Institut für Mikrobiologie

Zentralinstitut für Ernährungs-und Lebensmittelforschung Weihenstephan

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Development of a rapid identification system for *Listeria* at the species, and *Listeria monocytogenes* at the serovar level by Artificial Neural Network analysis of Fourier Transform Infrared Spectra

Cecilia A. Rebuffo-Scheer

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This paper represents chapter 3 of this thesis and constitutes the core of my research. It has been published in *Applied Environmental Microbiology*, a Journal of the American Society for Microbiology (ASM). The major part of the experimental work was performed and supervised by myself while Felix von Stetten sequenciated the strains and Jürgen Schmitt assisted me designing the ANN. I wrote the main part of the publication.

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This paper represents chapter 4 of this thesis and has been published in *Applied Environmental Microbiology*, a journal of the ASM. I carried out the experimental work and wrote the major part of the publication.

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Symbols and abbreviations

ANN	Artificial Neural Network
API-Listeria	Analytical Profile Index of Listeria
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion medium
CAMP	Christie–Atkins–Munch–Petersen test
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ELFA	Enzyme Linked Fluorescent Assay
FTIR	Fourier Transform Infrared Spectroscopy
Macro-samples	Fourier Transform Infrared macrospectroscopy method
Micro-samples	Fourier Transform Infrared microspectroscopy method
HCA	Hierarchical Cluster Analysis
iap gene	invasive associated proteins
IR	Infrared
L.	*Listeria*
LDA	Linear Discriminant Analysis
L. m.	*Listeria monocytogenes*
MIR	Mid-Infrared spectral Region
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
SLCC	Special Listeria Culture Collection
spp	species
subsp.	subspecies
thy gene	thymidylate synthase gene
TSA	Tryptone Soy Agar
WSLC	Weihenstephan Culture Collection

Preface

The genus *Listeria* consists of six different species: *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* of which *L. monocytogenes* is the species that has been involved in 99% of all human listeriosis cases caused by consumption of contaminated food products (Mead et al. 1999). The ubiquity of *Listeria* enables them to enter the food-processing environment and food chain. Their ability to grow under extreme conditions (refrigeration temperature, low pH, high salt concentration) increases the risk of food contamination. Although *Listeria* have been less frequently identified compared to other food-borne diseases they account for the majority of death of any food-borne pathogen (Lynch et al. 2006) resulting in a high mortality rate of about 30%. This makes *L. monocytogenes* a serious human pathogen (Mead et al. 1999).

Usually, the presence of any *Listeria* species in food is an indicator of poor hygiene in the food production chain and reflects the potential risk of contamination with *L. monocytogenes* strains. Therefore, during the last decade interest has grown to develop high discriminatory methods for species to be used in the food industry in order to create an effective control strategy. Although several methods have been proposed, they are still of limited potential to routine laboratories incorporating high costs, complexity and unreliable differentiation of all species.

Fourier Transform Infrared Spectroscopy (FTIR) is a physicoquemical method that fingerprints the whole microbial cells allowing their differentiation at different taxonomic levels with high-resolution power (Helm et al. 1991a). One advantage of this technique is the use of extensive reference libraries containing spectra from well-identified microbes which, combined with adequate computer data processing systems, such as Hierarchical Cluster Analysis and Artificial Neural Network (ANN), enables a rapid and semi automated identification of unknown strains.

In the present work, a classification system (*Listeria* database) based on FTIR combined with ANN which integrates the differentiation of *Listeria* at species (chapter 3) and *L. monocytogenes* at subspecies (serovar) level (chapter 4) has been established. Therefore, biochemical, microbiological, molecular biological, immunological and physicoquemical methods were applied. A detailed study and strict standardization of the parameters which influence the differentiation of *Listeria* by FTIR was carried out (chapter 2). The construction, optimization as well as the validation of the *Listeria* database is extensively described and their high potential in the routine laboratory is discussed.

Summary

Fourier Transform Infrared Spectroscopy combined with extensive reference microbial databases is advantageous for the routine identification of microorganisms compared with conventionally available methods. Although a number of studies have used FTIR spectroscopy to identify microbes, the extraction of complex pattern information from the microbial infrared spectra remains difficult when a large amount of data has to be analysed. Therefore, the successful in the identification of microorganisms using this computer-based method requires not only the use of comprehensive reference spectral databases but also effective data-processing methods capable of extracting the subtle discriminant information "encoded" in the spectra.

The present thesis comprises the application of the FTIR spectroscopy to (i) decide, by comparative analysis, between two different vibrational spectroscopic techniques, FTIR-macrospectroscopy (further on called macro-sample) and FTIR-microspectroscopy (further on called micro-sample), which of both is the most convenient method to apply for the differentiation of *Listeria* at species level; (ii) establish, optimize and validate a new classification system for *Listeria* at the species level using the pattern recognition and supervised method known as Artificial Neural Network (ANN) analysis. Moreover, their performance has been compared to univariate analysis and the standard API identification system for *Listeria*, and (iii) develop an ANN based FTIR subnet in order to additionally identify *L. monocytogenes* serogroups and serovars.

(i) The first part of this work was aimed to compare the FTIR macro- and micro-sample methods when used for the identification of *Listeria* species. For this purpose and once the growth and measurement conditions were optimized two model spectral databases, the one obtained with FTIR macro-sample and the other with the FTIR-micro-sample technique, were created using 25 well-identified strains of *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. The identification capacity at the species level of these model libraries was then evaluated at the species level in an internal validation. Results showed higher correct identification values for the macro-sample (92.8%) than for the micro-sample (79.2%) model. Moreover, the worst performance of the micro-sample model was yielded with the species *L. innocua*, *L. ivanovii* and *L. monocytogenes* in part due to the influence of the spectral heterogeneity of microcolonies as well as due to the insufficient discrimination features expressed in exponential growth cells in the microcolony. Thus, the optimal performance in respect to identification accuracy was achieved by the macro-sample model.

(ii) Based on the results from the first part of this work, FTIR- macrospectroscopy was applied for the construction of a new classification system for *Listeria* at the species level based on a comprehensive reference spectral database including 243 well-defined reference strains of *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. Two different analysis models, the multivariate ANN- and univariate-based FTIR, were developed using the same reference data set of 243 strains. After optimising the parameters for data analysis, the potential of these two models was evaluated in an external validation procedure using a data set of 277 isolates not included in the reference database. The univariate FTIR analysis model allowed a correct identification of 85.2% of all strains and 93% of the *L. monocytogenes* strains. Thereby the use of the ANN-based analysis significantly enhanced the differentiation success to 96% for all *Listeria* species including a success rate of 99.2% for *L. monocytogenes* identification. Besides this, the 277 test isolates were also identified by the standard phenotypical API *Listeria* system. This commercial kit was able to identify 88% of all strains and 93% of *L. monocytogenes* strains. These results demonstrate that ANN-based FTIR identification of *Listeria* species is the superior method. Starting from a pure culture, this technique allows the cost-efficient and rapid identification of *Listeria* species within 25 h and is suitable for the use in a routine food microbiological laboratory.

(iii) Furthermore, FTIR spectroscopy combined with ANN was also applied to design a classification system for the differentiation between 12 serovars of the pathogen *L. monocytogenes* using a database of 106 well-defined strains. The validation of the classification system was performed using a test set of 166 *L. monocytogenes* isolates. This yielded into a 98.8% correct identification rate at serogroup and a 91.6% at the serovar level. Importantly, 40 out of 41 potentially epidemic serovar 4b strains were unambiguously identified. The comparison of the FTIR- and PCR-based serovar differentiation method indicated that the former is superior for serogroup as well as for serovar determination. This part of the work clearly proves the high potential of the ANN based FTIR technique to discriminate *L. monocytogenes* to species, serogroup and serovar level. The FTIR technique may be further used as a tool to gain additional information on the pathogenic potential of isolates located in the food-processing chain.

Finally, the integration of both, the *Listeria* species and serovars classification system offers the possibility to simultaneously identify *Listeria* at species level, *L. monocytogenes* at serogroup and serovar level in a single step by simply measuring an infrared spectrum of an unknown isolate.

Zusammenfassung

Die Fourier Transform Infrarot Spektroskopie (FTIR), in Verbindung mit vollständigen mikrobiellen Referenzdatenbanken, ist für die Routineidentifizierung von Mikroorganismen den herkömmlichen Methoden überlegen.

Obwohl in einigen Studien die FTIR Methode zur Identifizierung von Mikroben verwendet wurde ist die Extrahierung komplexer Musterinformationen von mikrobiellen Infrarotspektren schwierig wenn dafür ein großer Datensatz analysiert werden muss.
Demnach benötigt es zur erfolgreichen Identifizierung von Mikroorganismen, unter Verwendung der komputerbasierten FTIR Methode, nicht nur umfangreiche Referenzdatenbanken sonder auch Datenverarbeitungsmethoden, die dazu fähig sind auch feine Informationen aus den Spektren zu extrahieren.

Thema der vorliegenden Doktorarbeit war die Anwendung der FTIR Spektroskopie um (i), unter Durchführung einer komparativen Analyse, zu analysieren welche der beiden schwingenden spektroskopischen Methoden, die FTIR-Makrospektroskopie (im Weiteren Makro-Probe genannt) oder die FTIR-Mikrospektroskopie (im Weiteren Mikro-Probe genannt), die bevorzugte Methode zur Differenzierung von *Listerien* auf Speziesebene ist sowie um (ii) ein neues Klassifizierungssystem, unter Verwendung einer *pattern recognition* Überwachungsmethode, der Künstlichen Neuronalen Netze (KNN) Analyse, für *Listerien* auf Speziesebene zu entwickeln, zu optimieren sowie zu validieren. Des Weiteren wurde die Leistungsfähigkeit der FTIR Methode, unter Verwendung der KNN Analysetechnik, mit der univariaten Analysemethode sowie der Standard API Differenzierungsmethode für *Listerien* verglichen sowie (iii) ein zusätzliches KNN, basierend auf FTIR entwickelt um *L. monocytogenes* Serogruppen sowie Serovaren zu identifizieren.
(i) Der erste Teil dieser Arbeit diente dem Vergleich der Makro- und Mikro-Probemethode, die zur Identifizierung von *Listerien* verwendet wurden. Sobald die Wachstums- und Messkonditionen erfüllt waren, wurden für diesen Zweck zwei Modelspektraldatenbanken kreiert, eine unter Verwendung der FTIR-Makro- und eine andere unter Verwendung der FTIR-Mikro-Probemethode. Bei beiden Methoden wurden 25 gut identifizierte Stämme der Arten *Listerien monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri* und *L. welshimeri* verwendet. Die Identifizierungsfähigkeit dieser Modelbibliotheken, auf Speziesebene, wurde in einer internen Validierung bestimmt. Der Vergleich der Ergebnisse erwies höhere korrekte Identifizierungswerte für die Makro- (92.8%) als für die Mikro-Probemethode (79.2%). Des Weiteren wurden die schlechtesten Ergebnisse der Mikro-Probemethode in Verbindung mit

den Spezies, *L. innocua*, *L. ivanovii* und *L. monocytogenes,* erzielt was wiederum sowol auf die starke Beeinflussung von der Kolonienheterogenität und deren Diskriminierung als auch auf die wenniger ausgeprägten Diskriminierunsmerkmale der Mikrokolonienzellen in Wachstumphase basierte. Demnach ist die Makro-Probe die leistungsstärkere Methode.

(ii) Basierend auf den Ergebnissen des ersten Teils dieser Arbeit wurde die FTIR Makrospektroskopiemethode angewendet um ein Klassifizierungssystem für *Listerien* auf Speziesebene zu entwerfen. Grundlage dessen wurde eine komplexe Spektraldatenbank, welche 243 gut definierte Stämme von *Listerien monocytogenes, L. innocua, L. ivanovii, L. seeligeri* sowie *L. welshimeri* enthielt. Zwei Modelle, das multivariate KNN sowie das univariate FTIR Model, wurden unter Anwendung des gleichen Referenzdatensatzes von 243 Stämmen, entwickelt. Nachdem die Parameter für die Datenanalyse optimiert waren wurde das Potential beider Modelle bewertet. Dies geschah in Form eines externen Validierungsverfahrens unter Anwendung eines Datensatzes von 277 Isolaten, welche nicht Bestandteil der Referenzdatenbank waren. Das univariate FTIR Analysemodel ermöglichte eine Identifizierung von 85,2% aller Stämme sowie 93% aller *L. monocytogenes* Stämme. Unter Anwendung der KNN verbesserte sich die Differenzierungsrate beträchtlich auf 96% aller Listerienarten. Die Identifizierungsrate bei den *L. monocytogenes* Stämmen erhöhte sich sogar auf 99,2%. Die 277 Testisolate wurden ebenfalls von dem standardphänotypischen API Listerien System identifiziert, welches 88% aller Stämme sowie 93% aller *L. monocytogenes* Stämme identifizierte. Anhand dieser Ergebnisse zeigte sich, dass die KNN, basierend auf FTIR, die überlegende Methode zur Identifizierung von *Listerien* ist. Diese Methode ermöglicht eine kostengünstige- sowie schnelle Identifizierung von *Listerien*, innerhalb von 25 h basierend auf Reinkulturen, und ist demnach zur Anwendung in der Routine von Lebensmittelmikrobiologielabors geeignet.

(iii) Des Weiteren wurde die FTIR Spektroskopie, in Verbindung mit KNN, auch für die Gestaltung eines Klassifizierungssystems zur Differenzierung zwischen 12 Serovaren der pathogenen *L. monocytogenes* verwendet. Diese basierten auf eine Datenbank von 106, gut definierten, Stämme. Die Validierung des Klassifizierungssystems wurde unter Anwendung eines Testsets bestehend aus 166 *L. monocytogenes* Isolaten durchgeführt. Diese ergab eine korrekte Identifizierung von 98,8% auf Serogruppen- sowie 91,6% auf Serovarebene. Hierbei gilt es zu unterstreichen, dass von 41 potentiel epidemienverursachender Stämme der Serovar 4b 40 Stämme richtig identifiziert wurden. Der Vergleich der FTIR- mit der PCR Methode zeigte, dass die FTIR Methode der PCR Methode sowie in der Identifizierung der Serogruppen als auch der Serovaren überlegen ist. Dieser Teil der Arbeit bestätigt das hohe

Potential der KNN, basierend auf FTIR, zur Identifizierung von *L. monocytogenes* auf Spezies-, Serogruppen- und Serovarebene. Eine weitere Anwendung könnte die FTIR Methode in der Lebensmittelindustrie finden wo sie als Instrument zur Identifizierung pathogener Isolate dienen kann.

Basierend auf der Messung eines einzelnen Spektrums der unbekannten Isolate bietet die Integration des *Listerien* Spezies- und des Serovarklassifizierungssystems die Möglichkeit, zur gleichen Zeit, in einem Vorgang, *Listerien* auf Speziesebene sowie *L. monocytogenes* auf Serogruppen- und Serovarebene zu identifizieren.

1 General Introduction

1.1 General characteristics of the genus *Listeria*

The genus *Listeria* comprises six species: *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. grayi* (Rocourt 1999). Two subspecies of *L. ivanovii* have been described: *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis* (Rocourt 1999). *L. murrayi*, which was once a separate species in the genus *Listeria*, is now included in the species *L. grayi* (Rocourt et al. 1992). All of these species are widespread in the environment though only *L. monocytogenes* is considered to be a significant human and animal pathogen. In very rare cases human infections have been reported due to *L. ivanovii* and *L. seeligeri* (Rocourt 1986, Snapir et al. 2006).

Listeria species are phenotypical and genetically closely related bacteria which make their differentiation very difficult. This drawback reflects a potential risk for persistent contamination of processing facilities with the pathogen *L. monocytogenes* since it can withstand a variety of environmental stresses including low temperatures (4°C) as well as extreme pH and osmolarity conditions.

Moreover, *L. monocytogenes* has recently been associated with a wide variety of foods (e.g. hot dogs (Donnelly 2001), fresh meats and fish, raw vegetables, diary products, including cheeses, butter, and milk (Jemmi and Stephan 2006).

1.2 Differentiation of *Listeria* at species level and detection of the human pathogen *L .monocytogenes*

L. monocytogenes is a significant public health threat and among all pathogens the bacterium responsible for the majority of death and food recalls as reported by the Centre of Disease Control in Atlanta last year covering all foodborne-disease outbreaks in the United States (Lynch et al. 2006). The disease caused by this bacterium, which is named listeriosis, is acquired by ingesting contaminated food products and does mainly affect immunocrompromised persons, pregnant women and newborns. The ability of the bacterium to grow under extreme conditions increases the risk of food contamination. Moreover, *L. monocytogenes* has recently received increasing attention not only due to large–scale outbreaks associated with a wide variety of foods, but also due to the increasing occurrence of international foodborne disease outbreaks related to world-wide distribution of long-life products raising new microbial safety problems and demands. Consequently, the monitoring

of food-production at all levels will contribute to detect potentially pathogenic strains and will therefore lead to decreased contamination in the food supply.

Many of the official methods used to identify *Listeria* are regulated by international agencies, such as the Food and Drug Administration (FDA), cannot differentiate between *Listeria* species. The lack of this capacity is critical in particular from the perspective of food hygiene since the presence of non-pathogenic species has frequently been found to be a marker of a subsequent *L. monocytogenes* contamination in diary plants (Loessner 2002, Rudolf and Scherer 2001). Furthermore, an unreliable control of *Listeria* may produce a risk of persistent contamination in the food processing environment for an extended period of time (Tompkin 2002). Therefore, a rapid and reliable differentiation of *L. monocytogenes* from the other species of the genus is necessary for the food industry in order to adopt an effective quality assurance strategy.

A comprehensive review of the methods developed in the last two decades for the identification and typing of *Listeria* and *L. monocytogenes* in food and environmental samples is summarized in Table 1.1. This table shows that expensive, time-consuming and laborious biochemical and phenotypical standard methods, i.e. the sugar fermentation and CAMP test or commercial systems which are unable to differentiate between species, such as chromogenic media, are often used. The review also shows that rapid molecular and immunological procedures have been developed, though they are limited to the detection of the genus or the pathogen *L. monocytogenes* only. Furthermore, sensitive microarrays have been proposed. However, they are of limited potential for routine laboratories due to high costs.

Table 1.1 Literature overview: Comparison of identification methods for food, environmental, and clinical for *Listeria* spp.

Method	Sensitivity (%)[a]	Level of identification	Cost/test	Labour	Enrichment time (h)	Identification time[b]	Auto-mation	Sample Types	Reference
Sugar fermentation and CAMP test	≤ 90	Differentiation of *Listeria* species	$1- $2	High	~ 48	1-2 days	No	Food and environmental samples	(Jones 1992) (Vazquez-Boland et al. 1990)
Chromo-genic media	99.2	*L. monocytogenes*. No differentiation of other species	$1- $3	Medium	24-30	1-2 days	No	Variety of foods and environmental samples	(Jinneman et al. 2003) (Hegde et al. 2007)
Immuno-assay (e.g. ELISA, ELFA)	92 98.1	Genus *Listeria* Differentiation of *L. monocytogenes*	$6- $10	Low-medium	48	1-2 h 52 h	Yes	Food and environmental samples	(Gangar et al. 2000) (Sewell et al. 2003)
Molecular (e.g. DNA hybridization), PCR	100	Differentiation of *L. monocytogenes* Differentiation of *Listeria* species	$10	Low	40- 48	2-4 h	Yes	Food samples Food and environmental samples	(Duvall and Hitchins 1997) (Norton 2002)
Microarray	100	Differentiation of *Listeria* species	≥ $10	Low	-	1h	Yes	Food and environmental samples	(Volokhov et al. 2002)
FTIR	96 99.2	Differentiation of *Listeria* species Differentiation of *L. monocytogenes*	$1- $2	Low	24- 48	25 h	Yes	Food and environmental samples	(Rebuffo et al. 2006) (this study)

[a] is calculated as TP/(TP + FN); TP: true positives; FN: false negatives.

[b] approximate time it takes to perform the test excluding enrichment times.

1.3 **Serovars of *Listeria***

The first studies on the chemical structure of the *Listeria* cell walls undertaken by Ullmann and Cameron (1969) revealed the presence of specific antigenic structures (O- and H-antigens) (Ullmann and Cameron 1969) which were later biochemically described by Fiedler et al. (Fiedler 1984). More recent studies found out that the O antigenic structures are defined by the nature and quantitative ratios of sugars substituents of the teichoic acid. Thus, based on the serological properties of these O antigens, six different serogroups (1/2, 3, 4, 5, 6, and 7) could be assigned to *Listeria* (Fiedler 1988). Additionally, *Listeria* species express flagellar (H) antigens which by its unique combination with somatic O antigens determine the serovar (serotype) of individual strains. Therefore, 15 different serovars have been identified for all species by serological typing using specific and standardized sera. A total of 15 O-antigens (subtypes I-XV) and H-antigens (subtypes A-D) were described by Seeliger & Jones, 1986 (table 1.2). In *L. monocytogenes* 13 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7) have been recognized, 7 serotypes (1/2a, 1/2b, 3b, 4a, 4b, 4c, and 6b) in *L. seeligeri*, 3 serotypes (1/2b, 6a, and 6b) in *L. innocua* and *L. welshimeri* and one in *L. ivanovii* (i.e. 5) (Seeliger and Jones 1986).

Table 1.2 Compositions of somatic (O) and flagellar (H) antigens in *Listeria* serotypes (based on Seeliger & Jones, 1986)

Serotype	O antigens	H antigens
1/2a	I, II	A, B
1/2b	I, II	A, B, C
1/2c	I, II	B, D
3a	II, IV	A, B
3b	II, IV	A, B, C
3c	II, IV	B, D
4a	(V), VI, IX	A, B, C
4b	V, VI	A, B, C
4c	V, VII	A, B, C
4d	(V), VI, VIII	A, B, C
4e	V, VI, (VIII), (IX)	A, B, C
7	XII, XIII	A, B, C
5	(V), VI, (VIII), X	A, B, C
6a	V, (VI), (VII), (IX), XV	A, B, C
6b	(V), (VI), (VII), IX, X, XI	A, B, C

1.4 Serovar differentiation of *L. monocytogenes* strains

We note, as mentioned above, that serotypes are shared among *Listeria* species and therefore can not be used to differentiate between species. Besides the identification of *Listeria* species, the serovar differentiation is particularly useful to determine the prevalence of specific serotypes in epidemiological studies and for tracking the source of contaminations. Current serotyping of *L. monocytogenes* in the routine laboratory using the methods available is quite rare because of the limited availability of commercial systems and the costs their use inherits (Seeliger and Langer 1979) as well as the time needed for their application (Palumbo et al. 2003). Furthermore, many PCR-based methods have been developed, however, they are limited to differentiate to the serogroup level (Comi et al. 1997, Doumith et al. 2004a, Jinneman and Hill 2001, Manzano et al. 1998), or only differentiate few serovars (Zhang and Knabel 2005). The implementation of a rapid and inexpensive serotyping method will allow a more accurate survey of foods and food processing facilities for the presence of potential pathogenic serovars of *L. monocytogenes*.

It is known that the repertoire of strains capable of contaminating food is wider than that of strains recovered from patients with listeriosis. On the other hand, it has been observed that most clinical isolates belong to serovars 1/2a, 1/2b and 4b. Among these, the majority of strains which have caused large outbreaks are serovar 4b (Kathariou 2000). In contrast, most food strains belong to serovar 1/2c (Jacquet et al. 2002, Yildirim et al. 2004). This controversy in the distribution of the serovars in clinical and food isolates arouses the interest to study their origin. Thus it is especially interesting that all serotypes posses the same virulence factors and hence have the same potential to cause diseases which suggests that there may exist a variation in the regulation of expression among serotypes (Kathariou 2002). Several findings support this opinion such as the existence of two different forms of a cell-surface protein internalin in clinical and food serovars that determine biomarkers for pathogenic potential (Jacquet et al. 2004), as well as the expression of specific genetic markers by epidemic serovar 4b clones which confer special virulence attributes to these strains involved in food contamination (Liu et al. 2006, Yildirim et al. 2004).

Although a lot of studies about the virulence of *L. monocytogenes* have contributed to a better understanding of its pathogenesis, they have not sufficiently elucidated the virulence related with food safety. Furthermore, at this time, limited information exists concerning the virulence of *L. monocytogenes* since only a few well-known strains of serovar 1/2a and 4b have been studied. Therefore, in order to discover a potential relationship between serovars

and pathogenic potential, new differentiation methods for serovars will help to develop science-based approaches to regulate the presence of *L. monocytogenes* in food.

Thus, the serovar differentiation of the species *L. monocytogenes* implicated in major food-borne listeriosis outbreaks is not only necessary to confirm sources of outbreaks and monitor reservoirs of potentially epidemic strains, but also to gain additional knowledge regarding the relation between the serovar and pathogenic potential in *Listeria monocytogenes* strains.

1.5 **FTIR spectroscopy of microorganisms**

The use of infrared spectroscopy for the identification of microorganisms was first suggested in the 1950's by the groups of Bordner and Thomas (Bordner et al. 1956, Thomas and Greenstreet 1954). However, due to technical limitations at that time the method could only be implemented in the 90ies when Naumann and his co-workers introduced the Fourier Transform Infrared Spectroscopy (FTIR) to the field of microbiology (Naumann et al. 1991).
FTIR spectroscopy is used to characterize microorganisms based on measurements of their whole chemical composition (Naumann et al. 1991, Naumann et al. 1996). Considering microorganisms as complex chemical systems, the FTIR spectroscopy uses the vibrational spectroscopic properties of all cellular compounds to obtain their chemical fingerprint. Indeed, the characteristic absorption of energy by the different constituents of microbial cells determines their characteristic spectrum when they are irradiated with infrared light. This infrared spectrum shows a complex pattern of band contours which represent the fingerprint of the bacterium. In this study, we use the mid-infrared spectral region (MIR) of the electromagnetic spectrum which covers the range between 4,000-500 cm^{-1}. For an easier interpretation of the infrared spectra of complex biological materials the group of Naumann defined in the MIR range five spectral regions dominated by different functional groups. Thus, for the major cellular components (nucleic acids, proteins, carbohydrates, and lipids) specific spectral regions were assigned (Helm et al. 1991a).

All phenotype-based method may suffer by the fact that the expression of some cellular constituents often varies with changing external conditions. Therefore, the characterization of microbial cells by FTIR spectroscopy requires the establishment of a standardized protocol for cultivation, sampling procedure and sample preparation, as well as the determination of data acquisition and evaluation in order to obtain reliable and reproducible results (Naumann 2001).

FTIR spectroscopy has frequently been applied for differentiations at various taxonomic levels of microorganisms. Nevertheless, in many of such studies the inclusion of few strains, covering only part of biodiversity within a species, lead to insufficient separation between the different groups i.e., the differentiation of *Listeria* species was not always possible (Holt et al. 1995, Lefier et al. 1997), or the differentiation of all serovars in *Escherichia coli* and *Salmonella enterica* was not resolved (Helm et al. 1991a, Kim et al. 2005). However, the identification of microorganisms by this method is advantageous since it allows the application of large spectral libraries of well-characterized microorganisms. For instance, for identifications at the species level a good representation of the intra-group (intra-species) microbiodiversity increases the inter-group (inter-species) separation and thus enhances the species differentiation (Helm et al. 1991b, Kümmerle et al. 1998, Maquelin et al. 2003), (Oberreuter et al. 2002a). Therefore, the creation of a comprehensive and universal reference database for all species and serovars of *Listeria* will allow reproducible identification results in routine analysis.

1.6 **Methods of analysis of the FTIR data of microorganisms**

The collection of thousands of spectra from different microorganisms needed for the construction of a comprehensive and valid database requires the use of appropriated statistical analysis methods for their analysis and interpretation. Chemometrics are mathematical, statistical or graphical methods used to improve the understanding of chemical information. These methods extract, in an efficient way, unique and redundant information from complex data such as spectra. There are two different categories of chemometric procedures. The first, the so called univariate statistical analysis, considers only a single property of a spectrum (e.g. a single intensity at a given wave number) leaving a wealth of information stored in the spectra unused whereas the second method, the multivariate statistical analysis method, is more advantageous since several properties of the spectra can be evaluated at the same time maximising thereby the information extracted. This pattern recognition technique aims at the classification of pattern based on either *a priori* knowledge or on statistical information extracted from the patterns. Among these techniques unsupervised and supervised learning methods are of particular interest when IR spectra of microorganisms have to be analized (Schmitt and Udelhoven 2001).

Unsupervised methods provide grouping schemes within complex data sets indicating similarity or relationships between the members of the group without the need to assign *a priori* the identity of the data. In this category of techinques Hierarchical Cluster Analysis

(HCA) and Principal Component Analysis (PCA) are included which are frequently used for classification problems. Since the HCA was used in this work it needs further explaination.

HCA: This method groups spectra in clusters based on their similarity. Their results are represented by a hierarchical tree called *dendrogram*. The distances between the spectra in a cluster as well as between the different clusters in a dendrogram are depending on the method or algorithm used for the cluster analysis. In this work the following methods were used:

Average linkage: In this method the distance between two clusters is calculated as the average distance between all pairs of objects in these two different clusters (Sneath and Sokal 1973).

Ward's algorithm: This method is distinctive from the previous one because it uses an analysis of variance approach to evaluate the distances between clusters. In short, this method attempts to minimize the Sum of Squares (SS) of any two (hypothetical) clusters that can be formed at each step (Ward 1963).

In contrast, supervised methods use a data set of well-known data for the establishment of a model which then permits the assignment of the identity of the unknown data. In this category of techniques are included the Linear Discriminant Analysis (LDA) and the Artificial Neural Network (ANN) (Naumann 2001). The ANN was used in this work and is therefore further explained here below.

Artificial Neural Networks: are data-processing methods based on an analogy with the structure and function of the brain. They are built from simple units, called *neurons*, which are linked by connections called *synapses*. Each neuron corresponds to a pattern that we want to analyse and different neurons are placed in different layers which are connected to each other by synapses forming the networks. The information to be analysed, called *inputs*, flows from the neurons of the first layer to the others in the second layer and so on until the neurons in the last layer. Finally, the inputs will be converted in *outputs* as a result of this processing.

The goal of the network is to learn or to find the structure of the classification system to be developed by repetitive exposure to data, estimation of output error and subsequent feedback. When a desired output is known the difference between the actual input and the desired output will be used as error signal for the so called *supervised* learning process. This learning process is made by iterative changes of the values of the neuronal network parameters. For supervised learning the most widely known algorithm is the

backpropagation error and its improved form, the *resilient propagation (Rprop)*, which was used in this work (Riedmiller and Braun 1993).

The pattern of connectivity or architecture of the network reflects their complexity and behaviour. The optimal network architecture has to be established empirically and their accuracy must be validated using an independent data set. In this work two and four layers were needed to design the optimal schemes for the reliable classification of *Listeria* to species and serovar level respectively.

The combination of FTIR spectroscopy with computer-based data evaluation is particularly required for an effective extraction of the information contained in the spectra. Unsupervised methods such as HCA are insufficient for the complete information extraction when a great amount of data has to be analysed. Therefore, advanced supervised methods such as ANN are definitely required to improve the discrimination capacity since they present a great potential to handle multiclass problems. Thus, the ability of ANN to classify infrared spectra of biological samples provides a means to improve the identification of unknown samples.

Aim and achievement of the work

The application of FTIR spectroscopy for the identification of microorganisms in the routine microbiological laboratory has become important during the last years since large spectral databases for the identification of microorganisms were constructed. Even though these libraries constitute a better alternative than conventionally available methods, the optimal extraction of the huge amount of information contained in the spectra is generally not possible when univariate methods are used for analysis.

The main intention of this PhD work was to assess whether the application of advanced supervised methods of data-analysis, such as the Artificial Neural Network (ANN), improve the discrimination capacity of the FT-IR technique. For this purpose, the following had to be investigated:

- first of all the adequate IR spectroscopic technique had to be defined and optimized for the classification of *Listeria* species.

  In order to achieve this, the results obtained by the application of both the FTIR macro- and –micro-sample methods should be compared. In this context the standardization of the sampling procedure and sample preparation for both methods, a detailed analysis of the factors which influence the success in the identification, namely the addition of more strains per species, the colony heterogeneity as well as age of the culture, among others, had to be carried out.

- Thereafter, (i) in order to evaluate the success in the differentiation of *Listeria* at species level on the basis of their spectral differences two different data-analysis methods had to be used.

  Having in mind these aims, univariate and the multivariate classification models had to be established for species classification using the same data set of *Listeria* reference strains. Further, by using a large set of independent strains not contained in the reference database, it had to be evaluated whether an improvement in the identification of *Listeria* species, and especially of the pathogen *L. monocytogenes*, could be achieved when multivariate ANN methods are applied instead of univariate methods.

  (ii) Furthermore, the identification capacity of the ANN had to be compared with already established and officially recognised methods for routine identification of *Listeria*.

Specifically, the superiority of ANN based FTIR methods should be compared with the API *Listeria* identification system based on physiological characteristics and which is widely used in routine microbiological laboratories.

- Thereafter, the established FTIR based ANN classification method for *Listeria* species had to be extended to allow further the subspecies differentiation of the pathogen *L. monocytogenes* at the serovar level.

To achieve this aim ANN-based classification models had to be established for sub-species typing of *Listeria monocytogenes* that allow the accurate detection of potentially pathogenic strains and provide insights into the pathogenic potential of strains isolated from the food processing chain.

2 Identification of five *Listeria* species based on infrared spectra (FTIR) using macro-samples is superior over a micro-sample approach

2.1 Summary

Based on infrared spectra (FTIR), microorganisms can be identified by using both macro-samples and micro-samples. This work is aimed to compare identification of the five closely related *Listeria* species *L. innocua, L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri* by both methods. The overall identification success for 25 strains was 92.8% for the former and 79.2% for the latter method, respectively. The worst performance of the micro-samples method was found for *L. innocua, L. ivanovii*, and *L. monocytogenes,* while *L. seeligeri* and *L. welshimeri* did not show significant differences between the techniques. Identification success was mainly influenced by the age of cells and spatial heterogeneity of the microcolonies as analyzed by the micro-samples method. Spectra of *Listeria* cells near stationary phase exhibited more species-specific markers and, therefore, allowed for a better discrimination than the spectra of growing cells. Furthermore, heterogeneity of cell composition at different locations in microcolonies of *L. innocua, L. ivanovii* and *L. monocytogenes* resulted in a limited discrimination success of the micro-samples method. We conclude that, at least in case of *Listeria*, the macro-samples method is superior over the micro-samples method, although the latter is the faster technique.

2.2 Introduction

The accurate differentiation of *Listeria* species in general and *Listeria monocytogenes* in particular, which is a human foodborne pathogen (Gasanov et al. 2005, Liu 2006), has become an important task for the food industry and public health authorities (Kathariou 2002, Rudolf and Scherer 2001). The differentiation of microorganisms in routine laboratories requires sensitive, reproducible, rapid, automated and inexpensive methods. Although many phenotypic and genetic methods have been developed (Liu 2006) few have so far met all of the above-mentioned criteria.

Naumann and coworkers introduced Fourier Transform Infrared Spectroscopy (FTIR) to microbiology (Naumann et al. 1991). Since then, various contributions have been published, aiming to improve FTIR based identification of microbes [summarized in (Maquelin et al. 2002, Naumann et al. 1991, Wenning et al. 2007)]. Among them, FTIR macrospectroscopy (called macro-sample method in this paper) is already used in routine laboratories. This

method uses infrared spectra of dried films of pure microorganism cultures, which are carried by sample wheels or on a microtiter plate format for measurement of the spectra. These are then identified by comparison with extensive reference libraries, containing spectra from a large variety of well-defined microbes. This method allows the identification of microbes in only 25 hours using a suspension of cells scraped off from a confluent lawn of a pure culture (Helm et al. 1991, Kümmerle et al. 1998, Oberreuter et al. 2002b). Recently, we applied this technique to develop a superior artificial neural network for the reliable identification of *Listeria* species, even down to the serovar level (Rebuffo et al. 2006, Rebuffo-Scheer et al. 2007b).

Another, promising FTIR technique is FTIR microspectroscopy (called micro-sample method in this paper) which combines FTIR spectroscopy with microscopy, thus allowing direct measurement of spectra of small microcolonies obtained by a replica stamping technique directly from agar plates after dilution plating. One of the major advantages of this approach is that the identification time can be significantly reduced, thus allowing the identification of clinically relevant bacteria after 6- 10 h (Choo-Smith et al. 2001, Ngo-Thi et al. 2003, Sandt et al. 2006) and yeasts after 10 h (Essendoubi et al. 2005, Ngo-Thi et al. 2003), instead of 24 h needed for the macro-sample technique. More important, this method can differentiate microorganisms without the need to produce pure cultures (Maquelin et al. 2003, Wenning et al. 2002, Wenning et al. 2006), thus further speeding up identification. Moreover, identification of bacteria down to the subspecies level can be achieved by the micro-sample method (Kirschner et al. 2001, Rebuffo-Scheer et al. 2007a). However, there are also reports which state that differentiation of some species of *Staphylococcus* and *Pseudomonas* could not be achieved by the micro-sample method (Sandt et al. 2006).

Until now, limited data are available concerning the factors which influence the results obtained by both methods and no comparison of the performance of both methods has been published. Therefore, we decided to compare the macro-sample and the micro-sample methods in order to evaluate which approach is more suitable for the identification of *Listeria* species. Two spectral databases were compiled and their identification capacity to the species level was compared. Additionally, a systematic analysis of the factors which influence the identification capacity was carried out including the heterogeneity of microcolony growth and the age of the cells. We suggest that the macro-sample technique is more reliable for identification of the closely related species of *Listeria* than the micro-sample technique.

2.3 Materials and methods

2.3.1 Bacterial strains

A collection of 25 well-identified strains belonging to *Listeria monocytogenes*, *L. innocua, L. ivanovii*, *L. seeligeri* and *L. welshimeri* was used. Each species was represented by five strains from the American Type Culture Collection, the Special Listeria Culture Collection and the Weihenstephan Culture Collection as summarized in Table 1.

Table 2.1 *Listeria* strains included in this study

***Listeria* strains**	**Reference Strains**	**WSLC[(1)]**
L. innocua		2214
L. innocua	ATCC 33090; SLCC 3379	2011
L. innocua	-	2390
L. innocua	-	2394
L. innocua	-	2521
L. ivanovii	SLCC 3773	3062
L. ivanovii	SLCC 5755	30163
L. ivanovii	SLCC 8431	30166
L. ivanovii	SLCC 7927	30168
L. ivanovii	SLCC 6965	30160
L. monocytogenes	-	1266
L. monocytogenes	-	1285
L. monocytogenes	-	1303
L. monocytogenes	-	1361
L. monocytogenes	-	1118
L. seeligeri	SLCC 8624	40128
L. seeligeri	SLCC 8610	40136
L. seeligeri	SLCC 8598	40134
L. seeligeri	SLCC 8621	40138
L. seeligeri	SLCC 8604	40127
L. welshimeri	-	5891
L. welshimeri	SLCC 5828	50150
L. welshimeri	-	5917
L. welshimeri	ATCC 35897; SLCC 5334	5013
L. welshimeri	SLCC 7625	50148

ATCC American Type Culture Collection; SLCC Special Listeria Culture Collection

[(1)] WSLC Weihenstephan Culture Collection

2.3.2 **Sample preparation and growth conditions**

In this study all *Listeria* strains were grown on tryptone soy agar (TSA) culture media for both the infrared-macro and –micro measurements by optimizing the temperature and growth time.

For the macro-sample method the standardized parameters 24 h and 30°C were applied. The sample preparation was performed as described elsewhere (Rebuffo et al. 2006). Per measurement, approximately 10^9 cells were used which is equivalent of approximately 1 mg wet weight. For the infrared micro-sample measurements, one full platinum loop of bacteria cells was suspended in 100 µl sterile water and then used for a dilution series down to 10^{-4}. 100 µl of this dilution were plated onto TSA agar (Merck, Germany) using a Drigalski spatula to allow single colony growth for 18 h at 25°C. These growth conditions were established for all *Listeria* species to obtain microcolonies with adequate biomass for reproducible measurements as described previously for strains of *Mycobacteria sp*. with different generation times (Rebuffo-Scheer et al. 2007a). These microcolonies were transferred by replica stamping from the agar plate onto an infrared transparent ZnSe carrier and air dried for 15 min for the microscopic measurements. A microcolony of 120 µm diameter contains roughly 10^5 cells which is equivalent to approximately 10^{-4} mg wet weight.

2.3.3 **Recording of spectra and data evaluation**

Data acquisition and evaluation of the macro-sample method were performed as described previously (Rebuffo et al. 2006). For the micro-sample measurements microcolonies of 80 to 180 µm in diameter were used to collect the IR-spectra with an IRscope II coupled to an IFS 28B spectrometer (Bruker Optics, Karlsruhe, Germany) in the spectral region between 700 and 4,000 cm^{-1}. These measurements were performed in transmission mode using the following parameters: 6 cm^{-1} resolution, zero-filling factor of 4 and Blackmann-Harris-3 Term apodization, 15x Cassegrain-objective and 128 interferograms which were averaged for each spectrum.

All strains were stamped three times from independent bacterial cultures. From each of these replicas 10 microcolonies were measured. Evaluation and processing of the spectral data were performed using the software OPUS 4.2 (Bruker Optics, Germany) as described previously for the macro-sample measurements (Rebuffo et al. 2006). Prior to data processing and in order to ensure an adequate quality of the spectra for identification, values of minimum and maximum absorbance as well as values of noise and water vapor content of the spectra were determined for each spectrum.

To establish the micro- and macro-sample databases, ten independent spectra measurements, obtained for each of the strains were included in each library (Rebuffo et al. 2006). First derivatives of the spectra and spectral regions from 900 to 1,200, 1,250 to 1,650 and 2,830 to 3,030 cm^{-1} were used. Afterwards, the identification performance of the two libraries was evaluated in an internal validation process.

In an internal validation process the capacity to correctly identify a given model can be evaluated. The so called "leave-one-out" method was used in this work. To apply this method to the macro- and micro-samples, we had to build various spectral libraries. When one spectrum was left out from the library containing n spectra, this spectrum was used to test against the new library comprising n-1 spectra. This process was repeated in a way that each spectrum in the library was used once as the validation data. The results of this validation procedure were expressed in terms of identification capacity of the model. Thus, the results for correct identification were determined at the species level in the following spectral windows: 900-1,200, 1,250-1,650 and 2,830-3,030 cm^{-1} using a cut-off value for the spectral distance SD < 0.5 (using a repro-level of 30). This implies that the SD value between a left out spectrum and the spectra comprised in the library must be less than 0.5 to be counted as a correct identification at the species level. Finally, the results of identification of all spectra tested were averaged for strains belonging to the same species in order to give the correct identification at species level.

2.3.4 **Heterogeneity of the microcolonies**

Heterogeneity within the microcolonies was measured by linear mapping across the microcolony imprints using a PC-controlled x, y-stage with 10 µm steps in x and y direction. At each marked position on the colony imprints the spectra were collected with a resolution of 6 cm^{-1}, aperture size of 30 µm, and 128 scans. First derivative of the spectra from each measured position was calculated and subjected to cluster analysis using the average linkage algorithm and scaling to first range method in the spectral region between 800 and 1,800 cm^{-1} to calculate the spectral distance (SD) value. This value determines the spectral heterogeneity of the microcolony.

2.3.5 **Variation of the number of strains per species in the library**

A total of twelve spectral libraries containing different strain sets of the five *Listeria* species were compiled for both the macro- and micro-sample technique. When constructing three libraries, by using the micro-sample method, we did start with 10 different strains per species

followed by three consecutive additional sets of 10 strains per species, in order to finally arrive at 40 strains per species. This allowed the construction of 12 different libraries containing different strain combinations. The same has been done by using the macro-sample method. Then, each of these libraries was internally evaluated as described in the above data evaluation paragraph. The results of these internal validations were then averaged for each strain set separately.

2.4 **Results and discussion**

Figure 2.1 shows typical first derivative of infrared macro-sample measurements of the five *Listeria* species included in this study. The most relevant differences observed between the five species are displayed. As can be seen, the polysaccharide region between 900 and 1,200 cm^{-1} exhibits the most important differences among the *Listeria* species.

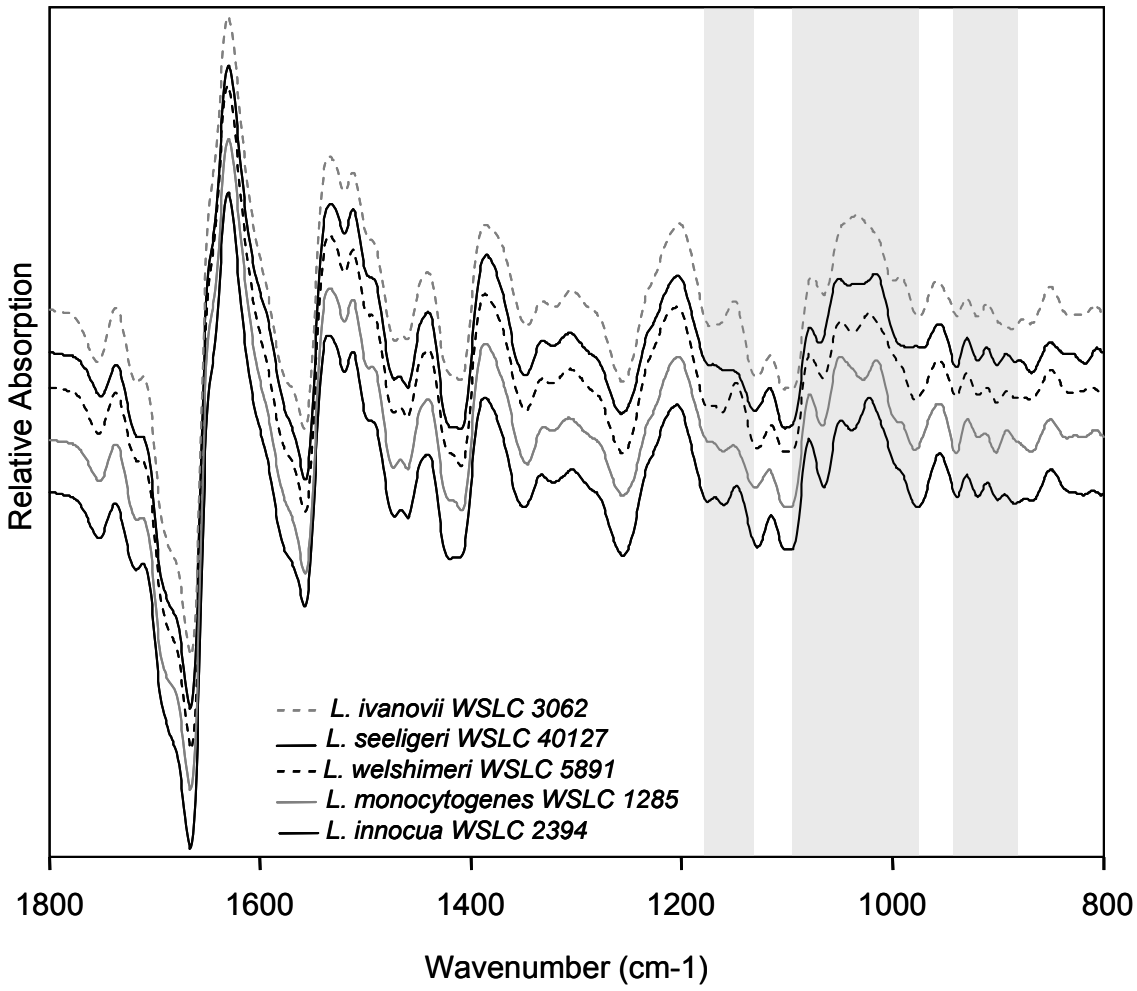

Fig. 2.1 Typical first derivative spectra of five *Listeria* species: *L. ivanovii WSLC 3062, L. seeligeri WSLC 40127, L. welshimeri WSLC 5891, L. monocytogenes WSLC 1285, and L. innocua WSLC 2394*

measured with macro-samples. The most important spectral differences found between the species are highlighted in the polysaccharide region between 900 and 1,200 cm^{-1}.

Identification accuracy at the species level by both methods is shown in table 2, based on an internal validation assay as described in the material and methods section. A higher identification rate was achieved with the macro-sample (92.8%) to the micro-sample (79.2%) method. The worst performance of the micro-sample method was found for *L. innocua* (72%), *L. ivanovii* (64%) and *L. monocytogenes* (80%) as compared to the species *L. seeligeri* (100%) and *L. welshimeri* (88%). Based on these differences and considering the fact that a standardized protocol for cultivation, conditions of measurement, and data interpretation was used throughout, we suggest that biological factors such as growth phase or colony heterogeneity may influence identification success.

Table 2.2 Correct identification of *Listeria* species by FTIR micro- and macro- sample methods

Species	FTIR-macro Correct identification*	FTIR-micro Correct identification*
L. innocua	100%	72%
L. ivanovii	100%	64%
L. monocytogenes	88%	80%
L. seeligeri	92%	100%
L. welshimeri	84%	88%

* Values have been calculated based on an internal validation procedure as described in the material and methods section.

The reproducibility of the macro- and micro-sample measurements was studied for each strain by using ten independent measurements carried out for each strain under standard conditions over a few weeks. The first derivative of the average (μ) and the average ± standard deviation ($\mu \pm \sigma$) spectra as well as their normalization were calculated for all strains using the software OPUS 4.2 (Bruker Optics, Germany) in order to highlight the spectral variances. These variances are displayed in Fig. 2.2 for some *Listeria* strains in the spectral region from 900 to 1,680 cm^{-1} where the most evident spectral variances were observed.

When the macro-sample measurements were analysed in the whole infrared spectra (from 700 to 4,000 cm^{-1}) very slightly or even no differences were found between the μ spectrum and the $\mu \pm \sigma$ spectrum for the same strain. These results were achieved for macro-sample measurements of all *Listeria* strains and indicate a high spectral reproducibility which is illustrated in the Fig. 2.2.

On the other hand, the reproducibility of the micro-sample measurements was found to be different in the different species of *Listeria*. Reproducibility of measurements in *L. innocua* and *L. monocytogenes* was dependent on the strain and the spectral region analysed. In Fig. 2.2, for example, strain *L. innocua WSLC 2214* presents less spectral variance than strain *L. innocua WSLC 2394*. Furthermore, the reproducibility of the last strain varies with the spectral region analysed. This was also found for *L. monocytogenes* strains, whereas *L. ivanovii* strains exhibit, in general, less reproducibility irrespectively of the strain. Conversely, all *L. seeligeri* and *L. welshimeri* strains present the same good spectral reproducibility of the micro-sample measurements as the strain *L. seeligeri WSLC 40136* which is shown in Fig. 2.2.

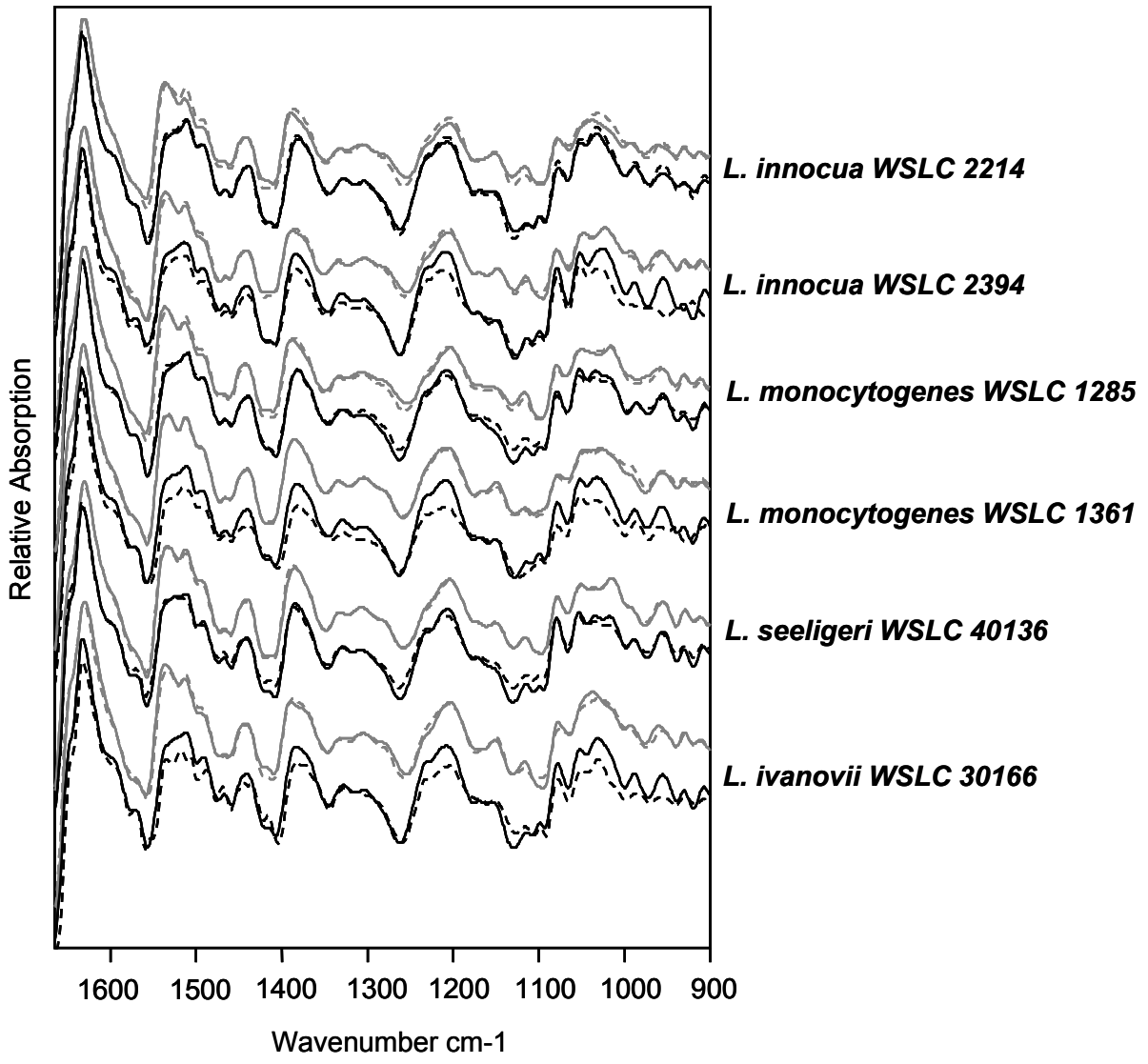

Fig. 2.2 First derivative of the average spectra (μ) (solid line), and average ± standard deviation (μ ± σ) spectra (dashed line) were normalized. The spectra in black represent the μ and μ ± σ spectra of the micro-sample measurements, and the spectra in grey represent the μ and μ ± σ spectra of the macro-

sample measurements. Both were calculated by using ten independent measurements carried out for each strain.

By using the macro-sample method a confluent and dense bacterial lawn on the agar plate was obtained for all species of *Listeria* when they were cultivated 24 hours at 30°C. During this bacterial growth, both, the modification of the medium composition (i.e. starvation for nutrients, oxygen, etc) as well as the increment of the bacterial density, lead to some variations in the bacterial environment on the agar plates. Such limitations may induce bacterial cells to enter the stationary phase and undergo phenotypical changes. This culture phase was described by Sandt and coworkers as characterized by a high degree of interspecies discrimination which is due to the expression of differential features of species (Sandt et al. 2006). Unlike the macro-sample method, the micro-sample method measures *Listeria* microcolonies grown on agar plates at 25°C and 18 hours which involves cells in exponential growth. It was previously described that young microcolony cells present a lower interspecies diversity compared to bacterial cells from a 24 hours old stationary phase lawn culture (Sandt et al. 2006). The same author also suggested that the age of the culture is the only factor that influences the discrimination power of the micro-sample method.

The cellular composition and, therefore, the infrared spectrum, depends on the composition of the medium which was kept constant in our assay. Also, it varies with temperature and time. Thus, the same 25 strains were grown for 18 h to a nearly confluent lawn at 25°C, and their infrared macro-sample spectra collected. These infrared macro-sample spectra were compared with cells grown at 30°C for 24 h to a confluent lawn. In parallel, spectra of microcolonies were measured which were grown under the same conditions. While only negligible variation was observed between the infrared macro-sample spectra, the infrared micro–sample spectra showed large differences when the same strains were compared (results not shown). These results indicate that the structure of microcolonies and the composition of cells within the colonies may affect the identification success of the micro-sample method. Furthermore, additional peaks were observed in the infrared macro-sample spectra of all *Listeria* species in the species discriminative region (polysaccharide region 900-1,200 cm^{-1}) and in the region from 1,500 to 1,550 cm^{-1}, when grown at 30°C for 24 h, which is in line with the conclusions of Sandt and co-workers (Sandt et al. 2006), who found such changes to increase the discriminative potential of older cell's spectra in other microorganisms.

FT-IR spectra obtained from microbial microcolonies exhibit some variance which may be indicative of the heterogeneity within the microcolonies (Choo-Smith et al. 2001, Ngo-Thi et

al. 2003, Rebuffo-Scheer et al. 2007a, Sandt et al. 2006, Wenning et al. 2002). Therefore, we evaluated whether the colony heterogeneity has an influence on the identification success. A linear mapping across the microcolony imprint was performed for all *Listeria* species. For example, Fig. 2.3A1 and Fig. 2.3A2 display the linear mapping procedure across the microcolony imprint of *L. monocytogenes WSLC 1929* and *L. seeligeri WSLC 40134*, respectively, in order to study the spectral heterogeneity within a colony. Spectra were collected at several positions separated by 10 µm steps. As can be seen in the dendrograms of Fig. 2.3, the spectra collected at different positions of *L. monocytogenes* microcolonies showed a higher heterogeneity (Fig. 2.3B1) compared to those derived from *L. seeligeri* microcolonies (Fig. 2.3B2). Strains of the species *L. innocua* and *L. ivanovii* were similar to *L. monocytogenes* while *L. welshimeri* strains exhibited the same characteristics as *L. seeligeri* strains (data not shown).

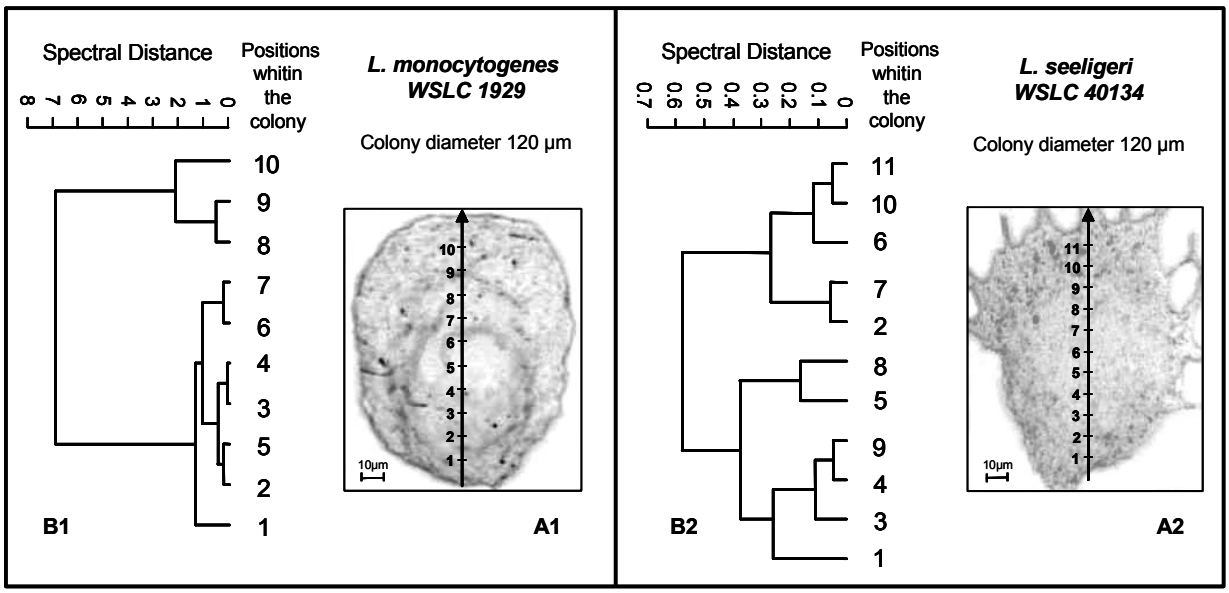

Fig. 2.3 Spectral heterogeneity within two different microcolonies of *L. monocytogenes WSLC 1929* (A1) and *L. seeligeri WSLC 40134* (A2). Each colony imprint obtained by the replica technique was approximately 120 µm in diameter. The marked positions on the colony imprints are the centers of various measuring positions. B1 and B2 display the dendrograms calculated from the first derivative of the spectra collected at the different positions within the microcolonies. Spectra were recorded between 800 and 1,800 cm^{-1}, dendrograms were constructed using the average linkage algorithm and the "scaling to first range" method.

Interestingly, the microcolony shape in *Listeria* varies among the species. We observed that different strains of *Listeria* exhibit two different types of microcolony shapes when growing on the same solid medium. This is visualized in the microcolony imprints of Fig. 2.3A1 and

A2. *L. innocua* and *L. ivanovii* microcolonies exhibit a characteristic halo in the centre similar to the *L. monocytogenes* strains (Fig. 2.3A1) whereas *L. seeligeri* and *L. welshimeri* strains show a slightly plan microcolony shape (Fig. 2.3A2).

In addition, spectral heterogeneity was measured for microcolony sizes between 70 to 280 µm. In general, the heterogeneity as indicated by the spectral distance value increased with colony size (Fig. 2.4). Spatial spectral heterogeneity therefore increases with the age of a colony, indicating different cellular compositions at different locations of the colonies.

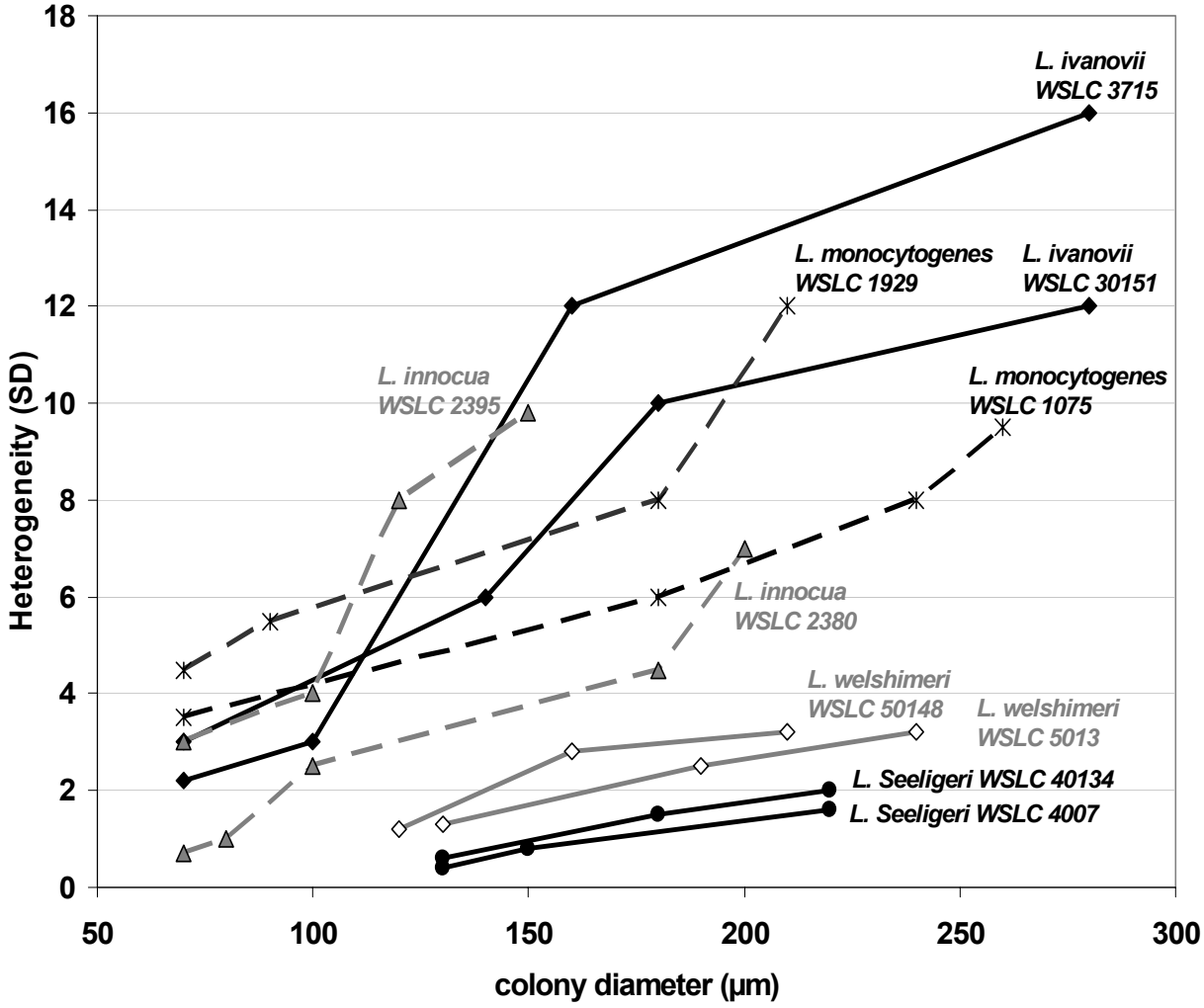

Fig. 2.4 Spectral heterogeneity expressed as spectral distance (SD) versus size of the microcolony for different *Listeria* species. Two different strains per species are shown. For details see Materials and methods.

In this study, the heterogeneity values achieved for *Listeria* species are comparable with those reported for other microorganisms with low colony heterogeneity (Ngo-Thi et al. 2003, Rebuffo-Scheer et al. 2007a). This indicates the existence of small differences in the

biochemical composition between different zones of the microcolony. However, qualitative characteristics specific to the *L. innocua, L. ivanovii and L. monocytogenes* microcolonies are evident from their colony shapes as well as their spectral reproducibility.

Microcolonies are microbial populations developing by the proliferation of individual cells on a solid medium. During bacterial growth different colony architectures have been observed for different *Listeria* strains growing on the same medium, probably correlating with physiological adaptations of the cells in response to nutrients and O_2 availability which are different in different locations of the colony. Examples for such adaptation processes are the reduction of the cells sizes (Herbert and Foster 2001) and changes in gene expression (Gray et al. 2006, Mauder et al. 2006). Thus, it may be possible that in the microcolony stage a complex superposition of peculiar cellular components hides species differencing spectral features. For this reason, the influence of the colony shape in *L. innocua*, *L. ivanovii* and *L. monocytogenes* strains may determine in part the low identification success (table 2). This is supported by the low spectral reproducibility which was observed for these three species when using the micro-sample method.

It is well known that FTIR based identification of microbes strongly depends on the number of strains per species contained in the spectral reference library which must reflect as much of the intraspecific biodiversity of a species as possible (Oberreuter et al. 2002b, Rebuffo et al. 2006). Therefore, a total of twelve libraries containing different strain sets of the five *Listeria* species (10, 20, 30, and 40 strains per species with three different strain combinations, respectively) were compiled for both techniques under consideration. (See materials and methods for technical details). As expected, a clear improvement of differentiation success was observed when more strains per species were included (Fig. 2.5). However, this improvement differs between the macro- and the micro-sample methods. Based on these results we do not expect that the micro-sample method will yield satisfactory identification results for the pathogen *Listeria monocytogenes*, even if large numbers of different strains are included in the spectral reference libraries. This is in part due to the influence of the spectral heterogeneity of microcolonies as well as to the insufficient discrimination features expressed in exponential growth cells.

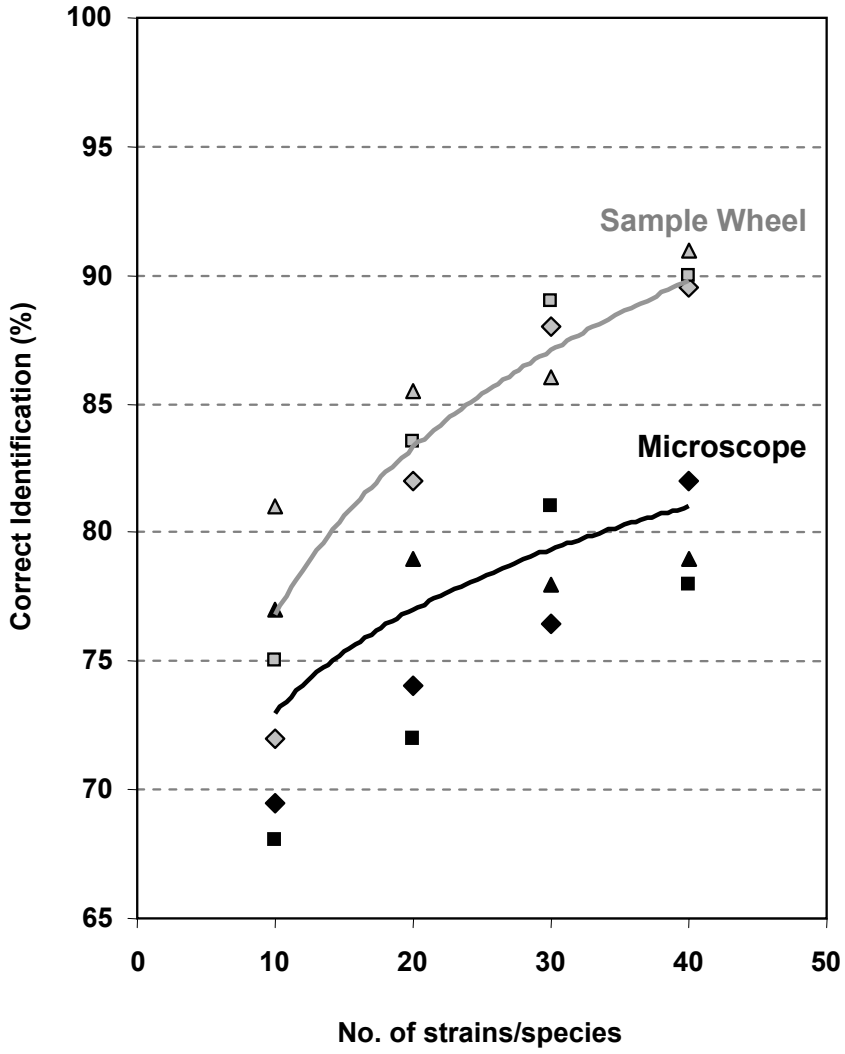

Fig. 2.5 Identification success depends on the number of strains per species in the library. A total of twelve libraries containing different strain sets of the five Listeria species were compiled for each technique. The results obtained for each library of the macro-sample method (Sample Wheel) are represented by grey symbols while those of the micro-sample method (Microscope) are represented by black symbols. When constructing three libraries, by using the micro-samples method, we did start with 10 different strains per species followed by three additional, consecutive sets of 10 strains per species to arrive at 40 strains per species. This allowed the construction of 12 different libraries containing different strain combinations. The same has been done by using the macro-sample method. Then, each of these libraries was internally evaluated as described in the material and methods section. The results of the internal validations are represented by the symbols triangle, rhombus and square. The values of these internal validations were then averaged for each strain set separately for both, the macro- and micro-sample methods. The curves represent an average of the correct identification results for each library.

2.5 **Conclusions**

In this work we have applied the FTIR micro- and macro-sample method to the differentiation of five very closely related bacterial species, one of them being an important human pathogen. In this case, the micro-sample method yields unsatisfactory results, even if spectral reference libraries with considerable coverage of intraspecific and interspecific biodiversity are available. This may hold generally if very similar microorganisms are to be differentiated. However, in case of a larger biodiversity of a taxonomic group, the micro-sample method may well be the method of choice. For instance, it was used successfully for the rapid analysis of complex microbial multi-species consortia mainly composed of coryneform bacteria (Wenning et al. 2006) and for the identification of atypical *Mycobacterium* species (Rebuffo-Scheer et al. 2007a), which display a considerably larger interspecific biodiversity than *Listeria* species.

2.6 **Acknowledgement**

This work was supported in part by the FEI (Forschungskreis der Ernährungsindustrie e. V., Bonn), the AiF (Arbeitskreis für industrielle Forschung), and the Ministry of Economics and Technology, project no. 14126

3 Reliable and Rapid Identification of *Listeria monocytogenes* and *Listeria* Species by Artificial Neural Network-Based Fourier Transform Infrared Spectroscopy

3.1 Summary

Differentiation of the species within the genus *Listeria* is important for the food industry but only few reliable methods are available so far. While a number of studies have used Fourier Transform Infrared (FT-IR) spectroscopy to identify bacteria, the extraction of complex pattern information from the infrared spectra remains difficult. Here, we apply the artificial neural network technology (ANN), which is an advanced multivariate data-processing method of pattern analysis, to identify *Listeria* infrared spectra at the species level. A hierarchical classification system based on ANN analysis for *Listeria* FT-IR spectra was created based on a comprehensive reference spectral database including 243 well-defined reference strains of *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. In parallel, a univariate FT-IR identification model was developed. To evaluate the potential, a set of 277 isolates from diverse geographical origin, but not included in the reference database, were assembled and used as an independent external validation for species discrimination. Univariate FT-IR analysis allowed the correct identification of 85,2% of all strains, and 93% of the *L. monocytogenes* strains. ANN based analysis enhanced differentiation success to 96% for all *Listeria* species, including a success rate of 99.2% for correct *L. monocytogenes* identification. The identity of the 277 strain test set was also determined using the standard phenotypical API *Listeria* system. This kit was able to identify 88% of the test isolates and 93% *L. monocytogenes* strains. These results demonstrate the high reliability and strong potential of ANN-based FT-IR spectra analysis for identification of the five *Listeria* species under investigation. Starting from a pure culture, this technique allows the cost-efficient and rapid identification of *Listeria* species within 25 hours and is suitable for use in a routine food-microbiological laboratory.

3.2 Introduction

The genus *Listeria* currently embraces six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi*, based on DNA homology, 16S rRNA homology, chemotaxonomic properties, and multilocus enzyme analysis (Rocourt 1999). All of these species are widespread in the environment, but only *L. monocytogenes* is considered to be an

opportunistic pathogen for humans and animals. Occasionally, human infections due to *L. ivanovii* and *L. seeligeri* have also been reported (Cummins et al. 1994, Lessing 1994, Rocourt 1986). Since most of the *Listeria* species are found as food contaminants, which may indicate a potential risk for subsequent contamination by *L. monocytogenes*, their presence require immediate action by the food company. For instance, *L. innocua* has frequently been found as a marker organism of a *L. monocytogenes* contamination in dairy plants (Hahn 1990, Loessner 2002, Rudolf and Scherer 2001). Therefore, a rapid and reliable differentiation of *L. monocytogenes* from the other species of the genus is particularly important for the food industry with respect to an effective quality assurance strategy.

For the identification of *Listeria* at the species level in routine laboratories, time-consuming, laborious and sometimes unreliable biochemical and phenotypical standard methods such as sugar fermentations and the CAMP test are often used (Bubert et al. 1997, Jones 1992). Easy and rapid identification systems commercially available often fail to accurately identify atypical strains, due to the lack of basic classification marker reactions (Bille et al. 1992, Gracieux et al. 2003, Johnson 1993, Paillard et al. 2003). Therefore, fast molecular methods and immunological procedures have been developed. However, most of them are limited to detect only the genus *Listeria* or only *L. monocytogenes* (Allerberger et al. 1997, Bubert et al. 1994, Deneer and Boychuk 1991, Ninet et al. 1992, Norton 2002, Olsen et al. 1995). Some other methods are laborious for species discrimination (Cocolin et al. 2002, Farber and Addison 1994, Jersek et al. 1996) or failed to identify all *Listeria* species (Howard et al. 1992, Manzano et al. 2000, Vaneechoutte et al. 1998). Recently developed sensitive and specific microarray techniques are still of limited potential for routine laboratories due to high cost and the requirement of highly skilled personnel (Volokhov et al. 2002).

Fourier transform-infrared spectroscopy (FT-IR) is a vibrational spectroscopic technique with high resolution power which is able to distinguish microbial cells at different taxonomic levels (Helm et al. 1991a). One attractive application of this inexpensive and rapid technique is the identification of unknown strains using an extensive reference library containing spectra from well-identified microbes (Goodacre et al. 1996, Helm et al. 1991, Kümmerle et al. 1998, Maquelin et al. 2003, Oberreuter et al. 2002b, Wenning et al. 2002). The identification is achieved by calculating the over-all difference between a test spectrum and all reference spectra. A test strain is assigned to the source of the nearest reference spectrum (Helm et al. 1991). However, such a procedure is univariate and does not consider patterns of individual differences at different wavelengths leaving a wealth of information stored in the spectra

unused. In case of the differentiation of closely related species within the same genus, advanced multivariate methods for data analysis are therefore required.

Investigation of *Listeria* species using FT-IR spectroscopy has been undertaken previously (Holt et al. 1995, Lefier et al. 1997). However, these studies included only a single strain per species and even in this simple case the unequivocal clustering of different spectra of the same strain was not always possible. It remained therefore unclear whether FT-IR spectroscopy would have the capacity to differentiate the *Listeria* species, especially if the technique is applied to a strain selection covering at least a significant part of the natural microbiodiversity of the species. If many strains from several species are included in the analysis, self learning systems such as artificial neural networks (ANNs) may be able to extract the information stored in the spectra of such a broad database and greatly enhance the species-specific differentiation of bacterial isolates, when a comprehensive reference dataset is used (Maquelin et al. 2003, Schmitt and Udelhoven 2001, Udelhoven et al. 2000). In the present study, ANNs have therefore been applied to establish a classification system for *Listeria* FT-IR spectra and its performance has been compared to univariate -FT-IR analysis and the standard phenotypical API differentiation of *Listeria*. We report that the semi-automated, ANN-based FT-IR technique allows reliable identification of *Listeria* species in 25 hours and is suitable for use in a routine microbiological laboratory.

3.3 Materials and Methods

3.3.1 Bacterial strains

A list of all 520 *Listeria* strains used in this study can be found in Appendix I and II in the Appendix section.

3.3.2 Reference strain set: Sequence analysis of the *iap* and *thy* gene

A reference strain set of 243 well-defined strains of *Listeria monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri* from international culture collections (ATCC and SLCC) and the Weihenstephan Listeria collection (WSLC) served to establish a spectral reference dataset (see Appendix I of chapter 7). A total of 164 strains of this set have been identified by DNA-sequence analysis of the complete *iap* gene and thymidylate synthase gene (the *thy* gene) and the remaining strains have been identified by a multiplex PCR system developed by Bubert et al. (1999).

For the sequence analysis of the *iap* and *thy* genes 9 ml of liquid culture grown in 10 ml of BHI (Brain Heart Infusion, Oxoid, England) broth at 30°C was harvested, resuspended in 2 ml of purified water and kept at –20°C. Eight microliters of this lysat served as a template for a 100 µl PCR reaction. Thermal cycling was performed in a Techne Cyclone Gradient cycler (Pequlab, Erlangen, Germany). The *iap* gene was PCR amplified and sequenced using the primers iap-P-V/57 (5'-ATG AAT ATG AAA AAA GCA ACT ATC GC), and iap-P-R/57 (5'-TTA TAC GCG ACC GAA GCC AA). These primers bind at the 5' and 3' ends of the *iap* gene, covering the entire *iap* sequence and were designed by ClustalW (http://www.ebi.ac.uk/clustalw/) alignments of Entrez Nucleotides database *Listeria* sequences (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide). The internal primer iap_F700/58 (5'-GTC ATG GAA TAA TTT ATC T[G/T]C TTC TTC) was used for DNA sequencing. PCR was performed using 50 µl AB-Gene 2x Reddy Mix with 1.5 mM MgCl2 (AB-Gene, Hamburg), 8 µl of lysate, 1 µl of each primer [50 pmol/µl], and 40 µl of purified water. Thermal cycling conditions were 5 min at 95°C, followed by 29 cycles of 20 s at 95°C, 30 s at 50°C, and 1 min 40 s at 72°C.

The *thy* gene was PCR amplified and sequenced using the primers thy_2_F/62 (5'-GAG GAA ATG ATG GAA CGC TGG GA), and thy_1_R/60 (5'-TAT T[G/C]C C[A/G]G CGC GGT CTT GTG). These primers bind in the non coding region of the *thy* gene, and were designed based on one *L. monocytogenes thy* sequence provided by Pascale Cossart (Institute Pasteur, France), and homologous GenBank sequences identified by Blast search (http://www.ncbi.nlm.nih.gov/BLAST/). All primers were checked using Netprimer (http://www.premierbiosoft.com/netprimer/netprlaunch/netprlaunch.html). PCR was performed as described for the *iap* gene, using the following thermal cycling conditions: 5 min at 95°C, followed by 27 cycles, each consisting of 20 s at 95°C, 30 s at 54°C, and 1 min 50 s at 72°C. PCR products were purified using QIAquick 96 PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was performed at Sequiserve (Willi Metzger, Vaterstetten, Germany). The complete coding region of the *thy* gene was sequenced. Sequences were aligned using ClustalW (http://genius.embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/ or http://www.ebi.ac.uk/clustalw/) and edited using Jalview (http://genius.@embnet.dkfz-heidelberg.de/menu/w2h/w2hdkfz/ or http://www.ebi.ac.uk/~michele/jalview/). Dendrograms were constructed using Jalview.

3.3.3 **Identification of the validation strain set isolates**

A set of 277 strains representing five species of *Listeria* of diverse habitats (foods, environment, animals and humans from South and North America, Central, North and South Europe) were used for the external validation of the ANN model. This strain set will be referred to in the rest of this paper as the external validation strain set (see Appendix II of chapter 7). These strains have been identified by a phenotypic API-*Listeria* test (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Additionally, these strains were examined for the presence of hemolysis activity using a tube test with an erythrocyte suspension. Briefly, the serum was carefully removed from fresh, sterile and defibrinated sheep blood (Oxoid, England), and a 2% (vol/vol) suspension of the erythrocytes was made in phosphate-buffered saline (pH 7.2). A total of 1ml of *Listeria* culture grown overnight at 37°C in brain heart infusion broth was mixed with 1ml of the erythrocyte suspension. After incubation for 24 h at 37°C, the presence or absence of hemolysis was observed.

The Multiplex PCR system developed by Bubert et al. (1999) was used for the differentiation of *Listeria* spp. belonging to the external validation strain set when API and FT-IR identification was discordant. DNA was prepared by using one loop of bacterial cells which were homogenized in 200 µl Mili-Q water. Cells were disrupted using 0.5 g zirconia/silica-beads (0.1 mm diameter, Roth, Karlsruhe, Germany) by shaking two times at 6.5 m s-1 for 45 s in a Hybaid RiboLyser-cell disrupter™ (Middlesex, UK). Afterwards, the lysate was separated by centrifugation at 13,000 x g for 3.5 min. DNA amplification reactions were carried out in 50 µl final volume containing 25 µl Reddy Mix [75 mM Thris-HCL (pH 8.8), 20mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM of each deoxyribonucleoside triphosphate, 1.25 U of Taq polymerase] from PCR Master Mix, ABgene (Surrey, UK), 8 µl of lysate, and 1mM of each primer. The primer combination and PCR conditions were as described previously (Bubert et al. 1999).

3.3.4 **Measurement of FT-IR spectra**

The strains stored at – 80°C were streaked and subcultured on agar plates of tryptone soy agar (TSA) (Oxoid, Basingstoke, Hampshire, England) for 24 h. The growth temperature was 37 ± 2°C. The sample preparation for the IR absorbance measurements was performed as described by Oberreuter et al. (Oberreuter et al. 2002b). Prior to the spectral measurements, the sample holder was sealed with a KBr cover plate to prevent contamination of the spectrometer. The spectra were recorded and evaluated according to Oberreuter et al. (Oberreuter et al. 2002b).

For data processing such as calculation of derivatives and normalization, the software OPUS version 4.2 for Windows NT (Bruker, Germany) was used. First derivatives of the original IR spectra were calculated using a 9-point Savitzky-Golay filter to minimize problems from unavoidable baseline shifts. It was observed that the identification quality increases with the inclusion of additional repetitive measurements per strain in the reference dataset. Best identification was achieved with ten independent measurements (from independent bacterial cultures) of each strain being included in the reference database to ensure a sufficient coverage of biological variance of growth and sampling procedure, although a standard and strict operation procedure was established (data not shown). The success of ANN modeling strongly depends on the quality of the spectra (Schmitt and Udelhoven 2001). Therefore, the thresholds for minimum absorbance (0.25) and maximum absorbance (1.20) for detector linearity, signal-noise (S/N) ratio with a noise maximum of 1.5×10^{-4} U, and a water vapor content of the spectral measurements of $<3 \times 10^{-4}$ U were predefined, and this quality test procedure was applied to each spectrum.

3.3.5 **Univariate FT-IR analysis**

The selection of relevant spectral ranges and establishment of the cutoff values of spectral distance (SD) for the identification of *Listeria* at the species level were done. In order to establish the SD value, three independent measurements of five strains of each *Listeria* species were used. The calibration of their SD threshold value for a correct identification of an isolate at species level was done using a procedure based on Oberreuter et al. (Oberreuter et al. 2002b). In our case the windows from 900 to 1,200, 1,250 to 1,650, and 2,830 to 3,030 cm^{-1} (all weight factors were 30) and a cutoff value of the SD 0.5 were used. This implies that the spectral distance between an isolate and the first hit of the identification hit list must be <0.5 to yield a valid identification at the species level. Then, 10 repetitive measurements from independent sample preparations of all reference strains were performed, resulting in 2,430 spectra, which were added to the reference spectral library.

3.3.6 **Artificial Neural Network based FT-IR identification**

Before artificial neural network analysis, hierarchical cluster analysis (HCA) of the spectra in the reference library was used as a first step in developing the *Listeria* species identification scheme based on ANN. The HCA was performed using the first derivative of the original spectra as input in the regions 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 according to the OPUS software

(Bruker). The two major groupings resulting from this HCA were used to establish the first layer of the two-layered neural network. Afterwards, the subsequent subnets were optimized for the respective classification at the species level.

For the ANN analysis, 2,430 spectra of the reference dataset were randomly distributed into a training set (8 spectra of each strain), pre-validation set (1 spectrum of each strain), and test set (1 spectrum of each strain). Prior to the artificial neural network analysis the spectral windows between 700 and 1,800 cm^{-1}, and 2,800 and 3,100 cm^{-1} were predefined in a data pre-processing step. For spectral feature selection, the most discriminative 61 wavelengths (Fig. 3.1) were selected based on the calculation of the covariance of the spectra data points (Schmitt and Udelhoven 2001). The Synthon NeuroDeveloper® software (Synthon, Heidelberg, Germany) was used to perform feature selection and to establish a two-layer neural network with 61 input neurons, one hidden unit, and two output units. For each classification level, a fully-connected feed-forward neural network was trained using the Rprop algorithm (Schmitt and Udelhoven 2001).

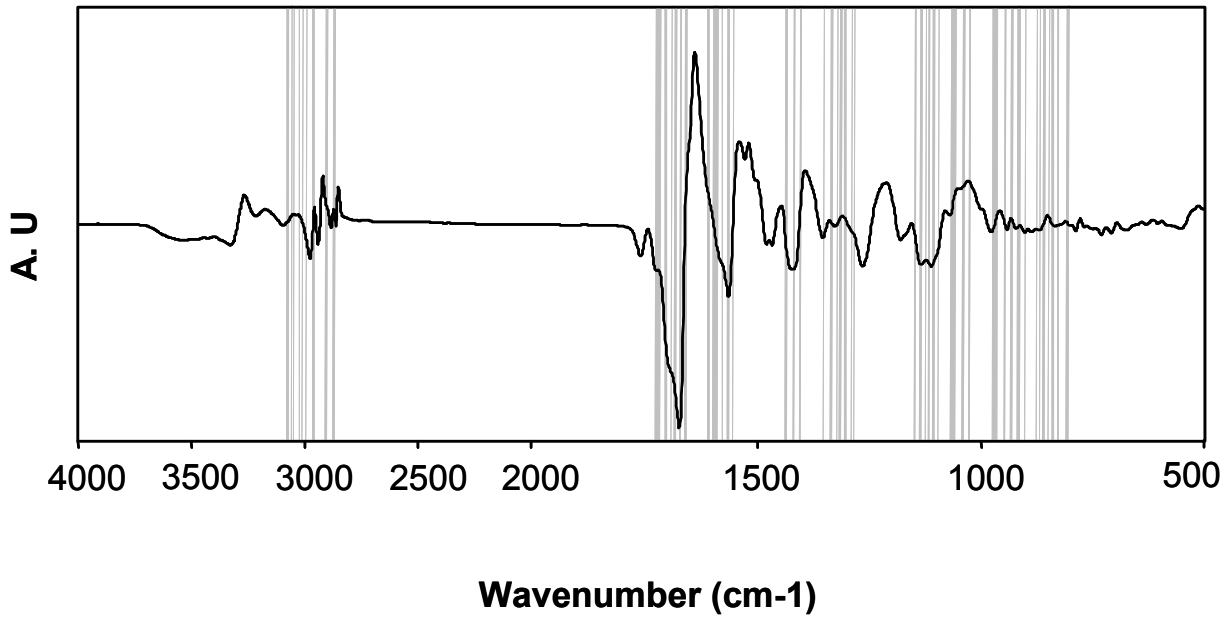

Fig. 3.1 First derivative of a *Listeria* FTIR spectrum. The regions of the infrared spectra contributing most significantly to the differentiation of the five *Listeria* species are highlighted. A.U., arbitrary units.

3.3.7 **Validation of FT-IR identification procedures**

To test both FT-IR univariate and FT-IR artificial neural network identification models, an internal validation was performed. One randomly selected spectrum of each strain in the

database was excluded and used to construct a test set. This test set containing independent spectra of each reference strain was used to test the reference dataset and the results determined at species level.

As a final test of performance, the validation strain set of 277 independent *Listeria* isolates, whose spectra were not included in the reference database, was identified by both univariate and ANN methods in an external validation.

3.4 **Results and discussion**

3.4.1 **Modular architecture of the Artificial Neural Network**

A modular ANN model was constructed for species identification at the basis of Hierarchical Cluster Analysis (HCA) groupings. HCA is a technique which groups IR spectra based on the overall similarity to other spectra. This technique can be applied "unsupervised", due to its ability to perform the comparisons mathematically without predetermined information. In contrast, the ANN model was used as a "supervised" method of analysis based on a learning procedure which can classify unknown samples into predetermined groups. The similarity between the species observed in the HCA analysis, representing the *Listeria* reference spectra dataset, provided information to develop modules of ANN with optimized classification performance through individual feature selection and network architecture. The modules are later integrated in one ANN classification system (Schmitt and Udelhoven 2001). Figure 3.2a shows the two major groups resulting from the cluster analysis which were used to establish the first level of the ANN architecture comprising the *L. innocua*-*L. ivanovii*-*L. welshimeri* net and the *L. monocytogenes*-*L. seeligeri* net. Then, according to the outputs of this first level in the ANN classification scheme, specialized networks were activated at a second level, determining the species-specific subnetworks (Fig. 3.2b). Based on this classification scheme, the discrimination of *Listeria* down to the species level resulted from the projection of an unknown *Listeria* spectrum from the first level to the second level. When, in the first level, this spectrum is predicted to belong to one of the nets at this level, the output from this first level is projected to the second level to distinguish between the respective species available in the respective subnet.

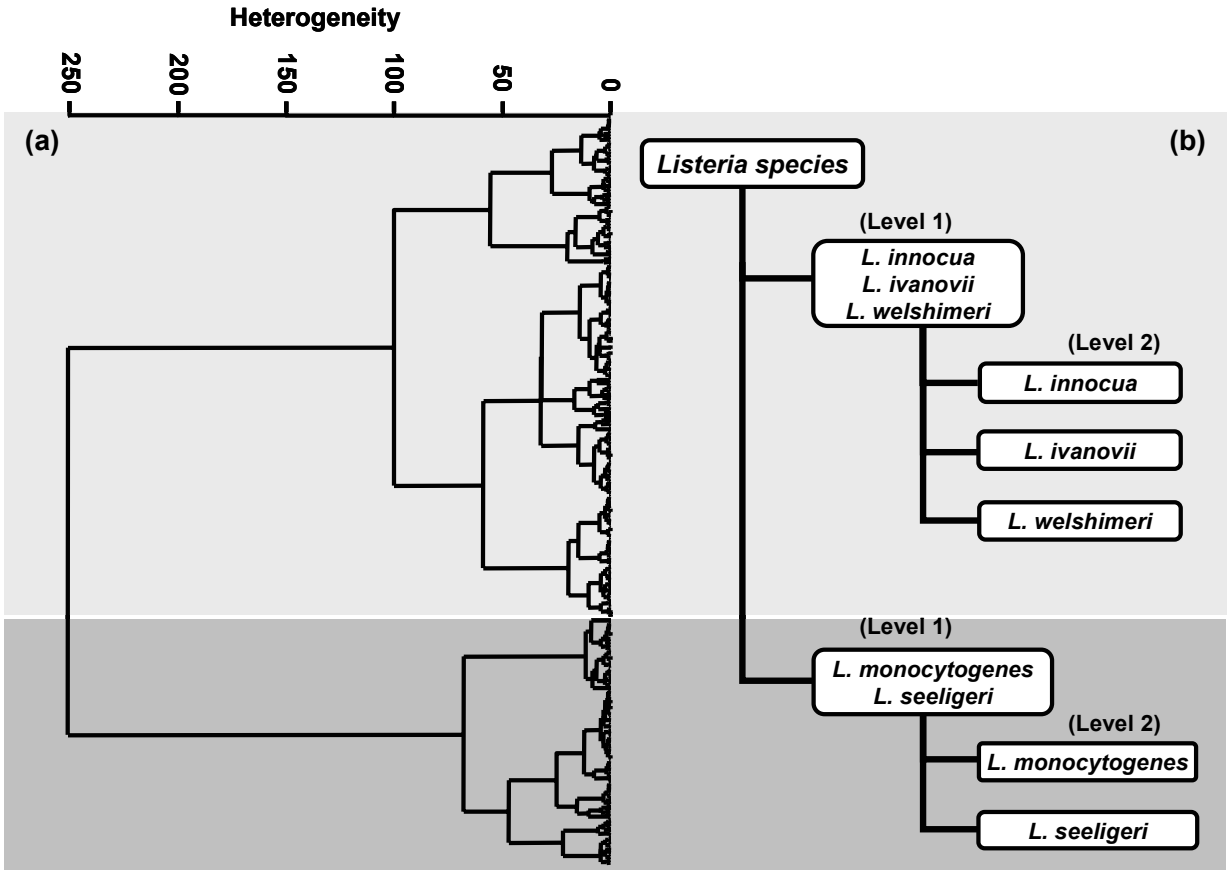

Fig. 3.2 (a) Hierarchical cluster analysis of the first derivative of 243 *Listeria* spectra included in the reference data set. It was performed by using the regions from 700 to 1,200, 1,500 to 1,800, and 2,800 to 3,100 cm^{-1}, correlation with scaling to first range, and Ward's algorithm. (b) The two major groups resulting from the cluster analysis (a) were used to establish the first level of the architecture of the neural network for the identification of *Listeria* species. In the first level, the *L. innocua-L. ivanovii-L. welshimeri* net and the *L. monocytogenes-L. seeligeri* net were established. In the second level of this classification scheme, the species-specific subnetworks (*L. innocua*, *L. ivanovii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*) were activated.

3.4.2 **Validation of the spectral reference databases**

Univariate based FT-IR and ANN based FT-IR identification procedures were internally validated based on 243 reference strains contained in the database (Table 3.1). The overall correctness of identification using the spectral window combination described in Material and Methods was 88.9% at the species level for the univariate method. Less satisfactory results were obtained for *L. seeligeri* (77.6%) due to the relatively high degree of misidentification as *L. monocytogenes*. The overall performance of the ANN model was 96.3% correct identification. The worst performance for both methods was observed with *L. welshimeri*, which showed the same misidentification results for the same two strains as *L. ivanovii* and *L. innocua* for the univariate and multivariate methods, respectively. This fact reveals that for

these two particular strains the intraspecific biodiversity represented in the database with only 19 *L. welshimeri* strains is limited.

Once the FT-IR univariate and the ANN models using a 243 reference strain data set were established, an external validation comprising 277 isolates not included in the reference database was used to challenge both FT-IR models. According to Table 3.2, 129 out of 130 strains of *L. monocytogenes* were correctly identified using the neural network method, whereas the univariate approach identified only 121 strains correctly. Similarly, *L. innocua*, *L. ivanovii* and *L. seeligeri* reached better identification results by ANN than by the univariate model. Only *L. welshimeri* showed the same poor identification accuracy by both methods. This indicates that the low number of *L. welshimeri* strains included in the database limited their identification. While the univariate FT-IR analysis procedure allowed the correct identification of 85.2% (236 of 277) of all test strains, the ANN method was able to identify 96.0% (266 of 277) of the strains correctly at the species level. Furthermore, comparable results of the prediction accuracy in the internal (96.3%) and external (96.0%) validation of the ANN reveals the stability of this model, indicating that a significant part of the microbiodiversity of the *Listeria* species was covered by the reference database and represented by the ANN classification system.

Table 3.1 Internal validation of the *Listeria* infrared spectral reference database

Species	N° of Strains tested	Univariate FT-IR analysis			ANN identification		
		Correct Identification % (No)	Mis-identification[a] % (No)	No identification[b] % (No).	Correct identification % (No)	Mis-identification[c] % (No)	No identification[d] % (No)
L. innocua	65	95.4 (62)	3.1 (2)	1.5 (1)	98.5 (64)	1.5 (1)	-
L. ivanovii	41	87.8 (36)	12.2 (5)	-	95.1 (39)	4.9 (2)	-
L. monocytogenes	69	91.3 (63)	8.7 (6)	-	97.1 (67)	2.9 (2)	-
L. seeligeri	49	77.6 (38)	22.4 (11)	-	96.0 (47)	4.0 (2)	-
L. welshimeri	19	89.5 (17)	10.5 (2)	-	90.0 (17)	10.0 (2)	-
Total	243	88.9 (216)	11.0 (26)	0.1 (1)	96.3 (234)	3.7 (9)	-

[a] Strains yielding an Spectral Distance (SD) value below or equal to the threshold value of 0.5 used for correct identification of *Listeria* species, but their identification corresponded to a different species.

[b] Strains yielding an SD value higher than the threshold value of 0.5 used for correct identification of *Listeria* species.

[c] Strains yielding identification results corresponding to a different species.

[d] Strains not yielding identification.

Table 3.2 External validation of the identification potential of univariate FT-IR, ANN and API using 277 *Listeria* strains not included in the reference dataset

Species	N° of Strains tested	Univariate FT-IR analysis			ANN identification			API Listeria		
		Correct Identification % (No.)	Mis-identification[a] % (No.)	No Identification[b] % (No.)	Correct Identification % (No.)	Mis-identification[c] % (No.)	No identification[d] % (No.)	Correct identification % (No.)	Mis-identification[e] % (No.)	No identification[f] % (No.)
L. innocua	60	91.7 (55)	8.3 (5)	-	93.3 (56)	6.7 (4)	-	90.0 (54)	8.3 (5)	1.7 (1)
L. ivanovii	28	71.4 (20)	28.6 (8)	-	96.4 (27)	3.6 (1)	-	89.3 (25)	3.6 (1)	7.1 (2)
L. monocyto genes	130	93.0 (121)	7.0 (9)	-	99.2 (129)	0.8 (1)	-	93.1 (121)	6.9 (7)	1.5 (2)
L. seeligeri	48	64.6 (31)	35.4 (17)	-	93.8 (45)	6.2 (3)	-	75.0 (36)	2.1 (1)	22.9 (11)
L. welshimeri	11	81.8 (9)	18.2 (2)	-	81.8 (9)	18.2 (2)	-	72.7 (8)	-	27.3 (3)
Total	277	85.2 (236)	14.8 (41)	-	96.0 (266)	4.0 (11)	-	88.0 (244)	5.1 (14)	6.9 (19)

[a] Strains yielding a Spectral Distance value SD below or equal to the threshold value of 0.5 used for correct identification of *Listeria* species, but their identification corresponded to an incorrect species.

[b] Strains yielding a Spectral Distance value SD higher than the threshold value of 0.5 used for correct identification of *Listeria* species.

[c] Strains yielding identification results corresponded to an incorrect species.

[d] Strains yielding not identification results.

[e] Strains yielding identification results corresponded to an incorrect species.

[f] Strains yielding none or multiple results.

3.4.3 **Influence of the number of the reference strains on the identification success**

Three ANN reference databases with 100, 171 and 243 randomly chosen strains, respectively, were compiled. The same 277 validation strain set described above was identified by all three databases (Fig. 3.3). In general, as expected, the inclusion of more biological intraspecies variability leads to an improvement of interspecies differentiation. This is in accordance with previous studies for other microbes (Maquelin et al. 2003, Oberreuter et al. 2002b). Oberreuter et al. (Oberreuter et al. 2002b) reported for the coryneform bacteria that, on average, 5 to 10 strains of a species are needed to achieve an identification success of approximately 90%. We have observed that the species of the genus *Listeria* require more strains per species (around 20 to 25) to better cover the natural intraspecies variability range (see chapter 2). Hence, a good representation of the species biodiversity in the reference database will allow a reasonable identification capacity. We have observed that the addition of more *L. innocua* strains not only improved the identification results of *L. innocua*, but also improved those of *L. ivanovii*. This is in agreement with the observation that *L. ivanovii* was mostly misidentified as *L. innocua* by the ANN models based on analysis of 100 and 171 strains (data not shown). This was supported by the noticeable increase of correct *L. ivanovii* identification from 89.3% to 96.4% when only a single strain of *L. ivanovii* was added to the ANN model with 171 strains. It was also noted that inclusion of more *L. monocytogenes* and *L. seeligeri* strains yielded a large improvement of their correct identifications. While for *L. monocytogenes* the identification success is near to perfect (99.2%), *L. welshimeri* identification results did not improve when ANN based on 171 strains was compared to ANN comprising all 243 strains. This led us to speculate that the species *L. welshimeri* contains strains whose FT-IR absorption differences are not mainly due to specific cellular structures. Therefore, the database must include more strains for *L. welshimeri* to cover the entire biological variance of this species.

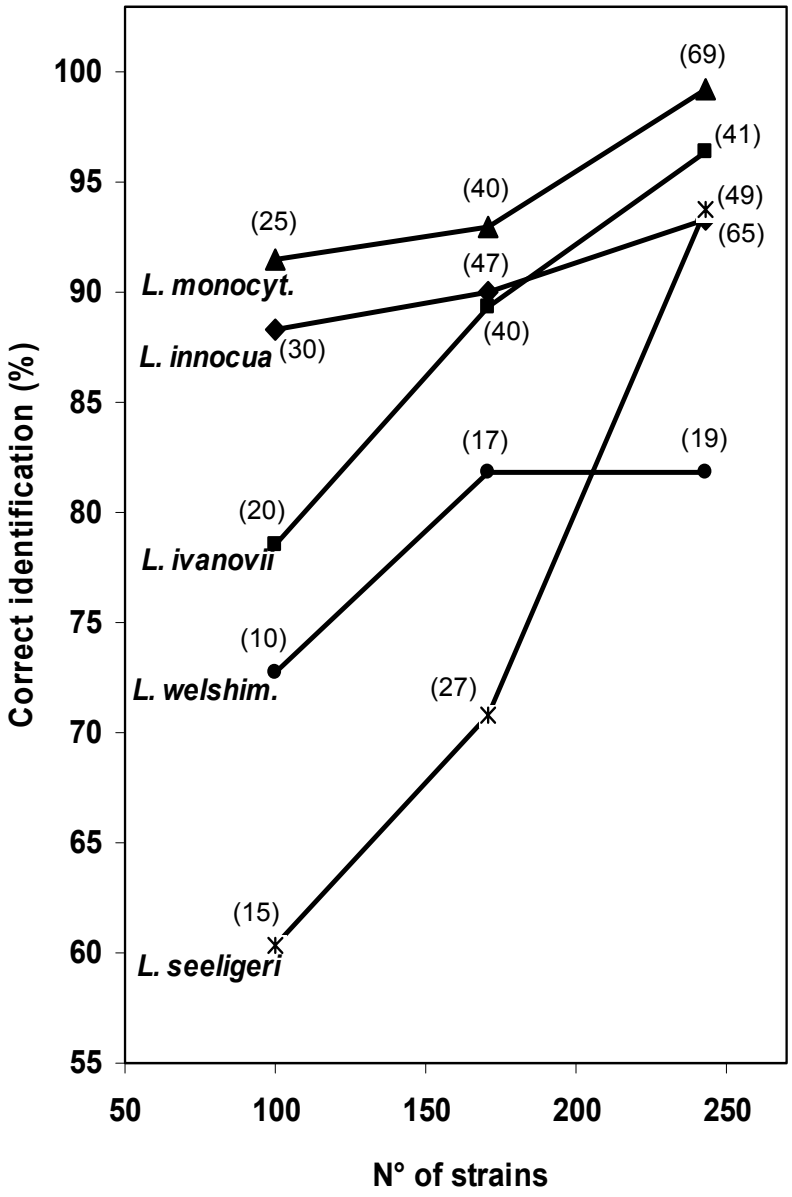

Fig. 3.3 Comparison of the external validation of the ANN model using three different reference data sets including 100, 171, and 243 strains. The number of strains per species included in each data set is indicated in parentheses.

3.4.4 **Comparison of API and FT-IR/ANN based *Listeria* identification**

Considering that the API *Listeria* system has been listed as one of the preferred rapid methods for the biochemical identification of *Listeria* species in the routine microbiology food laboratory (Hitchins 2003), we applied this technique as a second identification method to the 277 isolates of the validation strain set. This system allows a 24-h identification of all *Listeria* species, based on 10 sugar fermentation reactions and enzymatic reactions in microtubes, usually without the need for additional tests (Bille et al. 1992). Discordances between the FT-

IR-ANN method and API analysis were found for 39 out of the 277 strains. To resolve this conflicting data, multiplex PCR of the *iap* gene was performed (Bubert et al. 1999). This method confirmed the FT-IR-ANN results for 28 of the 39 discordances. On the other side, the multiplex PCR confirmed the API test for 6 strains only. The remaining 5 of the 39 discordant strains were unidentified by the API kit and were misidentified by the ANN method. The API *Listeria* test kit therefore provided a correct identification for 244 of the 277 isolates (88.0%), while the FT-IR-artificial neural network correctly identified 266 of the 277 isolates (96.0%) (Table 3.2). In this study, the API *Listeria* system misidentified 14 (5.1%) of the isolates. Most important, seven strains of the pathogenic species *L. monocytogenes* were misidentified as the nonpathogenic *L. innocua,* due to ambiguous results from the DIM reaction of the API test system. Additionally, for 10 strains the hemolysis test has been used as a supplementary test when the API *Listeria* system indicated inconclusive results. Several publications on the identification capacity of the API for *Listeria* species reported similar limitations (Bille et al. 1992, Johnson 1993, Paillard et al. 2003).

Table 3.3 Comparison of the Sensitivity, Specificity and Accuracy of ANN and API identification procedures.

Method	No. of strains*	TP	TN	FP	FN	Sensitivity (%)	Specificity (%)	Accuracy (%)
ANN	277	129	142	5	1	99.2	96.6	97.8
API *Listeria*	277	121	144	3	9	94.6	96.0	95.7

Abbreviations: TP, true positives; TN, true negatives; FP, false positives; FN, false negatives. Sensitivity is calculated as TP/(TP+FN), specificity is TN/(TN+FP), and accuracy is (TN+TP)/(TN+TP+FN+FP)

***** These strains were not included in the reference database.

Based on the identification of the 277 validation-strain set, the sensitivity, specificity and accuracy of the two methods in terms of their reliability to detect the human pathogen *L. monocytogenes* were evaluated. Sensitivity is defined as the ability of a test to detect a true *L. monocytogenes* sample when it is truly present. Specificity is defined as the ability of the test to detect the presence of *L. monocytogenes* in the sample when it is truly not present; accuracy relates to the closeness of the results to the true identification (American College of Physicians 2005, Dytham 1999). The data in Table 3.3 clearly demonstrate for all three parameters that ANN-based FT-IR identification is the superior method. No other phenotypical method so far described in the literature provides an overall correct identification of 96% for all *Listeria* species and a success rate of 99.2% for correct *L. monocytogenes* identification. ANN-based FT-IR identification therefore appears to be a

promising technique for the semiautomated and rapid identification of *Listeria* species in 25 h in a routine food microbiological laboratory.

3.5 **Acknowledgement**

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4 Differentiation of *Listeria monocytogenes* serovars by using Artificial Neural Network Analysis of Fourier-Transformed Infrared Spectra

4.1 Summary

A classification system based on Fourier Transform Infrared (FTIR) spectroscopy combined with artificial neural network (ANN) analysis was designed to differentiate 12 serovars of *Listeria monocytogenes* using a reference database of 106 well-defined strains. External validation was performed using a test set of another 166 *L. monocytogenes* strains. The O antigens (serogroup) of 164 strains (98.8%) could be identified correctly, and H antigens were correctly determined in 152 (91.6%) of the test strains. Importantly, 40 out of 41 potentially epidemic serovar 4b strains were unambiguously identified. FTIR analysis is superior to PCR-based systems for serovar differentiation and has potential for the rapid, simultaneous identification of both species and serovar of an unknown *Listeria* isolate by simply measuring a whole-cell infrared spectrum.

4.2 Introduction

Human listeriosis, caused by the pathogen *Listeria monocytogenes*, mainly is a consequence of the ingestion of contaminated food products and remains a significant public health problem (Gray et al. 2006, Kathariou 2002, Rudolf and Scherer 2001). Since many different contamination routes for this ubiquitous bacterium have been reported (Lemunier et al. 2005, Paillard et al. 2005, Wagner et al. 2005), the development of rapid and accurate typing methods is of particular importance. A substantial number of sensitive, discriminatory and reproducible typing technologies have therefore been developed (Liu 2006).

L. monocytogenes expresses O-somatic and H-flagellar antigens which, by their unique combination, determine the serovar (serotype) of individual strains. Thirteen serovars have been found in this species by using specific and standardized sera (Seeliger and Langer 1979). However, traditional serotyping presents a number of limitations such as the commercial availability and high cost of sera, as well as limited reproducibility. Palumbo et al. (Palumbo et al. 2003) therefore proposed a low-cost enzyme-linked immunosorbent assay combined with commercial antisera for the differentiation of *L. monocytogenes* serovars. This method, nevertheless, is of limited potential for application in routine laboratories due to the complex and laborious protocol. Consequently, very few diagnostic laboratories offer conventional serotyping of *L. monocytogenes*.

Most clinical isolates belong to serovars 1/2a, 1/2b and 4b; among these, the majority of strains which have caused large outbreaks are serovar 4b (Kathariou 2000). In contrast, most food strains belong to serovar 1/2c (Jacquet et al. 2002, Yildirim et al. 2004). There is now considerable evidence that epidemic serovar 4b clones express specific virulence biomarkers (Jacquet et al. 2004) and carry distinct genetic markers (Liu et al. 2006, Yildirim et al. 2004).

So far, only a few well-known strains of serovar 1/2a and 4b have been used to study the virulence of *L. monocytogenes*. Virulence attributes specific to other clinical or food related serovars cannot yet effectively be addressed. Although there are no specific legal specifications for serotyping in terms of food safety or risk assessment at this time, serovar differentiation may be helpful in the future when additional knowledge concerning the relation between the serovar and pathogenic potential of *Listeria monocytogenes* becomes avalailable (Kathariou 2002).

Infrared spectra of microorganisms reflect the overall structure of the molecular constituents of the cell (Naumann et al. 1991, Naumann et al. 1996), and Fourier transform infrared spectroscopical (FTIR) analysis of intact cells has been used to distinguish bacteria at different taxonomic levels (Helm et al. 1991a). Extensive reference libraries containing thousands of spectra of well-characterized microorganisms can be used for the rapid identification of unknown isolates at the species level (Helm et al. 1991, Kümmerle et al. 1998, Maquelin et al. 2003, Oberreuter et al. 2002b, Wenning et al. 2002). A powerful method for data-processing to interpret these complex spectral patterns is an important key for a successful identification. Advanced multivariate methods such as artificial neural networks (ANN) have been shown to be especially advantageous for the analysis of subtle differences at, and partially below, the species level (Goodacre et al. 1996, Schmitt and Udelhoven 2001, Udelhoven et al. 2000). Recently, Rebuffo et al. (Rebuffo et al. 2006) described a superior ANN-based FTIR method for the identification of *Listeria monocytogenes* and related *Listeria* species.

FTIR studies on *Escherichia coli* and *Salmonella enterica* for differentiation of serotypes based on variations in their lipopolysaccharide have been undertaken (Helm et al. 1991a, Kim et al. 2005). However, neither study used a sufficient number of strains to be statistically relevant or resolved the differentiation of all serovars. Therefore, it remains unclear whether FTIR spectroscopy potentially may differentiate the serovars of pathogens, in particular if the technique is applied to a large number of strains covering a significant part of the intraspecific biodiversity. In this study, we therefore applied FTIR combined with ANN to a large and

diverse collection of strains representing 12 serovars of *L. monocytogenes* in order to evaluate whether this method is suitable to discriminate specific spectral patterns which differentiate between *L. monocytogenes* serovars.

4.3 FTIR spectra of *Listeria monocytogenes* reflect serogroup and serovar specific markers

The *Listeria monocytogenes* strains used in this work were grown under standardized conditions on tryptone soy agar plates, and spectra were measured as described elsewhere in chapter 3 (Rebuffo et al. 2006). Fig 4.1 shows typical first derivatives of infrared spectra of 12 *Listeria monocytogenes* serovars.

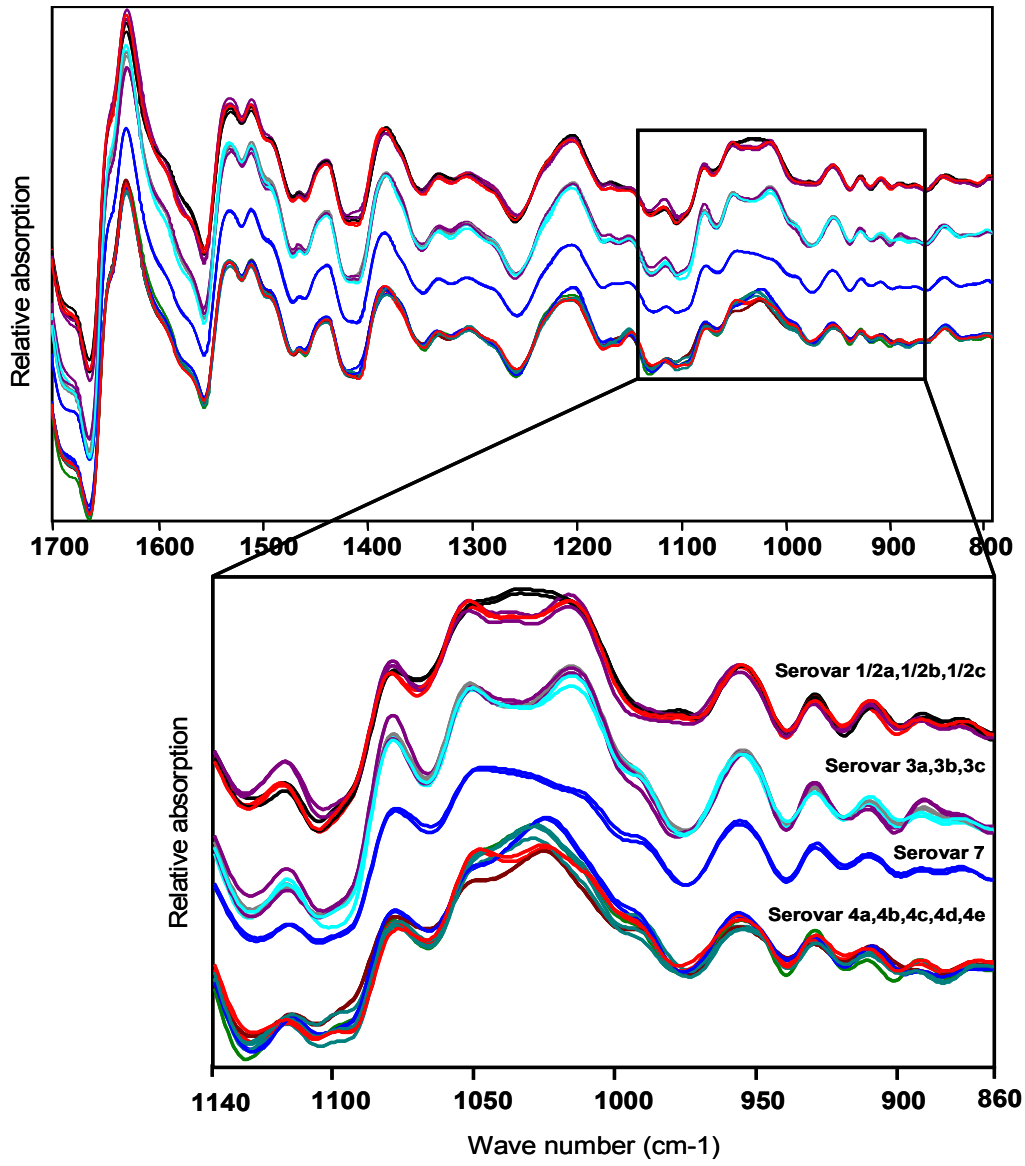

Fig. 4.1 Typical first derivatives of infrared spectra of 12 *Listeria monocytogenes* serovars: (black) *L. m. WSLC 1427* (1/2a), (violet) *L. m. WSLC 1030* (1/2b), (red) *L. m. WSLC 1377* (1/2c), (gray) *L. m.*

WSLC 1485 (3a), (violet) *L. m. WSLC 1444* (3b), (sky-blue) *L. m. WSLC 11082* (3c), (brown) *L. m. WSLC 1939* (4a), (red) *L. m. WSLC 1634* (4b), (blue) *L. m. WSLC 11094* (4c), (gray) *L. m. WSLC 1045* (4d), (green) *L. m. WSLC 1761* (4e), (blue) *L. m. WSLC 1932* (7). Each strain is represented by two independent spectra to show the reproducibility of the measurements. Spectra are stacked to clearly show the spectral differences among the four serogroups (1/2, 3, 7 and 4). The polysaccharide region (1,200-900 cm^{-1}) has been expanded to show the subtle differences between and within the serogroups. *L. m.* (*Listeria monocytogenes*); WSLC (Weihenstephan Listeria Collection).

At a first glance, the polysaccharide region between 900 to 1,200 cm^{-1} displays the most prominent spectral differences among the four serogroups (1/2, 3, 7 and 4). This indicates that carbohydrate containing structures are involved in serogroup discrimination. Moreover, subtle spectral differences within each serogroup due to serovar-specific markers were observed.

FTIR spectra of 106 *L. monocytogenes* strains, including the 69 reference strains of *L. monocytogenes* studied by Rebuffo et al. (Rebuffo et al. 2006) and comprising all known serovars (see Appendix III of chapter 7) were used for hierarchical cluster analysis (HCA) (Fig. 4.2A). This HCA was performed as described by Rebuffo et al. (Rebuffo et al. 2006) using the first derivative of the original spectra covering the regions from 900 to 1,200 and 1,400 to 1,800 cm^{-1}. One major cluster corresponded to the serogroups 1/2, 3 and 7, while another cluster corresponded to serogroup 4. This observation is in accordance with two distinct structural types of teichoic acids found previously for serogroups 1/2, 3 and 7 versus serogroup 4 (Fiedler 1984).

A serogroup 4-specific gene cassette which is absent in serovar 1/2b strains has been found (Lei et al. 2001), and DNA array studies of 13 genes involved in cell wall biosynthesis revealed the same grouping of *L. monocytogenes* serovars (Doumith et al. 2004b) .
These two major serovar groups were used to establish level 1 of a four-layered neural net (Fig. 4.2B). The construction and optimization of the ANN are described in more detail in the section below (4.4 of this chapter).

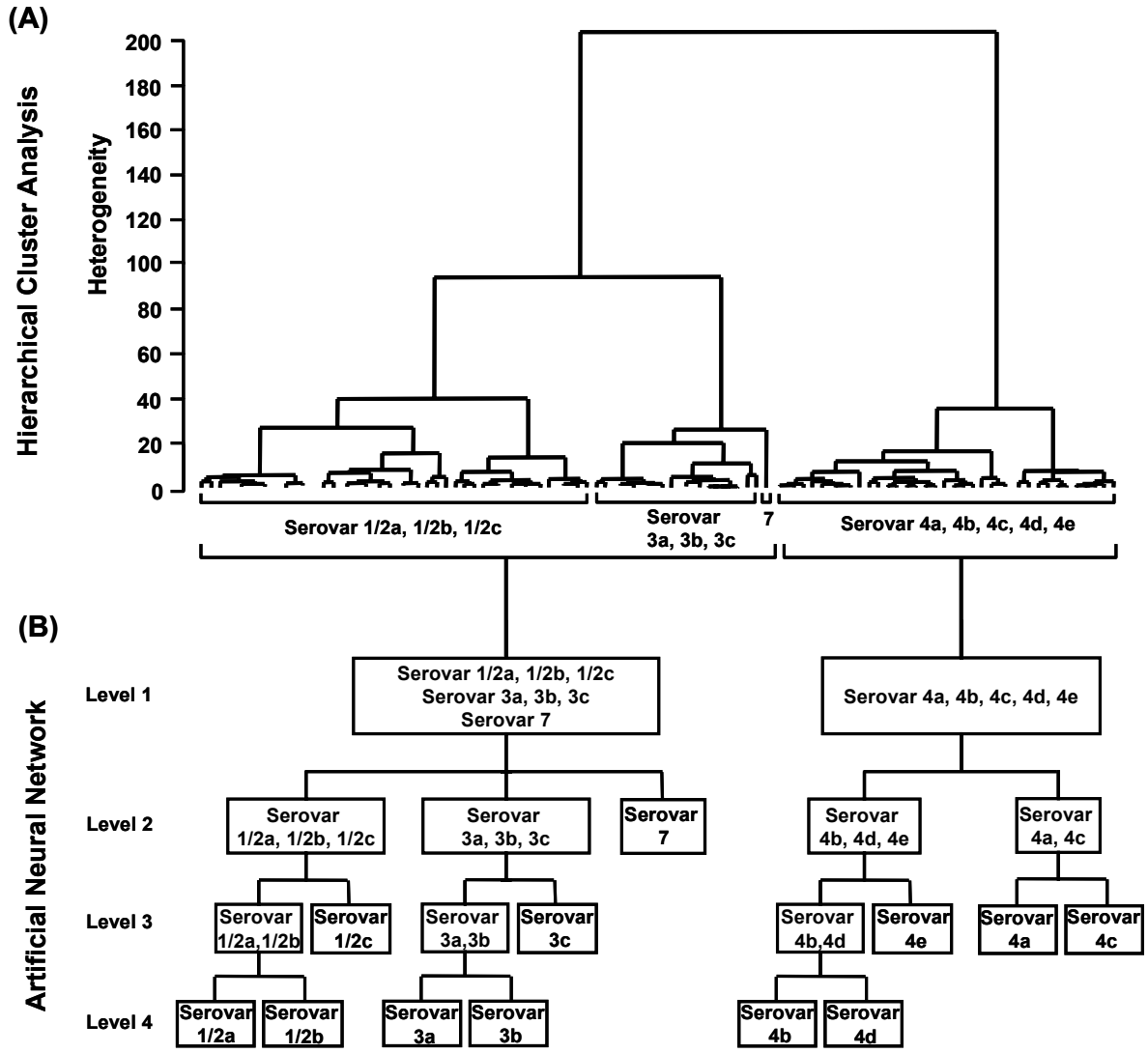

Fig. 4.2 (A) Hierarchical cluster analysis of the first derivative of 106 *L. monocytogenes* strains belonging to 12 serovars, and included in the reference dataset. Spectral regions used: 900- 1,200 cm^{-1}, and 1,400- 1,800 cm^{-1}, correlation with scaling to first range, and Ward's algorithm. (B) Artificial Neural Network classification scheme for the discrimination of serogroups and serovars.

4.4 **Construction and optimization of the Artificial Neuronal Nets (ANN)**

For the ANN development, 1,060 spectra of independent cultures (10 spectra per strain) were randomly distributed into a training set (8 spectra of each strain), a prevalidation set (1 spectrum of each strain) and a test set (1 spectrum of each strain). The NeuroDeveloper™ software (Synthon GmbH, Heidelberg, Germany, www.synthon-analytics.com) was used in order to perform spectral preprocessing and to establish a modular, hierarchical ANN with four levels of spectral classification (Udelhoven et al. 2003).

Spectral preprocessing was performed prior to the artificial neural network development by using spectral windows between 1,800- 1,400 and 1,200- 900 cm^{-1} based on first derivates containing differential information of the molecular composition in FT-IR spectra of bacteria (Fig. 4.1). This pre-selection was further enhanced by a subsequent feature selection algorithm based on covariance analysis followed by a ranking, selecting the most discriminative wavelengths. These wavelengths have been applied to a fully connected feed-forward artificial neural network training using the Rprop (Resilient back-propagation) algorithm (Schmitt and Udelhoven 2001). Each module was trained individually and optimized using individual feature selection procedure and network architecture. Module one, for first level classification, was established with 15 input and 2 outputs neurons, module 2 for level 2 with 30 input and 3 output neurons and 20 input and 2 output, module 3 for level 3 with 90 input and 2 output and 7 input and 2 output neurons, and module 4 for level 4 with 122 input and 2 output neurons.

The information flow during classification runs from the first to the fourth level, the outputs of the first level, in the ANN classification scheme, determine the activation of the specialized subnetworks at a second level. The same procedure is used for the subsequent subnetworks until the serovar-specific subnets are obtained. Finally, all optimized specific subnetworks are integrated in a single ANN classification system as shown in Fig. 4.2B. Based on this classification scheme, success of prediction of the serovars and serogroups resulted from the projection of an unknown *L. monocytogenes* spectrum from the first level to the lower levels of the ANN scheme and were externally validated with 166 independant spectra using the fully established modular system.

4.5 **Validation of FTIR based serovar differentiation**

The identification potential of this ANN was evaluated by an internal validation (compare Rebuffo et al. (Rebuffo et al. 2006). This internal validation (Table 4.1) resulted in a correct identification of 100 % of the somatic antigens (serogroup level). However, only 94.3 % of the flagellar antigens (serovar level) could be identified.

Then, an external validation was performed using a test set of 166 *Listeria monocytogenes* strains isolated from food, the environment, animals and humans. These represented 12 serovars and included the 130 *L. monocytogenes* strains used for external validation by Rebuffo et al (Rebuffo et al. 2006) (see Appendix IV).

Table 4.1 Internal validation of the infrared spectral reference database

***Listeria monocytogenes* Serovars**	**No. of strains:**			
	Tested	**For which O-somatic antigens (serogroup) correctly identified**	**For which H-flagellar antigens (serovar)**	
			Correctly identified	Misidentified
1/2a	23	23	21	2[a]
1/2b	7	7	7	-
1/2c	11	11	10	1[b]
3a	8	8	8	-
3b	12	12	12	-
3c	4	4	4	-
4a	3	3	3	-
4b	24	24	22	2[c]
4c	2	2	1	1[c]
4d	9	9	9	-
4e	2	2	2	-
7	1	1	1	-
Total	106	106 (100%)	100 (94.3%)	5.7 (6%)

[a] Strains misidentified as serovar 1/2c.

[b] Strain misidentified as serovar 1/2a.

[c] Strains misidentified as serovar 4d.

These strains were either identified at the species level according to chapter 3 (Rebuffo et al. 2006) and serotyped based on agglutination reactions with antisera for *L. monocytogenes* (Denka Seiken Co., Japan) according to the instructions of the manufacturer or had been previously serotyped by a reference laboratory. The external validation (Table 4.2) resulted in the correct typing rate of 98.8 % at the serogroup level and 91.6 % at the serovar level.

Importantly, 40 out of 41 potentially outbreak-causing serovar 4b strains were identified correctly. From 87 serovar 1/2a plus 1/2b strains, 84 were assigned to either one of these two serovars. Five serovar 1/2b strains were misidentified as 1/2a. In total, only 3.1 % of the potentially pathogenic serovars 1/2a, 1/2b and 4b were grouped with a potentially nonpathogenic serovar. However, both internal and external validations were somewhat biased since only a few strains of serovars 3c, 4a, 4c, 4e, and 7 were available, which is due to the fact that these are rarely isolated from food and never from patients.

Table 4.2 External validation of the infrared spectral reference database

Listeria monocytogenes Serovars	No. of strains:				
	Tested	For which O-somatic antigens (serogroup)		For which H-flagellar antigens (serovar)	
		Correctly identified	Misidentified	Correctly identified	Misidentified
1/2a	59	57	2[a]	56	3[b]
1/2b	36	36	-	31	5[c]
1/2c	11	11	-	10	1[c]
3a	3	3	-	3	-
3b	5	5	-	5	-
3c	2	2	-	1	1[d]
4a	1	1	-	1	-
4b	41	41	-	40	1[e]
4c	3	3	-	2	1[f]
4d	2	2	-	1	1[f]
4e	2	2	-	1	1[f]
7	1	1	-	1	-
Total	166	164 (98.8%)	2 (1.2%)	152 (91.6%)	14 (8.4%)

[a] Strains misidentified as serogroup 3.

[b] Strains misidentified as serovar 1/2c.

[c] Strain(s) misidentified as serovar 1/2a.

[d] Strain misidentified as serovar 3b.

[e] Strain misidentified as serovar 4d.

[f] Strain misidentified as serovar 4b.

4.6 Comparison of FTIR and PCR based serovar differentiation

Due to the importance of serotyping *L. monocytogenes*, a few PCR-based methods have been proposed. However, some are limited to differentiation of strains into only two or three serovar groups (Comi et al. 1997, Jinneman and Hill 2001, Manzano et al. 1998) or to differentiation of only two serovars from the others (Zhang and Knabel 2005). Others are more complicated since two or even three independent PCRs reactions are needed (Borucki and Call 2003, Jinneman and Hill 2001). In contrast, the PCR system developed by Doumith et al. (Doumith et al. 2004a), in a one-step multiplex PCR, allows the differentiation of *L. monocytogenes* strains into four "serovar groups". Group 1 comprises serovars 1/2a and 3a; group 2 comprises serovars 1/2c and 3c; group 3 contains serovars 1/2b, 3b and 7; and group 4 serovars 4b, 4d, and 4e. However, individual serovars cannot be separated. We applied this PCR system to the 166 strains of our external-validation strain set. The PCR-based method correctly differentiated 159 (4 not typeable and 3 incorrectly typed [see table in Appendix IV of chapter 7]) out of the 166 strains (95.8%) to the serovar group level, which is comparable to its previous validation (Doumith et al. 2005). FTIR-based serotyping was able to correctly

discriminate 164 out of the 166 strains (98.8%) at the serogroup level. In addition, FTIR is much more discriminatory since 91.6% of the individual serovars can be determined (Table 4.2).

4.7 **Conclusion**

Rebuffo et al. (Rebuffo et al. 2006) have previously described an ANN-based FTIR method for the reliable identification of all *Listeria* species in only 25 hours (chapter 3). Here, we report on the development of a *L. monocytogenes* ANN subnet in order to additionally identify serogroups and serovars. The integration of both classification systems now offers the possibility to simultaneously identify *Listeria* at the species level, *L. monocytogenes* at the serogroup level, and most *L. monocytogenes* at the serovar level in a single step by simply measuring an infrared spectrum of a pure *Listeria* culture. Our data indicate that this method is superior to molecular approaches for *L. monocytogenes* serovar determination. We suggest that FTIR identification and serotyping constitute a rapid and inexpensive tool which may be suitable for diagnostic laboratories. This tool may be used routinely in food control to gain additional information on the pathogenic potential of strains isolated from the food-processing chain.

4.8 **Acknowledgments**

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5 General conclusions

A comparative analysis of the identification results of *Listeria* to the species level using FTIR-macro- and FTIR–micro-samples methods demonstrated that the FTIR-macro-samples method is the most adequate to apply for *Listeria*. The study of the factors which influence the discrimination of *Listeria* species using FTIR-micro-samples method showed a limitation when cells of young colonies were used since these present a lower interspecies diversity, compared to bacterial cells from a lawn culture being 24 hours old, and in stationary phase such as in the FTIR –macro-samples method. Additionally, the heterogeneity of the cell growth increased with the colony size and is species and strain dependent. This interferes with the discrimination of *L. innocua, L. ivanovii*, and *L. monocytogenes*. We therefore state that the age of the culture does represent an important factor that influences the discrimination power of the method and that the cell growth heterogeneity represents an additional factor to be considered when FTIR- micro-samples method is used. Furthermore, in order to obtain an identification success of approximately 90% each species of *Listeria* should be represented in the database by 20 to 25 strains to cover the natural intraspecies variability.

FTIR spectroscopy combined with a large and standardized microorganism database presents a great potential for the identification of *Listeria* species in routine analysis. Particularly, for the differentiation of closely related species of *Listeria* the use of the advanced multivariate and supervised analysis method, the Artificial Neural Network improves the discrimination capacity due to a better extraction of information contained in the spectra. So far, no other phenotypical method has been described in the literature that provides an overall correct identification rate of 96% for all *Listeria* species and a success rate of 99.2% for correct *L. monocytogenes* identification in a rapid, simple and cost-efficient procedure. Therefore, we conclude that the use of a database including a number of strains which cover the entire biological variance of each species in combination with ANN analysis will allow a reliable interspecies differentiation.

The observation of serogroup- and serovar-specific markers in the FTIR spectra of *L. monocytogenes* strains has revealed the sensitivity of the FTIR method for the discrimination of specific patterns below the species level. Despite the closeness of the chemical structure of the different serogroups of *L. monocytogenes*, the FTIR method combined with ANN allows a successful discrimination of all serogroups and serovars at a success rate of 98.8% and 91.8% respectively. Additionally, a correct identification rate of 97% for the potentially pathogenic serovars 1/2a, 1/2b, and 4b demonstrates the capability of this method to be used for effective

detection and control of contamination sources. Furthermore, the comparison of these results with those obtained by the most discriminatory PCR-based method showed the superiority of the ANN based of FTIR when used for the serogroup and independent serovar differentiation.

Finally, the most attractive feature of this new technology is the capability of a simultaneous identification and typing of *Listeria* strains in one test, a powerful feature that none of the other tests can offer. Furthermore, unambiguous *Listeria* species and *Listeria monocytogenes* serovar differentiation from isolates with different geographical origins demonstrate the capacity of the ANN based FTIR method to be used in different laboratories as universal reference database. Moreover, this standardized classification system lends itself to high-throughput and semi-automation, and shows a great promise for future routine applications in food control and the epidemiological identification of *Listeria*.

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7 Appendixes

7.1 Appendix I: List of reference strains for the FTIR database for *Listeria* species

Alphabetical list of *Listeria* species contained in the reference FTIR spectral database in Chapter 3

ATCC, strains fom the American Type Culture Collection;
SLCC, strains fom the Special Listeria Culture Collection;
WSLC, strains fom the Weihenstephan Collection;
API, API Listeria test
M. PCR, multiplex PCR
Seq iap-thy, sequentiation of the *iap* and *thy* genes;
Seq iap, sequentiation of the *iap* gen;
Seq thy, sequentiation of the *thy* gen;
type strain T

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria innocua	2011	33090 T	3379	Seq iap-thy
Listeria innocua	2025		2745	Seq iap-thy
Listeria innocua	2054		4286	Seq iap-thy
Listeria innocua	2097		8799	Seq iap-thy
Listeria innocua	2101			Seq iap-thy
Listeria innocua	2110			Seq iap-thy
Listeria innocua	2160			Seq iap-thy
Listeria innocua	2214			Seq iap-thy
Listeria innocua	2310			Seq iap-thy
Listeria innocua	2339			Seq thy
Listeria innocua	2368			Seq iap-thy
Listeria innocua	2371			Seq iap-thy
Listeria innocua	2380			Seq iap-thy
Listeria innocua	2390			Seq iap
Listeria innocua	2394			Seq iap-thy
Listeria innocua	2395			Seq iap-thy
Listeria innocua	2412			Seq iap-thy
Listeria innocua	2414			Seq iap
Listeria innocua	2463			Seq iap-thy
Listeria innocua	2521			Seq iap-thy
Listeria innocua	2522			Seq iap-thy
Listeria innocua	2523			Seq iap-thy
Listeria innocua	2536			Seq iap-thy
Listeria innocua	2567			Seq iap-thy
Listeria innocua	2568			Seq iap-thy
Listeria innocua	2653			Seq iap
Listeria innocua	2668			Seq iap-thy
Listeria innocua	2693			Seq iap-thy
Listeria innocua	2698			Seq iap-thy
Listeria innocua	2699			Seq iap-thy
Listeria innocua	2711			Seq iap-thy
Listeria innocua	2736			Seq thy
Listeria innocua	2738			Seq iap-thy

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria innocua	2739			Seq iap-thy
Listeria innocua	2740			Seq iap-thy
Listeria innocua	2743			Seq iap-thy
Listeria innocua	2745			Seq iap-thy
Listeria innocua	2760			Seq iap-thy
Listeria innocua	5908			Seq iap-thy
Listeria innocua	5909			Seq iap-thy
Listeria innocua	5910			Seq iap-thy
Listeria innocua	5911			Seq iap-thy
Listeria innocua	5912			Seq iap-thy
Listeria innocua	5914			Seq iap-thy
Listeria innocua	5918			Seq iap-thy
Listeria innocua	5919			Seq iap-thy
Listeria innocua	5920			Seq iap-thy
Listeria innocua	5921			Seq iap-thy
Listeria innocua	5922			Seq iap-thy
Listeria innocua	5923			Seq iap-thy
Listeria innocua	5924			Seq iap-thy
Listeria innocua	20109		8818	Seq iap-thy
Listeria innocua	20125		8844	Seq iap-thy
Listeria innocua	21058			API, M.PCR
Listeria innocua	21059			API, M.PCR
Listeria innocua	21060			API, M.PCR
Listeria innocua	21061			API, M.PCR
Listeria innocua	21062			API, M.PCR
Listeria innocua	21063			API, M.PCR
Listeria innocua	21064			API, M.PCR
Listeria innocua	21065			API, M.PCR
Listeria innocua	21066			API, M.PCR
Listeria innocua	21107		7298	API, M.PCR
Listeria innocua	21108			API, M.PCR
Listeria innocua	21109			API, M.PCR
Listeria ivanovii	3009		4769	Seq iap-thy
Listeria ivanovii	3010	19119 T	2379	Seq iap-thy
Listeria ivanovii	3027		4713	Seq iap-thy
Listeria ivanovii	3050		4719	Seq iap-thy
Listeria ivanovii	3058		3584	Seq iap-thy
Listeria ivanovii	3059		3706	Seq iap-thy
Listeria ivanovii	3060		3765	Seq thy
Listeria ivanovii	3061		3772	Seq iap-thy
Listeria ivanovii	3062		3773	Seq iap-thy
Listeria ivanovii	3716			Seq iap-thy
Listeria ivanovii	3718			Seq iap-thy
Listeria ivanovii	3867			Seq iap-thy
Listeria ivanovii	3870			Seq iap-thy
Listeria ivanovii	3873			Seq iap-thy
Listeria ivanovii	3874			Seq iap-thy
Listeria ivanovii	3875			Seq iap-thy
Listeria ivanovii	3876			Seq iap-thy
Listeria ivanovii	3877			Seq iap-thy
Listeria ivanovii	3878			Seq iap-thy

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria ivanovii	3879			Seq iap-thy
Listeria ivanovii	3881		2028	Seq iap-thy
Listeria ivanovii	3883		2102	Seq iap-thy
Listeria ivanovii	3884		2379	Seq iap-thy
Listeria ivanovii	3885		4706	Seq iap-thy
Listeria ivanovii	3886		4770	Seq iap-thy
Listeria ivanovii	3906			Seq iap-thy
Listeria ivanovii	30151		8854	Seq iap-thy
Listeria ivanovii	30152		5638	Seq iap-thy
Listeria ivanovii	30153		4121	Seq iap-thy
Listeria ivanovii	30154		5579	API, M.PCR
Listeria ivanovii	30155		2443	API, M.PCR
Listeria ivanovii	30156		6967	API, M.PCR
Listeria ivanovii	30157		7926	API, M.PCR
Listeria ivanovii	30158		5843	API, M.PCR
Listeria ivanovii	30159		6966	Seq iap-thy
Listeria ivanovii	30160		6965	Seq iap-thy
Listeria ivanovii	30161		5486	API, M.PCR
Listeria ivanovii	30162		5756	API, M.PCR
Listeria ivanovii	30163		5755	Seq iap-thy
Listeria ivanovii	30166		8431	Seq iap-thy
Listeria ivanovii	30168		7927	Seq iap-thy
Listeria monocytogenes	1034		2482	API, M.PCR
Listeria monocytogenes	1040	15313 T	53	API, M.PCR
Listeria monocytogenes	1075		8811	API, M.PCR
Listeria monocytogenes	1118			Seq thy
Listeria monocytogenes	1153			Seq thy
Listeria monocytogenes	1175			Seq thy
Listeria monocytogenes	1182			Seq thy
Listeria monocytogenes	1211			Seq thy
Listeria monocytogenes	1266			API, M.PCR
Listeria monocytogenes	1303			Seq iap-thy
Listeria monocytogenes	1361			Seq iap-thy
Listeria monocytogenes	1363			API, M.PCR
Listeria monocytogenes	1364			API, M.PCR
Listeria monocytogenes	1370			Seq iap-thy
Listeria monocytogenes	1377			API, M.PCR
Listeria monocytogenes	1383			Seq iap-thy
Listeria monocytogenes	1399			API, M.PCR
Listeria monocytogenes	1400			API, M.PCR
Listeria monocytogenes	1411			API, M.PCR
Listeria monocytogenes	1413			API, M.PCR
Listeria monocytogenes	1416			Seq iap-thy
Listeria monocytogenes	1425			API, M.PCR
Listeria monocytogenes	1427			API, M.PCR
Listeria monocytogenes	1451			API, M.PCR
Listeria monocytogenes	1452			API, M.PCR
Listeria monocytogenes	1456			API, M.PCR
Listeria monocytogenes	1670			Seq thy
Listeria monocytogenes	1685			API, M.PCR
Listeria monocytogenes	1856			Seq iap-thy

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria monocytogenes	1857			Seq iap-thy
Listeria monocytogenes	1858			API, M.PCR
Listeria monocytogenes	1859			Seq iap-thy
Listeria monocytogenes	1860			API, M.PCR
Listeria monocytogenes	1861			API, M.PCR
Listeria monocytogenes	1862			API, M.PCR
Listeria monocytogenes	1863			Seq iap-thy
Listeria monocytogenes	1864			Seq iap-thy
Listeria monocytogenes	1865			Seq iap-thy
Listeria monocytogenes	1929			Seq iap-thy
Listeria monocytogenes	1931			Seq iap-thy
Listeria monocytogenes	1932			Seq iap-thy
Listeria monocytogenes	1933			Seq iap-thy
Listeria monocytogenes	1935			Seq iap-thy
Listeria monocytogenes	1936			Seq iap-thy
Listeria monocytogenes	1937			Seq iap-thy
Listeria monocytogenes	1938			Seq iap-thy
Listeria monocytogenes	1940			Seq iap-thy
Listeria monocytogenes	1941			Seq iap-thy
Listeria monocytogenes	1942			Seq iap-thy
Listeria monocytogenes	11042			API, M.PCR
Listeria monocytogenes	11043			API, M.PCR
Listeria monocytogenes	11044			API, M.PCR
Listeria monocytogenes	11046			API, M.PCR
Listeria monocytogenes	11047			API, M.PCR
Listeria monocytogenes	11078		5543	API, M.PCR
Listeria monocytogenes	11079		7140	API, M.PCR
Listeria monocytogenes	11080		7381	API, M.PCR
Listeria monocytogenes	11081		7392	API, M.PCR
Listeria monocytogenes	11082		6753	API, M.PCR
Listeria monocytogenes	11083		6848	API, M.PCR
Listeria monocytogenes	11084		7207	API, M.PCR
Listeria monocytogenes	11086		5069	API, M.PCR
Listeria monocytogenes	11087		5070	API, M.PCR
Listeria monocytogenes	11088		4925	API, M.PCR
Listeria monocytogenes	11089		4954	API, M.PCR
Listeria monocytogenes	11090		6277	API, M.PCR
Listeria monocytogenes	11102		7093	API, M.PCR
Listeria monocytogenes	11103		7002	API, M.PCR
Listeria monocytogenes	11105		7069	API, M.PCR
Listeria seeligeri	4268			Seq iap-thy
Listeria seeligeri	4345			Seq iap-thy
Listeria seeligeri	4389			Seq iap-thy
Listeria seeligeri	4449			Seq iap-thy
Listeria seeligeri	4450			Seq iap-thy
Listeria seeligeri	4453			Seq iap-thy
Listeria seeligeri	4457			Seq iap-thy
Listeria seeligeri	4462			Seq iap-thy
Listeria seeligeri	4491			Seq thy
Listeria seeligeri	4600			Seq iap
Listeria seeligeri	4601			Seq iap
Listeria seeligeri	4664			Seq iap-thy

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria seeligeri	4669			Seq iap-thy
Listeria seeligeri	4701			Seq iap-thy
Listeria seeligeri	4709			Seq iap-thy
Listeria seeligeri	4731			Seq iap-thy
Listeria seeligeri	5913			Seq iap-thy
Listeria seeligeri	40127		8604	Seq iap-thy
Listeria seeligeri	40128		8624	Seq iap-thy
Listeria seeligeri	40129		8601	Seq iap-thy
Listeria seeligeri	40130		8591	Seq iap-thy
Listeria seeligeri	40131		8695	Seq iap-thy
Listeria seeligeri	40132		8587	Seq iap-thy
Listeria seeligeri	40133		8600	Seq iap-thy
Listeria seeligeri	40134		8598	Seq iap-thy
Listeria seeligeri	40135		8615	Seq iap
Listeria seeligeri	40136		8610	Seq iap-thy
Listeria seeligeri	40137		8623	Seq iap-thy
Listeria seeligeri	40138		8621	Seq iap-thy
Listeria seeligeri	40139		8378	Seq iap-thy
Listeria seeligeri	40141		8127	Seq thy
Listeria seeligeri	40144		8609	Seq thy
Listeria seeligeri	40145		8626	Seq iap-thy
Listeria seeligeri	41051			API, M.PCR
Listeria seeligeri	41052			API, M.PCR
Listeria seeligeri	41053			API, M.PCR
Listeria seeligeri	41054			API, M.PCR
Listeria seeligeri	41112		7379	API, M.PCR
Listeria seeligeri	41113		7380	API, M.PCR
Listeria seeligeri	41114		6735	API, M.PCR
Listeria seeligeri	41115		7124	API, M.PCR
Listeria seeligeri	41116		6284	API, M.PCR
Listeria seeligeri	41117		6595	API, M.PCR
Listeria seeligeri	41118		6598	API, M.PCR
Listeria seeligeri	41120		6745	API, M.PCR
Listeria seeligeri	41121		6746	API, M.PCR
Listeria seeligeri	41123			API, M.PCR
Listeria seeligeri	51123		5870	API, M.PCR
Listeria seeligeri	51124		5873	API, M.PCR
Listeria welshimeri	5008			API, M.PCR
Listeria welshimeri	5013	35897 T	5334	Seq iap-thy
Listeria welshimeri	5639			API, M.PCR
Listeria welshimeri	5708			Seq iap-thy
Listeria welshimeri	5720			Seq iap-thy
Listeria welshimeri	5721			Seq iap
Listeria welshimeri	5723			Seq iap
Listeria welshimeri	5729			Seq iap
Listeria welshimeri	5890			Seq iap-thy
Listeria welshimeri	5891			Seq iap-thy
Listeria welshimeri	5917			Seq iap
Listeria welshimeri	5928			API, M.PCR
Listeria welshimeri	5943			Seq iap-thy
Listeria welshimeri	5944			Seq iap-thy
Listeria welshimeri	5945			Seq iap-thy

Strains	WSLC	ATCC	SLCC	Identification methods
Listeria welshimeri	50146		7622	Seq iap-thy
Listeria welshimeri	50147		6199	Seq iap-thy
Listeria welshimeri	50148		7625	Seq iap-thy
Listeria welshimeri	50150		5828	Seq iap-thy

7.2 Appendix II: List of strains for the external validation for *Listeria* species

Alphabetical list of *Listeria* isolates in the test strain dataset in chapter 3

Gly, strains in the Glycerin- stock in Weihenstephan;
WSLC, strains fom the Weihenstephan Collection;
API; strains differentiated using the API Listeria test
Hemol., hemolysis test
FT-IR, strains identified by the Infrared spectroscopic method;
M. PCR, strains differentiated using the Multiplex PCR method.

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. innocua		3040	API, FT-IR	Germany	gully
L. innocua		3041	API, FT-IR	unknown	unknown
L. innocua		3044	API, FT-IR	Germany	environmental
L. innocua		3045	API, FT-IR	Germany	wash water
L. innocua		3046	API, FT-IR	Germany	cheese
L. innocua		3047	API, FT-IR	Germany	swabs
L. innocua		3048	API, FT-IR	Germany	gully
L. innocua		3049	API, FT-IR, M.PCR, Hemol.	Germany	gully
L. innocua		3061	API, FT-IR	Germany	gully
L. innocua		3062	API, FT-IR	Germany	gully
L. innocua		3063	API, FT-IR	Germany	floor
L. innocua		3065	API, FT-IR	Germany	gully
L. innocua		3066	API, FT-IR	Germany	gully
L. innocua		3067	API, FT-IR, M.PCR	Germany	swabs
L. innocua		3076	API, FT-IR	Germany	swabs
L. innocua		3078	API, FT-IR	Germany	swabs
L. innocua		3079	API, FT-IR	Germany	swabs
L. innocua		3080	API, FT-IR	Germany	swabs
L. innocua		3085	API, FT-IR	Germany	cheese
L. innocua		3086	API, FT-IR	Germany	cheese
L. innocua		3087	API, FT-IR	Germany	cheese
L. innocua		3088	API, FT-IR	Germany	cheese
L. innocua		3089	API, FT-IR	Germany	swabs
L. innocua		3090	API, FT-IR	Germany	swabs
L. innocua		3091	API, FT-IR	Germany	swabs
L. innocua		3093	API, FT-IR	Germany	swabs
L. innocua		3096	API, FT-IR, M.PCR, Hemol.	Germany	cheese
L. innocua		3097	API, FT-IR	Germany	cheese
L. innocua	1636		API, FT-IR	United states	unknown
L. innocua	1705		API, FT-IR	Germany	unknown
L. innocua	2012		API, FT-IR	Denmark	unknown
L. innocua	2014		API, FT-IR	unknown	unknown
L. innocua	2021		API, FT-IR	unknown	unknown
L. innocua	2022		API, FT-IR	unknown	unknown
L. innocua	2023		API, FT-IR, M.PCR	unknown	unknown
L. innocua	2024		API, FT-IR	unknown	unknown
L. innocua	2035		API, FT-IR, M.PCR, Hemol.	unknown	unknown
L. innocua	2051		API, FT-IR	unknown	unknown
L. innocua	2052		API, FT-IR	unknown	unknown

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. innocua	2053		API, FT-IR	unknown	unknown
L. innocua	2055		API, FT-IR	unknown	unknown
L. innocua	2056		API, FT-IR	unknown	unknown
L. innocua	2057		API, FT-IR	unknown	unknown
L. innocua	2093		API, FT-IR	unknown	unknown
L. innocua	2095		API, FT-IR	unknown	unknown
L. innocua	2358		API, FT-IR	Hungria	chicken
L. innocua	2369		API, FT-IR	Netherlands	chicken
L. innocua	2410		API, FT-IR	Germany	chicken
L. innocua	2466		API, FT-IR	Austria	cheese
L. innocua	2481		API, FT-IR	Austria	cheese
L. innocua	2675		API, FT-IR	Germany	food
L. innocua	3907		API, FT-IR, M.PCR, Hemol.	Spain	environment
L. innocua	4974		API, FT-IR	Germany	food
L. innocua	5927		API, FT-IR, M.PCR	Austria	cheese
L. innocua	5889		API, FT-IR, M.PCR	unknown	unknown
L. innocua	21067		API, FT-IR	Argentina	sausage
L. innocua	21068		API, FT-IR	Argentina	sausage
L. innocua	21069		API, FT-IR	Argentina	sausage
L. innocua	21070		API, FT-IR	Argentina	sausage
L. innocua	51139		API, FT-IR, M.PCR	Germany	poultry salad
L. ivanovii	3026	1473	API, FT-IR	unknown	unknown
L. ivanovii		1522	API, FT-IR	unknown	unknown
L. ivanovii		1650	API, FT-IR	unknown	unknown
L. ivanovii		1652	API, FT-IR	unknown	unknown
L. ivanovii		2386	API, FT-IR	unknown	unknown
L. ivanovii		2416	API, FT-IR	Spain	animal
L. ivanovii		2455	API, FT-IR	Germany	food
L. ivanovii	3695		API, FT-IR	unknown	unknown
L. ivanovii	3717		API, FT-IR	Germany	unknown
L. ivanovii	3866		API, FT-IR	Germany	Milk
L. ivanovii	3868		API, FT-IR	Germany	smear bath
L. ivanovii	3872		API, FT-IR	Germany	smear bath
L. ivanovii	3887		API, FT-IR, M.PCR, Hemol.	unknown	unknown
L. ivanovii	3892		API, FT-IR	unknown	unknown
L. ivanovii	3893		API, FT-IR, M.PCR	unknown	unknown
L. ivanovii	3894		API, FT-IR, M.PCR	unknown	unknown
L. ivanovii	3895		API, FT-IR	Austria	unknown
L. ivanovii	3896		API, FT-IR	Austria	raw milk
L. ivanovii	3897		API, FT-IR, M.PCR	Austria	raw milk
L. ivanovii	3898		API, FT-IR	Austria	raw milk
L. ivanovii	3899		API, FT-IR	Austria	smear bath
L. ivanovii	3901		API, FT-IR	Austria	smear bath
L. ivanovii	3902		API, FT-IR	Austria	smear bath
L. ivanovii	3903		API, FT-IR	Austria	smear bath
L. ivanovii	3904		API, FT-IR	Austria	smear bath
L. ivanovii	3905		API, FT-IR	Austria	unknown
L. ivanovii	30164		API, FT-IR	Austria	unknown
L. ivanovii	30165		API, FT-IR	unknown	unknown
L. monocytogenes		3042	API, FT-IR	Germany	gully
L. monocytogenes		3043	API, FT-IR	Germany	floor

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. monocytogenes		3064	API, FT-IR, M.PCR, Hemol.	Germany	wash water
L. monocytogenes		3068	API, FT-IR	Germany	gully
L. monocytogenes		3071	API, FT-IR, M.PCR	Germany	soft cheese
L. monocytogenes		3072	API, FT-IR	Germany	soft cheese
L. monocytogenes		3073	API, FT-IR	Germany	gully
L. monocytogenes		3074	API, FT-IR	Germany	smear bath
L. monocytogenes		3075	API, FT-IR	unknown	unknown
L. monocytogenes		3077	API, FT-IR, M.PCR	Germany	gully
L. monocytogenes		3092	API, FT-IR	Germany	smear bath
L. monocytogenes	1042		API, FT-IR	unknown	unknown
L. monocytogenes	1043		API, FT-IR	unknown	unknown
L. monocytogenes	1044		API, FT-IR	unknown	unknown
L. monocytogenes	1046		API, FT-IR	unknown	unknown
L. monocytogenes	1116		API, FT-IR	unknown	unknown
L. monocytogenes	1117		API, FT-IR	unknown	unknown
L. monocytogenes	1119		API, FT-IR	unknown	unknown
L. monocytogenes	1120		API, FT-IR	unknown	unknown
L. monocytogenes	1121		API, FT-IR	unknown	unknown
L. monocytogenes	1122		API, FT-IR	unknown	unknown
L. monocytogenes	1376		API, FT-IR	Netherlands	soup hen
L. monocytogenes	1475		API, FT-IR	Austria	cheese
L. monocytogenes	1476		API, FT-IR	Austria	cheese
L. monocytogenes	1482		API, FT-IR	Austria	cheese
L. monocytogenes	1483		API, FT-IR	Austria	cheese
L. monocytogenes	1484		API, FT-IR	Austria	cheese
L. monocytogenes	1486		API, FT-IR	Austria	cheese
L. monocytogenes	1487		API, FT-IR	Austria	cheese
L. monocytogenes	1489		API, FT-IR	Austria	cheese
L. monocytogenes	1490		API, FT-IR	Austria	cheese
L. monocytogenes	1493		API, FT-IR	Austria	cheese
L. monocytogenes	1497		API, FT-IR	Austria	cheese
L. monocytogenes	1498		API, FT-IR	Austria	cheese
L. monocytogenes	1499		API, FT-IR	Austria	cheese
L. monocytogenes	1504		API, FT-IR	Austria	cheese
L. monocytogenes	1506		API, FT-IR	Austria	cheese
L. monocytogenes	1510		API, FT-IR	Austria	cheese
L. monocytogenes	1511		API, FT-IR	Austria	cheese
L. monocytogenes	1512		API, FT-IR	Austria	cheese
L. monocytogenes	1514		API, FT-IR	Austria	cheese
L. monocytogenes	1516		API, FT-IR	Austria	cheese
L. monocytogenes	1551		API, FT-IR	Germany	unknown
L. monocytogenes	1552		API, FT-IR	Germany	unknown
L. monocytogenes	1556		API, FT-IR	Germany	unknown
L. monocytogenes	1557		API, FT-IR, M.PCR, Hemol.	Germany	unknown
L. monocytogenes	1558		API, FT-IR	Germany	unknown
L. monocytogenes	1559		API, FT-IR	Germany	unknown
L. monocytogenes	1564		API, FT-IR	Germany	unknown
L. monocytogenes	1571		API, FT-IR	Germany	unknown
L. monocytogenes	1574		API, FT-IR	Germany	unknown
L. monocytogenes	1575		API, FT-IR	Germany	unknown
L. monocytogenes	1576		API, FT-IR, M.PCR	Germany	unknown

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. monocytogenes	1577		API, FT-IR	Germany	unknown
L. monocytogenes	1580		API, FT-IR	Germany	gully
L. monocytogenes	1582		API, FT-IR	Germany	unknown
L. monocytogenes	1584		API, FT-IR	Germany	unknown
L. monocytogenes	1585		API, FT-IR	Germany	unknown
L. monocytogenes	1592		API, FT-IR	Germany	unknown
L. monocytogenes	1595		API, FT-IR	Germany	unknown
L. monocytogenes	1596		API, FT-IR	Germany	unknown
L. monocytogenes	1602		API, FT-IR	Germany	unknown
L. monocytogenes	1607		API, FT-IR	Germany	unknown
L. monocytogenes	1609		API, FT-IR	Germany	unknown
L. monocytogenes	1612		API, FT-IR	Germany	unknown
L. monocytogenes	1614		API, FT-IR	Germany	unknown
L. monocytogenes	1618		API, FT-IR	Germany	unknown
L. monocytogenes	1619		API, FT-IR	Germany	unknown
L. monocytogenes	1621		API, FT-IR	Germany	unknown
L. monocytogenes	1625		API, FT-IR	Germany	unknown
L. monocytogenes	1626		API, FT-IR	Germany	unknown
L. monocytogenes	1629		API, FT-IR	Germany	unknown
L. monocytogenes	1647		API, FT-IR	Germany	silage
L. monocytogenes	1661		API, FT-IR	Germany	unknown
L. monocytogenes	1665		API, FT-IR	Germany	raw milk
L. monocytogenes	1703		API, FT-IR	Germany	unknown
L. monocytogenes	1704		API, FT-IR	Germany	unknown
L. monocytogenes	1713		API, FT-IR	Germany	unknown
L. monocytogenes	1714		API, FT-IR	Germany	unknown
L. monocytogenes	1763		API, FT-IR, M.PCR, Hemol.	France	unknown
L. monocytogenes	1766		API, FT-IR, M.PCR	Germany	animal
L. monocytogenes	1767		API, FT-IR, M.PCR	Germany	animal
L. monocytogenes	1768		API, FT-IR, M.PCR	unknown	animal
L. monocytogenes	1769		API, FT-IR	unknown	poult
L. monocytogenes	1770		API, FT-IR	unknown	goat
L. monocytogenes	1771		API, FT-IR	unknown	goat
L. monocytogenes	1773		API, FT-IR	unknown	sheep
L. monocytogenes	1774		API, FT-IR	unknown	lamb
L. monocytogenes	1775		API, FT-IR	Germany	corn
L. monocytogenes	1776		API, FT-IR	Germany	silage
L. monocytogenes	1778		API, FT-IR	Germany	grass
L. monocytogenes	1780		API, FT-IR	Germany	grass
L. monocytogenes	1845		API, FT-IR	Germany	grass
L. monocytogenes	2360		API, FT-IR	Germany	poult
L. monocytogenes	2397		API, FT-IR	Germany	unknown
L. monocytogenes	4448		API, FT-IR	Germany	unknown
L. monocytogenes	4978		API, FT-IR	unknown	unknown
L. monocytogenes	4981		API, FT-IR	unknown	unknown
L. monocytogenes	11005		API, FT-IR	France	milk
L. monocytogenes	11006		API, FT-IR	France	cheese
L. monocytogenes	11007		API, FT-IR	France	cheese
L. monocytogenes	11008		API, FT-IR	France	cheese
L. monocytogenes	11009		API, FT-IR	France	cheese
L. monocytogenes	11010		API, FT-IR	France	cheese

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. monocytogenes	11011		API, FT-IR	France	cheese
L. monocytogenes	11012		API, FT-IR	France	cheese
L. monocytogenes	11013		API, FT-IR	France	cheese
L. monocytogenes	11014		API, FT-IR	France	cheese
L. monocytogenes	11015		API, FT-IR	France	cheese
L. monocytogenes	11016		API, FT-IR	France	cheese
L. monocytogenes	11017		API, FT-IR	France	cheese
L. monocytogenes	11018		API, FT-IR	France	cheese
L. monocytogenes	11019		API, FT-IR	France	feed
L. monocytogenes	11021		API, FT-IR	France	feces
L. monocytogenes	11022		API, FT-IR	France	cheese
L. monocytogenes	11023		API, FT-IR	France	cheese
L. monocytogenes	11024		API, FT-IR	France	cheese
L. monocytogenes	11025		API, FT-IR	France	milk filter
L. monocytogenes	11026		API, FT-IR	France	teat
L. monocytogenes	11027		API, FT-IR	France	cheese
L. monocytogenes	11028		API, FT-IR	France	cheese
L. monocytogenes	11030		API, FT-IR	France	ground cellar
L. monocytogenes	11031		API, FT-IR	France	cheese
L. monocytogenes	11032		API, FT-IR	France	cheese
L. monocytogenes	11033		API, FT-IR	France	cheese
L. monocytogenes	11034		API, FT-IR	France	cheese
L. monocytogenes	11035		API, FT-IR	France	ground cellar
L. monocytogenes	11039		API, FT-IR	France	cheese
L. monocytogenes	11040		API, FT-IR	France	cheese
L. monocytogenes	11071		API, FT-IR	France	cheese
L. seeligeri		1482	API, FT-IR	unknown	unknown
L. seeligeri		1483	API, FT-IR	unknown	unknown
L. seeligeri		1534	API, FT-IR, M.PCR	unknown	unknown
L. seeligeri		1535	API, FT-IR	unknown	unknown
L. seeligeri		1604	API, FT-IR	unknown	unknown
L. seeligeri		1605	API, FT-IR	unknown	unknown
L. seeligeri		1607	API, FT-IR	unknown	unknown
L. seeligeri		1608	API, FT-IR	unknown	unknown
L. seeligeri		2447	API, FT-IR	unknown	unknown
L. seeligeri		2453	API, FT-IR	unknown	unknown
L. seeligeri		2454	API, FT-IR	unknown	unknown
L. seeligeri		2464	API, FT-IR	unknown	unknown
L. seeligeri	3900		API, FT-IR, M.PCR	Austria	smear bath
L. seeligeri	4007		API, FT-IR	unknown	unknown
L. seeligeri	4386		API, FT-IR	unknown	raw milk
L. seeligeri	4387		API, FT-IR	unknown	raw milk
L. seeligeri	4433		API, FT-IR	Germany	unknown
L. seeligeri	4441		API, FT-IR, M.PCR, Hemol.	Germany	unknown
L. seeligeri	4454		API, FT-IR, M.PCR	Germany	unknown
L. seeligeri	4546		API, FT-IR	unknown	unknown
L. seeligeri	4547		API, FT-IR	unknown	cheese
L. seeligeri	4548		API, FT-IR, M.PCR	unknown	cheese
L. seeligeri	4549		API, FT-IR	unknown	cheese
L. seeligeri	4550		API, FT-IR	unknown	cheese
L. seeligeri	4666		API, FT-IR, M.PCR	unknown	raw milk

Strains	WSLC	Gly	Identification methods	Origin	Isolated from
L. seeligeri	4972		API, FT-IR	unknown	unknown
L. seeligeri	4973		API, FT-IR	unknown	unknown
L. seeligeri	4975		API, FT-IR, M.PCR	unknown	unknown
L. seeligeri	4976		API, FT-IR	unknown	unknown
L. seeligeri	4980		API, FT-IR	unknown	unknown
L. seeligeri	40126		API, FT-IR	unknown	unknown
L. seeligeri	40140		API, FT-IR, M.PCR, Hemol.	unknown	unknown
L. seeligeri	40142		API, FT-IR	unknown	unknown
L. seeligeri	40143		API, FT-IR	unknown	unknown
L. seeligeri	41109		API, FT-IR	unknown	unknown
L. seeligeri	41110		API, FT-IR	unknown	unknown
L. seeligeri	41111		API, FT-IR, M.PCR	unknown	unknown
L. seeligeri	41119		API, FT-IR	unknown	unknown
L. seeligeri	41125		API, FT-IR	unknown	unknown
L. seeligeri	41126		API, FT-IR, M.PCR	unknown	unknown
L. seeligeri	41127		API, FT-IR	unknown	unknown
L. seeligeri	41128		API, FT-IR, M.PCR	unknown	unknown
L. seeligeri	41129		API, FT-IR	unknown	unknown
L. seeligeri	41132		API, FT-IR	Germany	salmon
L. seeligeri	41133		API, FT-IR	Germany	salmon
L. seeligeri	41134		API, FT-IR, M.PCR	Germany	salad
L. seeligeri	41135		API, FT-IR, M.PCR	Germany	junket
L. seeligeri	41136		API, FT-IR	Germany	sausage
L. welshimeri		2491	API, FT-IR	Austria	environmental
L. welshimeri		2499	API, FT-IR, M.PCR	unknown	unknown
L. welshimeri		2502	API, FT-IR, M.PCR	Germany	food
L. welshimeri		2470	API, FT-IR	Germany	environmental
L. welshimeri		2471	API, FT-IR	Germany	environmental
L. welshimeri	51130		API, FT-IR, M.PCR	unknown	unknown
L. welshimeri	51131		API, FT-IR	unknown	unknown
L. welshimeri	51137		API, FT-IR	Germany	sausage
L. welshimeri	51138		API, FT-IR	Germany	pork sausage
L. welshimeri	51140		API, FT-IR, M.PCR	Germany	salmon
L. welshimeri	51141		API, FT-IR	Germany	salmon

7.3 Appendix III: List of reference strains for the FTIR database for *Listeria monocytogenes* serovar

***Listeria monocytogenes* strains in the reference dataset, listed according to serovar in chapter 4**

ATCC, American Type Culture Collection;
SLCC, Special Listeria Culture Collection;
WSLC, Weihenstephan Listeria Collection; ZIEL, TU München
Type strain T

(a) strains provided by Prof. Hof, Klinikum Mannheim, Germany
(b) strain provided by Dr. Pellicer, CIDCA, Argentina

Misidentifications are highlighted in red

					Results of FTIR analysis	
Strains	**WSLC**	**other number**	**Origin**	**serovar**	**O- Antig**	**H- Antig**
L. monocytogenes	1040	SLCC 53 ATCC 15313 T	(a)	1/2a	1/2.	a
L. monocytogenes	1118		unknown	1/2a	1/2.	a
L. monocytogenes	1153		Germany	1/2a	1/2.	a
L. monocytogenes	1182		unknown	1/2a	1/2.	a
L. monocytogenes	1211		Germany	1/2a	1/2.	a
L. monocytogenes	1370		chicken, Hungary	1/2a	1/2.	a
L. monocytogenes	1383		meat	1/2a	1/2.	a
L. monocytogenes	1399		chicken	1/2a	1/2.	a
L. monocytogenes	1400		chicken	1/2a	1/2.	a
L. monocytogenes	1411		chicken, Germany	1/2a	1/2.	**c**
L. monocytogenes	1413		cheese	1/2a	1/2.	a
L. monocytogenes	1416		cheese	1/2a	1/2.	**c**
L. monocytogenes	1427		unknown	1/2a	1/2.	a
L. monocytogenes	1452		unknown	1/2a	1/2.	a
L. monocytogenes	1670		raw milk, Germany	1/2a	1/2.	a
L. monocytogenes	1856		milk, Germany	1/2a	1/2.	a
L. monocytogenes	1864		cheese	1/2a	1/2.	a
L. monocytogenes	1865		cheese	1/2a	1/2.	a
L. monocytogenes	1940		unknown	1/2a	1/2.	a
L. monocytogenes	1942		EGD	1/2a	1/2.	a
L. monocytogenes	11080	SLCC 7381	unknown (a)	1/2a	1/2.	a
L. monocytogenes	1933		unknown	1/2a	1/2.	a
L. monocytogenes	1936		unknown	1/2a	1/2.	a
L. monocytogenes	1266		unknown	1/2b	1/2.	b
L. monocytogenes	1425		unknown	1/2b	1/2.	b
L. monocytogenes	1857		pasta, Italy	1/2b	1/2.	b
L. monocytogenes	1863		milk	1/2b	1/2.	b
L. monocytogenes	1937		unknown	1/2b	1/2.	b
L. monocytogenes	11046		unknown, Argentina (b)	1/2b	1/2.	b
L. monocytogenes	11047		salami, Argentina (b)	1/2b	1/2.	b
L. monocytogenes	1075	SLCC 8811	(a)	1/2c	1/2.	c
L. monocytogenes	1361		turkey cock, Hungary	1/2c	1/2.	c

					Results of FTIR analysis	
Strains	**WSLC**	**other number**	**Origin**	**serovar**	**O- Antig**	**H- Antig**
L. monocytogenes	1377		soup hen, Germany	1/2c	1/2.	c
L. monocytogenes	1445		unknown	1/2c	1/2.	c
L. monocytogenes	1451		unknown	1/2c	1/2.	c
L. monocytogenes	1859		milk, France	1/2c	1/2.	**a**
L. monocytogenes	1935		unknown	1/2c	1/2.	c
L. monocytogenes	1938		unknown	1/2c	1/2.	c
L. monocytogenes	10177	SLCC 7290	unknown	1/2c	1/2.	c
L. monocytogenes	10180	SLCC 7355	unknown	1/2c	1/2.	c
L. monocytogenes	10183	SLCC 7356	unknown	1/2c	1/2.	c
L. monocytogenes	1485		cheese, Austria	3a	3	a
L. monocytogenes	1780		grass, Germany	3a	3	a
L. monocytogenes	11072	SLCC 4949	unknown (a)	3a	3	a
L. monocytogenes	11073	SLCC 7135	unknown (a)	3a	3	a
L. monocytogenes	11074	SLCC 7179	unknown (a)	3a	3	a
L. monocytogenes	11075	SLCC 7131	unknown (a)	3a	3	a
L. monocytogenes	11076	SLCC 7143	unknown (a)	3a	3	a
L. monocytogenes	11077	SLCC 7144	unknown (a)	3a	3	a
L. monocytogenes	1163		Germany	3b	3	b
L. monocytogenes	1229		Germany	3b	3	b
L. monocytogenes	1294		cheese	3b	3	b
L. monocytogenes	1444		unknown	3b	3	b
L. monocytogenes	1456		unknown	3b	3	b
L. monocytogenes	1460		unknown	3b	3	b
L. monocytogenes	1469		cheese, Austria	3b	3	b
L. monocytogenes	1557		unknown, Germany	3b	3	b
L. monocytogenes	1679		food	3b	3	b
L. monocytogenes	11078	SLCC 5543	unknown (a)	3b	3	b
L. monocytogenes	11079	SLCC 7140	unknown (a)	3b	3	b
L. monocytogenes	11081	SLCC 7392	unknown (a)	3b	3	b
L. monocytogenes	1175		unknown	3c	3	c
L. monocytogenes	11082	SLCC 6753	unknown (a)	3c	3	c
L. monocytogenes	11083	SLCC 6848	unknown (a)	3c	3	c
L. monocytogenes	11084	SLCC 7207	unknown (a)	3c	3	c
L. monocytogenes	1020	SLCC 2374 ATCC 19114 T	(a)	4a	4	a
L. monocytogenes	1939		unknown	4a	4	a
L. monocytogenes	11086	SLCC 5069	unknown (a)	4a	4	a
L. monocytogenes	1303		unknown	4b	4	b
L. monocytogenes	1363		unknown	4b	4	b
L. monocytogenes	1364		unknown	4b	4	b
L. monocytogenes	1477		cheese, Austria	4b	4	b
L. monocytogenes	1685		milk, USA	4b	4	b
L. monocytogenes	1858		raw milk, Germany	4b	4	b
L. monocytogenes	1860		raw milk	4b	4	b
L. monocytogenes	1861		raw milk, France	4b	4	b
L. monocytogenes	1941		unknown	4b	4	b
L. monocytogenes	1999		unknown	4b	4	b
L. monocytogenes	10169	SLCC 7372	unknown	4b	4	b
L. monocytogenes	10170	SLCC 7371	unknown	4b	4	b
L. monocytogenes	10173	SLCC 7341	unknown	4b	4	b

					Results of FTIR analysis	
Strains	**WSLC**	**other number**	**Origin**	**serovar**	**O- Antig**	**H- Antig**
L. monocytogenes	10174	SLCC 7340	unknown	4b	4	b
L. monocytogenes	10175	SLCC 7339	unknown	4b	4	b
L. monocytogenes	10186	SLCC 7346	unknown	4b	4	b
L. monocytogenes	10189	SLCC 7351	unknown	4b	4	b
L. monocytogenes	10191	SLCC 6902	unknown	4b	4	b
L. monocytogenes	10194	SLCC 6609	unknown	4b	4	**d**
L. monocytogenes	10199	SLCC 6603	unknown	4b	4	b
L. monocytogenes	10205	SLCC 6606	unknown	4b	4	b
L. monocytogenes	11000		unknown	4b	4	b
L. monocytogenes	11044		soft cheese, Argentina (b)	4b	4	b
L. monocytogenes	11102	SLCC 7093	unknown (a)	4b	4	**d**
L. monocytogenes	11089	SLCC 4954	unknown (a)	4c	4	**d**
L. monocytogenes	11094	SLCC 5999	unknown (a)	4c	4	c
L. monocytogenes	1045	SLCC 1806	(a)	4d	4	d
L. monocytogenes	11090	SLCC 6277	unknown (a)	4d	4	d
L. monocytogenes	11091	SLCC 6813	unknown (a)	4d	4	d
L. monocytogenes	11092	SLCC 6821	unknown (a)	4d	4	d
L. monocytogenes	11097	SLCC 4926	unknown (a)	4d	4	d
L. monocytogenes	11098	SLCC 4952	unknown (a)	4d	4	d
L. monocytogenes	11099	SLCC 6833	unknown (a)	4d	4	d
L. monocytogenes	11100	SLCC 6832	unknown (a)	4d	4	d
L. monocytogenes	11105	SLCC 7069	unknown (a)	4d	4	d
L. monocytogenes	1761		unknown	4e	4	e
L. monocytogenes	1929		unknown	4e	4	e
L. monocytogenes	1034	SLCC 2482	(a)	7	7	-

7.4 Appendix IV: List of strains for the external validation for *Listeria monocyogenes* serovar

***Listeria monocytogenes* isolates in the external validation set, listed according to serovar in chapter 4**

ATCC, American Type Culture Collection;
SLCC, Special Listeria Culture Collection;
WSLC, Weihenstephan Listeria Collection; ; ZIEL, TU München
Gly, strains in the Glycerin- stock in the Weihenstephan Collection; ZIEL, TU München

(a) strains provided by Prof. Hof, Klinikum Mannheim, Germany
(b) strains provided by Dr. Montel, INRA, France
(c) strain provided by Dr. Pellicer, CIDCA, Argentina

Multiplex PCR was performed according to Doumith et al. (3)
Misidentifications and Non-identifications are highlighted in red

					Results of FTIR analysis		Results of Multiplex PCR
Strains	**WSLC**	**other number**	**Origin**	**Serovar**	**O-Antig**	**H-Antig**	
L. monocytogenes	1237		smear cheese, Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1271		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1297		unknown	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1376		soup hen, Netherlands	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1475		cheese, Austria	1/2a	1/2.	a	**1/2c,3c**
L. monocytogenes	1483		cheese, Austria	1/2a	**3**	a	1/2a,3a
L. monocytogenes	1484		cheese, Austria	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1486		cheese, Austria	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1487		cheese, Austria	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1490		cheese, Austria	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1558		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1559		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1571		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1575		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1582		gully	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1584		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1596		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1607		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1612		Germany	1/2a	1/2.	**c**	1/2a,3a
L. monocytogenes	1618		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1647		silage	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1665		raw milk	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1714		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1767	SLCC 7152	animal, Germany (a)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1768	SLCC 6447	animal (a)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1769	SLCC 6448	poult (a)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1770	SLCC 6449	goat (a)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1771	SLCC 6450	goat (a)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1774	SLCC 6445	lamb (a)	1/2a	1/2.	a	1/2a,3a

					Results of FTIR analysis		Results of Multiplex PCR
Strains	**WSLC**	**other number**	**Origin**	**Serovar**	**O-Antig**	**H-Antig**	
L. monocytogenes	1775	SLCC 6481	corn, Germany (a)	1/2a	**3**	a	1/2a,3a
L. monocytogenes	1776	SLCC 6540	silage, Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	4448		Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11006		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11007		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11011		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11014		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11015		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11016		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11021		feces, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11023		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11024		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11027		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11028		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11030		ground cellar, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11031		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11032		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11034		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11035		ground cellar, France (b)	1/2a	1/2.	**c**	1/2a,3a
L. monocytogenes	11071		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11147		Salami	1/2a	1/2.	**c**	1/2a,3a
L. monocytogenes	11150		smoked salmon	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11151		smoked salmon	1/2a	1/2.	a	1/2a,3a
L. monocytogenes		Gly 3042	gully, Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes		Gly 3068	gully, Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes		Gly 3073	gully, Germany	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	4978		unknown	1/2a	1/2.	a	**1/2b,3b,7**
L. monocytogenes	4981		unknown	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	11008		cheese, France (b)	1/2a	1/2.	a	1/2a,3a
L. monocytogenes	1065	SLCC 8797	(a)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1066	SLCC 8800	(a)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1069	SLCC 8798	(a)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1070	SLCC 8806	(a)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1472		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1482		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1493		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1499		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1510		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1514		cheese, Austria	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1516		cheese, Austria	1/2b	1/2.	**a**	1/2b,3b,7
L. monocytogenes	1551		Germany	1/2b	1/2.	**a**	1/2b,3b,7
L. monocytogenes	1552		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1556		Germany	1/2b	1/2.	**a**	1/2b,3b,7
L. monocytogenes	1564		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1602		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1614		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1619		Germany	1/2b	1/2.	**a**	1/2b,3b,7
L. monocytogenes	1621		Germany	1/2b	1/2.	b	1/2b,3b,7

					Results of FTIR analysis		Results of Multiplex PCR
Strains	**WSLC**	**other number**	**Origin**	**Serovar**	**O-Antig**	**H-Antig**	
L. monocytogenes	1629		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1661		animal feces	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1703		Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1763		France	1/2b	1/2.	**a**	1/2b,3b,7
L. monocytogenes	1766	SLCC 7151	animal, Germany (a)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11010		cheese, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11017		cheese, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11018		cheese, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11019		feed, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11039		cheese, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3043	floor, Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3071	soft cheese, Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3072	soft cheese, Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3074	smearing, Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3075	Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes		Gly 3092	smearing, Germany	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	11005		milk, France (b)	1/2b	1/2.	b	1/2b,3b,7
L. monocytogenes	1017	SLCC 5778	(a)	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1076	SLCC 8812	(a)	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1077	SLCC 8813	(a)	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1091	SLCC 8848	(a)	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1285		chicken	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1430		chicken, Germany	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1489		cheese, Austria	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1497		cheese, Austria	1/2c	1/2.	**a**	1/2c,3c
L. monocytogenes	1498		cheese, Austria	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1609		Germany	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	1625		Germany	1/2c	1/2.	c	1/2c,3c
L. monocytogenes	11152		smoked salmon	1/2c	1/2.	c	1/2a,3a
L. monocytogenes	1459		Germany	3a	3	a	1/2a,3a
L. monocytogenes	1461		Germany	3a	3	a	1/2a,3a
L. monocytogenes	11149		smoked salmon	3a	3	a	1/2a,3a
L. monocytogenes	1455		Germany	3b	3	b	1/2b,3b,7
L. monocytogenes	1464		Germany	3b	3	b	1/2b,3b,7
L. monocytogenes	1704		Germany	3b	3	b	1/2b,3b,7
L. monocytogenes	2397		Germany	3b	3	b	1/2b,3b,7
L. monocytogenes		Gly 3077	gully, Germany	3b	3	b	1/2b,3b,7
L. monocytogenes	1443		Germany	3c	3	**b**	1/2c,3c
L. monocytogenes	11085	SLCC 1693	(a)	3c	3	c	**1/2a,3a**
L. monocytogenes	11087	SLCC 5070	(a)	4a	4	a	**no result**
L. monocytogenes	1016	SLCC 5637	(a)	4b	4	b	4b,d,e
L. monocytogenes	1063	SLCC 8793	(a)	4b	4	b	4b,d,e
L. monocytogenes	1064	SLCC 8795	(a)	4b	4	b	4b,d,e
L. monocytogenes	1067	SLCC 8802	(a)	4b	4	b	4b,d,e
L. monocytogenes	1071	SLCC 8792	(a)	4b	4	b	4b,d,e
L. monocytogenes	1226		unknown	4b	4	b	4b,d,e
L. monocytogenes	1283		Germany	4b	4	b	4b,d,e
L. monocytogenes	1284		Germany	4b	4	b	4b,d,e

					Results of FTIR analysis		Results of Multiplex PCR
Strains	**WSLC**	**other number**	**Origin**	**Serovar**	**O-Antig**	**H-Antig**	
L. monocytogenes	1447		Germany	4b	4	b	4b,d,e
L. monocytogenes	1476		cheese, Austria	4b	4	b	4b,d,e
L. monocytogenes	1511		cheese, Austria	4b	4	b	4b,d,e
L. monocytogenes	1520		unknown	4b	4	b	4b,d,e
L. monocytogenes	1538		cheese, Germany	4b	4	b	4b,d,e
L. monocytogenes	1539		cheese, Germany	4b	4	b	4b,d,e
L. monocytogenes	1541		cheese, Germany	4b	4	b	4b,d,e
L. monocytogenes	1585		Germany	4b	4	b	4b,d,e
L. monocytogenes	1592		Germany	4b	4	b	4b,d,e
L. monocytogenes	1604		Germany	4b	4	b	4b,d,e
L. monocytogenes	1617		Germany	4b	4	b	4b,d,e
L. monocytogenes	1672		unknown	4b	4	b	4b,d,e
L. monocytogenes	1673		unknown	4b	4	b	4b,d,e
L. monocytogenes	1690		unknown	4b	4	b	4b,d,e
L. monocytogenes	1713		Germany	4b	4	b	4b,d,e
L. monocytogenes	1772	SLCC 6439	sheep (a)	4b	4	b	4b,d,e
L. monocytogenes	1777	SLCC 6464	salad, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1778	SLCC 6396	grass, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1779	SLCC 6375	grass, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1783	SLCC 6253	grass, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1785	SLCC 6214	grass, Germany (a)	4b	4	**d**	4b,d,e
L. monocytogenes	1786	SLCC 6376	grass, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1787	SLCC 6369	grass, Germany (a)	4b	4	b	4b,d,e
L. monocytogenes	1862		raw milk	4b	4	b	4b,d,e
L. monocytogenes	1930		unknown	4b	4	b	4b,d,e
L. monocytogenes	1931		unknown	4b	4	b	4b,d,e
L. monocytogenes	11012		cheese, France (b)	4b	4	b	4b,d,e
L. monocytogenes	11013		cheese, France (b)	4b	4	b	4b,d,e
L. monocytogenes	11022		cheese, France (b)	4b	4	b	4b,d,e
L. monocytogenes	11040		cheese, France (b)	4b	4	b	4b,d,e
L. monocytogenes	11043		soft cheese, Argentina(c)	4b	4	b	4,b,d,e
L. monocytogenes	11088	SLCC 4925	(a)	4b	4	b	**no result**
L. monocytogenes	11104	SLCC 7065	(a)	4b	4	b	4b,d,e
L. monocytogenes	1652		silage	4c	4	**b**	**no result**
L. monocytogenes	11093	SLCC 6823	(a)	4c	4	c	4b,d,e
L. monocytogenes	11103	SLCC 7002	(a)	4c	4	c	4b,d,e
L. monocytogenes	1033	SLCC 2377 ATCC 19117	(a)	4d	4	**b**	4b,d,e
L. monocytogenes	1048	SLCC 1652	(a)	4d	4	d	4b,d,e
L. monocytogenes	1018	SLCC 2378 ATCC 19118	(a)	4e	4	e	4b,d,e
L. monocytogenes		Gly 3064	washing water, Germany	4e	4	**b**	**no result**
L. monocytogenes	1932		unknown	7	7	-	**no result**

Curriculum Vitae

Cecilia A. Rebuffo-Scheer

Personal details

Date of Birth:	December 10th, 1968
Civil status:	Married
Nationality:	Argentine
E-Mail:	cecilia@scheergroup.com
Languages:	English and German: good, Spanish: mother tongue

Education

Ph.D. in Microbiology	Institute of Microbiology, Prof. Dr. S. Scherer, ZIEL Weihenstephan, Technical University of Munich, Freising, Germany.	March 2003 – Sept. 2007
Diploma Thesis in Microbiology	Department of Biophysics, Prof. Dr. D. Naumann, Robert Koch Institute, Berlin, Germany	Nov. 2001 - Feb. 2003
Lic. Biochemistry	National University of La Plata (UNLP), Buenos Aires, Argentine	Oct. 1998 – April 2001
Lic. Chemistry	National University of La Plata (UNLP), Buenos Aires, Argentine	April 1988 – Sept. 1998

Work experience

Co-Head of Medical trial for Schering AG. Multicenter study in an open-label trial to investigate the efficacy of an experimental drug on patients	Clinical laboratory, La Plata, Buenos Aires, Argentine	May 2001 - Nov. 2001
Trainee in department of analytical chemistry and microbiology	Clinical laboratory, La Plata, Buenos Aires, Argentine	March 1999 - March 2001 (part time)
Trainee in laboratory of bacterial vaccines. Characterization of extracellular metabolites during the growing of *Bordetella pertussis* as biofilm by chemical analysis and FT-IR spectroscopy	National Institute of Biotechnology (CINDEFI), La Plata, Buenos Aires, Argentine	May 2001 - Oct. 2001 (part time)
Intern in food centre. Study of the cryoscopic effect of calcium in the stability of yoghurt	Centre of food conservation (CIDCA) and Institute of physical chemistry (INIFTA), La Plata, Argentina	Feb.1995 – Dec. 1996 (part time)