Research Square

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Validation of the REBEL - BioMedix Listeria Test Kit for Rapid Detection of Environmental Listeria

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Research Article

Keywords: Listeria, environmental swab, detection, lateral flow device kit, validation, inclusivity, exclusivity

Posted Date: November 18th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11448703/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

The persistence of *Listeria* species in food processing establishments is a serious food safety concern, highlighting the need for prompt and effective monitoring systems of environmental samples. A novel method using the one-step Rapid Enrichment Broth for Environmental *Listeria* (REBEL Media) followed by screening with the BioMedix *Listeria* Test Kit and/or CHROMagar™ *Listeria* were investigated and its effectiveness in detecting *Listeria* from swabs collected from multiple food contaminated surface materials was compared to the USDA reference method.

Findings

Our results show that the REBEL Method with BioMedix *Listeria* Test Kit is statistically equivalent to the reference method for detecting *Listeria* in swabbed samples from food contaminated plastic, stainless steel, or tile surfaces. In addition, CHROMagar™ *Listeria* was used as an alternative screening method for the tile-swabbed samples, showing that the REBEL Method with CHROMagar™ *Listeria* is statistically equivalent to the reference method. Detection specificity was determined using the randomized inclusivity and exclusivity panels and the results show that the REBEL Media and the BioMedix *Listeria* Test Kit successfully enriched and detected 100% of the 52 *Listeria* strains and 0% from the 35 strains in the exclusivity panel.

Conclusions

Our study demonstrates the effectiveness of the REBEL Method using the BioMedix *Listeria* Test Kit and the CHROMagar™ *Listeria* for rapid and specific detection of *Listeria* on various surface materials. The REBEL Method with the BioMedix *Listeria* Test Kit requires minimal setup and significantly reduces the testing time to less than 24 hours. This cost-effective and rapid method for detecting environmental *Listeria* could be readily integrated into food safety systems across Europe and worldwide.

Introduction

Listeria monocytogenes causes listeriosis, a serious foodborne illness in humans. *Listeria* species are abundant in soil, vegetations, and intestines of some animals. The microorganism can withstand substantial environmental hardship and persist in the natural and built environment for an extended amount of time (Burnett et al. 2025). In addition, *Listeria* species can reproduce at refrigeration temperature, posing a serious food safety concern especially for refrigerated ready-to-eat food products (Tirloni et al. 2024). Outbreaks of listeriosis have been associated with the ingestion of a variety of raw and processed foods, including ready-to-eat meats, salads, and dairy products. *Listeria monocytogenes* is among one of the costliest foodborne pathogens owing to its frequent involvement in large outbreaks, product recalls, and hospitalisations (Hoffmann et al. 2025).

The European Union One Health 2024 Zoonoses Report (17 March 2026 – amended version) is a comprehensive report on the zoonoses monitoring and surveillance activities that were conducted in 2024 in 27 EU Member States (MSs), the United Kingdom (Northern Ireland) and eight other non-MSs. The report ranked Listeriosis as the fourth most reported zoonoses in humans and the most severe zoonotic disease that resulted in the highest percentage of hospitalisations and the highest case fatality rate (EFSA & ECDPC 2025). According to the report, the EU recorded 3,041 confirmed invasive human listeriosis cases in 2024. These cases corresponded to a notification rate of 0.69 per 100,000 population, which was 3% higher than the 2023 rate of 0.67 per 100,000 population. The report also identified 2024 as the year with the greatest notification rate and number of reported listeriosis cases since 2007 (EFSA & ECDPC 2025). The data from the same report implicated *Listeria monocytogenes* in 38 foodborne outbreaks in 2024 that resulted in 210 cases of illness, 149 hospitalisations (72.3%) and 17 deaths (8.1%). The findings and supporting data that were presented in the European Union One Health 2024 Zoonoses Report have clearly established *Listeria monocytogenes* as a pathogen of concern in ready-to-eat products (EFSA & ECDPC 2025).

In view of this upsurge of Listeriosis cases in the Union, it is crucial that food safety criteria for *Listeria monocytogenes* ensure a high and consistent level of protection of consumers throughout the food chain. On 1 July 2026, the requirements stipulated in Commission (EU) Regulation 2024/2895 became binding and applicable to all member states of the Union. This set of requirements amends Regulation (EC) No 2073/2005 to add additional stipulations regarding *Listeria monocytogenes* in ready-to-eat products. In order to guarantee the same level of public health protection from production to distribution for ready-to-eat foods other than those intended for infant and for special medical purposes that are able to support the growth of *Listeria monocytogenes*, the food safety criterion "*Listeria monocytogenes* not detected in 25g" should apply to all situations where foods are placed on the market during their shelf-life and for which the producing food business operator has not been able to demonstrate, to the satisfaction of the competent authority, that the level of *Listeria monocytogenes* will not exceed the limit of 100 cfu/g throughout their shelf-life (EU 2024). These added requirements stipulated in Commission (EU) Regulation 2024/2895 invariably increase the importance of applying more stringent controls in mitigating the risks associated with the introduction and persistence of *Listeria monocytogenes* in a food processing environment where ready-to-eat products are being manufactured.

The food safety risk associated with the presence of *Listeria monocytogenes* in food manufacturing environment was further elucidated in the study "The Persistence of Microbiological Hazards in Food and Feed Manufacturing Environment", that was commissioned and published by the European Food Safety Authority (EFSA) in 2024. The study identified *Listeria monocytogenes* as a bacterial food safety hazard most relevant to public health that is associated with persistence in food processing environment in the meat, fish and seafood, dairy and fruit and vegetable sectors. The study identified inadequate zoning and hygiene barriers; lack of hygienic design of equipment; and inadequate cleaning and disinfection, as common risk factors that could make a food processing environment conducive to persistence of *Listeria monocytogenes* (EFSA BIOHAZ Panel et al. 2024). It is therefore critical for a food business operator to be able to assess in a timely manner if the risk associated with the persistence of *Listeria monocytogenes* exists in the processing environment where ready-to-eat products are being manufactured. The study has concluded that a well-designed environmental sampling and testing programme is the most effective

strategy to identify contamination sources and to monitor and detect the presence and potential persistence of *Listeria monocytogenes* in a food processing environment (EFSA BIOHAZ Panel et al. 2024).

The regulatory requirement for testing a ready-to-eat food processing environment for *Listeria monocytogenes* is stipulated in Article 5 of Commission Regulation (EC) 2073/2005.

Food business operators manufacturing ready-to-eat foods which may pose a *Listeria monocytogenes* risk for public health, shall sample the processing areas and equipment for *Listeria monocytogenes* as part of their sampling scheme. In the framework of Regulation (EC) No 2073/2005 (plus amendments), defining microbiological criteria for foods, food chain environmental sampling (including processing areas and equipment) for *L. monocytogenes* detection on a routine basis according to a sampling programme is mandatory. Such sampling programmes aim at detecting and eliminating a persistent strain or, if elimination is impossible, implementing corrective actions to avoid food contamination by the pathogenic bacteria (Midelet et al. 2023).

In view of the consequential need to conduct more environmental testing, a reliable and efficient environmental *Listeria* monitoring method is essential for compliance to current food safety regulations, as well as for the protection of consumer safety. The lengthy testing time commonly associated with traditional testing methods can cause delays in product release from food processing facilities into general distribution as well as in the timeliness by which potential food safety problems can be addressed, should they be detected. There is an increased demand for more reliable and rapid methods that can be done on-site, if needed, without the use of expensive instrumentations. Article 5 of the Regulation (EC) No 2073/2005 stipulates that the use of alternative methods is allowed when testing for *Listeria monocytogenes*. If the food business operator wishes to use analytical methods other than those described in paragraph 3, the methods shall be validated according to internationally accepted protocols and their use authorized by the competent authority (EC 2005).

In this study, we validated a proprietary enrichment medium, REBEL Media, for Rapid Enrichment Broth for Environmental *Listeria* (BioMedix, Pomona, California, USA), coupled with one of the two screening assays, the BioMedix *Listeria* Test Kit (International Point of Care, Toronto, Canada) or the CHROMagar™ *Listeria* (CHROMagar, Paris, France), for the detection of environmental *Listeria*. The REBEL Media supports rapid and selective growth of *Listeria monocytogenes, Listeria innocua, Listeria seeligeri, Listeria welshimeri, Listeria ivanaii, Listeria marthii, Listeria booriae* and *Listeria fleischmannii* without the need of secondary enrichment. The BioMedix *Listeria* Test Kit is a lateral flow rapid test that incorporates two complementary monoclonal antibodies, one of which is labelled with colloidal gold and the second immobilized on a nitrocellulose substrate. Together, these antibodies form a visually readable test line when *Listeria* is present in the sample being evaluated. The two monoclonal antibodies recognize a common flagellar antigen epitope expressed within the genus *Listeria*. The REBEL Method was compared side-by-side with the United States Department of Agriculture/Food Safety Inspection Services Microbiology Laboratory Guidebook - Method 8.11 reference method (USDA/FSIS MLG 8.11) on three types of surface materials commonly used in food processing facilities. Plastic and stainless steel were used to represent food contact surfaces, while tiles were used to represent non-food contact surfaces such as the floor. The REBEL Method with BioMedix *Listeria* Test Kit was validated for all three types of surface materials, while the REBEL Method with CHROMagar™ *Listeria* was validated on tiles only to provide an additional screening assay for environmental monitoring of *Listeria*. CHROMagar™ *Listeria* is an AFNOR validated selective and differential plate that develops blue-green colonies for all *Listeria* spp., while *L. monocytogenes* and *L. ivanovii* will show the additional opaque white halo surrounding the colony (Curtis at al. 1989; Hedge et al. 2007). This study was designed following the guidelines for the microbiological method validation (AOAC 2012; FDA 2019; USDA 2010) and is illustrated in Fig. 1. Our results show that the REBEL Method with BioMedix *Listeria* Test Kit is statistically equivalent to the reference method when tested on any of the three surface materials. The REBEL Method with CHROMagar™ *Listeria* tested on tile surface also shows statistical equivalence to the reference method. The REBEL method provides a reliable and easy-to-use alternative for detecting environmental *Listeria* spp. and results can be obtained within 24 hours using the BioMedix *Listeria* Test Kit and 48 hours when screened by the CHROMagar™ *Listeria*.

Results

Comparison of the REBEL method with the reference method for detecting environmental Listeria

Detection of *Listeria* species in environmental samples was compared between the REBEL Method and the reference method from USDA/FSIS MLG 8.11. Three food relevant surface materials—plastic, stainless steel, and epoxy-sealed tile—were tested in the presence of the quaternary ammonium sanitizer and food residues as potential interfering substances. Two screening assays were employed for the REBEL enrichment. The REBEL - BioMedix *Listeria* Test Kit method was validated for all three surface materials, while the REBEL - CHROMagar™ *Listeria* method was added for the tile samples to provide an additional option for screening. Results are summarized in Table 1 and Table 2, and the raw data and individual trial data analyses are provided in the **Supplemental Tables S1 - S3**.

Table 1
Comparison of the REBEL Method and the reference method in detecting *Listeria* on environmental surfaces

Surface Material			REBEL-Test Kit		Reference		Mantel-Haenszel	Validation Performance Indicators			
	Inoculation Level	No. of Samples	Positive (a)	Negative (b)	Positive (c)	Negative (d)	Chi Square[a]	Sensitivity (p+)[b]	Specificity (p-)[c]	False Negative (pf-)[d]	False Positive (pf+)[e]
Plastic	High (20–50 cfu)	7	7	0	7	0		100%		0%	
	Low (< 1 cfu)	60	28	32	33	27	**0.827**				
	Zero (0 cfu)	7	0	7	0	7			100%		0%
Stainless Steel	High (20–50 cfu)	6	6	0	6	0		100%		0%	
	Low (< 1 cfu)	60	34	26	34	26	**0.000**				
	Zero (0 cfu)	6	0	6	0	6			100%		0%
Tile	High (20–50 cfu)	6	6	0	6	0		100%		0%	
	Low (< 1 cfu)	60	42	18	47	13	**1.078**				
	Zero (0 cfu)	4	0	4	0	4			100%		0%
			REBEL-CHROMagar™								
Tile	High (20–50 cfu)	6	6	0	6	0		100%		0%	
	Low (< 1 cfu)	60	46	14	47	13	**0.047**				
	Zero (0 cfu)	4	0	4	0	4			100%		0%

[a] Mantel-Haenszel Chi Square for unpaired samples is defined as $((n-1)(ad-bc)^2)/((a+b)(a+c)(b+d)(c+d))$ where a = samples confirmed positive by alternative method, b = samples negative by alternative method, c = samples positive by reference method, d = sample negative by reference method, and n = a + b + c + d. **A Chi Square value > 3.84 indicates significance at P < 0.05.** When the Chi Square numerator and denominator are both 0, the Chi Square statistic has the value of 0 [(8)].

[b] Sensitivity (p+) is the % of alternative tested positive / reference positive.

[c] Specificity (p-) is the % of REBEL tested negative / total known negative.

[d] False negative rate (pf-) is 100-sensitivity rate. When the value is negative, 0% is used.

[e] False positive rate (pf+) is 100 – specificity rate.

Table 2
Pearson chi square analysis of the REBEL Method and the reference method

Surface Material	REBEL-Test Kit		Reference	Pearson Chi Square[a]
Plastic	Positive	28	33	
	Negative	32	27	
	Percent Positive	46.67%	55.00%	**0.8336**
Stainless Steel	Positive	34	34	
	Negative	26	26	
	Percent Positive	56.67%	56.67%	**0.0000**
Tile	Positive	42	47	
	Negative	18	13	
	Percent Positive	70.00%	78.33%	**1.0874**
	REBEL-CHROMagar™			
Tile	Positive	46	47	
	Negative	14	13	
	Percent Positive	76.67%	78.33%	**0.0478**

[a] Pearson Chi Square for unpaired samples is defined as $(a\text{-}c)^2 / (a + c) + (b\text{-}d)^2 / (b + d)$ as described in the FSIS Guideline (2010). Please refer to Table 1 for definitions of a, b, c, and d. The Pearson *Chi* square value **> 2.7055** indicates the rejection of the null hypothesis where the alternative and the reference methods are equivalent at alpha = 0.05.

The statistical analysis was based on the factionary recovery of the 60 replicates of the low inoculum level for both the tested method and the reference method (Table 1). For plastic surface, the REBEL-BioMedix Test Kit Method and the reference method show fractional recovery positive results at 28/60 (46.7%) and 33/60 (55%), respectively. For stainless steel surface, both methods yield the same fractional recovery at 34/60 (56.7%). For the tile surface, the REBEL-BioMedix Test Kit Method, the REBEL-CHROMagar method, and the reference methods show a fractional positive at 42/60 (70%), 46/60 (76.6%), and 47/60 (78%), respectively. The Mantel-Haenszel Chi Square for unpaired samples were used to compare the fractional recovery data between the REBEL Methods and the reference method. The Chi Squares range from 0 to 1.078, which is well below the cutoff value at > 3.84 for significant difference at $P < 0.05$, indicating the REBEL Method is equivalent to the reference method. All positive and negative controls worked as expected, with 0% false negative and 0% false positive, respectively (Table 1).

The comparison was also analyzed using the Pearson Chi Square for unpaired samples as described in the FSIS Validation Guideline (USDA 2010). The Pearson Chi Square uses a different formula from Mantel-Haenszel Chi Square in Table 1, but it yields comparable Chi Square values ranging from 0 to 1.087 (Table 2). These Chi Square values are below the cutoff of 2.7055 (alpha = 0.05) resulting in the acceptance of the null hypothesis where the alternative method is equivalent to the reference method.

Detection specificity of the REBEL Media and the BioMedix Listeria Test Kit

Detection specificity of the new method was evaluated according to the AOAC Guideline, requiring blinded testing on a 50 inclusivity strain panel and a 30 exclusivity strain panel (AOAC, 2012). Our inclusivity panel comprises 52 target organisms, consisting of *Listeria monocytogenes* and eight other *Listeria* species; with the exception of *L. grayi*. The exclusivity panel included 35 bacterial species commonly found in food and/or environmental samples, as well as closely related strains; such as *L. grayi* and other firmicutes. The enrichment capability of the REBEL Media and the detectability of the BioMedix *Listeria* Test Kit were evaluated separately to determine the detection specificity of each step. For the REBEL Media, each blind-coded culture was inoculated, followed by enrichment before plating on the MOX plate for *Listeria* and the TSA plate for general growth. For the BioMedix *Listeria* Test Kit, cultures were suspended in the REBEL Media to ensure sufficient cell numbers before performing the test kit.

After decoding of the blinded samples, both the REBEL Media and the BioMedix *Listeria* Test Kit showed consistently positive results for all inclusivity strains and consistently negative results for the exclusivity strains (Table 3 and Table 4). Of the 52 inclusivity strains tested, all 52 strains showed strong growth on the MOX and TSA plates, indicating successful enrichment of these *Listeria* species. The result of the BioMedix *Listeria* Test Kit also shows positive on all 52 strains in the inclusivity panel (Table 3). Of the 35 exclusivity strains tested, all 35 (including *L. grayi*) showed negative growth on the *Listeria*-selective MOX plates as expected. When examining the general growth on the non-selective TSA plates, 33 of the 35 exclusivity strains show no growth, indicating the capability of the REBEL Media to suppress the growth of most of the exclusivity strains (Table 4). Two of the 35 exclusivity strains, *Micrococcus luteus* ATCC 379 and *Staphylococcus aureus* PFGE 2000, showed weak growth after REBEL enrichment, as evidenced by some scattered colonies on the non-selective TSA plates. Both strains belong to the same firmicute family as *Listeria* and may share some common growth requirements with *Listeria*. Despite this, it did not contribute to the readings on the selective MOX plates as well as the BioMedix *Listeria* Test Kit as all 35 exclusivity strains tested negative on the device (Table 4). Based on the blinded inclusivity and exclusivity study, the REBEL Media and the BioMedix *Listeria* Test Kit successfully enriched and detected 100% of the 52 *Listeria* strains and 0% from the 35 strains in the exclusivity panel.

Table 3
Inclusivity panel for detection by the REBEL Media and BioMedix *Listeria* Test Kit

No.	Culture	Strain	REBEL Enrichment[1]		BioMedix *Listeria* Test Kit
			MOX	TSA	
1	*Listeria booriae*	FSL A5-0281	+	+	+
2	*Listeria fleischmannii subsp. coloradensis*	FSL F6-1016	+	+	+
3	*Listeria innocua*	ATCC33090	+	+	+
4	*Listeria innocua (hemolytic)*	FSL J1-023	+	+	+
5	*Listeria innocua Seeliger*	ATCC 51742	+	+	+
6	*Listeria ivanaii*	ATCC19119	+	+	+
7	*Listeria ivanovii*	FSL C2-011	+	+	+
8	*Listeria marthii*	FSL S4-965*	+	+	+
9	*Listeria monocytogenes*	PF16 #34	+	+	+
10	*Listeria monocytogenes*	F2365 = MFS 53	+	+	+
11	*Listeria monocytogenes*	ATCC19115	+	+	+
12	*Listeria monocytogenes*	2417 = V37CE	+	+	+
13	*Listeria monocytogenes*	2355 = LALM-2	+	+	+
14	*Listeria monocytogenes*	2632 = F1109	+	+	+
15	*Listeria monocytogenes*	OB001171	+	+	+
16	*Listeria monocytogenes*	OB1779	+	+	+
17	*Listeria monocytogenes*	OB40043	+	+	+
18	*Listeria monocytogenes*	OB001411	+	+	+
19	*Listeria monocytogenes*	Scott A	+	+	+
20	*Listeria monocytogenes*	OB000217B	+	+	+
21	*Listeria monocytogenes*	B184	+	+	+
22	*Listeria monocytogenes*	J.2629	+	+	+
23	*Listeria monocytogenes*	NCTC11994	+	+	+
24	*Listeria monocytogenes*	OB40037	+	+	+
25	*Listeria monocytogenes*	2223 = 81-1087	+	+	+
26	*Listeria monocytogenes*	B267	+	+	+
27	*Listeria monocytogenes*	PF23#23	+	+	+
28	*Listeria monocytogenes*	J.1768	+	+	+
29	*Listeria monocytogenes*	PF23#29	+	+	+
30	*Listeria monocytogenes*	FSL J1-158	+	+	+
31	*Listeria monocytogenes*	N/A	+	+	+
32	*Listeria monocytogenes*	H7858 = MFS 96	+	+	+
33	*Listeria monocytogenes*	2111 = 87-496-958	+	+	+
34	*Listeria monocytogenes*	14	+	+	+
35	*Listeria monocytogenes*	ATCC 15313	+	+	+
36	*Listeria monocytogenes*	OB10085	+	+	+
37	*Listeria monocytogenes*	PF23#28	+	+	+
38	*Listeria monocytogenes*	32771C=LMB0027	+	+	+

[1] A "+" symbol indicates growth and "-" indicates no growth on the streaked plates, while (+) indicates weak growth as the colonies only appeared on the first two quadrants of the streaked plates. All positive growth showed heavy smears for the first three quadrants and single colonies on the fourth quadrants of the streaked plates. The MOX plates showed the typical darkening around the colonies.

No.	Culture	Strain	REBEL Enrichment[1]		BioMedix *Listeria* Test Kit
			MOX	TSA	
39	*Listeria monocytogenes*	2364 = LALM-11	+	+	+
40	*Listeria monocytogenes*	35584A=LMB0291	+	+	+
41	*Listeria monocytogenes*	PF23#26	+	+	+
42	*Listeria monocytogenes*	ATCC 19111	+	+	+
43	*Listeria monocytogenes*	12459-01	+	+	+
44	*Listeria monocytogenes*	FSL C1-115	+	+	+
45	*Listeria monocytogenes*	PF23#38	+	+	+
46	*Listeria monocytogenes*	OB30849	+	+	+
47	*Listeria monocytogenes*	F6854 = MFS 110	+	+	+
48	*Listeria newyorkensis*	FSL M6-0635	+	+	+
49	*Listeria seeligeri*	FSL H6-169	+	+	+
50	*Listeria seeligeri*	ATCC35967	+	+	+
51	*Listeria welshimeri*	ATCC 35897	+	+	+
52	*Listeria welshimeri*	FSL H6-105	+	+	+

[1] A "+" symbol indicates growth and "-" indicates no growth on the streaked plates, while (+) indicates weak growth as the colonies only appeared on the first two quadrants of the streaked plates. All positive growth showed heavy smears for the first three quadrants and single colonies on the fourth quadrants of the streaked plates. The MOX plates showed the typical darkening around the colonies.

Table 4
Exclusivity panel for detection by the REBEL Media and BioMedix *Listeria* Test Kit

No.	Culture	Strain	REBEL Enrichment[1]		BioMedix *Listeria* Test Kit
			MOX	TSA	
1	*Acinetobacter sp.*	ATCC 49139	-	-	-
2	*Aeromonas hydrophilia*	ATCC 35654	-	-	-
3	*Alcaligenes faecalis*	ATCC 35655	-	-	-
4	*Bacillus cereus*	ATCC 11778	-	-	-
5	*Bacillus subtilis*	ATCC 6633	-	-	-
6	*Citrobacter freundii*	ATCC 8090	-	-	-
7	*Corynebacterium renale*	ATCC 19412	-	-	-
8	*E. coli*	ATCC 8739	-	-	-
9	*E. coli* O157:H7	TW14359	-	-	-
10	*E. coli* O157:H7	ATCC 43895 (CDC EDL933)	-	-	-
11	*Enterobacter aerogenes*	ATCC 13048	-	-	-
12	*Enterococcus faecalis*	CG110	-	-	-
13	*Enterococcus faecalis*	ATCC 19433	-	-	-
14	*Klebsiella pneumoniae*	ATCC 49472	-	-	-
15	*Lactobacillus casei*	ATCC 393	-	-	-
16	*Lactobacillus plantarum*	ATCC 14917	-	-	-
17	*Leclercia adecarboxylata*	ATCC 23216	-	-	-
18	*Listeria grayi*	ATCC 25401	-	-	-
19	*Micrococcus luteus*	ATCC 379	-	(+)	-
20	*Morganella morganii*	ATCC 25830	-	-	-
21	*Proteus mirabilis*	ATCC 29906	-	-	-
22	*Proteus vulgaris/hauseri*	ATCC 700826	-	-	-
23	*Pseudomonas aeruginosa*	ATCC 27853	-	-	-
24	*Salmonella abortus equi*	SEA 2575	-	-	-
25	*Salmonella choleraesuis*	ATCC 10708	-	-	-
26	*Salmonella diarizonae*	ATCC 12325	-	-	-
27	*Salmonella diarizonae*	ATCC 29934	-	-	-
28	*Salmonella enteritidis*	PFGE 2250	-	-	-
29	*Salmonella typhimurium*	PFGE 2029	-	-	-
30	*Shigella sonnei*	ATCC 25931	-	-	-
31	*Staphylococcus aureus*	PFGE 2000	-	(+)	-
32	*Staphylococcus aureus*	PFGE 1800	-	-	-
33	*Staphylococcus epidermidis*	ATCC 12228	-	-	-
34	*Vibrio parahaemolyticus*	ATCC 17802	-	-	-
35	*Yersinia enterocolitica*	ATCC 23715	-	-	-

[1] Symbols are as described in Table 3.

Discussion

Based on both the Mantel-Haenszel Chi Square analysis (Table 1) and the Pearson Chi Square analysis (Table 2), the one-step enriched REBEL methods show statistically equivalent to the 2-step enrichment reference method on various food related environmental surfaces. Both the REBEL Media and the BioMedix *Listeria* Test Kit showed 100% inclusivity for the tested *Listeria* species, except *L. grayi*, and exhibited no cross-reactivity with non-*Listeria* strains

(Table 3 and Table 4). It was anticipated that neither the REBEL Media nor the BioMedix *Listeria* Test Kit would support the growth or detection of *L. grayi*, as is the case with several validated media, including MOX and CHROMagar™. *L. grayi* belongs to the phylogenetically distinct *Listeria sensu lato* group and exhibits atypical growth and colony morphology compared with typical *Listeria* sensu stricto strains (Carlin et al. 2022). Since *L. grayi* is generally considered nonpathogenic, many methods prioritize *L. monocytogenes* and closely related species as food-safety indicators. For this reason, we included *L. grayi* in the exclusivity strain panel, and our results were consistent with expectations (Table 4).

The method evaluated in this study offers several major improvements: (1) rapid, one-step enrichment capable of enriching very low levels of *Listeria* contamination, as few as 1 cfu, to detectable levels within 24 hours; (2) rapid screening using a lateral-flow device, the BioMedix *Listeria* Test Kit, which targets a *Listeria*-specific flagellar antigen for additional confirmation; and (3) use of the CHROMagar™ *Listeria* to differentiate *Listeria* species and *L. monocytogenes*. The MOX agar plates recommended by the reference method are effective for detecting *Listeria* species but not specifically for identifying *L. monocytogenes*. Adding the CHROMagar™ *Listeria* enables simultaneous detection and differentiation of *Listeria* species, including *L. monocytogenes* (Curtis et al. 1989; Hegde et al. 2007). The REBEL Method with the BioMedix *Listeria* Test Kit is completed within 24 hours, the REBEL Method with CHROMagar™ *Listeria* within 48 hours, whereas the reference method requires 4–5 days.

Both the REBEL Media and the UVM – MOPS BLEB enrichment media used in this study include the esculin/ferric iron as their key selective and differential molecules. Blackening of the media is a result of esculin hydrolysis by *Listeria's* esculinase (Law et al. 2015). Interestingly, at the low inoculum level (approximately 1 cfu/test), positive samples typically blackened sooner in the REBEL Media than in the UVM medium, which often required a second enrichment step and up to 4 days to produce positive results (**Table S1**). These findings support the use of the REBEL Media for rapid enrichment of *Listeria* species. Different enrichment media have been shown to affect the growth rate and detection of *Listeria* in environmental samples (Ryser et al. 1996; Salazar et al. 2021). Conventional enrichment of environmental *Listeria* typically involves a two-step process, such as UVM followed by MOPS-BLEB in the FSIS MLG 8.11 protocol (USDA 2019) or Half-Fraser Broth followed by Fraser Broth in EN ISO 11290-1:2017. Although two-step enrichment has been shown to maximize the detectability of environmental *Listeria*, several one-step enrichment media have been evaluated or adopted in recent years. For example, the FDA's *Bacteriological Analytical Manual* (BAM) uses a short, 4-hour BLEB pre-enrichment, followed by the addition of a selective supplement before proceeding to full enrichment (Hitchins et al. 2022). The USDA/FSIS MLG 8.16 (2026) has recently incorporated a proprietary one-step enrichment medium, bioMèrieux® LPT *Listeria* enrichment broth, which may reduce the testing time to 24 hours when screening samples by polymerase chain reaction (PCR). This transition demonstrates the need for rapid screening methods for environmental *Listeria*. However, despite its popularity, PCR may not be feasible for many small food-processing establishments because of the cost of reagents and equipment and the need for skilled personnel. In this study, we evaluated the REBEL Method with the BioMedix *Listeria* Test Kit and the CHROMagar™ *Listeria*, and found it to be effective and specific for detecting environmental *Listeria*, providing food-processing facilities with a rapid, on-site alternative screening method.

Conclusions

Our study supports the effectiveness of the REBEL enrichment and BioMedix *Listeria* Test Kit in detecting environmental *Listeria* species on plastic, stainless steel, and tile surfaces, and it is statistically equivalent to the reference method. For the tile surface, the REBEL - CHROMagar™ *Listeria* method was also validated and shown statistical equivalence to the reference method. In addition, both the REBEL enrichment and the BioMedix *Listeria* Test Kit detected all 52 *Listeria* species in the inclusivity panel but none of the 35 strains in the exclusivity panel. Overall, our study demonstrates the effectiveness of the REBEL enrichment and the BioMedix *Listeria* Test Kit in detecting environmental *Listeria* with a high specificity and the test can be completed within 24 hours when screening with the BioMedix *Listeria* Test Kit and within 48 hours when screening with the CHROMagar™ *Listeria*. This cost-effective and rapid method for detecting environmental *Listeria* could be readily integrated into food safety systems across Europe and worldwide, helping address the increasing incidence of listeriosis and the persistence of *Listeria* in food-processing environments.

Materials and Methods

Environmental Surface Preparation

Three surface materials were tested: plastic, stainless steel, and epoxy-sealed ceramic tile. The first two materials represented common food-contact surfaces: polypropylene cutting boards (plastic) and stainless-steel workbenches, respectively. Ceramic tiles were used to represent floor surface and were epoxy-sealed to comply with flooring requirements of food processing facilities. Each of the surface materials was prepared to simulate operation in a food processing facility. The surface was first washed thoroughly with detergent (Versa-Clean #PVCLN04) and tap water. Quaternary ammonium was diluted to the appropriate ppm following the manufacturer's direction (Member's Mark #42939). The diluted quaternary ammonium solution was sprayed 6–8 inches away from surface, covering the entire surface, and was left on surface for 3 minutes to air dry. For plastic and stainless steel (food contact surface), 200 ppm of quaternary ammonium was used. After air-drying, pieces of cooked chicken were wiped on the entire surface in one single direction. This wiping step was repeated two more times, with fresh pieces of meat each time, to simulate 3 hours of food preparation and to introduce food residues on the food contact surface. For tile surface, the surface preparation was slightly different to simulate the non-food contact environment. The tiles were cleaned with detergent and rinsed with tap water, followed by spraying of 400 ppm of quaternary ammonium in a similar manner as above. To simulate food being dropped on the facility floor, each tile was wiped with pieces of cooked chicken and then rinsed with tap water to remove visible food particles. Excess liquid was removed by an industrial-grade rubber floor squeegee (AquaDozer, Unger #967560). The cooked chicken wiping, tap water rinsing, and squeegeeing process was repeated 2 more times to simulate 3 hours of operation with potential food spills on the floor. Following surface preparation, each surface will then be swabbed for a maximum of three times, with cooked chicken wiping and tap water rinsing/squeegeeing in between each swabbing. A 1 sq-ft area of the prepared surface was swabbed with moistened sponge or swab as described below. Inoculum will then be loaded directly to these sponges/swabs before proceeding to the enrichment step.

Inoculum Preparation

Listeria monocytogenes ATCC 19111 was used as the target strain to spike the environmental swabs and was selected based on the recommendation by FSIS Guidance for Test Kit (USDA 2010). Competitive microflora were obtained from multiple environmental swab samples collected from three local food processing facilities and had been tested negative for *Listeria* species. Frozen stock aliquots of *Listeria monocytogenes* ATCC 19111 and the competitive microflora were each reconstituted into 10 ml tryptic soy broth (TSB, Remel #R455052) tubes and incubated at 37°C for 24 hours. Each culture was then diluted to an OD_{600} of 0.2 in TSB broth to adjust to a uniform culture concentration. The tubes were then cold stressed at 4°C for 48 hours. Plate counts on TSA plates were performed to determine the actual viable cell concentration at 0 hour, before the cold stress, and after 48 hours of cold stress. The plate counts at 0 hour were used to estimate the cfu/ml of the 48-hour cold-stressed culture on the day of inoculation, using a multiplying factor pre-determined empirically from at least 5 replicates of the cell counts collected before and after cold stress. The plate count results from the 48-hour cold stress samples were reported as the actual concentration of the inoculum.

Inoculation and Study Design

After swabbing the prepared environmental surface, each sponge/swab was inoculated with the 48-hour cold-stressed *Listeria monocytogenes* at the designated level of inoculum, zero, low (< 5 cfu), and high (20–50 cfu). The cold-stressed competitive microflora were also added to each sponge/swab at 10 times the inoculum levels of the target organism, as well as the zero inoculum samples. For each surface material, at least 60 replicates of swab samples containing a low inoculum level were tested using both the reference method and the REBEL Method as an alternative method. Only the trials yielding fractional positive results were considered valid. The replicates were collected from two or three trials due to the limitations in space, time, and operational efficiency. The reference method and the alternative method were always performed side-by-side with the same number of replicates. At least five replicates each of the zero and high inoculum level were included as the negative and positive controls, respectively. The overall study design is shown in Fig. 1.

Detection of Listeria by the Reference Method

The USDA/FSIS Method MLG 8.11, "Isolation and Identification of *L. monocytogenes* from Red Meats, Poultry, Egg Products, Ready-to-Eat Siluriformes (Fish), and Environmental Samples", was used as the reference method. All reagents and media were prepared in-house or purchased from commercial sources as indicated below. Briefly, the environmental sampling sponge (Nasco #B01245) was pre-dampened with 10 ml D/E buffer (Hardy Diagnostics C7373) and wiped on a 1 sq-ft area of the prepared surface. After inoculation with the cold stressed *L. monocytogenes* and competitive microflora at the designated inoculum level, the sponge was aseptically inserted back into the Whirpak bag, followed by the addition of 225 ml of the University of Vermont Medium (UVM) (Neogen, NCM0012B) and incubated at 30°C for 24 hours. After 24 hours, 100 µl of each UVM-enriched sample was transferred to 10 ml MOPS-Buffered *Listeria* Enrichment Broth (BLEB) tube (Neogen, NCM0055A & VWR #97062-744 / VWR #97063-960) and incubate at 35°C for 18–24 hours. The presence of *Listeria* was determined by plating UVM-enriched samples on the Modified Oxford (MOX) agar plates (Difco #222530 & Supplement #211763). After incubation at 35°C for 18–24 hours, the MOX plates were examined for colonies typical of *Listeria* spp., with zone of darkening due to esculin hydrolysis. Negative plates are incubated for another 24 hours to confirm results. For samples that were determined negative on direct MOX plating, 10 ul of MOPS-BLEB-enriched samples were streaked on a fresh MOX plate (2nd MOX plating). A negative result is claimed if samples tested negative for both direct and 2nd MOX plating.

Detection of Listeria by the Alternative Method: the REBEL Method

All reagents and swabs used in the REBEL Method were provided by BioMedix (Pomona, California, USA). The complete REBEL Method includes the BioSwab with Butterfield's Medium (BioMedix, Pomona, CA), the Rapid Enrichment Broth for Environmental *Listeria* (REBEL Media), and the screening assays with the BioMedix *Listeria* Test Kit (Decision Point™ International Point of Care, Toronto, Canada) and/or the CHROMagar™ *Listeria* (CHROMagar, Paris, France). The BioSwab was pre-dampened by dipping into the Butterfield's Medium vial and swabbed on 1 sq-ft area of prepared environmental surface. The entire surface was thoroughly swabbed upwards, downwards, and side to side, flipping the swab between each direction to ensure adequate exposure of the swab to the area being sampled. After inoculation of the cold stressed *L. monocytogenes* and competitive microflora at the designated inoculum level as described above, the swab was inserted into the Butterfield's Medium vial and the swab handle was broken at its breaking point, dropping the swab into the vial. One milliliter of the REBEL concentrate was added directly into the Butterfield's Medium vial with the swab. The vial was capped and inverted three times to evenly mix contents. The vial cap was then loosened to allow aerobic growth at 30°C for 24–26 hours. After REBEL enrichment, the presence of *Listeria* was screened by the BioMedix *Listeria* Test Kit or the CHROMagar™ *Listeria*. For the BioMedix *Listeria* Test Kit, 2 ml of each enriched culture were heated in a glass tube at 80°C for 20 minutes. After cooling to room temperature for 15 minutes, 150 µl of cooled liquid was transferred to the Bead Vial provided in the kit and mixed thoroughly by gently pipetting until the bead was completely dissolved. The full content was then transferred to the loading window of the BioMedix *Listeria* Test Device at room temperature. Result was read up to 15 minutes. Positive results for *Listeria* spp. produce two lines in both the control and test areas, while negative results show only one line in the control area. No line observed in the control area indicated an invalid result. CHROMagar™ *Listeria* were also tested in pairs with the BioMedix *Listeria* Test Kit for the tile surface swabs. REBEL-enriched samples were streaked directly on the CHROMagar™ *Listeria* plates and incubated at 37°C for 22–26 hours. All *Listeria* spp. except *L. grayi*, produce blue-green colonies, whereas *L. monocytogenes* and *L. ivanovii* produce an additional opaque white halo surrounding the colonies (Hedge et al. 2007).

Data Analysis of the Validation Study

Comparison between the REBEL Method (alternative method) and the reference method were performed using two Chi Square formulas. The Mantel-Haenszel Chi Square for unpaired samples is defined as $((n-1)(ad-bc)^2)/((a+b)(a+c)(b+d)(c+d))$ where a = samples confirmed positive by the alternative

method, b = samples negative by the alternative method, c = samples positive by the reference method, d = samples negative by reference method, and n = a + b + c + d. A Chi Square value > 3.84 indicates a significant difference at $P < 0.05$. When the *Chi* Square numerator and denominator are both 0, the Chi Square statistic has a value of 0 (Fieldsine et al. 2008). The one-sided Pearson Chi-Square for unpaired samples is defined as $(a-c)^2 / (a + c) + (b-d)^2 / (b + d)$ as described in the FSIS Guideline (USDA 2010), where a - d are defined the same as above. The Pearson Chi Square value > 2.7055 indicates the rejection of the null hypothesis where the alternative and the reference methods are equivalent at alpha = 0.05.

The Inclusivity and Exclusivity Study for Detection Specificity of the REBEL Method

Strains for the *Listeria* inclusivity panel and the non-*Listeria* exclusivity panel were acquired from several sources, including the American Type Culture Collection (ATCC), ILSI North America *Listeria monocytogenes* Strains Collection at Cornell University, USDA ARS Culture Collection (NRRL), FDA PRLSW, as well as our in-house strain collection. All organisms were grown and maintained in the non-selective Tryptic Soy medium (TSB or TSA; Remel #R455052). To blind-code the cultures, each bacterial strain was first transferred to a TSA deep-agar tube. A third person then assigned blinded codes using Excel-generated random numbers. These tubes served as the working stocks for all subsequent blinded studies. The inoculum was prepared by streaking each bacterial culture to isolation on a TSA plate and incubated at 30°C for 18–24 hours. Isolated colonies were transferred to TSB to adjust to a fixed turbidity as described above. The inoculum for REBEL enrichment was set 10 times the detection limit of the REBEL Media at ~ 1 cfu/test. After enrichment at 30°C for 24–26 hours, the culture was streaked onto a TSA plate and a MOX plate to assess general growth and confirm the identity of the tested bacterial strain, respectively. To test the inclusivity and exclusivity panels on the BioMedix *Listeria* Test Kit, each bacterial culture was adjusted to 10^7 cfu/ml which is 100 times the detection limit for the device kit. The test was performed and recorded as described above. After the study was completed, a third person unblinded and sorted the strains into the inclusivity and exclusivity panels.

Abbreviations

CFU
colony forming unit
MOX
Modified Oxford agar
AFNOR
Association française de normalization (French Standardization Association)
REBEL
Rapid Enrichment Broth for Environmental *Listeria*
UVM
University of Vermont Medium

Declarations

Competing interests.

The authors declare that the funder had no role in the study design and outcome interpretation. All conclusions are based on scientific data collected and analyzed by the authors.

Funding.

The project is funded by BioMedix, Pomona, California, USA

Author Contribution

LAS performed experiments and preliminary data analyses and oversaw the project's progress. DKV and SL performed sample preparation and the subsequent testing and result readings. WJL is responsible for the study design, data analysis, and manuscript preparation.

Acknowledgement

The authors sincerely thank Dr. Claver Bundac for his insightful perspectives, practical expertise, and valuable input on current EU food-safety compliance requirements and regulations.

Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Figures

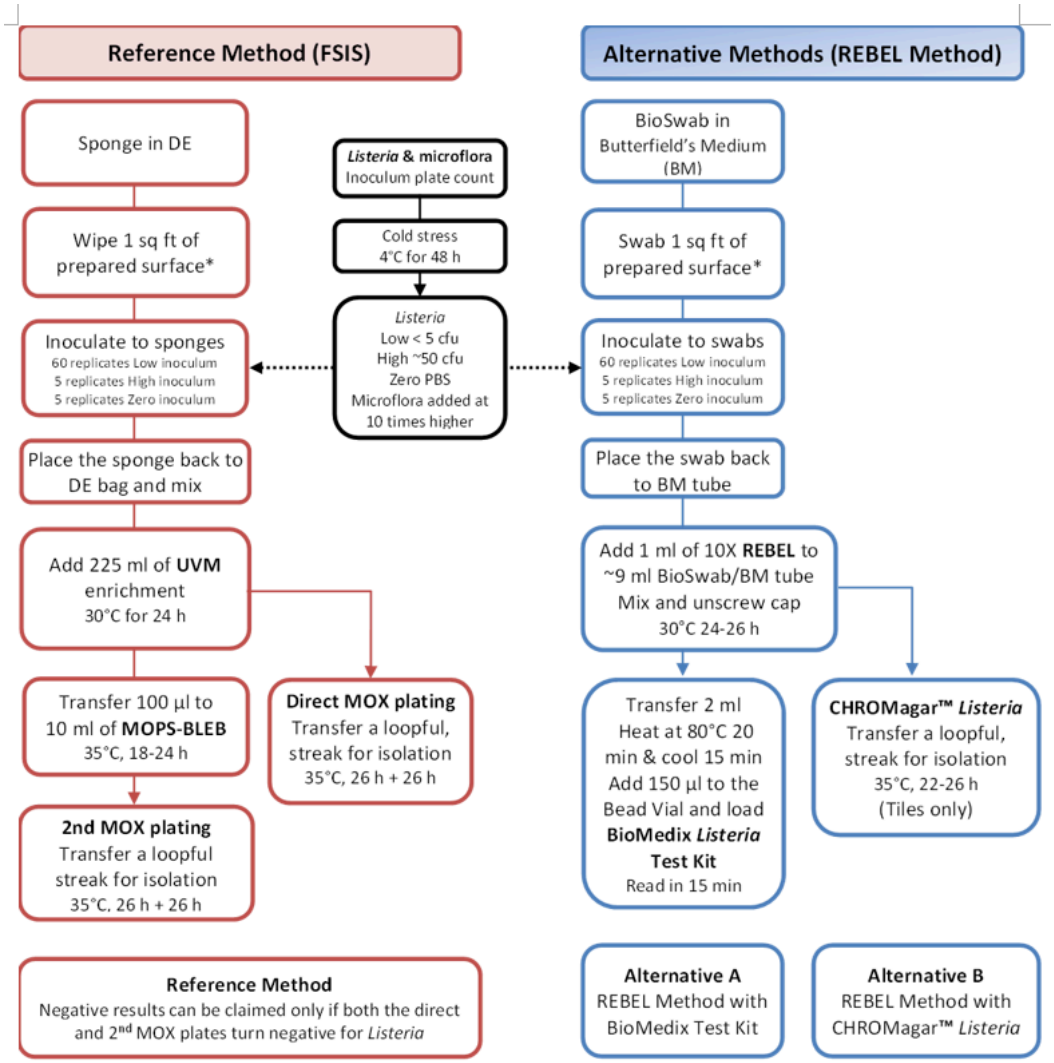


Figure 1

Study design for validation of the REBEL Method with BioMedix *Listeria* Test kit and CHROMagar™ *Listeria* for the detection of environmental *Listeria*. At least 60 replicates were collected from the low inoculum level and at least five replicates each of the high and zero inoculum levels were performed for each of the three surface materials, plastic, stainless steel, and tiles.
* Environmental surface preparation is described in **Methods**.

Supplementary Files

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