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Rapid identification of bacteria from agricultural environment using MALDI-TOF MS

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ABSTRACT

In this study, matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS) was used to identify bacteria from environmental matrices. The aim of this work was to determine the efficacy of this rapid technique and the bacterial community of agricultural samples. Environmental samples included the collection of irrigation waters and manures, and bacteria from the surface of vegetables were also investigated. From food safety point of view, the investigation of these microbial communities is inevitable considering their potential hazardous impact on the food production chain. Altogether 235 bacterial isolates were identified with the most frequent genera being *Pseudomonas*, *Bacillus*, *Acinetobacter* and *Aeromonas*. Our results indicated that MALDI-TOF MS can be used to identify causative agents of foodborne illnesses, food spoilage and common plant pathogens. However, limitations of the rapid identification technique were also encountered as we obtained correct identification at species level for 30.2% and at genus level for 69.8% of the isolates.

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1. INTRODUCTION

Consumption of raw vegetables and fruits has been responsible for various foodborne illnesses worldwide in the past decades (Kampmeier et al., 2018; Turner et al., 2019). Since many pathogenic bacteria (*Listeria monocytogenes,* verotoxigenic *Escherichia coli, Salmonella* spp., *E. coli* O157:H7) are able to survive and grow in low nutrient environment (Falardeau et al., 2017), the sources of the microbial hazards could be traced back to irrigation water as well. Therefore, to ensure food safety from farm to fork, the food production chain involving fresh produce should be monitored. Accurate and fast identification of the most common foodborne bacterial pathogens (*Campylobacter* spp., *L. monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Clostridium perfringens*, *E. coli* and other shiga toxin-producing *E. coli* strains (STEC) (Scallan et al., 2011)) could prevent the growing number of foodborne outbreaks.

Traditional microbiological methods (morphological, physiological, and biochemical tests) and/or staining (e.g. Gram-staining), or molecular methods (e.g. 16S rRNA gene sequence analysis) with the need of trained laboratory personnel, high costs, and being time-consuming, are inadequate for rapid bacterial identification (Böhme et al., 2011; Alnakip et al., 2020). In recent years, MALDI-TOF MS (matrix assisted laser desorption ionisation-time of flight mass spectrometry) has become a popular technique in microbiological identification. The application of MALDI-TOF MS in food microbiology offers a faster, less expensive, and labour-saving technique compared to the above mentioned methods (Böhme et al., 2011; Strejcek et al., 2018). Identification via MALDI-TOF MS is carried out by comparing the PMF (Protein Mass Fingerprint) of the tested microbe to the data of PMF databases, or by pairing the masses of the identified biomarkers of unknown organisms using proteomic databases. Two of the largest systems based on mass spectral microbial identification are BioTyper® (Bruker Daltonics GmbH & Co, Bremen, Germany) and VITEK® MS Plus (bioMérieux, Marcy l'Etoile, France) (Singhal et al., 2015).

MALDI-TOF MS is mostly used for the identification of clinically relevant pathogens. It has been reported that this system can effectively identify bacteria even at species level from clinical samples (van Veen et al., 2010; Ponderand et al., 2020; Chung et al., 2021). However, it is increasingly utilised to detect foodborne pathogens in the complex food chain as well (Böhme et al., 2011; de Koster and Brul, 2016; Horváth et al., 2020). Still, the application of MALDI-TOF MS to characterise the microbial composition of environmental samples such as from waste disposal sites (Kopcakova et al., 2014), soil samples (Strejcek et al., 2018), or from mining samples (Avanzi et al., 2017), seemed to be less competent compared to the results of clinical studies.

Therefore, the aims of our study were to describe the performance and limitation of MALDI-TOF MS using different types of agricultural samples and to characterise the bacterial communities of the environment.

2. MATERIALS AND METHODS

2.1. Bacterial isolation

In this study, irrigation water samples were collected and examined from different regions of Hungary. Sampling sites were chosen due to their utilisation as irrigation water. Sampling was

also done in different regions, where irrigation water contamination (e.g. by manure) could have occurred or from irrigated crops (e.g. corn, lettuce, onion, sorrel, spinach, and tomato), where irrigation water might have transmitted the microbes to them. Two groups were formed from these crops in accordance with their origins as Vegetables1 and Vegetables2. Group of Vegetables1 includes onion, corn, lettuce, spinach, while group of Vegetables2 contains tomato, spinach, and sorrel. Manure samples originated from different swine farms located in Hungary. Manure2 and Manure3 were from the same sample spot (Bátya, Southern Hungary), however, Manure2 was liquid sample. Three samples from wells in different towns are marked as Irrigation water samples. Details of samples and names of them as referred later are shown in Table 1.

Bacterial isolation was performed after preparing a ten-fold serial dilution in buffered peptone water (BPW) (Thermo Fisher Scientific Inc., Oxoid Ltd., Basingstoke, UK) up to dilution 10^{-3}. The isolates were plated on Trypticase Soy Agar (TSA, Biokar Diagnostics, Allonne, France) plates. Agar plates were incubated at 30 °C to grow overnight cultures. Extended direct transfer procedure was used to identify the isolates as outlined in the User Manual provided by the manufacturer (Bruker Daltonics GmbH & Co, Bremen, Germany). The identification process was done using MALDI Biotyper (Bruker Daltonics GmbH & Co, Bremen, Germany). Furthermore, beside MALDI-TOF MS identification, catalase and oxidase activities of the isolates were in concordance with the MALDI-TOF MS results.

Table 1. Origin of samples and bacterial isolates. All analysed samples were collected from the area of Hungary (HU)

Types	Sample name	Origin of samples*	Location (city, region)	Isolates	
				Gram-positive	Gram-negative
Still water	Lake1	Kavicsos-tó	Szigetszentmiklós (Central HU)	1	5
	Lake2	Szelidi-tó	Dunapataj (Central HU)	1	10
Running water	River1	Tisza	Tiszakécske (Eastern HU)	5	14
	River2	Tisza	Szolnok (Eastern HU)	0	13
	River3	Danube	Csepel (Central HU)	8	23
	River4	Danube	Kalocsa (Southern HU)	1	7
	River5	Vajas	Bátya (Southern HU)	0	14
Well	Irrigation water1	Soroksár	Soroksár (Central HU)	6	8
	Irrigation water2	Debrecen	Debrecen (Eastern HU)	3	4
	Irrigation water3	Nagykunsági-főcsatorna	Abádszalók (Eastern HU)	2	5
Liquid manure	Manure1	Békéscsaba	Békéscsaba (Eastern HU)	4	4
	Manure2	Bátya	Cegléd (Eastern HU)	7	28
Manure	Manure3	Bátya	Bátya (Southern HU)	7	13
	Manure4	Cegléd	Cegléd (Eastern HU)	8	0
Vegetables	Vegetables1	Soroksár	Soroksár (Central HU)	6	7
	Vegetables2	Debrecen	Debrecen (Eastern HU)	14	7

*: Two samples per sample spots were taken regarding every sample type.

2.2. MALDI-TOF MS data acquisition and processing

MALDI-TOF MS spectra of the samples were collected using a Microflex LT/SH (Bruker Daltonics GmbH & Co, Bremen, Germany) mass spectrometer equipped with a nitrogen laser (lambda = 337 nm) at a laser frequency of 60 Hz operating in linear positive ion detection mode under MALDI Biotyper Compass 3.0 and FlexControl 3.4 (Bruker Daltonics GmbH & Co, Bremen, Germany). Mass spectra were acquired in the range of 2,000–21,000 Da for each sample analysed for species level microbial identification. The spectra were generated from 240 single spectra that were created in 40-laser-shot steps from random positions of each isolate. The system was calibrated using *E. coli* ribosomal protein standard derived from the manufacturer (Bruker IVD Bacterial Test Standard). FlexControl 3.4 and FlexAnalysis 3.4 (Bruker Daltonics GmbH & Co, Bremen, Germany) were used for data acquisition and data processing, respectively.

Results of the identifications were categorised following the standard identification scores provided by the manufacturer. High-confidence identification indicates a score in the range of 2.00–3.00, which means reliable identification at species level. Low-confidence identification is accepted at genus level, with the score of 1.7–1.99. Scores below 1.7 are considered as not reliable identifications.

3. RESULTS AND DISCUSSION

Bacterial isolates in this study included 235 isolates from different water, vegetables, and manure samples. The samples contained more Gram-negative isolates than Gram-positive ones. In the case of Gram-negative bacteria, the software produced better results compared to Gram-positive bacteria, as a bigger proportion of those were identified both at species and genus levels. In this study, Biotyper could not identify, even at genus level, a significant part of total isolates (Table 2).

Kopcakova et al. (2014) used MALDI-TOF MS to identify the microbiota from waste disposal sites having an identification rate lower than 20% at species level, and Strejcek et al. (2018) reported 35% correct identifications at species level from soil samples. Similarly, we achieved 30.2% correct species identification, however, our study involved more isolates (235) than the aforementioned two (22 and 49, respectively).

Table 2. Identification results of MALDI-TOF MS of bacteria isolated from environmental samples

Organisms	Isolates	MALDI-TOF MS identification scores		
		Species identification (2–3)	Genus identification (>1.7)	Not reliable identification (0–1.69)
Gram-positive bacteria	73	18 (24.7%)	45 (61.6%)	28 (38.4%)
Gram-negative bacteria	162	53 (32.7%)	119 (73.5%)	43 (26.5%)
Total	235	71 (30.2%)	164 (69.8%)	71 (30.2%)

Among isolated bacteria, *Pseudomonas* was the most abundantly occurring genus as 45 isolates belonged to that group. It has been widely represented in the samples of River (1–5), Lake (1–2), Irrigation water1, and Manures (1–2) (Table 3). Plant pathogens belonging to *Pseudomonas fluorescens* group were isolated and identified such as *Pseudomonas marginalis*, which causes rots of plant tissues, and *Pseudomonas azotoformans*. *Pseudomonas alcaligenes* and *Pseudomonas veronii*, both used for bioremediation purposes, and *Pseudomonas extremorientalis*, usually found in drinking water, were also isolated from water samples (River1-5).

Table 3. Bacterial isolates identified by MALDI-TOF MS from environmental samples. Coloured cells indicate bacterial genera, within which more species were identified from the samples. Identified species are not listed in the table as those are presented in the text

Genera	Lakes		Rivers					Irrigation waters			Manures				Vegetables	
	#1	#2	#1	#2	#3	#4	#5	#1	#2	#3	#1	#2	#3	#4	#1	#2
Pseudomonas spp.	X	X	X	X	X	X	X	X				X	X			
Bacillus spp.		X	X		X			X	X		X	X			X	X
Acinetobacter spp.						X	X	X	X		X	X				X
Aeromonas spp.	X	X	X		X		X									
Staphylococcus spp.								X			X			X	X	X
Chryseobacterium spp.		X													X	X
Flavobacterium spp.				X	X			X								
Janthinobacterium spp.				X	X	X										
Pantoea spp.									X		X	X				X
Proteus spp.									X		X	X				X
Brevundimonas spp.										X	X					
Corynebacterium spp.													X	X		
Enterobacter spp.															X	X
Kocuria spp.				X												
Microbacterium spp.									X							X
Micrococcus spp.				X				X								
Providencia spp.										X	X					
Alcaligenes spp.											X					
Comamonas spp.											X					
Curtobacterium spp.															X	
Escherichia spp.											X					
Glutamicibacter spp.												X				
Moraxella spp.								X								
Paenibacillus spp.								X								
Paenochrobactrum spp.											X					
Psychrobacter spp.											X					
Rahnella spp.															X	
Ralstonia spp.		X														
Rheinheimera spp.		X														
Rhodococcus spp.			X													
Shewanella spp.		X														
Stenotrophomonas spp.								X								
Streptococcus spp.														X		
Vagococcus spp.											X					

As a ubiquitous genus in nature, species of *Bacillus* were also frequently found in most samples (Table 3). At least one isolate of genus *Bacillus* was found in every type of sample (irrigation water, vegetable, manure). However, most of the *Bacillus* isolates were found on vegetables. *Bacillus cereus* and *Bacillus licheniformis*, causative agents of foodborne illnesses or food spoilage, were found in both groups of Vegetables1 and 2.

The genus of *Acinetobacter*, a common one in soil and water, was identified from different types of samples (water, manure, vegetable). *Acinetobacter lwoffii*, an opportunistic human pathogen, and *Acinetobacter johnsonii*, part of the human skin flora, as well as *Acinetobacter pittii* and *Acinetobacter calcoaceticus* both belonging to *Acinetobacter baummannii* complex were also identified.

Members of the genus *Aeromonas* were also recurring with isolates identified from different water samples (Lake1-2, River1, River3, River5). *Aeromonas hydrophila*, a human and fish pathogen, and *Aeromonas salmonicida*, which infects salmon, were also identified.

In addition, MALDI-TOF MS could identify several Gram-negative ubiquitous bacteria from the environmental samples at species level. *Pantoea agglomerans*, a common plant and opportunistic human pathogen, was identified from several samples including Irrigation water2, Vegetables1, and Manure1. The genus *Brevundimonas*, commonly found in the environment, was also identified from different types of samples. One isolate was identified as *Brevundimonas diminuta* from sample Manure1, another isolate was identified as *Brevundimonas vesicularis* from sample Irrigation water3. Identified genera regarding each sample are shown in Table 3.

Nonetheless, as it can be seen in our results, the identification score of environmental isolates is lower compared to studies involving clinical isolates. Low identification rate can be explained by several factors. As Bruker's database is mostly made for clinically relevant microbes, environmental isolates regarding food safety and quality are underrepresented in it. This finding is similar to previously reported ones (De Koster and Brul, 2016; Strejcek et al., 2018). Moreover, several species of genus *Bacillus*, the most abundantly occurring Gram-positive genus in our study, such as *Bacillus drentensi*, *Bacillus pumilus*, and *Bacillus thuringiensis* have either been reported as missing from the database, misidentified, or identified with low confidence by other authors (Ashfaq et al., 2022). Another factor that contributes to poor identification result is that the database contains an inadequate number of reference spectra for a given species. As remarked by Edouard et al. (2012) and Kopcakova et al. (2014), who used MALDI-TOF MS to identify environmental isolates of *Propionibacterium* spp., a database expansion with environmental isolates would be vital.

4. CONCLUSIONS

The application of MALDI-TOF MS is gaining space for identification of food- and waterborne pathogens in the complex food production chain due to its faster and inexpensive identification process compared to traditional methods. Our analysis showed that Bruker Biotyper is able to identify causative agents of foodborne illnesses and food spoilage as well as common plant pathogens, however, its library still has room for improvement. Our research also revealed that the applicability of MALD-TOF MS in environmental bacteriology is limited due to the fact that the software failed to identify one third of the isolates even at genus level. However, according to identifications based on the library of Biotyper, Hungarian irrigation waters can be considered as microbiologically safe as no serious human pathogens were detected.

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