Evaluation of VITEK® 2 and MALDI-TOF/MS automated methodologies in the identification of atypical *Listeria spp.* isolated from food in different regions of Brazil

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A R T I C L E   I N F O

A B S T R A C T

Listeria monocytogenes is a pathogen responsible for listeriosis, a foodborne disease with high mortality rates (20–30%). It mainly affects the elderly, pregnant women, and immunocompromised people. Although not pathogenic, the isolation and identification of *Listeria innocua* are critical since they can indicate L. *monocytogenes*' presence as they are closely related and widely distributed in the environment and food processing plants. The objective of this study was to evaluate the effectiveness of the automated methods VITEK® 2 and MALDI-TOF/MS in identifying 94 strains of the genus *Listeria* with atypical identification profile. The resulting identification by Polymerase Chain Reaction (PCR), using specific primers for the most common species of *Listeria*, was considered the correct identification and presented a total of 31 strains identified as *Listeria innocua* (LI), 54 as L. *monocytogenes* (LM), 8 as *Listeria welshimeri* (LW) and 1 as *Listeria grayi* (LG). The VITEK® 2 automated system correctly identified, on average, 79% of the LI strains, 16% of the LM strains, and 88.0% of the LW strains. In the analysis by MALDI-TOF/MS, on average, 73% of LM strains were correctly identified, few LW strains were correctly identified, and all LI strains were incorrectly identified. Both VITEK® 2 and MALDI-TOF/ MS correctly identified the LG strain in both analyzes. The results demonstrate that automated methodologies could not discriminate atypical strains of the *Listeria* genus and point to the need for the use of complementary tests, such as PCR and chromogenic media, for the correct identification of these strains.

1. Introduction

L. monocytogenes is the etiologic agent of listeriosis, a predominantly foodborne disease that results in main clinical conditions: meningitis, sepsis and abortion. Gastrointestinal symptoms, such as nausea, vomiting, and diarrhea, precede or accompany the more severe manifestations of the disease. The main risk groups are represented by pregnant women, newborns, children, older adults with or without chronic degenerative processes, and immunosuppressed individuals, showing a high mortality rate (Gray et al., 2021). The species is recognized worldwide as one of the five main foodborne agents, however, listeriosis is a rare disease with a low incidence (CDC, 2019).

L. monocytogenes is able to grow under wide environmental conditions, such as wide ranges of pH (4.1–9.6), temperature (1–45 °C) and high salt concentrations (McLauchlin et al., 2013). *L. monocytogenes* escapes from the vacuole or phagosome under the action of a pore-forming hemolysin (listeriolysin O), encoded by the *hly* gene and two phospholipases. Once free in the cytoplasm, the bacterium multiplies and moves with the aid of the *ActA* protein, responsible for the polymerization of actin-rich structures and *ActA* gene products. The Listeria genus has specific surface proteins (invasins) called internalins A (*InlA*) and internalins B (*InlB*), which are responsible for the adhesion of L. *monocytogenes* on the surface of intestinal epithelial cells (Pizarro-Cerdá and Cossart, 2006; Vázquez-Boland et al., 2001).

From a bacteriological point of view, *L. monocytogenes* has a phenotypic profile similar to L. *innocua*, differing mainly by the presence of hemolysin, which is one of the main virulence factors in L. *monocytogenes* (Moreno et al., 2014). *L. innocua* is not pathogenic to humans,

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but its isolation and identification are critical. The species has the same habitat as L. *monocytogenes* and can be considered a risk of cross-contamination. Although non-pathogenic, rare cases of L. *innocua* septicemia and meningitis have been reported in humans.(Murray, 1995) Moura et al. (2019) cite the first report of atypical L. *innocua* and describe isolates of the species from seafood in Asia, swine in North America, and birds in Europe, suggesting a worldwide spread. In recent years, strains of L. *innocua* and L. *monocytogenes* with an atypical profile have been detected in food and the environment (Moreno et al., 2014). The existence of these atypical strains indicates that traditional methods of phenotypic characterization should be associated with genotypic methods to improve the identification of *Listeria spp.* The current trend is that new molecular and spectrometric methodologies are gradually introduced and propagated due to precision, sensitivity, and specificity criteria (Tsukimoto and Rossi, 2018).

The VITEK 2 Compact® is a fully automated microbial identification system that operates with barcode cards, ensuring complete traceability and a lower risk of transcription errors. The preparation time and the final result can be obtained in a range of 2 to 18 h, depending on the bacteria's metabolism and the card used. In the case of *Listeria sp.* identification, the GP card, used for Gram-positive bacteria, is based on biochemical methods that measure carbon utilization, resistance and enzymatic activity, totaling 43 biochemical tests (Moehario et al., 2021; Crowley et al., 2012). Although usually very precise, this equipment showed difficult in identifying Listeria strains with atypical phenotypic profile (De Lappe et al., 2014).

Currently, the use of Matrix-Assisted Laser Desorption Ionization technology – Time of Flight/Mass Spectrometry (MALDI-TOF/MS) has been gaining ground in the identification of bacterial species of clinical importance, considering the ease of execution, the immediate release of results, and the low cost per analysis. The technique consists of mixing the culture with a polymeric matrix that absorbs light and allows the ionization of proteins by excitation through a laser beam. Then, electric fields guide the generated ions inside a vacuum tube, where separation by mass/charge occurs according to the time spent through the tube to the detector. Each ionized particle generates a peak and the set of detected particles is converted into a spectral profile, which is compared with the profile of several species present in the database and interpreted as an identification result, associated with a confidence level. The relatively limited content of the database appears to be a limitation of the technique (Celandroni et al., 2016; Angeletti, 2017; Hou et al., 2019).

This study aimed to evaluate the potential of traditional, automated, and molecular techniques in identifying atypical *Listeria* species isolated from different food sources and different regions of Brazil.

2. Material and methods

2.1. Strains

Ninety-four strains of the genus *Listeria* with an atypical profile (negative hemolysis and serotyping for L. *innocua*), previously classified as non-typeable L. *innocua*, were selected for the study. All strains were of food origin (about 70% isolated from meat products) and come mainly from Brazil's south, southeast, and central-west regions. The selection criteria for these strains were the atypical profile and the difficulty of identification. The strains are deposited in the Listeria Collection (CLIST) of the Laboratory of Bacterial Zoonoses (IOC/Fiocruz), kept cryopreserved in Brain Heart Infusion broth (Oxoid) plus 20% glycerol and held at −20 °C. The strains were thawed and inoculated in Tryptone Broth (Oxoid) for 24 h at 37 °C. For isolation and verification of viability, 5% Blood Agar and Tryptone Agar (Oxoid) with the same incubation period and temperature were used.

2.2. Biochemical identification and antigenic characterization

The biochemical identification was carried out from the fermentation of carbohydrates (D-glucose, D-xylose, D-mannitol, D-rhamnose, and alpha D-Mannoside) (BD Difco, USA), motility at 25 °C (BD Difco, USA), catalase test, and hemolysis in Sheep Blood Agar at 5% (Merck, USA), according to the methodology described by Rocourt et al. (1983) (Table S1). Antigenic analysis to identify serogroups/serotypes was performed using the rapid agglutination slide technique, using somatic and flagellar polyclonal antisera, polyvalent and monovalent, according to the recommendations of Seeliger and Hönne (1979).

2.3. Assessment of phospholipase production C (PI-PLC)

The strains were inoculated on Listeria acc. Ottaviani & Agosti® - ALOA (Sigma-Aldrich, USA) and incubated for 24–72 h/37 °C (ISO 11290-1:2017, 2017) to evaluate the activity of phospholipase C (PI-PLC) production.

2.4. Automated biochemical identification (VITEK 2 Compact®)

Each tube with the prepared suspension was submitted to identification by the VITEK 2 Compact® equipment, VITEK® GP ID cards and VITEK 2 SYSTEM software (bioMérieux, France), according to the procedure indicated by the manufacturer, with variable incubation periods for each analyzed strain (up to 24 h).

The standard strains L. *monocytogenes* 1/2a (ATCC 15313, CDC F4561, 10403S), *L. monocytogenes* 1/2b (ATCC BAA-751), *L. monocytogenes* 3b (CDC F4540), *L. monocytogenes* 3c (CDC F6238), *L. monocytogenes* 4a (ATCC 19114), *L. monocytogenes* 4ab (CDC F1067), *L. monocytogenes* 4b (ATCC 7644, Scott A), *L. monocytogenes* 4c (ATCC 19116), *L. monocytogenes* 4d (ATCC 19117), *L. monocytogenes* 4e (ATCC 19118), *Listeria seeligeri* (CLIP 9529), *Listeria welshimeri* (CDC F4082), *L. innocua* (CLIP 12570, CLIP 12595, CLIP 12612, CLIP 12624, CDC F4078) e *L. grayi* (CDC F4076) were used as controls.

Two independent tests were made and the strains were analyzed in triplicate (three cards per strain), where only the result repeated in two of the three cards was considered.

2.5. Identification by MALDI-TOF/MS

Isolates previously cultivated in Blood Agar at 37 °C for 24/48 h were transferred to a spot on the Flexi-Mass-DS TO-430 model (bioMérieux, France) slide. With the aid of a micropipette, 0.5 μL of formic acid (70%) was applied to each smear, dried at room temperature, followed by the addition of 1 μL of alpha-cyano-4-hydroxycinnamic acid matrix solution (CHCA, bioMérieux, France). Formic acid was added to the strains for better results (SUWANTARAT, N., et al., 2016; Costa et al., 2022) Two replicates were performed per sample, and streaks of the strain of *Escherichia coli* ATCC 8739 (control strain and equipment calibrator) were performed in the central spots of each slide used. A spot without sample with CHCA matrix was included for the negative control, and the reference strains *Candida glabrata* (ATCC 2001), *Staphylococcus aureus* (ATCC 6538), and *Enterococcus faecalis* (ATCC 19433) were used for positive control of the test and evaluation of fine adjustment of the equipment. After matrix crystallization, the slides were introduced into the VITEK MS RUO equipment (MALDI-TOF/MS, model AXIMA) (Kratos/Shimadzu, USA), equipped with a nitrogen laser operating at 337 nm (nm). The results obtained were analyzed by the SARAMIS Premium software (Database version 4.10 and System version 4.0.0.4, 2010) and expressed through spectra generation. As method control, standard samples of the *Listeria* genus described in Section 2.3 were used. Two independent tests were made and the strains were analyzed in triplicate (three wells per strain), where only the result repeated in two of the three wells was considered.

2.6. Identification and molecular characterization – Polymerase chain reaction (PCR)

A bacterial suspension in 0.85% saline solution was subsequently centrifuged at 25,000*g* for DNA extraction for 5 min. According to the manufacturer's instructions, total DNA was extracted using the DNeasy Blood & Tissue® Kit (QIAGEN, USA) and stored at −20 °C.

The confirmation of genus and species was performed with two primers, one for the 23S ribosomal RNA subunit gene and the other for the *hly* gene, which encodes listeriolysin O (LLO) according to Hudson et al. (2001). To confirm the species L. *innocua* and L. *grayi*, primers in9 and lgr were used, respectively (Tao et al., 2017). And, according to Liu et al. (2004), the primer lwe7-571 was used to confirm the species L. *welshimeri*. Molecular serotyping of L. *monocytogenes* samples was performed using the primers according to Doumith et al. (2004). Table 1 shows the used primers. Standard samples of L. *monocytogenes* ATCC 19111 (serovar 1/2a), CDC F4976 (1/2b), CDC F6254 (1/2c), CDC F4555 (4b), CDC F4076 (L. *grayi*), CLIP 12612 (L. *innocua*) and CDC F4082 (L. *welshimeri*) were included as controls for all PCR reactions, according to species.

To each sample tube were added reaction buffer (1×), 2 mM magnesium chloride solution, dNTPs solution (0.2 mM of each nucleotide), primers (10 pmol/μL for 23S and *hly*; 1 pmol/μL for serotypes), 1 U/Ll of Taq DNA Polymerase and sterile deionized water to complete the final volume of 24 μL. After homogenizing, the reagent mix was poured into 200 μL tubes, and 1 μL of genomic DNA from each sample was added, totaling a final volume of 25 μL. All amplification reactions were performed in a model MG96G thermocycler (Long Gene, China).

The multiplex reaction with 23S and hly primers used a program with 35 cycles consisting of a denaturation step of 95 °C for 1 min, an annealing step of 62 °C for 1 min, and an extension step of 72 °C for 1 min, followed by a final step of 72 °C for 8 min (Hudson et al., 2001). This same cycle was used for multiplex reaction with primers in9, lgr, and lwe7-571 (Tao et al., 2017; Liu et al., 2004). For the molecular serotyping multiplex, 35 cycles were used, consisting of a 94 °C denaturation step for 40 s, an annealing step of 53 °C for 1 min and 15 s, an extension step of 72 °C for 1 min and 15 s, followed by a final step at 72 °C for 7 min (Doumith et al., 2004).

The PCR products were electrophoresed on a 1% agarose gel for 23S, hly, in9, lgr, and lwe7-571 for 50 min at 100 V and 2% for 80 min at 100 V for the serotyping multiplex, with buffer 0.5× TBE run (Tris-base (45 mM), boric acid (45 mM) and EDTA (1 mM), pH 8 - Bio-Rad, USA). In addition, the gels were stained with ethidium bromide solution (500 mL of deionized water +50 μL EtBr 10 μg/μL) for 15 min and visualized in a UV transilluminator coupled to a digital gel imaging system.

All PCR reagents were from Promega (USA). The primers were synthesized by IDT (USA).

2.7. Statistical analysis

Statistical analysis was performed by evaluating the sensitivity, specificity, and accuracy of the identification systems used in this study, calculated according to Greenhalgh (1997). They were determined by comparing the results obtained in the semi-automated system Vitek® 2, and MALDI-TOF/MS with the results obtained by the PCR technique (Doumith et al., 2004) considered the gold standard. The accuracy of a diagnostic test responds to how this test correctly discriminates a species (Borges, 2016). This discriminative ability can be quantified through sensitivity and specificity. According to Borges (2016), sensitivity is the ability of the diagnostic test to detect the genuinely positive, and specificity is the ability to detect true negatives.

3. Results

3.1. Conventional biochemical identification and molecular characterization

All strains negative in the hemolysis test had the same biochemical profile, characteristic of L. *monocytogenes* or L. *innocua,* except for one strain, positive for D-mannitol and identified as L. *grayi*. All strains were a positive for the 23S gene, confirming that all strains belonged to the *Listeria* genus.

Conventional biochemical tests, considered by ISO 11290-1:2017 as a standardized methodology, were combined to the PCR result and designated the "gold standard." Thus, 54 samples positive for the *hly* gene were identified as L. *monocytogenes*. Of the 40 negative samples for the *hly* gene, 31 were identified as L. *innocua*, eight as L. *welshimeri*, and one as L. *grayi*.

PCR for L. *monocytogenes* identified serovar 1/2a as predominant (44 strains), followed by serovar 1/2c and 4b (4 strains) and serovar 1/2b (2 strains). A compilation of the results, for each strain, is presented in Table S2, as supplementary data.

3.2. Proteomic identification and automated biochemical identification of standard strains

VITEK® 2 identified the samples L. *innocua, L. grayi, L. welshimeri,* and L. *seeligeri* with percentages above 90%. However, as for the L. *monocytogenes* species, the equipment was not able to correctly identify the serovars 3b *(L. monocytogenes* 3b CDC F4540)*, 4a (L. monocytogenes*

Table 1
Nucleotide sequence of primers.

Primer	Sequence (5′- 3′)	Size (bp*[1])	Target	Reference
23S rRNA	F: GGGGAACCCACTATCTTTAGTC R: GGGCCTTTCCAGACCGCTTCA	239	*Listeria*	Hudson et al., 2001
hly	F:GCCTGCAAGTCCTAAGACGCCAAC R:CTTGCAACTGCTCTTTAGTAACAC	706	*Listeriolisina O*	Hudson et al., 2001
Lgr	F: GCGGATAAAGGTGTTCGGGTCAA R: ATTTGCTATCGTCCGAGGCTAGG	201	*L. grayi*	Tao et al., 2017
In9	F: GGCTTCAGCGATTCTTCCG R: GCCCGATTTCCTCACTGTCTAA	421	*L. innocua*	Tao et al., 2017
lwe7-571	F: TCCCACCATTGGTGCTACTCA R: TTGGCGTACCAAAGAAATACG	608	*Listeria welshimeri*	Liu et al., 2004
*lmo*0737	F: AGGGCTTCAAGGACTTACCC R: ACGATTTCTGCTTGCCCATTC	691	*Listeria monocytogenes serovars 1/2a, 1/2c,3a, and 3c*	Doumith et al., 2004
lmo1118	F: AGGGGTCTTAAATCCTGGAA R: CGGCTTGTTCGGCATACTTA	906	*L. monocytogenes serovars 1/2c and 3c*	Doumith et al., 2004
ORF2819	F: AGCAAAATGCCAAAACTCGT R: CATCACTAAAGCCTCCCATTG	471	*L. monocytogenes serovars 1/2b, 3b, 4b,4d, and 4e*	Doumith et al., 2004
ORF2110	F: AGTGGACAATTGATTGGTGAA R: CATCCATCCCTTACTTTGGAC	597	*L. monocytogenes serovars 4b, 4d, and 4e*	Doumith et al., 2004

* bp: base pairs. F: forward. R: reverse.

4a ATCC 19114), 4b (*L. monocytogenes* 4b ATCC 7644) e 4d (*L. monocytogenes* 4d ATCC 19117).

Regarding MALDI-TOF/MS, the method correctly identified all serovars of L. *monocytogenes* and the species L. *grayi*. On the other hand, the equipment could not identify any strain of L. *innocua, L. seeligeri*, and L. *welshimeri*, classifying them as *Listeria sp.* (Table 2).

3.3. Automated biochemical identification (VITEK 2 Compact®)

Automated biochemical identification was performed at two different times (Table 3). The equipment could not identify, at the species level, 11 samples in the first analysis and eight samples in the second analysis, classifying them as low discrimination and showing a doubtful result between two species, maintaining the identification of the genus.

3.4. Assessment of phospholipase C production (PI-PLC)

The 94 strains were tested for phospholipase C production and only 54 samples showed positive activity for PI-PLC, requiring up to 72 h for the final result. After this period, 40 samples remained negative for PI-PLC.

3.5. Proteomic identification (MALDI-TOF/MS)

As well as automated biochemical identification, proteomic identification was performed at two different times (Table 4). As a result, some strains were incorrectly identified as L. *monocytogenes* (1st analysis: 16 and 2nd analysis: 21). Others identified only at the genus level (1st analysis: 15 and 2nd analysis: 10), classified as *Listeria sp.* by the

Table 2
Proteomic identification (MALDI-TOF/MS) and automated biochemical identification (VITEK® 2) of standard strains.

Strain	VITEK® 2	MALDI-TOF/MS
L. grayi CDC F4076	*L. grayi 95%*	*Listeria grayi 99.90%*
L. innocua CLIP 12570	*L. innocua 99%*	*Listeria sp. 99.90%*
L. innocua CLIP 12595	*L. innocua 99%*	*Listeria sp. 99.90%*
L. innocua CLIP 12612	*L. innocua 98%*	*Listeria sp. 99.90%*
L. innocua CLIP 12624	*L. innocua 98%*	*Listeria sp. 99.90%*
L. innocua CDC F4078	*L. innocua 98%*	*Listeria sp. 99.90%*
Listeria ivanovii CLIP 7842	*L. ivanovii 98%*	*Listeria sp. 99.90%*
Listeria monocytogenes 1/2a CDC F4561	*L. monocytogenes 96%*	*L. monocytogenes 84.20%*
L. monocytogenes 1/2a ATCC 15313	*L. monocytogenes 99%*	*Listeria sp. 99.90%*
L. monocytogenes 1/2a 10403S	*L. monocytogenes 99%*	*L. monocytogenes 90%*
L. monocytogenes 1/2b ATCC BAA-751	*L. monocytogenes 91%*	*L. monocytogenes 99.90%*
L. monocytogenes 3b CDC F4540	*L innocua 49%/L. monocytogenes 51%*	*L. monocytogenes 91.30%*
L. monocytogenes 3c CDC F6238	*L. monocytogenes 94%*	*L. monocytogenes 82.80%*
L. monocytogenes 4a ATCC 19114	*L innocua 49%/L. monocytogenes 51%*	*L. monocytogenes 90%*
L. monocytogenes 4ab CDC F1067	*L. monocytogenes 99%*	*L. monocytogenes 93.60%*
L. monocytogenes 4b ATCC 7644	*L innocua 50%/L. monocytogenes 50%*	*L. monocytogenes 86.40%*
L. monocytogenes 4b Scott A	*L. monocytogenes 99%*	*L. monocytogenes 93.60%*
L. monocytogenes 4c ATCC 19116	*L. monocytogenes 99%*	*L. monocytogenes 93.60%*
L. monocytogenes 4d ATCC 19117	*L innocua 49%/L. monocytogenes 51%*	*L. monocytogenes 97.20%*
L. monocytogenes 4e ATCC 19118	*L. monocytogenes 88%*	*L. monocytogenes 90%*
Listeria seeligeri CLIP 9529	*L. seeligeri 98%*	*Listeria sp. 99.90%*
Listeria welshimeri CDC F4082	*L. welshimeri 95%*	*Listeria sp. 99.90%*

Table 3
Identification of strains by VITEK® 2.[*1], [*2]

Species (identified by PCR)	Number of strains	Identification by VITEK® 2		
		Correct	Only *Listeria sp.*[*2]	Incorrect
1st test[*1]				
Listeria monocytogenes	54	8	4	42
L. innocua	31	23	7	1
Listeria welshimeri	8	6	0	2
L. grayi	1	1	0	0
Total	94	38	11	45
2nd test[*1]				
L. monocytogenes	54	10	3	41
L. innocua	31	26	5	0
L. welshimeri	8	8	0	0
L. grayi	1	1	0	0
Total	94	45	8	41

[*1] Two independent analyses were performed.
[*2] Number of strains that the equipment identified only at the genus level.

equipment.

3.6. Assessment of sensitivity, specificity, and accuracy of identification systems

The results obtained in the PCR were considered the gold standard in this evaluation, and the results of sensitivity, specificity, and accuracy (Greenhalgh, 1997) of the tests are presented in Table 5.

4. Discussion

Although L. *innocua* is not pathogenic to humans, Perrin et al. (2003) reported the first case of bacteremia involving the species and defined as a risk the distinction between L. *monocytogenes* and L. *innocua* based on hemolytic activity. This study's hemolytic profile of some strains became evident, probably due to the cryopreservation period (Alves et al., 2020). Thus, 35 strains were identified as L. *monocytogenes*, and the other 59 remained without hemolysin expression. The existence of atypical strains indicates that traditional phenotypic and genotypic methods should be used with caution. More studies and methodologies are needed to correctly identify the *Listeria* genus (Moreno et al., 2014).

Automated biochemical identification by VITEK® 2 Compact did not correctly identify L. *monocytogenes* samples. This fact can be explained by the slow phospholipase C activity. The equipment performs the reading within 24 h, and some samples took up to 72 h for phospholipase expression. All L. *monocytogenes* samples (*hly* positives) that showed late phospholipase activity in ALOA Agar were mistakenly identified as L. *innocua* by the equipment. In the two analyses performed by VITEK® 2, only the identification of the species L. *grayi* was maintained and considered correct compared to the gold standard. It is believed that the difference found in the characterization of the species in the two analyses, once again, is due to the reading time of the equipment and the fact that the strains have different metabolisms.

The strains in this study were considered atypical for not expressing hemolysin, the main virulence factor of the *Listeria* genus. The *hly* gene was detected through PCR and, for this reason, the samples were identified as L. *monocytogenes*. Rosimin et al. (2016), in studies carried out with food, described atypical strains of L. *innocua* for presenting virulence genes, agreeing with the hypothesis of Moreno et al. (2014), who cites the presence of the virulence gene in L. *innocua* as a stage in the evolution of a common ancestor of L. *monocytogenes*. In agreement with our results, De Lappe et al. (2014) cite VITEK® 2 as a limiting methodology for identifying the genus *Listeria*, as the method erroneously identified clinical and food samples of L. *monocytogenes* as L. *innocua*. The authors emphasize the negative phospholipase test for these samples.

Table 4

Identification of strains by MALDI-TOF/MS.*1, *2, *3

Species (identified by PCR)	Number of strains	Identification by MALDI-TOF/MS			
		Correct	Only *Listeria sp.*[*2]	Incorrect	No identification[*3]
1st test[*1]					
Listeria monocytogenes	54	40	14	0	0
L. innocua	31	0	15	16	0
Listeria welshimeri	8	0	4	0	4
L. grayi	1	1	0	0	0
Total	94	41	33	16	4
2nd test[*1]					
L. monocytogenes	54	39	15	0	0
L. innocua	31	0	10	21	0
L. welshimeri	8	2	6	0	0
L. grayi	1	1	0	0	0
Total	94	42	31	21	0

[*1] Two independent analyses were performed.

[*2] Number of strains that the equipment identified only at the genus level (could not distinguish between two or more *Listeria* species).

[*3] Few strains could not be identified even at the genus level, returning the "no identification" result.

Table 5

Sensitivity, specificity and accuracy of the analyses performed by VITEK® 2 and MALDI-TOF/MS, according to species.

Species (identified by PCR)	Parameter (%)	VITEK® 2[*1]		MALDI-TOF/MS[*1]	
		1st test	2nd test	1st test	2nd test
Listeria monocytogenes (n = 54)	Sensitivity	14.8	18.5	24.1	59.3
	Specificity	100.0	97.5	85.0	70.0
	Accuracy	51.1	52.1	50.0	63.8
L. innocua (n = 31)	Sensitivity	74.2	83.9	0.0	0.0
	Specificity	30.2	34.9	100.0	100.0
	Accuracy	44.7	51.1	67.0	67.0
Listeria welshimeri (n = 8)	Sensitivity	75.0	100.0	0.0	12.5
	Specificity	98.8	100.0	100.0	100.0
	Accuracy	96.8	100.0	91.5	92.6
L. grayi (n = 1)	Sensitivity	100.0	100.0	100.0	100.0
	Specificity	100.0	100.0	100.0	100.0
	Accuracy	100.0	100.0	100.0	100.0

[*1] Two independent analyses were performed.

The MALDI-TOF/MS was assertive in identifying 40 strains of L. *monocytogenes* in the first analysis and 39 in the second analysis. However, it was not able to identify any strain of L. *innocua*. This fact is probably due to only one reference spectrum of the species in the equipment's database (Rahi et al., 2016). In a previous study, using another MALDITOF/MS equipment (Brunker, USA), we noticed that the inclusion of more spectra in the database improved the precision of the equipment (Araújo et al., 2020). Unfortunately, the equipment used in this study does not allow editing of its database by the user. The only species that maintained the exact identification in both analyzes was L. *grayi*. It is noteworthy that the same behavior was also observed in the Vitek® 2 identification. Guo et al. (2014) obtained better performance in the MALDI-TOF/MS technique when compared to Vitek® 2 in identifying bacteria of clinical origin. The authors described the method as fast and inexpensive and classified it as having the potential to replace conventional phenotypic methods in identifying common bacterial isolates in clinical microbiology laboratories.

Febbraro et al. (2016) obtained better results with Vitek® 2 when compared to MALDI-TOF/MS in identifying bacteria of clinical origin. The authors highlighted favorable results only for Gram-negative strains and suggested inserting reference spectra in the MALDI-TOF/MS database for Gram-positive strains. In agreement with these results, Silva et al. (2017) analyzed Gram-positive samples of clinical origin. They obtained satisfactory results by VITEK® 2, while MALDI-TOF/MS could not identify all strains. Comparing the VITEK® 2 and MALDI-TOF/MS methodologies in identifying anaerobic microorganisms of clinical origin, Tsukimoto and Rossi (2018) described both methodologies with excellent performance in identifying isolates. The authors highlighted significant differences in terms of cost-effectiveness. MALDI-TOF/MS allowed significant savings beyond result release five days before VITEK® 2, contributing to successful clinical resolution. On the other hand, the spectrometric technique requires highly qualified labor and the investment cost to obtain the equipment is much higher.

In this study, MALDI-TOF/MS showed 100% specificity for L. *innocua*. However, the method had low sensitivity, representing false-negative results and causing harm to public health when misidentifying a pathogen. On the other hand, VITEK® 2 showed high sensitivity in identifying this species. Regarding L. *monocytogenes*, Vitek® 2 showed low sensitivity, with false-negative results for the pathogen.MALDI-TOF/MS showed greater sensitivity to the species but showed repeatability problems, probably because the method does not have a standard inoculum. The low accuracy demonstrated by the methods is because when the test sensitivity was high for a species, the specificity was down and *vice versa*. It is essential to emphasize the low number of strains used in this study in interpreting the statistical results.

Despite the successful results reported using MALDI-TOF/MS and the wide range of scenarios in which the equipment can be used, more studies are needed to standardize the applied procedures and confirm the reproducibility of the results (Rodríguez-Sánchez et al., 2019).

5. Conclusion

VITEK® 2 presented as one of the limiting factors in identifying only 6 species of the genus *Listeria*, as currently, the genus comprises 19 species and 4 subspecies. In addition, the equipment reads the biochemical and enzymatic tests within an interval of 8 to 24 h, depending on the card used, while the atypical strains present a late metabolism for some phenotypic characteristics evaluated. MALDI-TOF/MS limited database hinder its ability to correctly identify atypical *Listeria* strains. The addition of spectra from local sources could improve its performance. As a future perspective, it becomes interesting to evaluate the effectiveness of other popular systems, such as the Biolog OmniLog® or BD Phoenix®. *Listeria sp.*, especially atypical ones, in the food industry is a significant concern. It requires rigorous detection and identification methods to prevent the transmission of listeriosis through food.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2022.106434.

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