.8% of success. However, one year later, Coutinho et al.[46] showed that FTIRS could lead to a high rate of misidentifications of *B. cepacia* complex species. In 2009, Kuhm and collaborators[47] successfully discriminated 4 *Yersinia* species based on their IR spectra and ANN. In 2004, seven cyanobacterial strains belonging to five different genus and/or species were also successfully discriminate by FTIRS-ATR and ANN (Bounphanmy and co-workers[48]). Several other works aiming at species discrimination available in the literature consider both Gram-positive and -negative species. It should be noted that the discrimination between bacterial species belonging to different Gram type and/or genera, is naturally easier to accomplish. Rodriguez-Saona et al.[49] successfully discriminated several *Escherichia*, *Bacillus*, *Pseudomonas*, and *Listeria* species with FTIRS and PCA. Whittaker et al. 2003,[50] distinguished several species (*S. aureus*, *Listeria monocytogenes*, *Bacillus anthracis*, *B. cereus*, *Yersinia enterocolitica*, *Salmonella* typhimurium, *Shigella sonnei*, *Escherichia coli*, *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahemolyticus*) with SIMCA and PCA through their fatty acid methyl esters profile obtained by FTIRS. Sandt et al.[51] obtained 100% of correct species discrimination for the most frequent Gram positive bacteria tested in their study (*S. aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Enterococcus faecium*) and about 80% for Gram-negative bacteria (*Enterobacter aerogenes*, *Enterobacter cloacae*, *Klebsiella* spp., *Citrobacter koseri*, *Proteus mirabilis*, and *E. coli*. *Salmonella enterica*, *E. coli*, *Yersinia*, and *Shigella* species[52] with FTIRS and PCA or CVA. Garip and co-workers[53] also noticed several

differences in the IR spectra of mesophilic and thermophilic bacteria. Bombalska et al.[54] discriminated several *Bacillus* species, *E. coli*, *Micrococcus luteus*, and *Pantoea agglomerans* by a combined FTIRS and chemometric approach as well as bacterial spores from vegetative forms. Maity et al.[55] successfully used FTIRS to discriminate between *S. maltophilia* and *Bacillus flexus*. It is of note that this study has a strong limitation in the number and diversity of the collection (could bias the reported results).

3.3. Bacterial Typing at the Subspecies Level

Several genotypic methods, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), multilocus variable number of tandem repeats analysis (MLVA), real-time polymerase chain reaction (PCR), and amplified fragment length polymorphism (AFLP), are currently used to identify particular bacterial lineages at both local and global levels. Despite their high accuracy, these methods are laborious and time consuming, preventing immediate interventions in an outbreak context, and difficult to apply on a large-scale basis. Bacterial serotyping through their IR spectra was one of the most explored topics. *Listeria monocitogenes* and *S. enterica* were the targets of most of the reported studies. In 2007, Lefier et al. discriminated five *L. monocytogenes* serotypes and concluded that the results were strongly influenced by the culture medium, growing time, temperature, and washing procedure before the FTIRS analyses.[21] About 10 years later, a more comprehensive study proposed a discrimination method for twelve *L. monocytogenes* serotypes with percentages of correct assignments ranging from 91.6 to 98.8% depending upon the serogroup.[56] Two more studies were found in the literature considering *L. monocytogenes* serotyping, both achieving 96.6% of correct assignments considering 4[57] and 11[25] serotypes. Regarding *S. enterica*, Kim and co-workers[58] noticed that FTIRS was able to discriminate serotypes from lipopolysaccharide extracts (100% of correct assignments) but not from intact cells. In 2006, the same authors were able to discriminate between serotypes analyzing exclusively the outer membrane proteins.[59] Despite the success of this approach, a very poor bacterial collection was used comprising only one isolate of each serotype which could compromise the results generalization. Baldauf et al.[60] successfully discriminated six *S. enterica* serotypes considering three distinct sampling procedures (transmission with ZnSe, transmission with disposable polyethylene membranes, and reflectance through ATR). They processed the spectral data with SIMCA. Other studies were found aiming at *Salmonella* isolates serotyping with high rates of correct assignments. However, they considered in the analysis only one isolate per serotype which might be unsufficient considering the chemometric-based approach.[61–63] FTIRS combined with appropriate chemometric methods were also able to discriminate several serotypes of *Streptococcus pneumonia*,[64] *S. aureus*,[65] and *Y. enterocolitica*[47] and three *Legionella pneumophila* serogroups.[66] *E. coli*[67,68] and *A. baumannii*[69,70] were successfully discriminated according to their sequence types by IR spectroscopy and chemometrics, *E. faecium* isolates according their PFGE type[71] and *E. coli* according to their MLVA profiles.[72] Several studies were also found in the literature aiming to discriminate specific bacterial lineages. Several *E. coli* strains were correctly identified and/or discriminated by FTIRS and appropriate chemometric methods namely, PCA, SIMCA and CVA.[49,50,52,73] Five strains of the epidemic Canadian methicillin-resistant *S. aureus* (MRSA) were discriminated by PCA and k-nearest neighbor (KNN) algorithm with 87.8 and 97% of correct assignments, respectively.[74] *Alicyclobacillus* strains,[75] epidemic clones of *L. monocytogenes*[76] and *S. enterica* serovar enteritidis[77] outbreaks strains were also successfully discriminated by this technique. IR spctroscopy proved also to be able to discriminate *Brucella* spp. isolates according to their biovars,[78] *Xanthomonas oryzae* pathovars,[79] *B. cepacia* complex species ribopatterns,[46] *L. monocytogenes* halotypes,[57] *Y. enterocolitica* and *Yersinia ruckeri* biotypes,[47,80] *S. enterica* serovar Enteritidis phage types,[81] and *Campilobacter* spp. genotypes.[82] Other studies successfully discriminated vegetative from sporulated *Bacillus*,[83] live and dead *Salmonella* cells,[63] intact and injured *L. monocytogenes*,[76,84] and *P. mirabilis* according to the lipopolyssacharide structure.[85]

4. Advantages and Limitations of Infrared-Based Techniques

Spectroscopic techniques started to be explored in the context of bacterial typing principally due to the limitations of the molecular biology techniques, which are mostly related with time, cost, and laboriousness. Additionally, closely related species are often difficult to distinguish by molecular biology. Globally, IR-based spectroscopic techniques main advantages are the analysis time, cost, laboratorial simplicity, need of chemical reagents (virtually none), and sample amount per analysis (very low). Also, an IR spectrum of a microbial contains relevant information about the biomolecular content of the microorganism including lipids, carbohydrates, proteins, and nucleic acids. However, IR spectroscopy for bacterial typing requires normally a high level of standardization, regarding growth and medium culture. IR is highly limited when it concerns the processing of aqueous samples (e.g., bacterial suspensions).[86] The lack of IR spectroscopy based libraries does not allow the utilization of this method for routine analysis. In general, the reported methods based on IR spectroscopy do not provide enough information for establishing the method for routine analysis essentially because they do not account for the intrinsic variability of clones. Despite these limitations, the interest on IR-based techniques for bacterial typing has substantially increased as proof of concept research works spread.

5. Competing Spectroscopic Techniques

Several spectroscopic techniques, such as IR, Raman, IF, or MALDI-TOF MS, have been applied in the context of bacterial typing with distinct success rates (depending on the microorganism considered or the taxonomic level). Most of these studies compare the discrimination level considering the standard molecular biology techniques as golden methods. Few studies were dedicated to a direct and exhaustive comparison between

the performances of different spectroscopic techniques. Despite being substantially different, FTIRS and mass spectrometry, mainly MALDI-TOF MS, are among the most directly compared techniques for typing purposes. Globally, mass spectrometry-based techniques are pointed as preferable for species level discrimination due to the need of a low degree of experimental standardization regarding growing conditions and the commercially available spectral databases.[44,87,88] On the other hand, FTIRS seems to have high potential for subspecies discrimination due to the information available in the IR spectra namely, several classes of biomolecules, including lipids, nucleic acids, and carbohydrates.[69,70,87,89] In 2004, Wenning et al.[90] studied the influence of the incubation time and medium type for typing bacteria at the genus, species, and strain level: FTIRS and MALDI-TOF MS were compared. The higher percentage of correct class assignments were achieved with the mass spectroscopic technique. In 2014, Sousa and colleagues[44,88] obtained 100% of correct *Acinetobacter* species assignments with MALDI-TOF MS. The results obtained with FTIRS were less satisfactory. However, there are some exceptions to this apparent advantage of MALDI-TOF over FTIRS. FTIRS and MALDI-TOF MS demonstrated a similar potential for the discrimination of two pathovars of *X. oryzae*[79] and Goodacre et al.[91] concluded that FTIRS was better than pyrolysis mass spectrometry and dispersive Raman microscopy for the species discrimination of 59 Gram-positive and -negative bacteria. Regarding the subspecies level, the discrimination of an *Acinetobacter* collection belonging to four distinct sequence types[69,70,89] was better with FTIRS than MALDI-TOF MS in. In contrast, similar results were obtained with FTIRS and MALDI-TOF MS regarding the discrimination of *Klebsiella oxytoca*[87] and *Campylobacter* strains.[92] FTIRS was compared with Raman, Raman micro-spectroscopy, atomic force microscopy (AFM), and IF. Raman spectroscopy exhibits some known advantages over IR spectroscopy such as the lower interference of water and narrower bands. However, Raman is a costly equipment and generally more difficult to operate. In 2001, Kirschner et al.[33] compared the potential of FTIRS and Raman spectroscopy to identify a collection of eighteen *Enterococcus* isolates belonging to six different species. The authors found a considerable consistency between the results obtained by both techniques. Later, Maquelin et al.[93] compared both techniques to identify bacterial and fungal pathogens from blood cultures. The results obtained with IR spectroscopy were slightly better (98.8% versus 92.2% of correct assignments). Tang and co-workers[86] compared FTIRS, Raman microspectroscopy, and atomic force microscopy to characterize a collection of mycobacteria and Gram-negative bacteria.[86] The authors concluded that a combined approach, including the three techniques, will lead to a more complete characterization of the isolates (making advantage on the distinct outputs of the techniques). This is a very important finding that should guide the selection of the most appropriate spectroscopic technique for a specific purpose. Regarding IF spectroscopy, several studies have been published claiming the high potential of the technique in the context of bacterial typing.[94–97] However, a recent study[98] directly compared the performance of IF spectroscopy with FTIRS and MALDI-TOF MS for *Acinetobacter* species identification. The study concluded that IF spectroscopy has a significant lower potential for bacterial typing. The percentage of correct species assignments considering IF data yielded values ranging from 38% to 65%. These results are significantly lower than the numbers obtained with IR and/or MS.

6. Conclusions and Futures Perspectives

Over the last three decades, the application of spectroscopic techniques for the analysis of biological materials has considerably grown[99] due to their versatility and accuracy. Regarding bacterial typing, a considerable effort has been made to develop reliable alternatives to molecular biology techniques. In this context, mass spectrometry gained popularity among researchers and clinicians due to the promising results obtained for species identification.[100] IR-based spectroscopic techniques gathered less attention. However, IR has proved already to be quite reliable for typing purposes. Noteworthy, in this review, it was shown that IR spectroscopy was successfully used for bacterial typing at different taxonomic levels. Applications covered a wide variety of microorganisms. IR spectroscopy demonstrated to have potential to become a real alternative for molecular biology and biochemical methods. However, additional developments and especially systematization are required. Validated spectral databases are of utmost importance as they will account for natural clonal variability and allow different laboratories to resource to the same reference for results comparison. Databases are absolutely crucial for example when dealing with outbreaks for which it is essential to have a quick and accurate response or when severe ill-condition patients are being monitored. Also, making future reported applications less sensitive to instrument's specifications, grow media, environmental grow conditions (e.g., cultivation time and temperature), sampling origin, and chemometric method can make this methodology more transparent and ready to use for routine applications. Similarly, new emerging technologies such as Quantum Cascade Lasers (QCLs) for infrared spectroscopy could improve the accuracy of the measurements, increasing the signal-to-noise ratio. Such lasers could be operated at room temperature. The interest of microbiology and health sciences field researchers and professionals in this theme is expected to grow in many directions other than the reported here. For instance, the ability of IR methods to be used in mixed cultures without resourcing to complex sampling methods (as those required for micro-spectroscopy) may be extremely important not only in the microbiology field, but also for bioengineering (e.g., high-throughput monitoring of bacteria strains within a fermenter), medical sciences (e.g., fast identification of bacteria from lung disease patients), or environmental sciences (e.g., monitoring of bacteria in an activated sludge wastewater treatment process) just to name a few.

Abbreviations

AFLP, amplified fragment length polymorphism; AFM, atomic force microscopy; ANN, artificial neural network; ATR, attenuated total reflectance; CVA, canonical variate analysis; CART, classification and regression trees; DFA, discriminant function analysis; FDA, factorial

discriminant analysis; FT-NIRS, Fourier transform near infrared spectroscopy; FTIRS, Fourier transform infrared spectroscopy; FTIRS-ATR, Fourier transform infrared spectroscopy with attenuated total reflectance; FTIRS-HATR, Fourier transform infrared spectroscopy with horizontal attenuated total reflection; HCA, hierarchical clustering analysis; IF, and intrinsic fluorescence; IR, infrared; KNN, k-nearest neighbor; KPLS, Kernel partial least squares; LDA, linear discriminant analysis; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MLST, multilocus sequence typing; MLVA, multilocus variable number of tandem repeats analysis; MRSA, methicillin-resistant *S. aureus*; NIRS, Near infrared spectroscopy; PC-DFA, principal component-discriminant function analysis; PCA, principal component analysis; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; PLSDA, partial least squares-discriminant analysis; PLSRDA, partial least squares regression discriminant analysis; QCLs, Quantum Cascade Lasers; SCDA, step-wise canonical discriminant analysis; SIMCA, soft independent modeling of class analogy.

Acknowledgements

This work received financial support from the European Union (FEDER funds POCI/01/0145/FEDER/007265, POCI-01-0145-FEDER-006684, and NORTE-01-0145-FEDER-000004) and National Funds (FCT/MCTES, Fundação para a Ciência e Tecnologia) under the Partnership Agreement PT2020 UID/QUI/50006/2013 and UID/BIO/04469/2013. Cristina Quintelas was supported by a postdoctoral grant (SFRH/BPD/101338/2014). Clara Sousa was funded through the NORTE-01-0145-FEDER-000024 – "New Technologies for three Health Challenges of Modern Societies: Diabetes, Drug Abuse and Kidney Diseases".

Conflict of Interest

The authors declare no commercial or financial conflict of interests.

Keywords

bacteria, bacterial typing, chemometrics, infrared spectroscopy, microbiology

Received: June 29, 2017
Revised: October 18, 2017
Published online: November 15, 2017

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