Listeria monocytogenes Identification by Subtyping MALDI-TOF MS Biotyper™ Analysis (CARTS # IR01049)

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Abstract

Listeria are motile, gram-positive rods that occur individually or form short chains. The bacterium is widely distributed throughout the environment, inhabiting soil and decaying vegetable matter. The majority of human infections follow consumption of food contaminated with *L. monocytogenes*. Listeriosis during pregnancy results in fetal loss in about 20% of cases and causes newborn deaths in approximately 3% of reported instances. Most people with invasive listeriosis require hospital care, and about one in five people with the infection die. The Centers for Disease Control and Prevention (CDC) estimates that *Listeria monocytogenes* is the third leading cause of death from foodborne illness in the United States. The fact that *Listeria monocytogenes* can survive and replicate at refrigerated temperatures makes it a particular concern for food manufacturers and processing plants. In the United States, listeriosis infections must be reported to local, state, territorial, or federal public health authorities who monitor epidemiology. Rapid, sensitive, and accurate microorganism identification methods are crucial for assessing the safety of foods and reducing the incidence of foodborne illness. Matrix-Assisted Laser Desorption/Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) enables the rapid identification of both gram-negative and gram-positive bacteria based on protein mass spectra profiles. Ionized proteins unique to each bacteria species reach the detector at different times based on their mass, yielding a characteristic "fingerprint" for each bacteria. *Listeria* species have nearly identical protein patterns to each other; however, looking closely at the MALDI-TOF mass spectra reveals some peaks that are unique to each *Listeria* species. These peak differences are being exploited to distinguish pathogenic *L. monocytogenes* from other *Listeria* species. We have demonstrated that MALDI-TOF MS analysis can distinguish pathogenic *L. monocytogenes* from other *Listeria* species by using the newly created in-house subtyping reference spectra library. This technology can reduce the time needed to identify *Listeria monocytogenes*, therefore, simplifying and speeding regulatory analysis of food products.

Introduction

The *Listeria* genus contains several species including *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri* and *L. grayi*. Of these species, *L. monocytogenes* is pathogenic to humans and animals making it the primary concern of the Food and Drug Administration (FDA). Although the overall incidence of listeriosis is low (0.24 per 100,000 population) [1], the Centers for Disease Control and Prevention (CDC) estimates that *Listeria* is the third leading cause of death from foodborne illness in the United States [2]. Healthy individuals may suffer only short-term symptoms such as high fever, severe headache, stiffness, nausea, abdominal pain and diarrhea; however, 20% of listeriosis cases result in death despite prompt treatment. This is particularly likely in older adults and in persons with weakened immune systems. At least 90% of people who get *Listeria* infections are in a higher risk group (expectant mothers, the elderly and immunocompromised individuals). Pregnant women are about 10 times more likely than the general population to get a *Listeria* infection which can cause fetal loss (miscarriage or stillbirth), preterm labor, and illness or death in newborn infants [2]. Most human infections follow consumption of contaminated food such as unpasteurized (raw) milk and cheeses, cooked or processed foods, processed (or ready-to-eat) meats, and smoked seafood. Outbreaks from 2015 to 2017 include ice cream, soft raw milk cheese, frozen vegetables, and raw milk [3]. *Listeria monocytogenes* is of particular concern for food manufacturers and processing plants because it is able to survive and replicate at refrigerated temperatures.

Listeria species other than *L. monocytogenes* are not considered to be foodborne hazards, however, they have similar growth characteristics and are often isolated from the same food and environmental sources. Typically, phenotypic assays, polymerase chain reaction (PCR), or genetic sequencing are performed. Phenotypic methods include the API® biochemical test strips and VITEK2®, an automated biochemical identification system. Identifications using these supplementary methods entail numerous consecutive steps and typically require an additional 24 hours after isolation before they can be reported.

Genotyping methods, including 16S sequencing, enable highly sensitive and specific identification but remain costly and time consuming thus discouraging its routine use on every isolate [4, 5]. MALDI-TOF MS has been used extensively as a research tool for protein analysis and was applied recently to clinical microbiology [6-12]. Compared with conventional phenotype or genotype-based identification, MALDI-TOF MS features rapid turnaround time, low sample volume requirements, and modest reagent costs. While the initial cost and maintenance fees of this instrument can be high, the functionality over several disciplines and high throughput capabilities make it viable for facilities with large

sample loads. Extracted proteins from an isolated bacterial colony are deposited on a sample target, dried, overlaid with a saturated organic acid solution, and analyzed by MALDI-TOF MS. Ionized peptides and proteins unique to each bacteria species reach the detector at slightly different times based on their mass; yielding species specific profiles of spectra. Bacteria identification is accomplished by comparing sample profiles with profiles from a database of reference strains. Unbiased species identifications and even genotyping have been achieved using MALDI-TOF MS [13-17].

Rapid, sensitive, and accurate microorganism identification methods are crucial to quickly assess the safety of foods and reduce the incidence of foodborne illness. Despite nearly identical protein patterns, there are peaks unique to *Listeria monocytogenes* in MALDI-TOF mass spectra [18]; these peak differences have been exploited to make a distinction between pathogenic *L. monocytogenes* and other *Listeria* species. This method specifically focuses on using Bruker BioTyper™ software version 3.1.65 to identify *Listeria* to the species level, referred to as subtyping by Bruker.

Materials and Methods

Media and Reagents Formic acid solution (LC-MS Ultra grade - Fluka ~98%), trifluoroacetic acid (reagent plus grade, 99%), acetonitrile (ACN; anhydrous, 99.8%), and Standard Solvent (acetonitrile 50%, water 47.5% and trifluoroacetic acid 2.5%) (Fluka) were purchased from Sigma-Aldrich (St Louis, MO). Ethanol (200 proof, 99.5% ACS reagent grade - ACROS Organics) was purchased from Fisher Scientific (Pittsburgh, PA).

Formic acid solution (FA) was prepared at 70% (v/v) with sterile water. MALDI Matrix (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid [HCCA]) and Bruker Bacterial Test Standard (both purchased from Bruker Daltonics – Billerica, MA) were each solubilized in standard solvent.

Tryptic soy agar (TSA), modified Oxford agar (MOXA), CHROMagar, PALCAM, Sheep blood agar (SBA), trypticase soy broth (TSB), monopotassium phosphate (anhydrous), disodium phosphate (anhydrous), pyruvic acid (sodium salt), sodium hydroxide (NaOH), hydrochloric acid (HCl), and nalidixic acid (sodium salt) were purchased from Fisher Scientific (Pittsburg, PA). RAPID'L.mono (RLM) was purchased from Bio-Rad (Hercules, CA) and yeast extract (YE) was purchased from Thermo Fisher Scientific (Waltham, MA). Cycloheximide, acriflavin HCl, and ethanol were purchased from Sigma Chemical Co. (St. Louis, MO).

Bacterial Strains Previously identified *Listeria* isolates (113) were obtained from the FDA Pacific Northwest Laboratory (PNL), the American Type Culture Collection (ATCC), the Winchester Engineering and Analytical Center (WEAC), and/or the Center for Food Safety and Applied Nutrition (CFSAN). The collection included 53 *L. monocytogenes* isolates,9 *L. ivanovii, 20 L. seeligeri, 20 L. innocua and 11 L. welshimeri.* Thirty other

bacteria isolates were obtained for exclusivity testing from CFSAN. Isolate details are listed in Supplementary Table 1 at the end of this document.

Enrichment Pure cultures were used when creating any spectra that was saved in a library for the identification of *Listeria* species. *Listeria* strains were grown on Tryptic Soy Agar with 5% Sheep Blood (SBA) for 24 or 48 h at 35°C.

Additionally, in order to examine potential media and temperature effects, *Listeria* cultures were streaked to the following selective agars: MOXA, PALCAM, CHROMagar, and RAPID'L.mono in addition to SBA and Tryptic Soy agar with 0.6% yeast extract (TSA-YE). Isolates were grown for 24 or 48 hours at 30°C and 35°C.

Sample Extraction Procedure The identification of bacteria by MALDI-TOF MS and Bruker Biotyper™ analysis has been detailed in LIB # 4624 [20]. A freshly grown, isolated colony was picked and transferred to a 1.5 mL Eppendorf microfuge tube containing 300 µL of sterile water and vortexed. To that, 900 µL of ethanol (100%) was added and the sample vortexed to thoroughly mix the contents. The sample was then centrifuged at 14,000 rpm for 2 min (Eppendorf MiniSpin Plus variable speed microcentrifuge; USA Scientific, Ocala, FL). The ethanol was decanted, and the sample centrifuged again for 2 min. Excess ethanol was removed using a pipette and residual ethanol was allowed to evaporate from the tube by standing at room temperature for several minutes. A solution of 70% formic acid (up to 50 µL depending on sample size) was added; the sample was vortexed and allowed to stand for approximately 5 minutes. An equal amount (up to 50 µL) of acetonitrile was added followed by vortexing. The sample was then centrifuged at 14,000 rpm for 2 minutes. The supernatant contains the ribosomal proteins that will undergo MALDI-TOF MS analysis. A ground steel target plate was spotted, in duplicate, with 1 µL of the extraction supernatant and was allowed to dry at room temperature. The protein film was then overlaid with 1 µl of HCCA MALDI matrix and allowed to dry at room temperature for approximately 5 minutes.

Direct Smear Procedure for Samples Alternatively, a portion of the isolated colony could be smeared directly onto the MALDI target plate. Thus, a small amount of a freshly grown colony was removed from the culture plate using a sterile disposable 1 µl loop. The sample was sequentially spread over the surface of three wells of the target plate. Each microbial film was then overlaid with 1µl of HCCA MALDI matrix and allowed to dry at room temperature. Too much material deposited as a direct smear may interfere with the identification. For this reason, the procedure to spread the picked colony sequentially over the surface of three wells of the target plate is followed whenever using the direct smear protocol. The sample amounts in each of the 3 wells are not necessarily equivalent. As a result, the highest of three scores was used. Scores ≥ 2.00 were considered highly confident to the species level, scores between 1.800 to 1.999 were considered to be

confident identifications to the genus level, scores between 1.600 and 1.799 were considered to be low confidence.

Instrument Parameters Mass spectra were acquired using the MALDI-TOF mass spectrometer (Autoflex Speed TOF/TOF MS; Bruker Daltonics Billerica, MA) equipped with a Smartbeam™–II, 2 kHz laser. Spectra were recorded in the positive linear mode (delay, 350 ns; ion source 1, 19.5 kV; ion source 2, 18.25 kV; lens, 6.5 kV; mass range, 2–20 kDa). Each spectrum was obtained after 675 shots in automatic mode at variable laser power. Each spot has a total acquisition time of 30 to 60 seconds. Sample data were automatically acquired using MALDI Biotyper™ Realtime Classification software, version 3.1.65 (Bruker Daltonics), and were analyzed by standard pattern matching (with default parameter settings) against the 4274 reference spectra in the BioTyper™ database (these spectra are an integrated part of the MALDI Biotyper™ software version 3.1.65, updated in Oct 2014).

Instrument Calibration An external calibration spot on the ground steel target plate was created by spotting 1 µL of Bacteria Test Standard (BTS), a DH5-Alpha *E. coli* spiked with two additional high molecular weight proteins designed to cover the entire protein range used in the Biotyper™ range. The spot was then allowed to dry before being overlaid with 1 µL of HCCA MALDI matrix. Prior to sample analysis, the BTS spot was analyzed resulting in *E. coli* protein peaks at 3637.80 Da, 5096.80 Da, 5381.40 Da, 6255.40 Da, 7274.50 Da, 10300.10 Da, plus the RNAse A peak $[M+H]^+$ at 13683.20 and myoglobin peak $[M+H]^+$ at 16952.30 Da. The acquisition method was calibrated on the known masses of the external BTS calibration standard.

In-house Library spectra creation Sample data were automatically acquired using AutoXecute acquisition control software. For reference library construction, 24 independent spectra were collected for each control isolate (three independent measurements of eight spots each). Isolates used in the subtyping library were all purchased from ATCC and additionally confirmed using VITEK2 Compact. At least 21 spectra were averaged by the Biotyper™ software to create a Main Spectra (MSP) record in the reference library, allowing for the removal of up to 3 outlier spectra from the final MSP.

Data Analysis Spectra of the isolates originally derived from different food, clinical and environmental samples, were acquired and analyzed by Biotyper™ RT (v 3.1.65 Bruker Daltonics) for identification against the Bruker library of 4274 reference spectra. Spectra of samples confidently identified as *Listeria* were then imported into Biotyper™ OC (v 3.1.66 Bruker Daltonics) for sub species identification by the newly created in-house *Listeria* subtyping reference spectra library. During the Biotyper™ analysis, each sample spectrum was compared to the reference spectra (“main spectra” or MSP) present in the

library using a pattern matching approach that's based on statistical, multi-variant analysis and takes into account peak position and intensity. The score value is defined by three components- the matches of the unknown spectrum against the MSP, the matches of the MSP peaks against the unknown spectrum, and the correlation of intensities of the matched peaks. This leads to a score, from 0 (no match) to 1,000 (perfect match), which is converted into a log score from 0 to 3. The integrated software generates an outcome list, in which species are ordered according to their logarithmic score value (log (score value)).

Variability Testing The quality of identification scores was evaluated for different enrichment media and growth temperatures for *Listeria monocytogenes*. Identification scores were compared for samples isolated on SBA, RLM, TSA-YE and MOXA at 30 and 35 °C for both extraction and direct smear sample preparation procedures. Additional media effects were evaluated by growing *L. monocytogenes* (ATCC 19115)*, L. innocua* (ATCC 33090)*,* and L. *ivanovii* (ATCC 19119) on CHROM agar, PALCAM and TSA-YE at 35 °C. After 48 hours, the plates were removed from the incubator and picked colonies underwent the sample preparation procedures described earlier.

Identification of Listeria to the Species Level (Subtyping) An identification process using subtyping MSPs enables accurate classification of closely related species. A subtyping MSP peak list has the same m/z values, intensities and frequencies as its parent standard MSP, but with the assignment of a different peak weighting system. Subtyping MSPs were created for a selection of *Listeria* reference spectra that were chosen as representative of the various species. Using FlexAnalysis software (v 3.4 Bruker), spectra of the different *Listeria* species were overlaid to identify peaks that occur in one to four of the five species evaluated (*L. ivanovii, L. innocua, L. monocytogenes, L. seeligeri, L. welshimeri*). Peak lists were compared and the weighted contribution of peaks to the identification was edited accordingly. The more specific a peak is to a particular *Listeria* species, the higher its weighted contribution was set. Sample spectra are first matched against the reference spectra in the Bruker library by Biotyper™ Realtime Classification (RTC; v 3.1.65 Bruker Daltonics). Samples identified as *Listeria* with a log score ≥ 2.000 (figure 1A) are then matched against the in-house *Listeria* library spectra by Biotyper™ Offline Classification (OC; v 3.1.66 Bruker Daltonics). The Biotyper™ software generates an outcome list of the ranked spectra matches and score values as seen in figure 1B. When subtyping with the newly created in-house *Listeria* library, log (score values) ≥ 2.000 are considered to be highly confident identifications. Final scores of 1.800 to 1.999 were considered to be confident identifications to the genus level. Confidence in the identification is considered low when the score is between 1.600 and 1.799. In this case, identification can still be considered accurate to the genus, however the species should be identified by other means. Additionally, in order to

differentiate between *Listeria* species, a minimum difference of 10% between the top score and next closest score was required for individual isolates (referred to as the 10% differential rule). Isolates which had conflicting species identifications that failed the 10% rule were considered correct to the genus level, but too ambiguous to accurately identify to the species level.

Results and Discussion

The Bruker library contains 12 *Listeria* reference spectra (2 *L. grayii*, 1 *L. innocua*, 2 *L. ivanovii*, 5 *L. monocytogenes*, 1 *L. seeligeri* and 1 *L. welshimeri*). A log (score value) higher than 2.000 is needed to be highly confident in the identification. A 10% difference between score values is required to identify to the species level. For example, if the top results for 1 sample show highly confident identifications of 2.584, 2.430 and 2.389 for *L. monocytogenes*, *L. innocua*, and *L. ivanovii*, respectively, then we can be very confident in the identification to the genus *Listeria*, but we cannot identify the sample to the species level. Highly confident *Listeria* identifications were obtained from MALDI Biotyper™ analysis of the 112 *Listeria* isolates under the tested growth conditions. An example of the BioTyper™ outcome lists is shown in Figure 1.

A

Detected Species	Log(Score)
Listeria monocytogenes Mb19348 1 CHB	2.584
L.mono 19115	2.519
L.innocua-33090	2.430
Listeria monocytogenes ser4B ATCC 19115 THL	2.427
Listeria innocua DSM 20649T DSM	2.420
Listeria monocytogenes MB 1601 05 THL	2.409
Listeria ivanovii ssp ivanovii DSM 20750T DSM	2.389
Listeria monocytogenes DSM 20600T DSM	2.382
L.seeligeri-35967	2.380
L.ivanovii 19119	2.364

B

Detected Species	Log(Score)
L.mono 19 (FNW 2492)	2.292
L.mono 19115	2.228
L.mono 15 (FNW 15c22)	1.867
L.ivanovii 19119	1.689
L.innocua-33090	1.688
L.innocua 56 (FNW 3516)	1.657
L.seeligeri-35967	1.448
L.mono 19114	1.306
L.Welshimeri 104 (FNW 2231)	1.097
L.welshimeri 109 (FNW 15b06)	0.860

Figure 1: Bruker BioTyper software generates an outcome list of the ranked spectra matches. Initial comparison of a Listeria monocytogenes isolate against the Bruker reference spectra database resulted in outcome list A which shows a highly confident Listeria identification, but fails the 10% difference between scores needed to identify to the species. Comparison of the same spectra against the in-house subtyping library resulted in outcome list B which shows a highly confident identification of Listeria monocytogenes.

The goal of this project was to improve the resulting scores so that a distinction between *Listeria* species is discernable as shown in figure 1B. It is important to note that because the difference between scores was < 10% for figure 1A, *Listeria* isolates could

not be identified to the species level, but all identifications were correct to the genus using only the Bruker reference library.

The quality of identification scores was evaluated for different enrichment media and temperatures. *Listeria monocytogenes* (ATCC 19115) was grown on SBA, RLM, TSA-YE, and MOXA at both 30 and 35 °C. Isolates were processed by the direct smear method and the sample extraction procedure for comparison. It's possible that inhibitors in some selective media could have an effect on the identification score quality. For the selective media MOXA, Figure 2 shows how the sample preparation procedure affected the resultant scores. A comparison of the 2 sample procedures shows that some peaks are missing when the direct smear procedure was used. The direct smears of *Listeria* grown on MOXA resulted in an average MALDI Biotyper™ score of 1.684 ± 0.232 (n = 6) compared to direct smears from RLM which had an average score of 2.143 ± 0.073 (n = 5). Identification scores of *Listeria* grown on MOXA were improved to 2.202 ± 0.202 (n = 5), if the isolated colony underwent the sample extraction procedure. Alternatively, an isolated colony can be picked from MOXA and streaked onto TSA-YE or SBA for purification before MALDI Biotyper™ analysis to improve the score. Results of the media comparison can be seen in Figure 3. Without accounting for growth temperature or how the isolate was prepared prior to analysis, SBA scores averaged 2.359 ± 0.121 and TSA-YE averaged 2.195 ± 0.130 (n = 12). The temperature did not have an apparent effect on the resulting scores for any of the media tested.

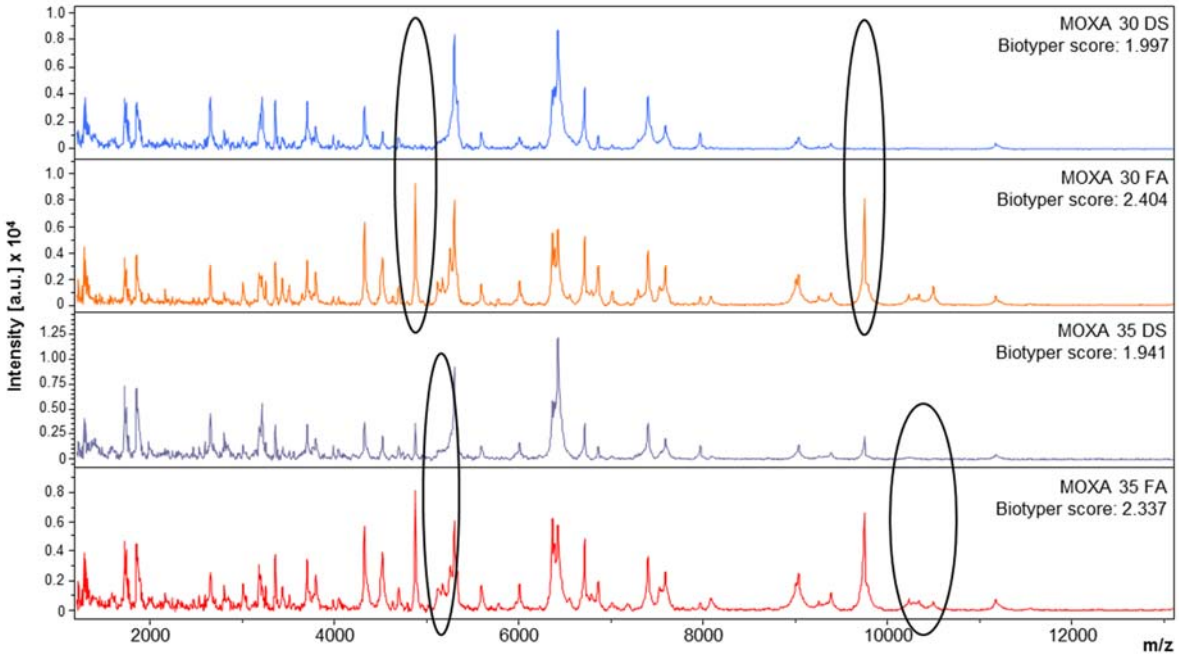

Figure 2: A *Listeria* isolate was enriched on MOXA at either 30 °C or 35 °C. At 24 h post enrichment, a colony was either directly smeared (DS) on the MALDI target plate or the protein extraction procedure was performed (FA). Areas on the MALDI-TOF mass spectra are circled where peaks are missing from the direct smear resulting in lower identification score values.

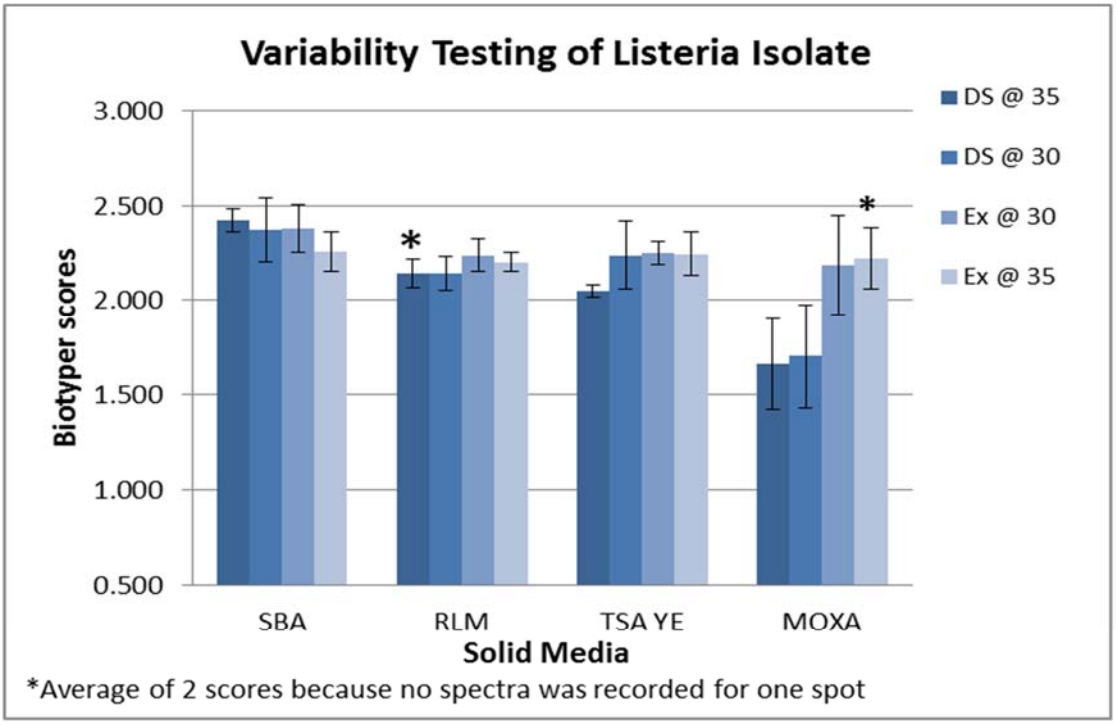

Figure 3: Comparison of average MALDI Biotyper scores (n = 3) shown for Listeria plated on different media at either 30 or 35 °C incubation temperatures and direct smear vs extraction sample preparation procedures.

Additional evaluations were performed by growing *Listeria monocytogenes* (ATCC 19115), *Listeria innocua* (ATCC 33090), and *Listeria ivanovii* (ATCC 19119) on CHROM agar, PALCAM and TSA-YE at 35 °C. Isolated colonies underwent the sample extraction procedure followed by MALDI Biotyper™ analysis. Highly confident scores identifying *Listeria* were obtained for CHROM agar, PALCAM and TSA-YE which ranged from 2.493 – 2.638, 2.541 – 2.715, and 2.509 – 2.618, respectively. In order to achieve more consistent results, our lab preferred using the sample extraction procedure. Additionally, the extracted proteins can be frozen and saved for future analysis whereas the direct smears need to be analyzed within 48 hours.

An in-house *Listeria* subtyping library was created with reference strains obtained from the FDA PNL to assess whether changing the weighted contributions from specific peaks in a spectrum would affect the outcome. Screening isolates against the Bruker library showed that the Biotyper™ software could not correctly discriminate between *Listeria* species, but did result in a highly confident identification of *Listeria*. FlexAnalysis (v 3.4 Bruker) evaluation of spectra and peak lists were used to assess peak differences between and within the *Listeria* species. Looking closely at overlaid spectra of the

different *Listeria* species reveals some peaks that are unique to each species. A *Listeria* subtyping library was created by identifying peaks that are characteristic to the different species (*L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*) as illustrated in Figure 4. The more specific a peak is, the higher its weighted contribution was set. Due to the low resolving power of linear mode TOF analysis, a tolerance of 1000 ppm was chosen as the mass error. This should result in a score differential ≥10% so that the species identification can be made with confidence. When comparing 2 spectra profiles, peaks were considered to be identical if:

$$\frac{peak\ mass\ diff\ (Da)}{peak\ mass\ (Da)} \times 10^6 \leq 1000\ ppm$$

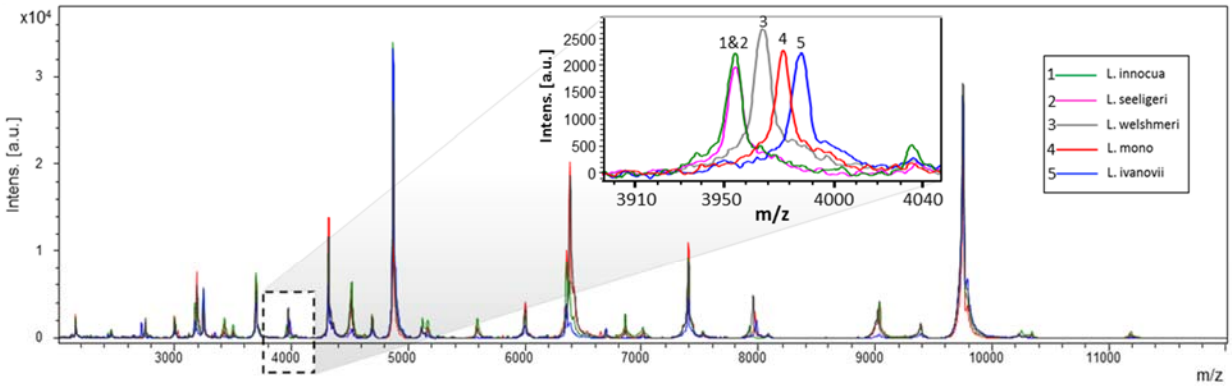

Figure 4: Illustration of subtle peak variations between different listeria species. These peaks are considered unique as the difference between species is over 10%.

Thus, at 4000 Da, a peak mass difference greater than 4 Da was required for the peaks to be considered unique from each other. This is illustrated in Figure 4, where the *L. monocytogenes* peak (red; peak 4) at m/z 3978 is unique from the other *Listeria* species with an 8 Da difference between it and the *L. welshimeri* peak (gray; peak 3) and the *L. ivanovii* peak (blue; peak 5).

It was important to prevent bias when creating the subtyping library. Thus, we did not include every *Listeria* strain in our collection, but included those found to be representative of the different species and varying serotypes. The MSPs were compared with respect to the peaks present and the discriminating power of the individual peaks was determined. For each standard MSP, a corresponding subtyping MSP was created with newly weighted values for the distinguishing peaks. A peak which is present in only one of the standard MSPs' peak lists receives a higher weight than a peak which occurs in all peak lists. Based on those evaluations, 11 MSPs were created for the in-house subtyping library: 4 *L. monocytogenes*, 2 *L. innocua*, 1 *L. ivanovii*, 2 *L. seeligeri* and 2 *L.*

welshimeri (detailed in Sup Table 1). The BioTyper™ Subtyping MSP Editor (v 3.1.66.0) was employed to create a subtyping database for these 11 reference isolates. Isolates were analyzed by the initial Bruker weighted subtyping library and the results were recorded. Weighting was then adjusted based on our analysis of the intensity relative to the base peak and the relative and absolute frequency in the peak lists contributing to that subtyping MSP. After reanalyzing the spectra with the in-house subtyping library, the results were compared to the Bruker results and summarized in Table 1.

Table 1: Comparison of Bruker subtyping library to in-house subtyping library for 113 test isolates.

	Bruker subtyping			In-House subtyping		
Species	correct species identification	# of isolates	% accuracy	correct species identification	# of isolates	% accuracy
L. monocytogenes	27	53	51	48	53	91
L. innocua	17	20	85	19	20	95
L. ivanovii	8	9	89	9	9	100
L. seeligeri/L. welshimeri	20	31	65	25	31	81
Total	72	113	64	101	113	89

Our main objective was to confidently discriminate pathogenic *L. monocytogenes* from other *Listeria* species. By utilizing BioTyper's™ subtyping option, we evaluated the ability of the software to differentiate the closely related species of *Listeria*. The inability to distinguish *L. monocytogenes* from *L. innocua* was frequently encountered when using the Bruker subtyping library (v 3.1.65 Bruker Daltonics). Recall that a score differential of ≥10% was needed to be highly confident in the identification to the species level. For *L. monocytogenes* isolates, the outcome list from the Bruker library scored >2.000 for both *L. monocytogenes* and *L. innocua* 10 times, thus a clear distinction could not be made. In addition, there were 7 instances of the resulting scores being <2.000. These results were considered ambiguous identifications and would require new spots to be deposited and reanalyzed. For *L. innocua* isolates, there were 2 instances in which the Bruker library generated outcome list scored <2.000 (low confidence scores) and 2 false positive identifications of *L. monocytogenes*. There was 1 occurrence in which the *L. ivanovii* spectra resulted in a score <2.000 with the Bruker subtyping library. Distinctions between *L. seeligeri* and *L. welshimeri* spectra patterns were difficult to distinguish, so it was decided to group them into a single identification. Thus, if Biotyper™ gave a highly confident identification of *L. welshimeri to a L. seeligeri* isolate, the identification was

considered correct. After subtyping, there were 10 instances of the *L. welshimeri/L. seeligeri* scores being < 2.000 using the Bruker subtyping peak library. In these instances, 16S sequencing or PCR will be necessary to distinguish species.

To improve the subtyping results, the weighting of peaks in the 11 MSP files was adjusted. After reanalyzing the spectra with the in-house subtyping library, the correct identification was achieved with high confidence (scores ≥2.000) for 48 out of 53 *L. monocytogenes* isolates. There were 2 times wherein the identification had results confident to the genus (scores 1.800-1.999) and 2 occasions in which the identification had low confidence (scores 1.600-1.799) in the results. On 1 occasion, the 10% differential rule was not met between the *L. monocytogenes* and *L. innocua* result scores. *L. innocua* was identified with high confidence for 19 out of 20 isolates utilizing the in-house subtyping library. There was 1 instance in which an *L. innocua* isolate spectra was identified with low confidence. Evaluation of *L. ivanovii* isolates using the in-house subtyping library resulted in highly confident results and no ambiguous identifications for the 9 tested isolates. Although distinctions between *L. seeligeri* and *L. welshimeri* spectra patterns were difficult, confident distinction between these 2 species and the 3 other *Listeria* species we tested was achieved by using the in-house Listeria subtyping library. Evaluation of these 30 isolates identified *L. welshmeri* or *L. seeligeri* with high confidence on 25 occasions, confident on 4 occasions, and low confidence on 1 occasion.

Overall, the in-house subtyping library correctly identified *L. monocytogenes* 91% of the time with high confidence. Using the newly developed peak weighting system has improved the accuracy of MALDI-TOF MS *Listeria* identification to the species level from 64% to 90%.

The spectra of different isolates from food, clinical and environmental samples, were acquired and analyzed by BioTyper RT™ (v 3.1.65 Bruker Daltonics) for identification against the Bruker library of 4274 reference spectra. None of the 30 exclusivity strains analyzed misidentified as *Listeria*. Most were correctly identified by BioTyper™ to the species level. Analysis of the exclusivity strains showed zero *Listeria* false positive results. Therefore, none of the exclusivity strains underwent further analysis with the subtyping library.

We have demonstrated that MALDI-TOF MS analysis can distinguish pathogenic *L. monocytogenes* from other *Listeria* species by using the newly created in-house subtyping reference spectra library. This technology can reduce the time needed to identify *Listeria monocytogenes*, therefore, simplifying and speeding regulatory analysis of food products.

Acknowledgements

The authors gratefully acknowledge Brian Baker, Patrick Regan and John Messana for their support. Special thanks to Karen Jinneman, Zonglin Hu and Mark Mammel for sharing their panels of isolates for this study.

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Supplementary Table 1: Listeria spp. Inclusivity Panel and Exclusivity Isolate Panel

Listeria Panel #	Organism	Original Designation	Isolate Information
			Food Isolates
1	*L. monocytogenes*	FNW 15b42	cucumber
2	*L. monocytogenes*	FNW 3365	mackerel
3	*L. monocytogenes*	FNW 3312	cheese
4	*L. monocytogenes*	FNW 15b27	radish
5	*L. monocytogenes*	FNW 2388	coleslaw
6	*L. monocytogenes*	FNW 2478	raw milk
7	*L. monocytogenes*	FNW 3313	shrimp
8	*L. monocytogenes*	FNW 3326	roast beef
9	*L. monocytogenes*	FNW 3358	milk product
10	*L. monocytogenes*	FNW 3363	cooked snow crab
11	*L. monocytogenes*	FNW 3756	beef & gravy Rh-
12	*L. monocytogenes*	FNW 15b72	apple juice
13	*L. monocytogenes*	FNW 15b85	cream ch. & veg
14	*L. monocytogenes*	FNW 15c14	avocado pulp
* 15	*L. monocytogenes*	FNW 15c22	fontina cheese
16	*L. monocytogenes*	FNW 15a90	turkey ham
17	*L. monocytogenes*	FNW 2450	veg. mix
18	*L. monocytogenes*	FNW 2475	cold cut sand.
* 19	*L. monocytogenes*	FNW 2492	ice cream
20	*L. monocytogenes*	FNW 3291	popsicle
21	*L. monocytogenes*	FNW 3318	lobster
22	*L. monocytogenes*	FNW 3321	raw shrimp
23	*L. monocytogenes*	FNW 3332	Mexican-style cheese
24	*L. monocytogenes*	FNW 3359	surimi scallops
25	*L. monocytogenes*	FNW 3362	pollack
26	*L. monocytogenes*	FNW 3558	cheese
27	*L. monocytogenes*	FNW 3644	red bean ice bar
28	*L. monocytogenes*	FNW 3662	cheese
29	*L. monocytogenes*	FNW 15b70	cheddar cheese
			Clinical Isolates
30	*L. monocytogenes*	FNW 2369	Clinical
31	*L. monocytogenes*	FNW 2370	Clinical
32	*L. monocytogenes*	FNW 15b55	Clinical-Blood
33	*L. monocytogenes*	FNW 15b65	Clinical-Blood

34	*L. monocytogenes*	FNW 3555	Clinical-amniotic fluid
35	*L. monocytogenes*	FNW 3664	Clinical
36	*L. monocytogenes*	FNW 3666	Clinical
37	*L. monocytogenes*	FNW 3668	Clinical
38	*L. monocytogenes*	FNW 15a82	Clinical-Blood
39	*L. monocytogenes*	FNW 15b56	Clinical-Blood
40	*L. monocytogenes*	FNW 15b58	Clinical-Nares
41	*L. monocytogenes*	FNW 15b81	Clinical-Blood
42	*L. monocytogenes*	FNW 15b82	Clinical-CSF
			Environmental Isolates
43	*L. monocytogenes*	FNW 3315	swab
44	*L. monocytogenes*	FNW 3286	swab
45	*L. monocytogenes*	FNW 3308	swab
46	*L. monocytogenes*	FNW 3360	swab
			Other Isolates
47	*L. monocytogenes*	KC 1710	
* 48	*L. monocytogenes*	ATCC 19114	
49	*L. monocytogenes*	V-7	Raw Milk Outbreak -CDC
50	*L. monocytogenes*	ATCC 15313	
51	*L. monocytogenes*	Scott A	Clinical
52	*L. monocytogenes*	ATCC 19116	
* 53	*L. monocytogenes*	ATCC 19115	
54	*L. innocua*	FNW 3107	LA-1 Mexican style cheese
55	*L. innocua*	FNW 3124	Brie
* 56	*L. innocua*	FNW 3516	Shrimp
57	*L. innocua*	FNW 3654	Scallops
58	*L. innocua*	FNW 3758	Smoked salmon
59	*L. innocua*	FNW 6273	lab control
60	*L. innocua*	FNW 3181	Brie
61	*L. innocua*	FNW 3270	Muenster cheese
62	*L. innocua*	FNW 3390	Crab
63	*L. innocua*	FNW 3392	Lobster tail
64	*L. innocua*	FNW 3552	Ground pork
65	*L. innocua*	FNW 3757	Chop suey
66	*L. innocua*	FNW 15a93	Soft cheese
67	*L. innocua*	FNW 15a94	Meat patties
68	*L. innocua*	FNW 15a95	Ham salad
69	*L. innocua*	FNW 15b30	Mushroom
70	*L. innocua*	FNW 15b31	radish
71	*L. innocua*	FNW 15b51	Queso blanco
72	*L. innocua*	FNW 15a92	Env. - ceiling condensate

* 73	*L. innocua*	ATCC 33090	
74	*L. ivanovii*	FNW 2244	Env. - swab smk.salmon processor
75	*L. ivanovii*	FNW 3106	KC1714- FDA Cinc.
76	*L. ivanovii*	FNW 3417	Environmental - water
77	*L. ivanovii*	FNW 6274	lab control
78	*L. ivanovii*	FNW 15a96	Brie
79	*L. ivanovii*	FNW 15a97	Clinical - CDC
80	*L. ivanovii*	FNW 15a98	Clinical - CDC
81	*L. ivanovii*	FNW 15b24	sheep
* 82	*L. ivanovii*	ATCC 19119	
83	*L. seeligeri*	FNW 2232	smoked salmon
84	*L. seeligeri*	FNW 2233	Matjes fillets
85	*L. seeligeri*	FNW 2243	in-line smk. seafood processor
* 86	*L. seeligeri*	ATCC 35967	
87	*L. seeligeri*	FNW 3110	Brie
88	*L. seeligeri*	FNW 3126	Brie
89	*L. seeligeri*	FNW 3389	Crab
90	*L. seeligeri*	FNW 3423	Environmental - water
91	*L. seeligeri*	FNW 3439	Environmental - water
92	*L. seeligeri*	FNW 3451	Environmental - water
93	*L. seeligeri*	FNW 3517	Shrimp
94	*L. seeligeri*	FNW 3531	Environemntal - water
* 95	*L. seeligeri*	FNW 3656	Scallops
96	*L. seeligeri*	FNW 6275	lab control
97	*L. seeligeri*	FNW 15b07	Milk
98	*L. seeligeri*	FNW 15b08	Brie cheese
99	*L. seeligeri*	FNW 15b09	Green peppers
100	*L. seeligeri*	FNW 15b26	Ice cream roll
101	*L. seeligeri*	FNW 15b28	Cherry nut ice cream
102	*L. seeligeri*	FNW 15b49	Ice
103	*L. welshimeri*	FNW 2230	Crabmeat
* 104	*L. welshimeri*	FNW 2231	Smoked Salmon
105	*L. welshimeri*	FNW 3425	Environmental - water
106	*L. welshimeri*	FNW 3441	Environmental - water
107	*L. welshimeri*	FNW 3659	Pollack roe
108	*L. welshimeri*	FNW 15b05	Limburger cheese
*109	*L. welshimeri*	FNW 15b06	Raw milk
110	*L. welshimeri*	FNW 15b16	Smoked salmon pate
111	*L. welshimeri*	FNW 15b46	radish
112	*L. welshimeri*	FNW 15b48	crabmeat
113	*L. welshimeri*	FNW 15b50	cucumber

			Exclusivity isolates
114	*Hafnia alvei*	ATCC 13337	
115	*E. coli*	FNW 6365	C-600
116	*Morganella morganii*	FNW 13b67	lettuce
117	*Shigella dysenteriae*	ATCC 29026	
118	*Citrobacter braakii*	FNW 13d26	Broccoli sprouts
119	*E. coli*	FNW 13d64	Porcino cheese
120	*Leclercia adecarboxylata*	FNW 13d65	Jack cheese
121	*Hafnia alvei*	FNW 13d66	Roquefort cheese
122	*Shigella sonnei*	FNW 13g01	Clinical
123	*Shigella boydii*	FNW 13g18	Clinical
124	*Shigella flexneri*	FNW 13g19	Clinical
125	*Citrobacter freundii*	FNW 6251	QC control
126	*Salmonella* Grp. 30	FNW 6269	lab control
127	*Salmonella lansing* Grp. P	FNW 6270	lab control
128	*Klebsiella pneumonia*	FNW 6271	lab control
129	*Vibrio cholerae*	FNW 6277	Clinical - 154
130	*Vibrio parahaemolyticus*	FNW 6278	Clinical - NY477
131	*Vibrio vulnificus*	FNW 6279	Shrimp
132	*Staphylococcus aureus*	ATCC 25923	
133	*Rhodococcus equi*	FNW 6281	lab control
134	*Lactobacillus* sp.	FNW 6282	salsa
135	*Lactobacillus* sp.	FNW 6286	canned artichokes
136	*Salmonella typhimurium*	FNW 6290	Clinical
137	*Streptococcus pyogenes*	ATCC 19615	
138	*Alcaligenes faecalis*	ATCC 8750	
139	*Salmonella choleraesuis*	ATCC 6539	
140	*Yersinia entercolitica*	FNW 1269	PT-18-1
141	*Yersinia entercolitica*	FNW 1270	WA+
142	*E. coli* O157:H16	FNW 13a80	CDC
143	*Enterobacter cloacae*	ATCC 2335	

*Control isolates that were placed in the subtyping library