Veterinarski Glasnik 2026, 00, 1-13
UDC:
https://doi.org/10.2298/VETGL251110005V

Short Communication

THE ABILITY OF MALDI-TOF MS TO IDENTIFY *SALMONELLA* ISOLATED FROM FOOD

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Received 10 November 2025; Accepted 12 March 2026
Published online: 16 April 2026

How to cite: Suzana Vidaković Knežević, Jelena Vranešević, Slobodan Knežević, Jasna Kureljušić, Sunčica Kocić-Tanackov, Dubravka Milanov. The ability of MALDI-TOF MS to identify *Salmonella* isolated from food. Veterinarski Glasnik, 2026. 00: 1-13. https://doi.org/10.2298/VETGL251110005V

Abstract

Salmonella is among the leading foodborne pathogens in the European Union, with *Salmonella* Enteritidis and *Salmonella* Typhimurium being the most frequently reported serovars. Traditional methods for *Salmonella* identification and serotyping, such as ISO standards, are time-consuming and labor-intensive. MALDI-TOF MS has emerged as a rapid and reliable tool for bacterial identification. This study evaluated the ability of MALDI-TOF MS in identifying *Salmonella* isolates collected from various stages of chicken and pork meat production. Eight pathogenic isolates (four *S*. Enteritidis and four *S*. Typhimurium) were analyzed using both direct colony transfer and protein extraction methods. Results showed that the direct transfer method yielded low-confidence or no identification for four isolates. However, all isolates were successfully identified with high-confidence scores (> 2.00) after protein extraction. Despite the good scores, all isolates were identified only at the genus level (*Salmonella* sp.), consistent with current limitations of the MALDI Biotyper database. Nevertheless, best database matches after protein extraction indicated potential for more detailed classification. This study confirms that MALDI-TOF MS, particularly when combined with protein extraction, is a valuable method for rapid screening and identification of *Salmonella* sp. in

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food production chain. However, its inability to perform accurate serotyping highlights the need for improved databases and computational approaches. Future integration of machine learning and expanded reference spectra may enhance the serotype-level resolution of MALDI-TOF MS.

Key Words: MALDI-TOF MS, protein extraction, *Salmonella* Enteritidis, *Salmonella* Typhimurium

INTRODUCTION

The trend of *Salmonella* infections in humans indicates a period of stability from 2019 to 2023 in the European Union. The five frequently reported serovars are *S*. Enteritidis (70.8%), *S*. Typhimurium (8.9%), monophasic *S*. Typhimurium (5.1%), *S*. Infantis (2.0%) and *S*. Coeln (0.77%) (EFSA and ECDC, 2024). Humans primarily acquire *Salmonella* infections via the fecal-oral route or through the consumption of contaminated foods (Mkangara, 2023). Contamination of foods can also result from the persistence of *Salmonella* on machinery and surfaces that come into contact with food (Vidaković Knežević et al., 2024).

The ISO 6579 standard is recognized as the reference method for the *Salmonella* identification (ISO, 2017). For epidemiological investigations, isolation and serotyping of *Salmonella* isolates serve as the initial steps in outbreaks studies, but they are often insufficient on their own, as further discrimination using molecular methods is usually required. Serotyping of *Salmonella* can also be labor-intensive and time-consuming, as it requires a wide range of specific antisera. Additionally, factors including observer error, unintended agglutination, auto-agglutination, and antigenic loss can contribute to classification inaccuracies (Persad et al., 2022).

Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) is based on the generation of profiles of the spectrum of proteomes, i.e., small proteins and peptides that mainly originate from ribosomes. Ribosomal proteins, in the range of molecular masses between 2 and 20 kDa, ionize well and give accurate spectra (Sparbier et al., 2012; Wieser et al., 2012; Pavlović et al., 2013). In addition to this, a smaller part of the peaks originates from structural proteins (Ryzhov and Fenselau, 2001). The recorded spectra are characteristic and form a fingerprint of the genus, species or strain under investigation (Böhme et al., 2016). Based on the similarity of the test spectrum and the database, the software expresses the result as a numerical value, i.e., the reliability coefficient. The numerical score indicates how reliable the identification is. Besides this, the software presents supplementary results together with the best match to facilitate validation (Wieser et al., 2012). A previous study demonstrated that the Bruker MALDI Biotyper system enables reliable identification of *Salmonella* isolates at the genus level, while its performance at the serotype level was limited (Bastin et al., 2019). Although these limitations of commercial MALDI Biotyper databases have been reported, data in the performance of this method for *Salmonella* isolates circulating in Serbia remain scarce.

Therefore, the aim of this study was to evaluate the capability of MALDI-TOF MS for the identification of *Salmonella* species and clinically relevant serotypes, particularly Enteritidis and Typhimurium.

MATERIALS AND METHODS

Salmonella Isolates

Pathogenic isolates of *S*. Enteritidis and *S*. Typhimurium, previously identified by PCR (data not shown), were collected from various stages of chicken and pork meat production. The collection comprised four isolates of *S*. Enteritidis (SE53, SE56, SE132 and SE144) and four isolates of *S*. Typhimurium (ST28, ST35, ST48 and ST49). Prior to MALDI-TOF MS analysis, all isolates of *Salmonella* were inoculated on Xylose Lysine Deoxycholate (XLD) (CM0469, Oxoid, Basingstoke, UK) using the streak plate method to obtain individual colonies, and incubated at 37 °C for 24 h.

Confirmation of *Salmonella* Isolates by MALDI-TOF MS

MALDI-TOF MS was performed using a Microflex BioTyper spectrometer (Bruker Daltonics, Germany) equipped with a nitrogen laser under the control of the operating system Flexcontrol ver. 3.4 (Bruker Daltonics, Germany) on all *Salmonella* isolates using two methods, i.e., direct colony transfer to the plate and protein extraction.

The direct transfer of colonies onto the MALDI-TOF MS target plate was performed as follows: using a sterile applicator stick, bacteria cultures were smeared as thin films onto polished steel plate with 96 spots (Bruker Daltonics GmbH, Leipzig, Germany). The applied bacteria were left at room temperature for one minute to air-dry. Then, 1 μL of matrix solution composed of saturated α-cyano-4-hydroxycinnamic acid (Bruker Daltonics, Germany) dissolved in 50% acetonitrile (Sigma-Aldrich, Germany) and 2.5% trifluoroacetic acid (Sigma-Aldrich, Germany) was added to each dried spot. The prepared plate was left for 10 minutes at room temperature to achieve co-crystallization of the bacteria sample and the matrix. After this, the plate was subjected to MALDI-TOF MS analysis.

For protein extraction, bacterial cultures were suspended in 300 μL of distilled water to achieve a turbidity equivalent to 2 McFarland standards. Subsequently, 900 μL of ethanol was added. The prepared suspension was then vortexed thoroughly and centrifuged at 20,000 × g for 2 minutes. The supernatant was discarded, and the resulting pellet was dried at 55 °C for 30 minutes. Following drying, 50 μL of formic acid was added and mixed well with a pipette. An additional 50 μL of acetonitrile was then added, and the sample was centrifuged again at 20,000 × g for 2 minutes. The supernatant in the amount of 1 μL was transferred to the MALDI-TOF MS target plate and left for 10 minutes at room temperature to dry. A matrix solution (1.5 μL), prepared as described in the direct transfer protocol, was then added to the dried spot.

In both cases, spectra were acquired in the mass range of 2-20 kDa using Auto Execute mode, accumulating 240 laser pulses (laser frequency: 60 Hz; first ion source voltage: 19.9 kV; second ion source voltage: 18.53 kV; lens voltage: 6 kV), with laser power set between 30% and 40% of maximum. The obtained spectra were then compared to the database of the integrated software that shows the identification and logarithmic value of the results between 0.00 and 3.00. The scores above 2.0 indicate high-confidence identification at the species level (+++), scores between 1.70 and 1.99 indicate identification at the genus level (++), and scores below 1.69 are considered insufficient for identification (+) (Anderson et al., 2012; Pavlović et al., 2013).

RESULTS AND DISCUSSION

Table 1 shows the best values of the results obtained by direct transfer of colonies to the plate and the application of protein extraction in the preparation of *Salmonella* isolates for MALDI-TOF MS.

Table 1. Results of MALDI-TOF MS analysis of *Salmonella* isolates by two different methods

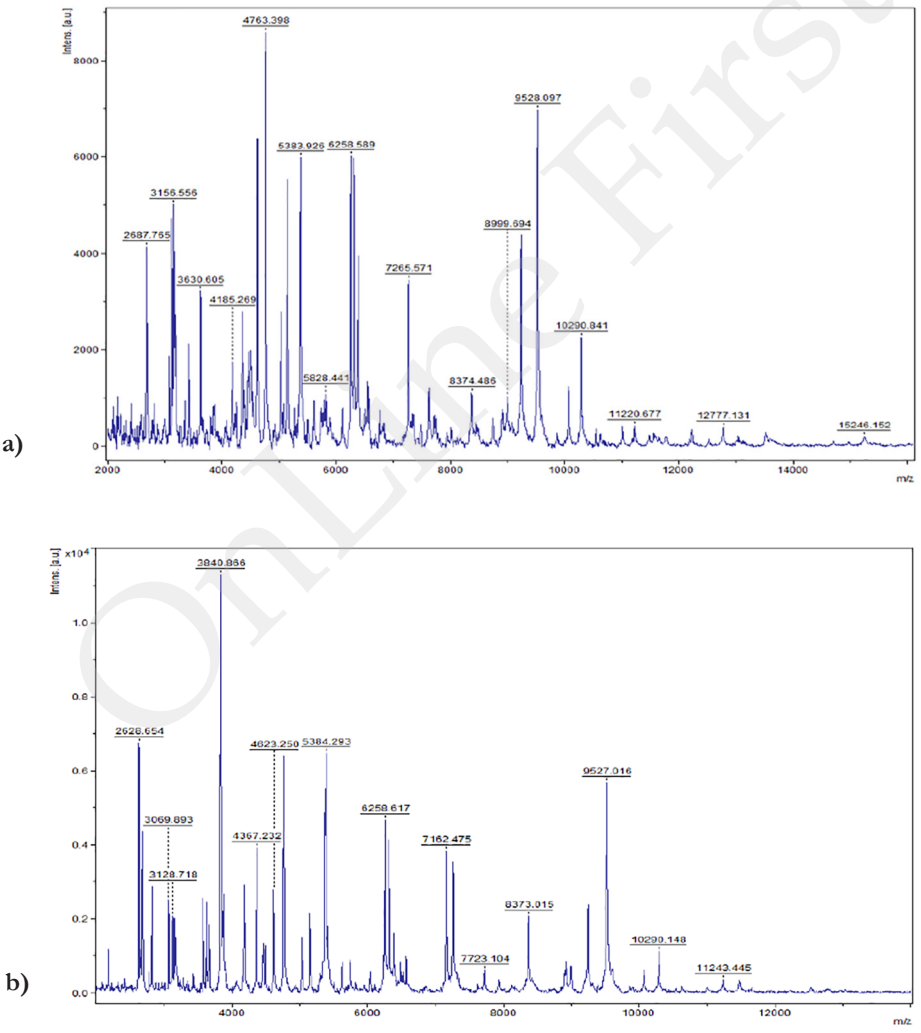
	Direct transfer method		Protein extraction method	
Isolates	**Organism (best match)**	**Score Value**	**Organism (best match)**	**Score Value**
SE53	*Salmonella* sp.	1.86	*Salmonella* sp.	2.18
SE56	No peaks found	0.00	*Salmonella* sp.	2.30
SE132	*Salmonella* sp.	2.15	*Salmonella* sp.	2.43
SE144	*Salmonella* sp.	2.19	*Salmonella* sp.	2.19
ST28	*Salmonella* sp.	1.99	*Salmonella* sp.	2.47
ST35	No Organism Identification Possible	1.43	*Salmonella* sp.	2.24
ST48	*Salmonella* sp.	2.32	*Salmonella* sp.	2.49
ST49	*Salmonella* sp.	2.22	*Salmonella* sp.	2.49

Using the direct colony transfer method, two tested isolates could not be confirmed as *Salmonella* sp., specifically one isolate of *S*. Enteritidis (SE56) and one of *S*. Typhimurium (ST35). All remaining isolates of both *S*. Enteritidis and *S*. Typhimurium were confirmed as *Salmonella* sp. Two isolates, SE53 (*S*. Enteritidis) and ST28 (*S*. Typhimurium) were confirmed with a low level of confidence (++), while the remaining isolates, on each of *S*. Enteritidis and *S*. Typhimurium, were confirmed with a high level of confidence (+++), with a score value > 2.15.

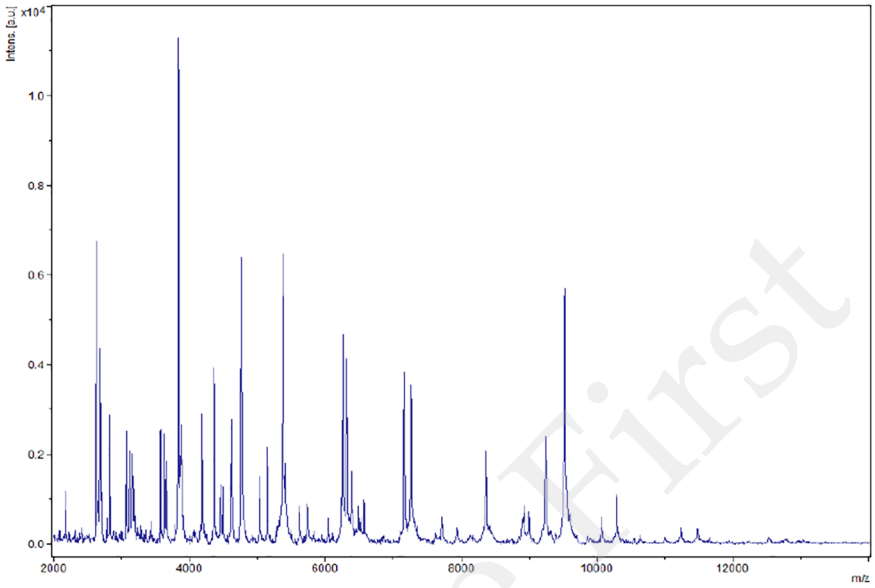
Protein extraction of bacterial cells produced better result scores than did direct colony transfer for all tested isolates. The MALDI-TOF MS spectra after protein extraction are shown in Figures 1 and 2 for *S*. Enteritidis and *S*. Typhimurium, respectively. Out of the two isolates that could not be identified by direct colony transfer, both (SE56 and ST35) had scores > 2.00, with a high level of confidence (+++). However, all

tested isolates of *S*. Enteritidis and *S*. Typhimurium were identified only to the genus level, as *Salmonella* sp., although the result scores were high (> 2.18). These results were expected, given that the MALDI Biotyper gives a warning that *Salmonella* can only be identified to the genus level. Despite this, the best matches of tested isolates with isolations from the database are also shown in the results. After protein extraction, *S*. Choleraesuis was the best match for four isolates (SE53, SE56, SE144 and ST35), *S*. Anatum for three isolates (SE132, ST28 and ST49), and *S*. Typhimurium for one isolate (ST48).

Figure 1. The MALDI-TOF mass spectra of *S*. Enteritidis isolates after protein extraction. (a) SE53, (b) SE56, (c) SE132, and (d) SE144.



c)



d)

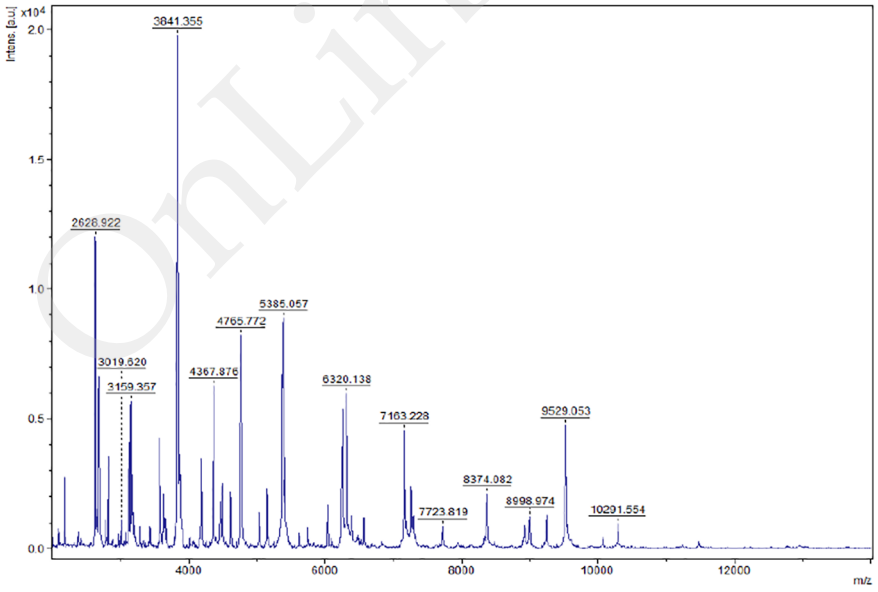
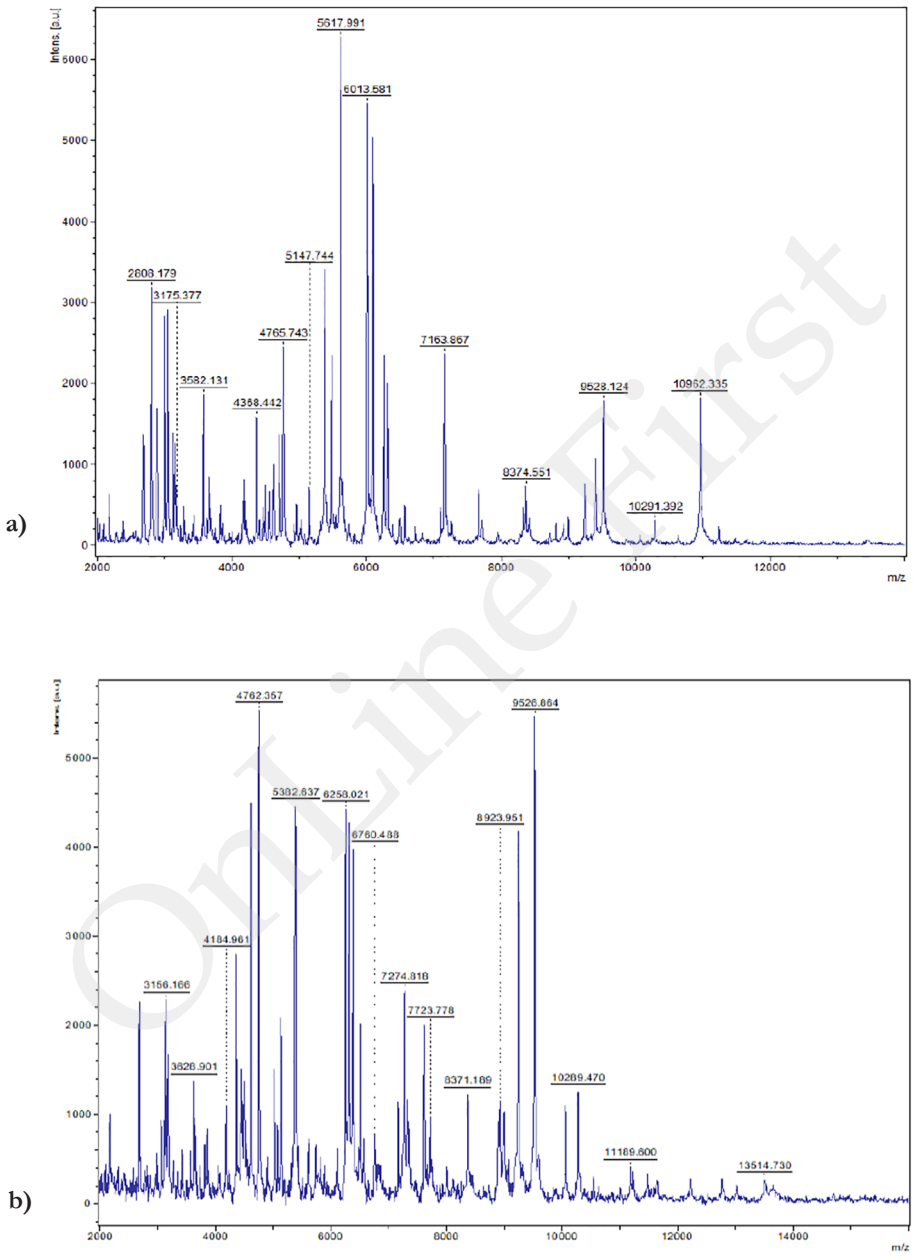
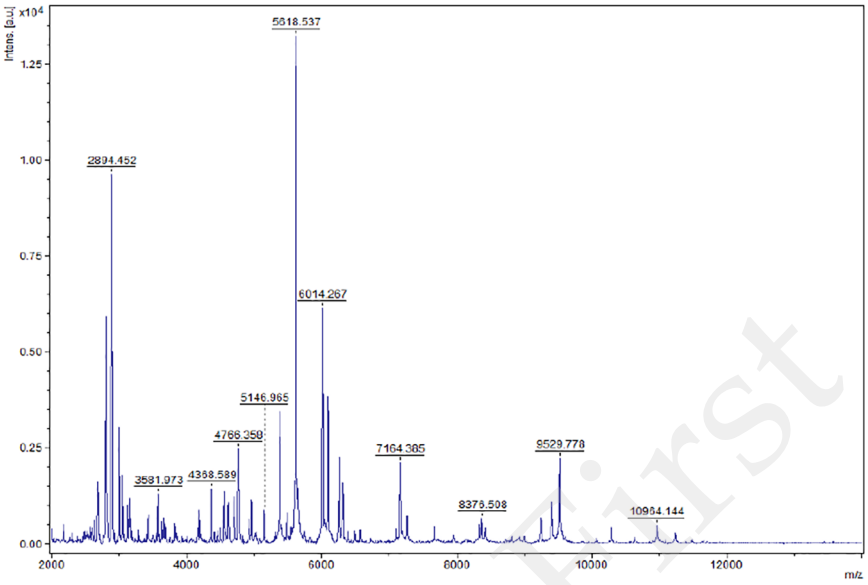


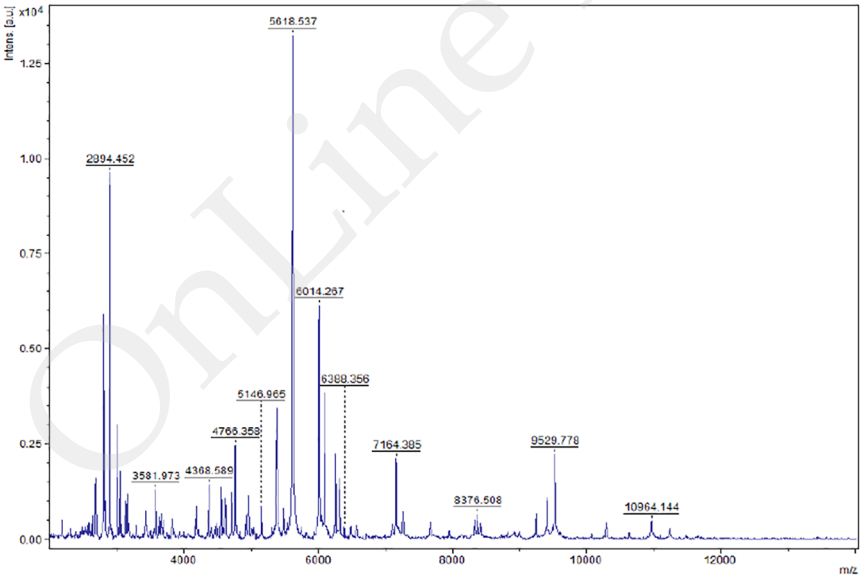
Figure 2. The MALDI-TOF mass spectra of *S*. Typhimurium isolates after protein extraction. (a) ST28, (b) ST35, (c) ST48, and (d) ST49.



c)



d)



Alatoom et al. (2011) identified 95% of isolates to genus level and 69% of isolates to species level using protein extraction, in contrast to direct colony transfer when they identified 56% of isolates to genus level and 20% to species level.

Extraction involves lysing bacterial cells using chemicals or enzymes in order to release proteins (Alatoom et al., 2011). This method is used for bacteria with a more complex cell wall, where applied formal acid lyses the cell, and acetonitrile affects protein

precipitation (Anderson et al., 2012). Preparing samples in this way requires additional time, reagents and equipment. In this study, two isolates (SE56 and ST35) required additional preparation, which was essential for their identification.

The use of MALDI-TOF MS has become revolutionary in the detection and identification of microorganisms (Elbehiry et al., 2017). The Bruker Biotyper database for MALDI-TOF MS was initially developed with a focus on clinical diagnostics. However, in recent years it has expanded to include foodborne pathogenic bacteria (Wenning et al., 2014). The obtained results, as well as literature data, show that MALDI-TOF MS is a reliable method for the identification of pathogenic foodborne bacteria, such as *Salmonella* spp. (Mazzeo et al., 2006; Elbehiry et al., 2017; Jadhav et al., 2018; Yan et al., 2020). However, the use of MALDI-TOF MS in the routine identification of foodborne bacteria depends on many factors. The advantages of MALDI-TOF MS are accuracy, speed, simplicity, sensitivity and reproducibility. The speed of this method is of key importance for the quality and safety of food, especially in situations of food poisoning, when rapid detection and identification are extremely important for human health. Depending on the quality and purity of the sample, as well as the volume of the database, the identification of isolates can be completed in a few minutes (Elbehiry et al., 2017). The small amount of affordably-priced reagents is another advantage of this method. However, the major disadvantages of MALDI-TOF MS are the price of the instrument (Pavlović et al., 2013), as well as the inability to identify *Salmonella* at the serotype level (Kang et al., 2017), which was also observed in this research, due to the use of characteristic prints, based on ribosomal proteins and not on specific biomarkers (Dieckmann et al., 2008). Amino acid polymorphisms, potentially useful for serotyping, are often found in classes of proteins other than ribosomal, with unknown functions. Dieckmann and Malorny (2011) reported that the mass peak at m/z (mass-to-charge ratio) 6,036 is unique for the identification of the *S*. Enteritidis serotype, while the mass peak at m/z 7,097 for the uncharacterized YaiA protein is unique for the identification of the *S*. Typhimurium serotype. This approach was also applied in the study by Al-Hindi et al. (2023), which focused on MALDI-TOF MS based identification of *Salmonella* isolated from retail chilled chicken in Saudi Arabia.

Although, commercially available databases and analysis packages that can successfully identify *Salmonella* bacteria at the serotype level are limited (Mangmee et al., 2020), recent studies showed that MALDI-TOF were capable of subtyping *Salmonella*. Persad et al. (2022) constructed reference spectra and combined them into a new reference library while Ren et al. (2025) used machine learning models for *Salmonella* serotype identification analysis, including *S*. Enteritidis and *S*. Typhimurium serotypes. Therefore, modern artificial intelligence technology could be a powerful computational tool that can enhance MALDI-TOF MS-based serotyping analysis of *Salmonella*. In contrast to these approaches, the present study evaluates the performance of the standard commercial MALDI Biotyper database using *Salmonella* isolates circulating in Serbia under routine laboratory conditions. This provides region-specific data on the

applicability and limitations of MALDI-TOF MS for the most prevalent serotypes in the region.

CONCLUSION

In conclusion, MALDI-TOF MS offers a fast, reliable, and affordable approach for detecting foodborne pathogens like *Salmonella*. However, its ability to differentiate *Salmonella* isolates at the serotype level remains limited. In this study, several isolates could not be identified using the direct transfer method but were successfully identified with high confidence following protein extraction. Still, all isolates were ultimately classified to the genus level, which is consistent with the current MALDI-TOF MS database limitations. A further limitation of this study is the relatively small number of isolates analyzed (four *S*. Enteritidis and four *S*. Typhimurium), which restricts the generalizability of the findings. Recent advances, including enhanced spectral libraries and the integration of artificial intelligence, show promising potential for improving serotype-level identification of *Salmonella*.

Acknowledgements

This research was funded by Ministry of Science, Technological Development and Innovation of Republic of Serbia by the Contract of implementation and funding of research work of NIV-NS in 2025, Contract No: 451-03-136/2025-03/200031.

Authors' contributions

Conceptualization: S.V.K.; data acquisition and analysis: S.K.; writing–original draft preparation: S.V.K.; writing–review and editing: J.V., J.K.; supervision: S.K.T.; providing technical and material support: D.M.

Competing interests

The authors declare that they have no competing interests.

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SPOSOBNOST MALDI-TOF MS METODE U IDENTIFIKACIJI VRSTA *SALMONELLA* IZ HRANE

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Kratak sadržaj

Salmonella i dalje predstavlja jedan od najčešćih uzročnika trovanja hranom u Evropskoj uniji, pri čemu su *Salmonella* Enteritidis i *Salmonella* Typhimurium najčešće prijavljeni serotipovi. Tradicionalne metode za identifikaciju *Salmonellae*, poput ISO standarda i serotipizacije, zahtevaju mnogo vremena i rada. Nasuprot tome, MALDI-TOF MS se pokazao kao brza i pouzdana metoda za identifikaciju bakterija. Ova studija ispitivala je mogućnost primene MALDI-TOF MS metode u identifikaciji izolata *Salmonella* prikupljenih iz različitih faza proizvodnje pilećeg i svinjskog mesa. Analizirano je osam patogenih izolata (četiri *S*. Enteritidis i četiri *S*. Typhimurium) korišćenjem dve metode: direktnog prenosa kolonija i ekstrakcije proteina. Rezultati su pokazali da metoda direktnog prenosa kolonije daje identifikaciju sa niskim stepenom pouzdanosti ili bez identifikacije kod četiri izolata. S druge strane, svi izolati su nakon ekstrakcije proteina uspešno identifikovani sa visokim stepenom poudanosti (ocena > 2,00). Iako su rezultati bili poboljšani, svi izolati su identifikovani samo na nivou roda (*Salmonella* spp.), što je u skladu sa postojećim ograničenjima MALDI Biotyper baze podataka. Ipak, najbolja poklapanja, nakon ekstrakcije proteina, ukuzuju na potencijal za detaljniju klasifikaciju. Ova studija potvrđuje da je MALDI-TOF MS, naročito u kombinaciji sa ekstrakcijom proteina, dragocena metoda za brzo otkrivanje i identifikaciju *Salmonella* spp. u lancu proizvodnje hrane, Međutim, njena trenutna nemogućnost da precizno razlikuje serotipove ističe potrebu za unapređenjem baza podataka i razvojem naprednijih računarskih pristupa. Buduća integracija mašinskog učenja i proširenih referentnih spektara mogla bi značajno poboljšati sposobnost MALDI-TOF MS metode da razlikuje serotipove na višem nivou tačnosti.

Ključne reči: MALDI-TOF MS, ekstrakcija proteina, *Salmonella* Enteritidis, *Salmonella* Typhimurium