Listeria Detection from Enrichment to Identification:

Evaluating 24 Species with Enrichment Media, Screening Media, and Rapid Identification Using MALDI-TOF MS Proteomics Method

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Introduction:

Listeria species have rapidly diversified, with 28 species (shown in table 1) currently proposed. While only *Listeria monocytogenes* is pathogenic to humans and the primary target for food safety testing, monitoring *Listeria* species has become crucial in food production environments. Various rapid testing kits are available, but few studies have addressed the detection capabilities of newly proposed species.

Aim:

- This study evaluated the growth of 24 *Listeria* species using standard enrichment media and screening medium CompactDry™ LS.
- Currently, commercial MALDI-MS microbial identification systems recognize only 17 *Listeria* species. We developed MicrobialTrack™, a proteomics-based identification software that utilizes theoretical protein masses from public databases. This software was evaluated for its ability to identify these *Listeria* species.

Table 1 The strains used in this study are indicated by the strain number

Strain No.	Species	Derivation	Type strain	Whether it is mentioned in each standard method: *sensu stricto Listeria*	ISO 11290 (2017)	FDA BAM Ch.10 (Apr.2022)	USDA MLG 8.15 (Dec.2024)	ISO 11290 (under consideration)
1	*Listeria ivanovii* subsp. *ivanovii*	JCM 7681 (ATCC 19119)	Yes	✓	✓	✓	✓	✓
2	*Listeria ivanovii* subsp. *londoniensis*	ATCC 49954	Yes	✓	✓	✓	✓	✓
3	*Listeria ivanovii*	Crinical	No	✓	✓	✓	✓	✓
4	*Listeria grayi*	ATCC 19120	Yes		✓	✓	✓	
5	*Listeria innocua*	ATCC 33090	Yes	✓	✓	✓	✓	✓
6	*Listeria welshimeri*	ATCC 35897	Yes	✓	✓	✓	✓	✓
7	*Listeria welshimeri*	Crinical	No	✓	✓	✓	✓	✓
8	*Listeria seeligeri*	ATCC 35967	Yes	✓	✓	✓	✓	✓
9	*Listeria marthii*	CCUG 56148T	Yes	✓	✓			✓
10	*Listeria fleischmannii*	DSM 24998	Yes		✓		✓	
11	*Listeria floridensis*	DSM 26687	Yes				✓	
12	*Listeria aquatica*	DSM 26686	Yes				✓	
13	*Listeria newyorkensis*	DSM 28861	Yes				✓	
14	*Listeria cornellensis*	DSM 26689	Yes				✓	
15	*Listeria rocourtiae*	CCUG 59857T	Yes		✓		✓	
16	*Listeria weihenstephanensis*	DSM 24698	Yes		✓		✓	
17	*Listeria grandensis*	DSM 26688	Yes				✓	
18	*Listeria riparia*	DSM 26685	Yes				✓	
19	*Listeria booriae*	DSM 28860	Yes				✓	
20	*Listeria thailandensis*	DSM 107638	Yes					
-	*Listeria goaensis*	-	-					
-	*Listeria costaricensis*	-	-				✓	
-	*Listeria valentina*	-	-					
21	*Listeria ilorinensis*	DSM 111566	Yes					
22	*Listeria cossartiae*	CCUG 74667T	Yes	✓				✓
23	*Listeria immobilis*	CCUG 74666T	Yes	✓				✓
24	*Listeria portnoyi*	CCUG 74671T	Yes					
25	*Listeria rustica*	CCUG 74665T	Yes					
26	*Listeria farberi*	CCUG 74668T	Yes	✓				✓
-	*Listeria swaminathanii*	-	-	✓				
27	*Listeria monocytogenes*	ATCC 15313	Yes	✓	✓	✓	✓	✓

Evaluation of Enrichment Media

Methods:

Pre-culture on sheep blood agar medium (35±1℃, 24h)

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Prepare 10-fold dilutions of McF No.1 in sterile saline solution (10^-4, -5, -6 dilutions)

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Dispense 200 µL of the culture medium (shown in Table 2) into 96-well plates

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Inoculate 10 µL of each diluted solution into each well and onto sheep blood agar (n=2)

▼

Culture (30±1℃ or 35±1℃).

- Measure 650 nm absorbance of 96-well plates at 0h, 24h and 48h
- Count the colonies on sheep blood agar to confirm the number of inoculated bacteria

Results:

✓ None of the enrichment media specified in the guidelines of various countries were able to support the growth of all 24 species of *Listeria*. In contrast, mMSB, like the non-selective medium SCD, successfully supported the growth of all 24 species of *Listeria*.

➢ However, since mMSB is a medium developed for the simultaneous enrichment of multiple species of bacteria, its exclusivity is weak. Therefore, it is difficult to combine mMSB with screening media. mMSB is considered effective in combination with highly specific screening methods such as gene detection kits.

✓ LPT broth (USDA FSIS MLG 8.15) did not support *L. grayi*, but it effectively supported a broad range of *Listeria* species.

➢ MLG 8.15 combines LPT broth with a screening kit with gene detection. Evaluation of exclusivity and combination with screening media should be considered for appropriate broad-spectrum detection of *Listeria* species.

Table 2 Enrichment media used in this study

Medium	Manufacturer	Abbreviation	Culture Condition	Source
Half-Fraser broth	Merck	HFB	30℃, 24-26h	ISO 11290:2017
Buffered Listeria Enrichment broth	Oxoid	BLEB	30℃, 24 and 48h	FDA BAM Ch.10
LPT broth	bioMerieux	LPTB	35℃, 22-26h	USDA MLG 8.15
Modified MSB broth	Shimadzu Diagnostics	mMSB	30℃, 24 and 48h	Modified from MSB (Costa-Ribeiro et al, 2024)
Trypto-Soya Broth	Shimadzu Diagnostics	TSB	30-35℃	JP, USP

Figure 1 Status of the rise in 650 nm absorbance at 24 or 48 hours of incubation at 30℃ incubation

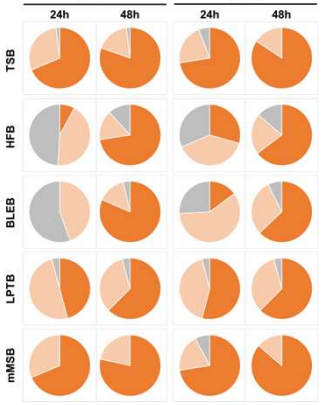
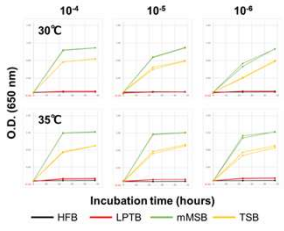


Table 3 Results of colony count of *L. grayi* on sheep blood agar

Dilution 10^n	Colony count CFU/10 µL (n=2)	
-4	+	+
-5	36	29
-6	5	3

Figure 2 Change in absorbance of wells inoculated with 10^{-4} dilution of *L. grayi*



rise in 6 wells
rise in 1-5 wells
no rise in any of the 6 wells

Evaluation of Screening Media

Methods:

Pre-culture on SBA (35±1℃, 24h)

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Prepare McF No.1 bacterial solution in sterile saline solution

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Prepare 10-fold step dilution from McF No.1 bacterial solution

▼

Add 1 mL solution to CD LS and inoculate 10 µL solution onto each medium

▼

Culture (30±1℃ or 37±1℃, 48h) and count number of colonies on each plate

Results:

✓ Some strains could not grow on these selective media at 37℃ incubation.

✓ In CD LS, all 24 species of *Listeria* were detectable as a typical colony (blue colony), while 8 species showed atypical colonies in ALOA at 30℃ incubation.

Figure 3 Example of growth on each medium (37°C, 48 h)

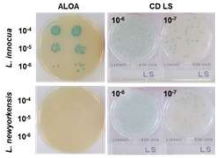

Table 4 Screening media used in this study

Medium	Manufacturer	Abbreviation	Culture Condition	Source
CompactDry™ LS	Shimadzu Diagnostics	CD LS	37℃, 24h and 48h	-
Agar Listeria according to Ottaviani and Agosti	Merck	ALOA	37℃, 24h and 48h	ISO 11290-2:2017
Trypticase soy agar with 5% sheep blood	Shimadzu Diagnostics	SBA	37℃, 24h and 48h	(non selective agar)

Table Results of colony count on screening media at 30℃ incubation

Cells with a Log CFU difference of -0.5 or less were filled in red.

Strain No.	Strain	Colony color on CD LS (30℃)		Log CFU/mL on CD LS	Colony color on ALOA (30℃)		Log difference CD LS - ALOA	Log difference CD LS - SBA
1	*Listeria ivanovii* subsp. *ivanovii*	Blue		8.79	Blue		0.02	0.04
4	*Listeria grayi*	Light-blue ~ Blue		8.35	Light-blue		-0.23	-0.16
5	*Listeria innocua*	Blue		8.53	Blue		-0.16	-0.10
6	*Listeria welshimeri*	Blue		8.38	Blue		-0.07	-0.04
8	*Listeria seeligeri*	Blue	Small colonies	6.37	Blue	Small colonies	-1.91	-2.05
9	*Listeria marthii*	Blue		8.51	Blue		-0.11	-0.11
10	*Listeria fleischmannii*	Blue		8.58	Light-blue		-0.03	-0.01
11	*Listeria floridensis*	Light-blue ~ Blue		8.35	Light-blue		0.04	0.06
12	*Listeria aquatica*	Blue		8.94	Blue		0.07	0.08
13	*Listeria newyorkensis*	Blue		8.77	White		0.19	0.22
14	*Listeria cornellensis*	Blue	Small colonies	5.48	White	Small colonies	-1.70	-1.92
15	*Listeria rocourtiae*	Blue		8.29	White		-0.02	0.03
16	*Listeria weihenstephanensis*	Light-blue		7.46	White		0.29	-0.60
17	*Listeria grandensis*	Light-blue		8.33	White		-0.21	-0.22
18	*Listeria riparia*	Blue		7.92	White		-0.06	-0.14
19	*Listeria booriae*	Blue		8.54	Light-blue		0.02	0.12
20	*Listeria thailandensis*	Blue		9.13	Blue		0.21	0.17
21	*Listeria ilorinensis*	Blue		7.99	Blue		-0.32	-0.16
22	*Listeria cossartiae*	Blue		8.54	Blue		0.07	0.02
23	*Listeria immobilis*	Blue		8.37	Blue		-0.11	-0.08
24	*Listeria portnoyi*	Light-blue ~ Blue		7.38	White		-0.10	0.38
25	*Listeria rustica*	Light-blue		7.38	White		-1.10	-1.07
26	*Listeria farberi*	Blue		8.53	Blue		-0.09	0.00
27	*Listeria monocytogenes*	Blue		8.80	NT*		NT	0.05

*NT: not tested

Identification with MALDI MS Proteomics Method

Methods:

Twenty-four *Listeria* species cultured overnight at 30℃ on SBA

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Samples were prepared in 4 wells each using the following 3 methods:

Direct smear method (DS); where microbial samples were applied to a sample slide, followed by the addition of CHCA solution and drying

Ethanol wash extraction method (EW); where microbial samples washed with high-concentration ethanol were combined with formic acid and acetonitrile to extract cytoplasmic components in microtubes, followed by dropping the extract onto a sample slide, drying, and adding CHCA solution

EW method with Bead-crash extraction (BC); which adds a bead disruption step during the extraction process of EW method

▼

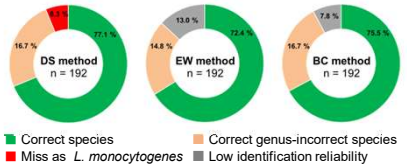
Using the MALDI-8020 (RUO, Shimadzu Corporation), mass spectra were obtained twice per well, and then analyzed using MicrobialTrack

Results:

✓ DS method; the identification was 100% accurate at the genus level. However, there were instances of misidentification as *L. monocytogenes* for *L. mathii* and *L. cossartiae*.

✓ EW and BC methods; lower reliability results were obtained compared to the DS method, but no misidentifications as *L. monocytogenes* occurred.

Figure 4 Results of identification with MicrobialTrack with each sample preparation method



✓ The results indicating "correct at the genus level but incorrect at the species level" (highlighted in light orange) were mainly attributed to 3 species; *L. farberi*, *L. riparia*, and *L. thailandensis*. *L. farberi* and *L. riparia* were misidentified as the closely related *L. innocua* and *L. booriae*.

➢ Given that the DS method had fewer issues with the identification reliability, it is suggested to first perform a simple identification using the DS method. If the identification is *L. monocytogenes*, the EW and BC methods with multiple measurements can be implemented to obtain more reliable results.

Conclusion

✓ **The use of LPT broth, CompactDry LS, and MicrobialTrack in *Listeria* testing is expected to enable the detection and identification of a wide range of *Listeria* species.**

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