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Original article

IDENTIFICATION OF *LISTERIA* SPECIES BY FOURIER-TRANSFORM INFRARED SPECTROSCOPY

A. Al-MARIRI[1], L. RAMADAN[1], A. ABOU YOUNES[2] & A. Al-LAHAM[1]

[1]Department of Molecular Biology and Biotechnology, Atomic Energy Commission, Damascus, Syria; [2]Faculty of Agriculture, Damascus University, Syria

Summary

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Differentiation of the genus *Listeria* is significant for food industry, but only few reliable methods are available so far. In the present study, 56 strains isolated from 345 samples of cow raw milk were used. The isolated pure cultures were defined by PCR-based method using specific primers of *16S-23S IGS* region of DNA. Bacterial strain samples were submitted to spectroscopic measurements by the transmission method at a wavelength of 3000–700 cm^{-1} using Fourier-transform infrared (FTIR) spectrophotometry. Hierarchical cluster analysis (HCA) was performed based on the identification of the 56 isolated strains. The utilisation of HCA in univariate-FTIR spectral analyses as the most progressive chemometric method was supported by the correct identification of 86.9% bacteria of the genus *Listeria* at the species level. These results explained the ability of univariate-FTIR spectrum analysis for determination of suspected *Listeria* species.

Key words: Fourier-transform infrared spectra, hierarchical cluster analysis, *Listeria*, polymerase chain reaction, raw milk

INTRODUCTION

Determination of agents involved in foodborne diseases started at the end of the 19th century with the explication of the etiology of botulism in humans (Silk *et al.,* 2012). However, in the early 1980s, the number of releases on *Listeria* involvement began to rise and in 1983, the first human listeriosis outbreak directly related to consumption of *Listeria* contaminated aliments was reported (Schlech *et al.*, 1983). The bacteria of the genus *Listeria* are widely spread in nature as well as in different food processing and storing environments. The genus includes motile, regular, non-sporing, non-capsulated, Gram-positive coccobacillus shaped bacteria. Currently, they are taxonomically subdivided into seven species including *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. grayi*, *L. ivanovii* and *L. marthii* (Graves *et al*., 2010). Only two are pathogenic and cause listeriosis (Giacometti *et al*., 2012). These are *L. ivanovii*, an animal pathogen, and *L.*

monocytogenes, an intracellular human food-borne pathogen which infects, in particular, the foetuses in the third trimester of pregnancy, the aged and immunocompromised persons, and is clinically deemed as causing meningitis and bacteraemia, with high fatality, because of its high mortality and morbidity (Grundling *et al*., 2004). The mortality rate of listeriosis is very high, approximately 30% (Conzen, 2006), and for this reason the FDA maintains a policy of zero-tolerance for *L. monocytogenes*, (Janbu *et al*., 2008). Most cases in human listeriosis are now ascribed to ingestion of *L. monocytogenes*-contaminated foods, mainly ready-to-eat foods (Aygun & Pehlivanlar, 2006) as soft cheese, particularly those made from unpasteurised milk (Rosshaug *et al*., 2012). Control of human food-borne listeriosis comprises reduction or elimination of food contaminated with *L. monocytogenes*, since this bacterium is capable to grow at refrigeration temperatures.

The rapid identification of food products contaminated with *Listeria* spp. is essential. Common microbiological culture methods depend on growth in culture media, followed by isolation, and biochemical and serological identification. However traditional and standard bacterial detection methods may take up to 7 or 8 days to distinguish *Listeria* spp. (Velusamy *et al.*, 2010). The utilisation of molecular typing methods has allowed further sub-typing of different isolates and survey on their distribution in the production sites and more specifically to observe the contamination sources. Among these, PCR has been increasingly utilised for the rapid, sensitive and specific detection of foodborne pathogens (Chen *et al.*, 2011). Yet, the prosperous applying of PCR assays to food samples has been blocked by the lack of a rapid and efficient method for preparation of PCR-amplifiable DNA (Rossmanith & Wagner, 2011). The PCR is significantly less time-consuming than culturing and plating requiring from 5 to 24 h to detection result but this relies on specific PCR alteration used and does not involve any preceding enrichment steps. One of the restrictions of PCR techniques is the inability to distinguish between viable and non-viable cells because DNA is always found no matter if the cell is dead or alive.

The implementation of new technologies, namely Fourier-transform infrared (FTIR) spectroscopy, offered new and promising approaches. The FTIR spectroscopy has been successfully enforced for detection, discrimination, determination, and classification of bacteria relating to different species, particularly food-borne pathogens such as *Listeria* (Cecilia *et al*., 2007; 2008). FTIR method is not only utilised for bacterial determination, but also supplies information about bacterial metabolism and growth phase (Becker *et al*., 2006). There is an increased demand for FTIR spectroscopy in the area of food microbiology due to its technical improvement, simplicity of sample preparation, and speed of analysis (Davis *et al*., 2012). FTIR spectroscopy could be an exquisite alternative to the available analytical techniques in food analysis because of its increased sensibility, resolution, high signal-to-noise ratio, multiple-component analysis and rapid measurement capabilities (Lin *et al*., 2004; Soto Beltran *et al*., 2015). The purpose of the present work to inquire the potential of FTIR microspectroscopy, in adjunction to PCR method, in the rapid determination of *Listeria* species in food.

MATERIALS AND METHODS

Raw milk samples and media

A total of 345 bovine raw milk samples collected from different provinces of Syria (from unorganized sectors) from August 2005 to December 2008 were analysed for presence of *Listeria* spp. All samples were gathered aseptically, brought to the laboratory and stored at 4° C.

Isolation and determination of Listeria spp.

For bacterial growth, *Listeria* enrichment broth base (LEB) (Oxoid –Basingstoke-UK) was used. Isolation of *Listeria* spp. from raw milk was directly inoculated into *Listeria* selective agar base (Oxford) supplemented with *Listeria* selective supplement (Oxford), and incubated at 37 °C for 48 h. Gray colonies with black centres from Palcam were suspected to be *Listeria* spp. These typical 5–7 colonies were further characterised. Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25 °C , Camp test with *Staphylococcus aureus*, nitrate reduction, fermentation of sugars (rhamnose, xylose, and methyl-α-D-mannopyranoside) and haemolysis on 5% sheep blood agar (SBA).

DNA extraction and amplification

The cell pellet, from 10 mL overnight culture, was utilisied for extraction of DNA. Genomic DNA was extracted using Qiagen DNA extraction kit (Qiagen, GmbH) and the DNA concentration was identified using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific GmbH, Germany) at 260 nm.

The PCR primers for amplification of DNA fragments targeting the *16S-23S IGS* regions were determined at highly conserved regions of the 16S and 23S DNA respectively, (Al-Mariri *et al*., 2013). Primers for detection of the *Listeria* spp. isolates are given in Table 1.

Five hundred ng of extracted genomic DNA were used for PCR amplification. PCR reaction mixture (25 µL) contained 5 µL PCR buffer HotStar HiFidelity (Qiagen, Germany), 200 µM of each dNTP (Promega, USA), 2.5 mM $MgCl_2$, 25 pmoL of each primer and 1.5 U HotStar HiFidelity DNA Polymerase (Qiagen, Germany). PCR amplification reactions were executed in a GeneAmp® PCR sys-

Table 1. Primers used for PCR and amplification of *16S-23S IGS* region of *Listeria* spp. isolates

Primer sequence (5'–3')	Product size (bp)	*Listeria* spp.
For1: TCATTCGCTCACACCGTAAA Rev1: TCATTAGCACCTGGTGTCAGA	380	*L. welshimeri*
For2: CTTAAAAGACCGCCTGCGCG Rev2: GATAAGAGTAACTGCTTGTCCCTT	200	*L. gravi*
For3: CTTAAAAGACCGCCTGCGCG Rev3: GGGAATCTTCCGCAATGGAC	200	*L. ivanovii*
For4: AATTCCCACAGGACACAACC Rev4: CGGGAATGCAATTTTTCACTA	380	*L. innocua*
For5: CCGTGCGCCCTTTCTAACTT Rev5: TTTGTTCAGTTTTGAGAGGT	400	*L. monocytogenes*

tem 9700 (Applied Biosystems, USA) – initial denaturation step at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 ºC for 30 s and extension at 72 ºC for 30 s followed by final incubation at 72 ºC for 10 min. PCR products were separated on a 2% agarose gel (Sigma, Germany) containing a 1% solution of ethidium bromide (Al-Mariri *et al.*, 2013).

Preparation of the samples for FTIR measurements

Samples were executed according to the methods of Oberreuter *et al.* (2002). The 56 isolates stored at 80 °C were streaked and subcultured on tryptone soy agar plates (TSA containing 15 g tryptone, 5 g soya peptone, 5 g sodium chloride and 15 g agar per litre, Oxioid, Basingstoke, United Kingdom), for 24 h at 30 °C. Colonies were transferred from the agar plate to an infrared-transparent ZnSe sample holder (25 mm in diameter) by replica stamping and were dried to a transparent film under mild vaccum (2.5–7.5 kPa), under a controlled atmosphere of 5–10% relative humidity for approximately 30 min. The spectra were registed and evaluated (Oust *et al*., 2004).

Phenotypic typing of FTIR spectra

Samples were run in replicates and analysed by FT-IR spectroscopy using a TENSOR spectrometer (Bruker Optik, Karlsruhe, Germany) in transmittance mode. All spectra were registed in the region between 4000 and 500 cm^{-1} with an IFS 28/B FTIR (Bruker Optik, Karlsruhe, Germany). Physical resolution was set to 6 cm^{-1}, a Blackman/Harris apodisation was used for Fourier transmutation and a zerofilling factor of 4 was applied to yield an encoding interval of approximately one data point per wave number. The specialty of each spectrum was evaluated using a quality test in the OPUS 6.5 software.

Univariate FTIR analysis

The selection of relevant spectral ranges and construction of the cutoff values of spectral distance (SD) for the determination of *Listeria* at the species level were done. To found the SD value, three individual measurements of six strains of each *Listeria* species were utilised. The calibration of their SD threshold value for exact determination of an isolate at species level was done using the procedure of Oust *et al*. (2004). In our case, the windows were from 3030–2830 cm^{-1}, 1650–1250 cm^{-1}, 1200–900 and 900–700 cm^{-1} (all weight factors were 30). A cutoff SD value of 0.5 was used (spectral distance between an isolate and the first hit of the identification hit list must be <0.5) for a valid identification at the species level.

Hierarchical cluster analysis (HCA) of Listeria spectra

HCA, an unsupervised classification technique, the Ward's algorithm, was utilised to establish the dendrograms. As distance measure, Pearson's product moment correlation coefficient was used as identified previously (Helm *et al*., 1991). The HCA was executed on the original spectra as input in the regions ranging from 700 to 1,200, 1,500 to 1,800, and 2,800 to 3,100 cm^{-1}, correlation with scaling to first range and Ward's algorithm due to the OPUS software (Bruker Optik, Karlsruhe, Germany). The two major groups resulting from this HCA served to construct the first layer of the two-layered neural network for particular classification at the species level.

Validation of univariate FTIR identification procedure

To test the FTIR univariate identification model, an internal validation was performed. However, as it was not possible to obtain the *Listeria* reference data set, a final assay of performance and the validation strain set of 147 individual *Listeria* isolates, whose spectra did not need the reference database, was identified by univariate method in an external validation.

Statistical analysis

Data were transported to a Microsoft Excel spreadsheet for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), chi-square test and Fisher's exact two-tailed test analysis were run and variances were considered important at $P<0.05$.

RESULTS

Isolation and PCR identification of bacteria

Listeria spp. were isolated and identified in only fifty six samples (16.23%). All *Listeria* spp. isolates were Gram positive, regular-short rods with parallel sides and blunt ends, catalase positive and oxidase negative. Twenty three isolates were considered as *L. monocytogenes*, 11 as *L. innocua*, 7 as *L. ivanovii,* 3 as *L. gravi* and 5 as *L. welshimeri* (Table 2).

Table 2. *Listeria* spp. isolates from raw cow milk samples

Listeria isolates	Number (%)
L. monocytogenes	23 (41.07%)
L. innocua	11 (19.64%)
L. ivanovii	7 (12.5%)
L. gravi	3 (5.35%)
L. weshimeri	5 (8.92%)
Listeria spp.	7 (12.5%)
Total	56 (100%)

The amplification results of the target genes for five *Listeria* strains using specific primer sets (Fig. 1) defined the 49 isolates as *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. gravi*, and *L. welshimeri* (except for seven *Listeria* spp. samples – Table 2).

Univariate-FTIR spectra of Listeria

The areas of the infrared spectra which participated in the differentiation between the five *Listeria* species were highlighted in Fig. 2. Representative FTIR spectra of five *Listeria* strains are shown. Each strain exhibited characteristic absorbance at waves between 500–3500 cm^{-1}. There were many important visible peaks in the spectra of *Listeria*, and most of them explained functional group vibrations in the major biomolecular constituents like protein, fatty acids, amino acids, and polysaccharides.

Hierarchical cluster analysis

Cluster analysis helped to determine analogies between the spectrum of microorganisms utilising the distances between spectrum and cumulation algorithms. The repeatedly utilised distances were the Pearson product moment correlation modulus and the Euclidian distance (Janbu *et al.,* 2008) (Fig. 3). Factorisation is also executed to the spectrum to break apart the spectral data into the most universal spectral variations (factors, loadings, principal components) and the corresponding scores (Conzen, 2006). HCA was applied to 49 samples (except seven *Listeria* isolates, Table 2).

Three individual measurements of the five strains for each *Listeria* species were utilised in order to complete the present

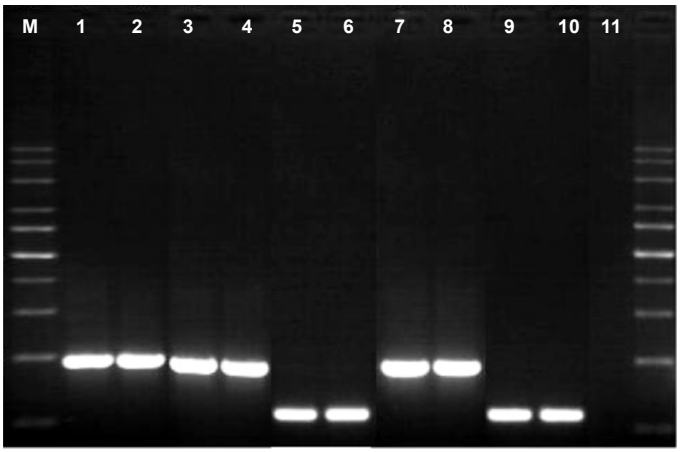

Fig. 1. Electrophoresis on 1.5% agarose gel showing the PCR products: MW=100-bp DNA ladder; lanes 1, 2= amplification of *L. monocytogenes*; lanes 3, 4= amplification of *L. innocua*; lanes 5, 6= amplification of *L. ivanovii*; lanes 7, 8= amplification of *L. welshimeri*; lanes 9, 10= amplification of *L. gravi*; lane 11= negative control (distilled water).

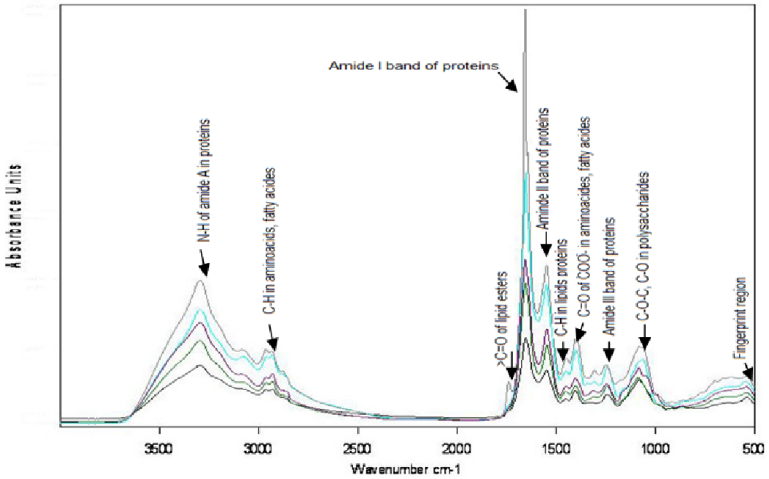

Fig. 2. FTIR spectra of five *Listeria* species: *L. monocytogenes* spectra; *L. ivanovii* spectra; *L. welshimeri* spectra; *L. ivanovii* spectra; *L. gravi* spectra (from the upper to lower spectrum, respectively), and the functional groups associated with major vibration bands in IR spectra (500–3500 cm^{-1}) of *Listeria*.

study. The dendrogram of a hierarchical cluster analysis performed with univariate FTIR spectra of different five strains of *Listeria* spp is illustrated on Fig. 3.

HCA and dendrograms were used to explain the analogies between spectra of *Listeria* representing the correlation with the species on the first level. It has been noticed that two major groups resulted from the cluster analysis and were used to construct the first level of the architecture of the neural network for *Listeria* spp.

determination. On the first level, the *L. monocytogenes, L. innocua, L. ivanovii* net and the *L. welshimeri, L. gravi* net were established.

Cluster analysis was run with vector normalised first derivative spectra using Ward's algorithm and squared Euclidian distance measurements and computer programme OPUS (Bruker Optik, Karlsruhe, Germany).

Validation of univariate FTIR identification procedure

Univariate-based FTIR identification procedure was not internally validated because it was hard to obtain *Listeria* reference data set, but the procedure was externally validated with 147 isolates of the validation strain set as shown in Table 3.

The FTIR univariate method exactly defined 128 of the 147 isolates (87%).

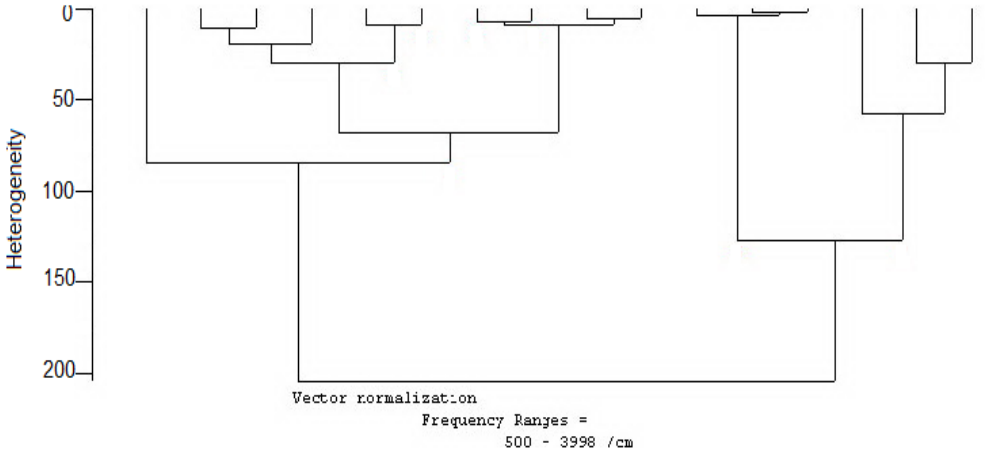

Fig. 3. Hierarchical cluster analysis of the univariate FTIR spectroscopy of 168 *Listeria* spectra by using the areas from 700 to 1,200, 1,500 to 1,800, and 2,800 to 3,100 cm^{-1}, correlation with scaling to first range, and Ward's algorithm.

Table 3. External validation of univariate FTIR spectroscopy for identification of *Listeria* spp.

Species of *Listeria*	Number of strains tested	Univariate FTIR analysis	
		Correct identification n (%)	Incorrect identification* n (%)
L. monocytogenes	69	63 (91.3)	6 (8.6)
L. innocua	33	30 (90.9)	3 (9.1)
L. ivanovii	21	15 (71.4)	6 (28.5)
L. gravi	9	8 (88.8)	1 (11.1)
L. welshimeri	15	12 (80.0)	3 (20.0)
Total	147	128 (87.0)	19 (12.9)

*strains yielding an SD value below or equal to the threshold value of 0.5 are used for exact determination of *Listeria* species.

The misidentified isolates of the this method were 19 (12.9%). In this study, 63 of 69 (91.3%) strains of *L. monocytogenes* were correctly identified, whereas seven samples were misidentified by this method. Similarly, *L. innocua*, *L. gravi*, and *L. welshimeri* exhibited good rates of correct identification, which were 30 (90.9%), 8 (88.8%), 12 (80%), respectively. Only *L. ivanovii* showed poor rate of correct identification: 71.4%.

All studied samples were defined and those with SD values below or equal to the threshold value of 0.5 were utilised for the exact determination of *Listeria* spp.

DISCUSSION

In general, detection, identification and classification of *Listeria* spp. are performed with traditional methods based on biochemical or serological tests. Identification of *Listeria* in laboratories needs sensitive, reproducible rapid automated and inexpensive methods as conventional culture-based methods are labour-intensive and time-consuming (Al-Mariri *et al*., 2013). Despite that the PCR method solves the problem of interpretation of classical biochemical and serological typing in only one step, the consistency of PCR detection methods relies, in part, on the clarity of the target template and the existence of sufficient numbers of target molecules. So, it is necessary to seek another method which is technologically simple, low-cost, highly specific, and reproducible with PCR results. Helm *et al*. (1991) recomposed FTIR methods for *in-situ* analysis of bacterial cells and spectral analysis to identify, differentiate, and classify bacteria.

In this study, univariate FTIR spectroscopy was used to distinguish *Listeria* species *(L. monocytogenes, L. innocua, L. ivanovii, L. gravi, L. welshimeri*). There are several interesting peaks emerging on univariate IR spectrum of bacteria, and most of them showed functional group vibrations in the main biomolecular constituents like protein, fatty acids, and carbohydrates. Naumann (2000) indicated that five main absorbance areas in IR spectra should be employed for differentation of bacteria: the 2800–3000 cm^{-1} spectral area is the fatty acid area (area I); 1700–1500 cm^{-1} comprises amide I and II bands of proteins and peptides (area II); 1200–1500cm^{-1} is a mixed area of fatty acid bending vibrations, proteins, and phosphate-carrying compounds (area III); 900–1200 cm^{-1} comprises absorption bands of carbohydrates in microbial cell walls (area IV ; and 700–900 cm^{-1} is the " fingerprint area" that comprises weak but very singular absorbance that are characteristic to specific bacteria (area V). Areas I and II are the most advantageous for routine bacterial identification. The fingerprint area is important for the distinction of microorganisms at the strain level (Wenning *et al.*, 2010). Therefore, specific recognition can be realised from spectra by focalisation on specific absorbance areas correlated to those compounds that are particular to five *Listeria* species and of diagnostic relevance to a specific pathogen (Davis & Mauer, 2011).

HCA and dendrograms were used to show the analogies between spectra of bacteria. The left vertical axis of a dendrogram describes the increasing variance or heterogeneity. The magnitude of this heterogeneity is based on the number of spectra in a cluster and the analogies between them. The five main clusters in this dendrogram conform with the five PCR strains of *Listeria* species. The subclusters within the major clusters correspond with different multilocus genotyping haplo-

types of *Listeria.* It has been noticed in this study that there was a correlation between both methods (PCR, FTIR) in distinguishing *Listeria* samples, but the FTIR method was more accurate and specific in differentiation at the species level in the neural network.

Schmitt & Udelhoven (2001) determined two networks of five *Listeria* isolates: *L. monocytogenes-L. inocuca-L. ivanovii* net and *L. welshimeri-L. gravi* net activated on the first level. These results were supported by Cecilia *et al*. (2008). PCR method was able to resolve the conflicting data in the wrong identification by univariate FTIR analysis, whereas *16S–23S* IGS regions were highly conserved, and of two different sizes: the small rRNA IGS region of *L. innocua, L. ivanovii, L. welshimeri,* and *L. grayi* showed 83–99% homology to that of *L. monocytogenes*; the large rRNA IGS region of *L. monocytogenes* demonstrated 81–96% similarity to those of non-*Listeria monocytogenes* species. On the other hand, univariate-based analysis enhanced differentiation success to 87% for all *Listeria* species, including a success rate of 91.3% for correct *L. monocytogenes* identification. Similarly, *L. innocua*, *L. gravi*, and *L. welshimeri* realised good rates of exact identification – 30 (90.9%), 8 (88.8%), 12 (80%), respectively. Only *L. ivanovii* (previously known as *L. monocytogenes* serotype 5) showed a poor rate of correct identification of 71.4%. These results agree with the results of Cecilia *et al*. (2008) on 277 isolates, where the exactly determined strains in univariate FTIR method enhanced differentiation success to 85.2% for all *Listeria* species, including a success rate of 93% for correct *L. monocytogenes* identification.

Nevertheless in this study, there were seven *L. monocytogenes* isolates which were misidentified because some true non-haemolytic *L. monocytogenes* isolates might be wrongly identified as *L. innocua* or as *L.ivanovii* on the basis of their phenotypical behaviour. Also, there were 19 of 147 (12.9%) wrongly identified *Listeria* vs all strains by this analysis, due to strains yielding an SD value equal to the threshold value of 0.5 utilised for exact differentation of *Listeria* species (Cecilia *et al*., 2008).

This result corresponds with the study of Dziuba (2011) about distinction of selected *Leuconostoc* species by means of FTIR spectroscopy. Mouwen *et al.* (2005) found that *Campylobacter coli* and *Campylobacter jejuni* strains could be identified mainly through the FTIR spectral range, 1,200 to 900 cm^{-1} and FTIR was utiliused as a method of differentation of *Coryneform* bacteria by Oberreuter *et al*. (2002). In another study, a set of well defined *Yersinia* strains from Switzerland and Germany were used to create a method for FTIR-based differentiation of *Yersinia* isolates at the species level (Kuhm *et al*., 2009). Wenning *et al*. (2010) promoted differentation systems based on FTIR spectra for species distinction of lactic acid bacteria. Also, FTIR was tested in a wide range of applications such as quantitative detection of microbial spoilage of beef and meat (Ellis *et al.*, 2002). Al-Holy *et al.* (2006) recruited FTIR along with pattern distinction techniques such as principal component analysis for identification of food-borne pathogens (*Bacillus cereus*, *Salmonella enterica*, *E. coli* and *Listeria* spp.). Application of FTIR was reported by Al-Qadiri *et al.* (2008); Soto Beltran *et al*., (2015) for detection of sublethal thermal damage in *Salmonella enterica* serotype *Typhimurium* and *L. monocytogenes*.

All previous studies supported our study in the employment of FTIR spectroscopy as a method for fast differentation of *Listeria* as well as other bacterial species (Yu & Irudayaraj, 2005). In future studies, it will be however important to process the univariate raw *Listeria* spectra for detecting the first derivative and using ANN-FTIR analysis in order to increase the ability of the FTIR system in the exact distinction of tested samples, in order to differentiate and determine the closely related species-specific subnetworks of five *Listerial* isolates at the second level, and to utilise FTIR system by best means, to make it a routine diagnostic device for bacterial analysis by the food industry, diagnostic laboratories, and public health authorities (Den Bakker *et al*., 2014).

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Correspondence:

A. Al-Mariri,
Department of Molecular Biology
and Biotechnology,
Atomic Energy Commission,
P.O. Box 6091, Damascus, Syria
tel. 963-11-213580;
fax: 963-11-6112289,
e-mail: ascientific1@aec.org.sy