MIR spectroscopy as alternative method for further confirmation of foodborne pathogens *Salmonella* spp. and *Listeria monocytogenes*

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Abstract Listeriosis and Salmonellosis are two of the most common foodborne diseases. Consequently, an early and accurate detection of *Listeria monocytogenes* and *Salmonella* spp. in food products is a critical concern of public health policies. Therefore, it is of great interest to develop rapid, simple, and inexpensive alternatives for pathogen detection in food products. In this study, mid-infrared spectroscopy has been successfully used to confirm *Listeria* species and the presence of *Salmonella* isolated from food samples. This methodology showed to be very sensitive and could be a rapid alternative to detect these important pathogens, allowing to obtain results in a few minutes after previous growth in selective media, avoiding the confirmation procedures that delay the achievement of the results for up to 2 days.

Keywords Infrared spectroscopy (IR) · Principal component analysis (PCA) · Foodborne bacteria · *Listeria monocytogenes* · *Salmonella* spp

Introduction

Nowadays, the microbiological quality of food is a very important factor for food industries and regulatory agencies. The accurate and reliable detection and identification of microorganisms in food is critical in order to detect microorganisms early on to prevent their transmission, avoiding infections and/or food poisoning.

Listeria monocytogenes and *Salmonella* spp. are some of the most important and common foodborne pathogenic microorganisms, causing public health problems in almost all industrialized countries. The multistate outbreak due to cheese contaminated with *L. monocytogenes* (Heiman et al. 2016) and the international outbreak due to chia seeds contaminated with *Salmonella* spp. (Harvey et al. 2017) are recent examples.

The genus *Listeria* is constituted by six species, of which *L. monocytogenes* is considered an opportunistic serious foodborne pathogen for humans, causing a disease named listeriosis which is a significant public health hazard (Rocourt and Buchrieser 2007). The identification of *Listeria* at the species level in routine laboratories is time-consuming, laborious, and expensive (Pusztahelyi et al. 2016). Selective primary and secondary enrichment steps, followed by isolation in *selective differential media*, and suspect colonies confirmation procedures by biochemical tests are needed (Pusztahelyi et al. 2016). OCLA agar medium allows the visual differentiation of the pathogenic species (*L. monocytogenes* and *L. ivanovii*) from the non-pathogenic species (*L. innocua*). Colonies from pathogenic species present blue to green colour surrounded by an opaque halo that does not exist in the case of the non-pathogenic species. However, ISO 11290-1 (1996) specifies that the biochemical confirmation based on haemolytic activity and carbohydrate fermentation should be

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performed. *L. monocytogenes* and *L. ivanovii* possess haemolytic activity, whereas *L. innocua* does not have this capacity. Regarding carbohydrate fermentation, it is known that *L. monocytogenes* and *L. innocua* use glucose, lactose, and rhamnose in aerobic conditions, while *L. ivanovii* ferments xylose (Pine et al. 1989). The confirmatory tests extend the detection for more 2 days, being the total identification completed only after 7 days (ISO 11290-1 1996). In addition, despite of the advances in the detection methodologies, the incidence of false-negative results is still a problem (Benetti et al. 2013; Bernardi et al. 2015).

The genus *Salmonella* includes two species: *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies, including *S. enterica enterica*, that includes more than 2500 serovars (Tindall et al. 2005). Salmonellosis disease typically resolves in about 6 days and does not require treatment with antibiotics, however, bacteremia occurs in 3–10% of the reported cases (Hoelzer et al. 2011). Cultivation methods for the detection of *Salmonella* include a non-selective pre-enrichment step, followed by a selective enrichment and posterior plating in selective and differential solid media. Posteriorly, suspect colonies need to be confirmed by biochemical or serological tests as referred in ISO 6579 (2002), Gracias and McKillip (2004). The majority of *Salmonella* are recognized as non-lactose fermenters (Lac^-) and hydrogen-sulphide producers (H_2S^+), thus, confirmatory testing of all H_2S^+ and/or Lac^- colonies is required, which extends the total identification time to 5 days. The majority of the H_2S^+ and Lac^- colonies turn out not to be *Salmonella enterica*, but related species such as *Citrobacter* (Brenner et al. 2000).

During the last years, molecular and immunological procedures have been developed to detect the presence of *Listeria* and *Salmonella* in food (Yoshida et al. 2016; Su et al. 2016; Aydin et al. 2018). These methods, despite being rapid are yet expensive, requiring high skilled personnel. Moreover, they can be affected by the presence of certain substances in food products, which can lead to an underestimation of bacterial concentration or false negative results (Hu et al. 2009). Additionally, the legislation requires that positive results for the presence of *Salmonella* and *Listeria* in foods must be confirmed by traditional culture methods and further confirmation (ISO 11290-1 1996; ISO 6579 2002). Taking these aspects into account, it is very important to develop sensitive and specific faster methods to detect/identify these two foodborne pathogenic microorganisms in order to prevent illnesses associated to food consumption.

Vibrational spectroscopic techniques, infrared spectroscopy (IR) [mid-infrared (MIR) and near-infrared (NIR)] and Raman spectroscopy, have been used since the 1980s as complementary methods for bacteria differentiation owing to their rapid “fingerprinting” capabilities and

the molecular information that they can provide (Ellis et al. 2012; Sahar and Dufour 2014; Kammies et al. 2016). These techniques present several advantages in the microbiological classification and identification fields. They are fast (requiring virtually no sample processing), non-destructive, multi-purpose (e.g., detection, enumeration, classification, identification) and discriminating at different taxonomic levels (serotype, strain, species or genus) (Lefier et al. 2006; Das and Agrawal 2011; Ferraro and Krischnan 2012; Moreirinha et al. 2016). Although both MIR and NIR have been used for bacterial identification purposes, NIR bands are 10 to 100 times less intense than the corresponding MIR bands. Upon comparison, MIR spectroscopy has a higher signal/noise ratio, resolution, sensitivity, and output than NIR. Therefore, MIR spectroscopy can easily elucidate the chemical composition as well as the secondary and tertiary structures of a given bacteria (S. Sivakesava et al. 2004; Ellis et al. 2012), facilitating its identification.

Identification of *Listeria* species and *Salmonella* serovars using IR spectroscopy has been undertaken previously (Lefier et al. 2006; Rebuffo et al. 2006; Brandily et al. 2011; Romanolo et al. 2015). However, these studies included some time consuming cell pre-treatments, such as growth of the bacteria in liquid medium with subsequent centrifugation and washing with distilled water. In this study, the potential of mid infrared spectroscopy (MIR) to confirm the presence of *L. monocytogenes* and *Salmonella* spp. in food products, after isolation in their selective media, was investigated. Moreover, the potential to differentiate *Listeria* species and to discriminate *Salmonella* from other bacteria that grew on XLD *Salmonella* selective medium and could be mistaken for *Salmonella* (e.g. *Shigella flexneri*, *Pseudomonas aeruginosa* and *Citrobacter freundii*) was also evaluated.

Materials and methods

Bacterial strains and growth conditions

Listeria spp. and *Salmonella* serovars were isolated from food products (cheeses, sausages and prepared dishes) according to ISO 6579 (2002) and ISO 11290-1 (1996), respectively. Three different species of *Listeria* were obtained: 3 isolates of *L. monocytogenes*, 2 isolates of *L. ivanovii*, and 3 isolates of *L. innocua*, as well as three serovars of *S. enterica*: 2 isolates of serovar *S. Nottingham*, 2 isolates of serovar *S. Anatum* and 2 isolates of serovar *S. Liverpool* (Table 1).

Briefly, for the isolation of *Listeria* spp., a primary enrichment of the samples in half-Fraser broth (1:10), for 24 h at 30 °C was done. Then, a secondary enrichment was performed by transferring 0.1 mL of the previous culture to

Table 1 Bacteria species/serovars used in this study and respective food sources

Bacteria species/serovar	Food product
<i>Listeria monocytogenes</i>	Soft cheese
<i>Listeria monocytogenes</i>	Minced meat
<i>Listeria monocytogenes</i>	Smoked pork sausage
<i>Listeria ivanovii</i>	Soft cheese
<i>Listeria ivanovii</i>	Soft cheese
<i>Listeria innocua</i>	Bovine meat burger
<i>Listeria innocua</i>	Cured cheese
<i>Listeria innocua</i>	Pasta with bovine meat
<i>Salmonella enterica</i> sv. Nottingham	Cured cheese
<i>Salmonella enterica</i> sv. Nottingham	Cured cheese
<i>Salmonella enterica</i> sv. Anatum	Bovine meat burger
<i>Salmonella enterica</i> sv. Anatum	Hotdog with pork sausage
<i>Salmonella enterica</i> sv. Liverpool	Smoked sausage
<i>Salmonella enterica</i> sv. Liverpool	Cured cheese

a tube with Fraser broth (Oxoid Ltd., UK) that was incubated for 48 h at 37 °C. The cultures obtained in the primary enrichment were transferred to *Listeria* identification agar base (PALCAM, Oxoid Ltd., UK) or Oxoid chromogenic *Listeria* agar (OCLA, Oxoid Ltd., UK) media. PALCAM and OCLA plates were incubated at 37 °C and examined after 24 h or, if necessary, 48 h to check for the presence of *Listeria* characteristic colonies. Confirmation of *Listeria* spp. was carried out by selecting 5 presumed *Listeria* colonies and plating them in tryptone soya yeast extract agar (TSYEA, Oxoid Ltd., UK). The plates were incubated at 37 °C during 18–24 h. Typical colonies, colourless with an opaque halo, were then confirmed with catalase test. If the morphological and physiological characteristics indicated the presence of *Listeria* spp. a haemolysis test was performed in Columbia agar 5% (Oxoid Ltd., UK) to investigate which species was present. Biochemical API *Listeria* and RAPIDEC *L. monocytogenes* identification kits (Biomérieux, France) were used to confirm the identification of the species.

For *Salmonella* spp. isolation, a pre-enrichment in buffered peptone water (BPW, Oxoid Ltd., UK) for 18 h at 37 °C was made. Then, 0.1 mL of the previous culture was transferred to Rappaport–Vassiliadis with soya agar (RVS, Oxoid Ltd., UK) and 1 mL to Muller-Kauffmann tetrathionate novobiocin (TTmk, Oxoid Ltd., UK). Samples were incubated for 24 h at 42 °C (RVS) and 37 °C (TTmk) respectively. After incubation, samples were plated in xylose lysine deoxycholate agar (XLD, Merck, Germany) and brilliant green agar (BGA, Merck, Germany) in order to select typical colonies of *Salmonella* spp. (red with a black centre). The positive colonies were identified by API

system (API 20E, Biomérieux, France). The OMNI-O antiserum test (Bio-Rad, USA) was also used to confirm the presence of *Salmonella* (*Salmonella* spp. shows positive agglutination).

Collection strains *L. innocua* NCTC-11288 and *L. monocytogenes* NCTC-1194 were also included in order to assess the robustness of the multivariate analysis, being used as an external validation.

Collection strains *S. flexneri* DSM-4782, *C. freundii* NCTC-6272 and *P. aeruginosa* isolated on our laboratory (Vieira et al. 2012) were also used in this study, as they also grow in XLD agar and can be mistaken for *Salmonella* due to possible colour similarities.

Mid-infrared spectroscopy

Spectroscopic acquisition was performed in an infrared spectrometer (Bruker Alpha Platinum, Germany) with a resolution of 4 cm⁻¹ and 32 scans, in the mid-infrared region (4000–600 cm⁻¹). Analyses were performed in a room with controlled temperature (25 °C) and humidity (29%). Bacterial colonies grew in agar medium, for 18 h at 37 °C: trypticase soy agar (TSA, Merck, Germany) for *Salmonella* Nottingham and *L. monocytogenes* preliminary analysis, XLD for *Salmonella*, *Shigella*, *Citrobacter* and *Pseudomonas* and OCLA for *Listeria*. The colonies were collected with a loop and placed directly on the crystal of a 2 mm × 2 mm horizontal single attenuated total reflectance (ATR) accessory and were dried under gentle cold air flow for 10 s. At least three replicate spectra were obtained for each sample, obtained from different plates in different days. The sampling accessory was cleaned with ethanol (70%) and distilled water between each measurement.

Statistical analysis

The spectra (obtained in OPUS format) were transferred via JCAMP.DX format to an in-house developed data analysis package (CATS build 97). Principal component analysis (PCA) was used to find the major sources of variability in data, detect outliers and detect the probable presence of clusters. Previous to PCA, the spectra were standard normal deviate (SNV) corrected.

Results and discussion

Listeria species discrimination

In this study, instead of the biochemical confirmation step, MIR was used to assess the species distinction.

MIR spectral profile of *Listeria* spp

Figure 1 shows the spectral profile of the three bacterial strains of *Listeria* obtained in OCLA agar. Despite their similarity, some spectral differences were visible in the region between 1800 and 980 cm^{-1} . Consequently, this region was selected to perform the discrimination by principal component analysis.

Principal component analysis of *Listeria* spp

PCA analysis (Fig. 2a) revealed 3 different clusters. *Listeria monocytogenes* is located on negative PC1, mainly characterized by a peak at 1517 cm^{-1} (Fig. 2b). *Listeria innocua* and *L. ivanovii* are located on positive PC1, both characterized by a broad band between 1700 and 1570 cm^{-1} and a peak at 1200 cm^{-1} . *Listeria innocua* is also located at positive PC2, characterized mainly by a peak at 1666 cm^{-1} and another at 1505 cm^{-1} , and *L. ivanovii* is located at negative PC2, with the most significant peaks at 1615, 1330, 1240 and 1035 cm^{-1} .

In this case, it was possible to discriminate the 3 different studied *Listeria* species after growth in OCLA agar, with the spectra from the collection strains *L. monocytogenes* and *L. innocua* being grouped along with the spectra of the isolated bacteria of the same species.

Regarding the chemical structure of the cell wall of the different species, there is little information. In consequence, it is difficult at this stage to make specific assignments to the discriminatory peaks (Romanolo et al. 2015). However, differences on the surface proteins, which influence the species virulence, would contribute to the found discrimination peaks (between 1700 and 1500 cm^{-1}). This is in accordance with studies showing that surface protein patterns are specific for species and even serovars (Su et al. 2016). Furthermore, our study indicates that not only the proteins may have a significant paper on the species distinction, but also the saccharides

and phosphates, that are also present in bacterial extracellular envelope, were responsible for the obtained distribution (peaks between 1300 and 980 cm^{-1}) (Orsini et al. 2000; Davis and Mauer 2010). Taking these results into account, MIR spectroscopy allowed the rapid differentiation of the 3 *Listeria* studied species without the necessity of performing the biochemical confirmation step, after obtaining the colonies in the selective medium. This could shorten the identification procedure in 2 days.

Salmonella identification

MIR spectral profile of *Salmonella* and other bacteria

Mid infrared spectra from the isolated bacterial strains of *Salmonella*, *C. freundii*, *P. aeruginosa* and *S. flexneri* obtained in XLD agar showed similar profiles (Fig. 3). However, as in the case of *Listeria* species, some spectral differences are visible at the region between 1800 and 980 cm^{-1} , and this was also the chosen region to perform principal component analysis.

Principal component analysis of *Salmonella* and other bacteria

The PCA of the three *Salmonella* serovars, *C. freundii*, *P. aeruginosa* and *S. flexneri* are shown in Fig. 4. Five distinct groups are visible: *S. Anatum* and *S. Liverpool* were grouped together and *S. flexneri*, *S. Nottingham*, *C. freundii* and *P. aeruginosa* formed distinct groups. *S. Anatum*, *S. Liverpool* and *S. flexneri* were located on negative PC1. *C. freundii*, *S. Nottingham* and *P. aeruginosa* were located on positive PC1. *C. freundii* on negative PC2 separates from *S. Nottingham* and *P. aeruginosa*, both on positive PC2 (Fig. 4a).

According to the loadings plot profile, the most significant peaks that contributed to the distinction of the studied species were located in the region between 1200 and

Fig. 1 MIR average spectra of *L. monocytogenes*, *L. ivanovii* and *L. innocua* obtained from OCLA agar in the spectral region between 4000 and 500 cm^{-1}

Fig. 2 Principal component analysis (PC1 vs. PC2) of *L. ivanovii*, *L. monocytogenes* and *L. innocua* spectra in the region between 1800 and 980 cm^{-1} . **a** Scores scatter plot, **b** loadings plot profile. *L. innocua* and *L. monocytogenes* from NCTC culture collection are surrounded by a circle

Fig. 3 MIR average spectra of *C. freundii*, *P. aeruginosa*, *S. flexneri*, *S. Nottingham*, *S. Liverpool* and *S. Anatum* obtained from XLD agar in the spectral region between 4000 and 500 cm^{-1}

Fig. 4 Principal component analysis (PC1 vs. PC3) of *S. enterica* serovars (Anatum, Nottingham and Liverpool), *S. flexneri* and *C. freundii* and *P. aeruginosa* spectra in the region between 1800 and 980 cm^{-1} . **a** Scores scatter plot, **b** loadings plot profile

980 cm^{-1} , region of saccharides and phosphates, compounds that may differ at the surface of the different studied species/serovars (Fig. 4b). As it is known, IR spectra of bacteria provide not only the absorption bands that describe molecular composition of the cells, but many of these bands are also sensitive to structure changes, numerous intra and inter-molecular interactions including the hydrogen bonding pattern, membrane constitution, lipid-protein interactions, and conformational states as the

secondary structures of proteins (Lasch and Naumann 2006). So, in this case, it is also difficult to make an accurate assignment of the spectral bands to structural compounds of bacteria external membrane or cell wall.

XLD agar offers the possibility of selecting *Salmonella*-like colonies because the medium contains indicators of H_2S production (typical of *Salmonella* spp.) and pH changes (*Salmonella* spp. exhaust the xylose of the medium and decarboxylate the lysine, altering the pH to alkaline).

Shigella spp. are genetically close to *Salmonella* spp. and *Citrobacter* spp. (Doyle and Buchanan 2013). *Citrobacter* generally grows as yellow colonies, different from *Salmonella*, however, there are some works reporting that *C. freundii* and other *Citrobacter* species produced false-positive colonies (Warburton et al. 1994; Coleman et al. 1995). Likewise, *P. aeruginosa* and *Shigella* spp. can produce colonies very similar to those produced by some *Salmonella* species on XLD media, leading to misinterpretation of the results (Maddocks et al. 2002). Taking this into account, MIR can help to overcome these problematics, as in this case these bacteria were easily differentiated with this methodology.

Despite the existence of distinct groups, it was intended to obtain a better separation of *Salmonella* serovars from *S. flexneri*. As the latter is distributed in the same quadrant of *S. Anatum* and *S. Liverpool*, a PCA with *Shigella* and *Salmonella* serovars was performed, and a good separation between *Shigella* and *Salmonella* was observed (Fig. 5).

Salmonella flexneri is separated from the other bacteria in the negative PC2. *S. Anatum*, *S. Liverpool* and *Shigella* are separated from *S. Nottingham* in the positive PC1 (Fig. 5a). The loadings plot profile (Fig. 5b) shows that the distribution of the species is mainly characterized by peaks in the region between 1700–1500 and 1200–980 cm^{-1} , that may have the contribution of protein, phosphate and saccharide differences between the studied species/serovars.

Conclusion

In general, the studied bacteria were discriminated by peaks corresponding mainly to polysaccharide (1200–980 cm^{-1}) and protein (1700–1500 cm^{-1}) spectral regions. This was expectable, as the cellular surface of the different species and serovars studied differ in these compounds. Some signals, corresponding to DNA phosphates,

could also be important in the discrimination of the bacteria.

In this study, mid-infrared spectroscopy has proved to be a very useful method for the confirmation of *Salmonella* spp. and *Listeria* spp. after their isolation in selective media. Our data are indicative that MIR can be used instead of the traditional biochemical methods, as it allows a very good discrimination of the analysed *Listeria*, *Salmonella* and other foodborne bacteria, by forming well discriminated groups separating the studied species in PCA analysis. Ideally, matching the spectra obtained for bacterial colonies with a previously obtained spectral library of different foodborne bacteria would allow the immediate identification of possible pathogens. Further studies using both MIR and traditional confirmatory methods in different real food samples are needed to evaluate the potential of MIR to confirm the presence of pathogenic bacteria. Moreover, MIR can also be a good substitute for the existent molecular identification methods that despite of being accurate, are expensive, complex and time consuming, delaying the detection of the microorganisms.

The methodology used in this work is very easy to perform (the colonies obtained in specific agar media are directly placed on the MIR sampling accessory without any handling), rapid (a few seconds to obtain a spectrum) and considerably inexpensive, taking in account that MIR spectrometers with very good quality and not expensive are available nowadays. Using this method, the time for the detection of *Salmonella* spp. and *Listeria* spp. can be shortened up to 2 days, comparing with traditional methods. Another advantage is to avoid the use of additional expensive chemicals needed for traditional biochemical confirmation procedures. MIR could be a new tool for laboratories and manufacturers to easily screen food pathogens, even though more research work is needed, namely further studies with more species/serovars should be performed to validate this methodology.

Fig. 5 Principal component analysis (PC1 vs. PC2) of *S. enterica* serovars (Anatum, Nottingham and Liverpool) and *S. flexneri* spectra in the region between 1800 and 980 cm^{-1} . **a** Scores scatter plot, **b** loadings plot profile

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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