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**Istotność rozwoju metod spektrometrycznych w procesie
identyfikacji mikroorganizmów**

Rozprawa doktorska

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The significance of developing spectrometric methods for microorganism's identification



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Wykaz akronimów i skrótów

AST	<i>Antimicrobial Susceptibility Testing</i>	Oznaczanie lekowrażliwości-
CE	<i>Capillary Electrophoresis</i>	Elektroforeza kapilarna
CFU	<i>Colony-Forming Unit</i>	Jednostka tworząca kolonię
CVD	<i>Chemical Vapour Deposition</i>	Chemiczne osadzanie z fazy gazowej
DHB	<i>2,5-dihydroxybenzoic acid</i>	Kwas 2,5-dihydroksybenzoesowy
DNA	<i>Deoxyribonucleic acid</i>	Kwas deoksyrybonukleinowy
ESβL	<i>Extended-Spectrum β-Lactamases</i>	β -laktamazy o rozszerzonym spektrum działania
FA	<i>Formic Acid</i>	kw. mrówkowy
FC	<i>Flow Cytometry</i>	cytometria przepływowa
FTIR	<i>Fourier-Transform InfraRed spectroscopy</i>	Spektroskopia w podczerwieni z transformacją Fouriera
HCCA	<i>α-cyano-4-hydroxybenzoic acid</i>	kw. α -cyjano-4-hydroksybenzoesowy
LDI	<i>Laser Desorption/Ionization</i>	Laserowa desorpcja/ionizacja
LMW	<i>Low-Molecular Weight compounds</i>	Związki o niskiej masie cząsteczkowej
MALDI	<i>Matrix Assisted Laser Desorption/ Ionization</i>	Laserowa desorpcja /jonizacja wspomagana matrycą
MSP	<i>Main Spectrum Profile</i>	Główny profil widmowy
MRSA	<i>Methicillin-Resistant Staph. aureus</i>	<i>Staph. aureus</i> oporny na metycylinę
MSSA	<i>Methicillin-Sensitive Staph. aureus</i>	<i>Staph. aureus</i> wrażliwy na metycylinę
MTBE	<i>methyl tert-butyl ether</i>	eter metylo-tert-butyłowy
NALDI	<i>Nanostructure Assisted Laser Desorption/Ionization</i>	Laserowa desorpcja/ionizacja wspomagana nanostrukturami
NP	<i>nanoparticle</i>	nanocząstka
OD	<i>Optical Density</i>	gęstość optyczna
PAGE	<i>PolyAcrylamide Gel Electrophoresis</i>	Elektroforeza żelowa
PCR	<i>Polymerase Chain Reaction</i>	Reakcja łańcuchowa polimerazy
RNA	<i>Ribonucleic Acid</i>	Kwas rybonukleinowy
SALDI	<i>Surface Assisted Laser Desorption/Ionization</i>	Wspomagana powierzchnią laserowa desorpcja/ionizacja
SPR	<i>Surface Plasmon Resonance</i>	Powierzchniowy rezonans plazmonowy
TOF	<i>Time-Of-Flight analyser</i>	Analizator czasu przelotu
VRE	<i>Vancomycin-Resistant Enterococci</i>	Enterokoki odporne na wankomycyn

Wprowadzenie

Współczesna diagnostyka mikrobiologiczna rozumiana jest jako zespół badań mających na celu wykrycie, identyfikację i określenie lekowrażliwości drobnoustrojów mających istotne znaczenie dla szerzenia się zakażeń i rozprzestrzeniania genów lekooporności. Stosowanie szybkich, tanich i dokładnych metodologii analiz mikrobiomu jest kluczowe dla zapewnienia bezpieczeństwa żywności, utrzymania higieny publicznej oraz wdrażania terapii i strategii prewencyjnych w ochronie zdrowia ludzi i zwierząt.

W rutynowych badaniach mikrobiologicznych do identyfikacji nadal głównie stosuje się metody konwencjonalne, które opierają się na analizie mikroskopowej i hodowli bakterii na podłożach selektywnych oraz wykorzystaniu najmniej czułych metod wykrywania antybiotykooporności. Techniki te są czasochłonne i w większości przypadków nie dają dokładnych wyników. Z kolei metody oparte na biologii molekularnej, pomimo wysokiej dokładności, są drogie, czasochłonne i mogą być wykonywane tylko w specjalistycznych laboratoriach przez wykwalifikowany personel. Rozwijanie nowoczesnych technologii analitycznych łatwych w użyciu tj. laserowa desorpcja/ionizacja wspomagana matrycą (MALDI, ang. *Matrix Assisted Laser Desorption/Ionisation*), choć nie zawsze dostarcza pełnych odpowiedzi, wykazuje wysoki potencjał w pogłębionej charakterystyce mikrobiomu. W ostatnim czasie techniki laserowej desorpcji/ionizacji (LDI, ang. *Laser Desorption/Ionisation*) znajdują coraz więcej zastosowań, m.in. o analizę związków o małej masie cząsteczkowej (np. lipidy), czy analizie oporności bakterii na wybrane klasy antybiotyków. Ponadto zastąpienie klasycznej matrycy nanomateriałami, m.in. nanocząstkami srebra (technika SALDI - wspomagana powierzchnią laserowa desorpcja/ionizacja, ang. *Surface Assisted Laser Desorption/Ionization*), poprawia selektywność i czułość pomiarów spektrometrycznych związków o niskich masach, które mają znaczenie biologiczne i diagnostyczne.

Tematyka badań zrealizowanych w ramach niniejszej rozprawy doktorskiej dotyczy problematyki identyfikacji oraz analizy bakterii i skupia się na znaczeniu rozwoju metod spektrometrycznych w kontekście podejścia omicznego. Przedmiot badań stanowią mikroorganizmy o znaczeniu klinicznym, których skuteczna diagnostyka może wpływać na jakość i bezpieczeństwo życia dużej części społeczeństwa (tj. stopa cukrzycowa, rak prostaty) i był ściśle powiązany z zakresem projektów, w które byłam zaangażowana. Przeprowadzone badania miały na celu rozwijanie bardziej skutecznych narzędzi diagnostycznych, które umożliwią precyzyjną identyfikację czynników patogennych oraz szczegółową analizę, zwłaszcza w kontekście wykrywania antybiotykooporności. Głównie skupiono się na doskonaleniu technik laserowej desorpcji/ionizacji. Wynikiem przeprowadzonych badań jest zbiór artykułów, stanowiący podstawę niniejszej pracy doktorskiej.

Nota informacyjna wraz z wykazem publikacji składających się na rozprawę

Na rozprawę doktorską składa się osiem prac naukowych, z czego siedem zostało opublikowanych w czasopismach w latach 2021 - 2024. Praca [P8] jest w trakcie oceny przez powołanych recenzentów czasopisma.

Poniżej zostały wskazane artykuły wchodzące w skład cyklu publikacji:

- [P1] B. Buszewski, **E. Maślak**, M. Złoch, V. Railean-Plugaru, E. Kłodzińska, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* **2021**, 39:116250 (IF = 14.908, PM = 140)
- [P2] **E. Maślak**, W. Kupczyk, V. Railean-Plugaru, P. Pomastowski, M. Jackowski, B. Buszewski, Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches, *Electrophoresis* **2022**, 43(6) (IF = 2.900, PM = 70)
Best Student Paper Award
- [P3] M. Złoch, **E. Maślak**, W. Kupczyk, M. Jackowski, P. Pomastowski, B. Buszewski, Culturomics Approach to Identify Diabetic Foot Infection Bacteria, *International Journal of Molecular Sciences* **2021**, 22(17):9574 (IF = 6.208, PM = 140)
- [P4] J. Walczak-Skierska, F. Monedeiro, **E. Maślak**, M. Złoch, Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS, *Analytical Chemistry* **2023**, 95(1) (IF = 7.400, PM = 200)
- [P5] **E. Sibińska**, A. Arendowski, P. Fijałkowski, D. Gabryś, P. Pomastowski, Comparison of Bruker Microflex LT and Zybio EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* **2023**, 116150 (IF = 2.900, PM = 70)
- [P6] M. Złoch, **E. Maślak**, W. Kupczyk, P. Pomastowski, Multi-Instrumental Analysis Toward Exploring the Diabetic Foot Infection Microbiota, *Current Microbiology* **2023**, 80(8):271 (IF = 2.600, PM = 40)
- [P7] **E. Maślak**, A. Arendowski, M. Złoch, J. Walczak-Skierska, A. Radtke, P. Piszczek, P. Pomastowski, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* **2023**, 12(874):1-17 (IF = 4.800, PM = 70)
- [P8] **E. Sibińska**, J. Walczak-Skierska, A. Arendowski, A. Ludwiczak, P. Piszczek, A. Radtke, D. Gabryś, P. Pomastowski, Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in Enhancing Detection of Low-Molecular-Weight Biomolecules, *Journal of the American Society for Mass Spectrometry*, Manuscript ID: js-2024-00071h – **praca w recenzji**

Σ IF = 41.7

Σ PM = 730

1. Mikroorganizmy i ich znaczenie dla człowieka

Mikroorganizmy do których zaliczamy bakterie, archeony, grzyby, pierwotniaki i wirusy przystosowały się do życia w większości środowisk i odgrywają kluczową rolę w wielu aspektach życia na Ziemi. Wśród nich najliczniejszą grupę stanowią bakterie będące prokariotycznymi organizmami jednokomórkowymi o rozmiarze 0,5-5µm. Bakterie utrzymują równowagę ekosystemów poprzez rozkład wszelkiej materii organicznej i obieg pierwiastków, takich jak tlen, azot czy siarka, co zapewnia recykling substancji odżywczych. Zdolności metaboliczne niektórych gatunków sprawiają, że są one powszechnie wykorzystywane w różnych gałęziach przemysłu, zwłaszcza spożywczego, farmaceutycznego i biotechnologicznego, np. w produkcji żywności fermentowanej, witamin, probiotyków, leków i antybiotyków. Wiele gatunków bakterii stanowi istotną część mikrobiomu organizmów żywych, w tym ludzi, przynosząc korzyści poprzez wspieranie układu odpornościowego czy wspomaganie procesów trawienia [1].

Choć czerpiemy wiele korzyści z ich obecności w naszym życiu, istotne jest skupienie uwagi na potencjalnie negatywnych aspektach, zwłaszcza w kontekście zdrowia. Spośród wszystkich mikroorganizmów bakterie stanowią najczęstszy czynnik chorobotwórczy oraz zagrażający życiu. W 2019 roku 33 rodzaje bakterii były odpowiedzialne za śmierć ponad 7,7 miliona ludzi, co stanowiło co ósmy zgon na świecie [2]. To sprawia, że infekcje bakteryjne są drugą najczęstszą przyczyną zgonów na świecie, tuż po chorobach niedokrwiennych serca. Według raportu, ponad 75% tych zgonów było spowodowanych przez pięć gatunków bakterii: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, oraz *Pseudomonas aeruginosa*. Izolaty kliniczne wymienionych powyżej gatunków stanowiły główny obiekt zainteresowania w przeprowadzonych przeze mnie badaniach [P2-P7].

Bakterie patogenne, będąc nieustannie obecne w otaczającym środowisku, mają zdolność do rozprzestrzeniania się na szereg różnych sposobów (Rysunek 1). Kontakt z zakażonymi osobami lub zwierzętami, skażona gleba, unoszące się w powietrzu cząstki czy skażona woda stanowią potencjalne źródła transmisji. Poza tym, obecność pewnych gatunków bakterii (np. z rodzaju *Salmonella*, *Listeria*, *Clostridium*) w żywności może prowadzić do procesów ich psucia, co skutkuje nie tylko utratą cennych wartości odżywczych, ale również może stanowić istotne zagrożenie dla konsumentów. Temperatura organizmu ludzkiego stanowi optymalne warunki do wzrostu i namnażania dla wielu patogenów, co w znacznym stopniu sprzyja rozwojowi infekcji. Bakterie poprzez produkcję toksyn nabycie mechanizmów oporności na antybiotyki i/lub tworzenie biofilmów, znacznie utrudniają eradykację chorób w warunkach klinicznych [3]. Nielezione zakażenia bakteryjne i przewlekłe stany zapalne zwiększają ryzyko wystąpienia poważnych stanów chorobowych tj. stopa cukrzycowa czy rak prostaty.

Krótko od momentu wprowadzenia do leczenia infekcji bakteryjnych pierwszego antybiotyku, penicyliny (lata 50. XX w.), bakterie zaczęły rozwijać mechanizmy lekooporności. Przez długi czas nie stanowiło to poważnego problemu, ponieważ opracowywano nowe klasy

leków, takie jak karbapenemy, tetracykliny, cefalosporyny, co sugerowało, że problem oporności można łatwo rozwiązać poprzez syntezę nowych związków przeciwdrobnoustrojowych. Niestety, w ciągu następnych dziesięcioleci bakterie rozwijały kolejne mechanizmy oporności względem wprowadzanych terapeutyków, w wyniku czego oporność na antybiotyki stanowi aktualnie poważne zagrożenie dla całego społeczeństwa.



Rysunek 1. Drogi transmisji mikroorganizmów w ekosystemie oraz udział człowieka w szczyrzeniu zjawiska antybiotykooporności.

Oporność na antybiotyki dzielimy na pierwotną, będącą cechą naturalną zakodowaną w genomie, oraz nabytą, która rozwija się w wyniku przenoszenia genu oporności (transdukcja) lub mutacji chromosomalnej. Oporność może dotyczyć konkretnego związku lub grupy związków o podobnym działaniu. Bakterie wykształciły różnorodne mechanizmy obronne przed działaniem antybiotyków, w związku z czym nie istnieje powszechne wytłumaczenie działania wszystkich antybiotyków. Skuteczność tych substancji wynika głównie z ich selektywnego oddziaływania na elementy komórek bakteryjnych, jednocześnie nie wpływając na komórki zwierzęce. Z tego powodu często dokonuje się podziału antybiotyków na grupy w oparciu o ich mechanizm działania [4]. W Tabeli 1 przedstawiono podstawowe mechanizmy działania antybiotyków.

W raporcie opublikowanym przez Światową Organizację Zdrowia (WHO, ang. *World Health Organization*) w 2017 r. wymieniono listę 12 gatunków bakterii uznawanych za patogeny o globalnym priorytecie – charakteryzujących się średnią lub krytyczną opornością na antybiotyki [5]. Do krytycznych szczepów na tej liście zaliczają się *Enterobacteriaceae*, które coraz częściej wykazują oporność na antybiotyki β -laktamowe. Największe zagrożenie stwarzają te gatunki, które uodporniły się na wiele antybiotyków, np. niektóre szczepy *E. coli* i *P. aeruginosa*, które nabywają oporność poprzez produkcję enzymów ES β L (β -laktamaz o rozszerzonym spektrum), odpowiedzialnych za oporność na większość klas antybiotyków β -laktamowych. Do patogenów bakteryjnych o wysokim priorytecie zaliczane są również

szczepy *S. aureus* odporne na metycylinę (MRSA, ang. *methicillin-resistant S. aureus*) i enterokoki odporne na wankomycynę (VRE, ang. *vancomycin-resistant enterococci*). Rozprzestrzenianie się tego zjawiska nie tylko obniża skuteczność leczenia powszechnych infekcji, ale również stanowi istotne ryzyko podczas wykonywania procedur ratujących życie, takich jak operacje, przeszczepy czy terapie onkologiczne.

Tabela 1. Sposoby działania antybiotyków na komórki bakteryjne oraz wytworzone przez bakterie najistotniejsze mechanizmy oporności [4].

Grupy antybiotyków (wraz z przykładami)	Mechanizm działania antybiotyku	Mechanizm oporności wykształcony przez bakterie
β-laktamy (karbapenemy, penicyliny, cefalosporyny)	Blokowanie biosyntezy ściany komórkowej	Produkcja enzymów β-laktamaz
Inhibitory B-laktamaz (kwas klawulanowy, awibaktam)	Blokowanie aktywność enzymów β-laktamaz	Produkcja enzymów ESβL o rozszerzonym spektrum działania
Aminoglikozydy (streptomycyna), Makrolidy (azytromycyna), Tetracykliny (tetracyklina)	Hamowanie syntezy białek poprzez wiązanie się z podjednostką 30S lub 50S rybosomu	Wieloczynnikowe (modyfikacja enzymatyczna, modyfikacja miejsca docelowego i pompy efflux)
Chinolony (kwas nalidyksowy), Fluorochinolony (ciprofloksacyna)	Hamowanie transkrypcji oraz replikacji DNA	Wieloczynnikowe (mutacje genów w miejscu docelowym, pompy efflux i enzym modyfikujący)
Sulfonamidy (sulfacetamid) Trimetoprym	Hamowanie metabolizm kwasu foliowego	Rozprzestrzenianie się genów oporności za pośrednictwem transpozonów i plazmidów

W związku z tym kontrola obecności mikroorganizmów, zarówno w kontekście przemysłowym, jak i zdrowotnym, jest kluczowa dla zapewnienia bezpieczeństwa żywności, utrzymania higieny publicznej oraz ochrony zdrowia ludzi i zwierząt. Szybka i dokładna identyfikacja mikrobiomu, oraz prawidłowe określenie lekooporności patogenów w rutynowych laboratoriach mikrobiologicznych, odgrywa kluczową rolę, szczególnie w procesie leczenia zakażeń i wdrożenia antybiotykoterapii. W niniejszej pracy opracowałam metody analizy lekooporności bakterii Gram(-) na antybiotyki β-laktamowe z zastosowaniem techniki MALDI [P6]. Jako pierwsza wykorzystałam to podejście do analizy antybiotykooporności towarzyszącej szczepom patogennym odpowiedzialnym za rozwój zakażenia stopy cukrzycowej.

Podejścia omiczne w analizie mikrobiomu

Koncepcja mikrobiomu odnosi się do złożonych konsorcjów mikroorganizmów, które zamieszkują określone środowisko, takie jak organizm ludzki i zwierzęcy, gleba czy woda. Celem opisu składu mikrobiomu jest zrozumienie wpływu mikroorganizmów na zdrowie i funkcjonowanie gospodarza. Badania nad mikrobiomem koncentrują się na poznaniu różnorodności, funkcji oraz interakcji między mikroorganizmami. Otwiera to nowe horyzonty w dziedzinie medycyny, umożliwiając rozwój terapii i interwencji z wykorzystaniem modyfikacji mikrobiomu, co ma na celu poprawę stanu zdrowia ludzi i zwierząt [6].

Analizy mikrobiomu w głównej mierze skupiają się na identyfikacji tworzącej go populacji mikroorganizmów i charakterystyce cech ważnych dla danego obszaru badawczego (medycyna, przemysł, środowisko). W przemyśle, analizy skupiają się na badaniu aktywnych szlaków metabolicznych, które umożliwiają produkcję substancji takich jak etanol, kwas mlekowy, witaminy czy też biorą udział w procesach bioremediacyjnych. Te informacje są kluczowe w optymalizacji procesów produkcyjnych i poprawie ich wydajności. W diagnostyce medycznej, istotnym aspektem analizy mikrobiomu jest identyfikacja wydzielanych do światła jelit oraz krwioobiegu metabolitów, zarówno tych naturalnie produkowanych przez mikrobiotę jelitową, jak i tych powstałych w odpowiedzi na zastosowany rodzaj leczenia m.in. antybiotykoterapię, radio- i chemioterapię, dietę w tym suplementację probiotykami. Obecnie dostępne techniki badania mikrobiomów, tzw. *multi-omiki*, obejmują analizy na poziomie całych komórek mikroorganizmów (wysokowydajna izolacja – kulturomika, wizualizacja – mikroskopia), oraz różnych cząsteczek składających się na i podtrzymujących życie komórek (DNA – genomika, mRNA – transkryptomika, białka – proteomika, lipidy – lipidomika, metabolity – metabolomika) [7]. W ramach pracy doktorskiej prowadziłam badania, które opierały się na podejściu proteomicznym [P3, P5, P6], lipidomicznym [P4, P7, P8], metabolomicznym [P6] oraz genomicznym [P6] w analizie mikroorganizmów.

Pomimo dostępu do tak znacznego rozwoju technologii umożliwiających analizę mikroorganizmów (patrz Tabela 2) w laboratoriach mikrobiologicznych nadal najczęściej stosuje się metody konwencjonalne. Obejmują one przygotowanie próbki, wstępne namnażanie, rozcieńczanie, posiew, zliczanie i izolację wyrosłych kolonii jednego gatunku w celu dalszej charakterystyki. Identyfikacja opiera się na cechach morfologicznych, biochemicznych, fizjologicznych (fenotyp) lub genetycznych (genotyp) mikroorganizmu. W podstawowych procedurach często stosuje się pożywki wzbogacane (np. poprzez dodatek krwi), które mają wspierać wzrost hodowalnej puli mikroorganizmów – przede wszystkim tych o wysokich wymaganiach pokarmowych. Do zliczania określonych klas bakterii i patogenów stosuje się pożywki selektywne lub różnicujące. Każda morfologicznie odrębna kolonia (różna wielkość, kształt, kolor kolonii) jest następnie dalej charakteryzowana m.in. metodą barwienia Grama, obserwacją pod mikroskopem i testami biochemicznymi lub serologicznymi [8]. Na tym etapie często dla wybranych izolatów określa się fenotypowo wrażliwość na działanie antybiotyków: przy zastosowaniu stałych pożywek lub podłoży płynnych (metoda rozcieńczeń w bulionie), krążkowo-dyfuzyjną lub stosując paski gradientowe (E-test) [9]. W pracy [P6] badałam wrażliwość szczepów bakteryjnych wyizolowanych ze stopy cukrzycowej, gdzie E-test stanowił metodę referencyjną, do opracowanych metod spektrometrycznych.

Chociaż w wielu przypadkach badanie właściwości fizjologicznych mikroorganizmów jest wystarczające, niemniej jednak należy pamiętać, iż mogą one znacznie różnić się nawet w obrębie tego samego gatunku, zwłaszcza gdy izolaty pochodzą z różnych środowisk.

Co więcej, identyfikacja fenotypowa zaliczana jest do metod pracochłonnych a jej interpretacja jest często subiektywna i niejednoznaczna w przypadku porównywania wyników pozyskanych za pomocą różnych technik. Istotnym wyzwaniem dla mikrobiologów są przede wszystkim nowe patogeny bakteryjne o jeszcze nieznanym lub słabo zdefiniowanym cechach fenotypowych. Choć wdrożenie półautomatycznych lub w pełni zautomatyzowanych systemów (np. API, BACTEC, BacT/ALERT¹) analiz fenotypowych przyczyniło się do skrócenia czasu uzyskania wyników, to wciąż dokładność przypisania taksonomicznego jest mocno ograniczona w stosunku do złotego standardu identyfikacji mikroorganizmów jakim jest sekwencjonowanie 16S rRNA. Pomimo tego, że tradycyjne metody (np. posiew na pożywkach selektywnych) są stosunkowo niedrogie i proste, to są one jednocześnie praco- i czasochłonne. Proces identyfikacji zazwyczaj trwa od 3 do 7 dni z powodu konieczności przeprowadzenia etapu wstępnego namnażania, inkubacji i przeprowadzenia testów biochemicznych [8]. W niektórych przypadkach np. *Mycobacterium* spp. czas analiz może wydłużyć się nawet do kilku tygodni [10].

Wyzwania te podkreślają znaczenie charakteryzowania bakterii za pomocą metod niezależnych od cech biochemicznych mikroorganizmu. Nowoczesne techniki genomiki oparte na amplifikacji kwasów nukleinowych (DNA i RNA) nie tylko skutecznie odpowiadają na te potrzeby, ale również stają się „złotym standardem” w laboratoriach, otwierając szeroki zakres możliwości w tym obszarze. Techniki biologii molekularnej wykorzystujące reakcję łańcuchową polimerazy (PCR, ang. *polymerase chain reaction*) i sekwencjonowanie, ułatwiają precyzyjną identyfikację patogenów. Ponadto, umożliwiają identyfikację genów oporności na środki przeciwdrobnoustrojowe oraz lokalizację ognisk infekcyjnych [11].

Tabela 2. Zestawienie zalet i wad technik najczęściej stosowanych w laboratoriach mikrobiologicznych [8], [11].

Metoda	Zalety	Wady	Granica wykrywalności
Metody konwencjonalne	- nie wymagają kosztownej aparatury, - stosunkowo prosta procedura analityczna	- czasochłonne (zależą od tempa wzrostu drobnoustrojów), - pracochłonne, - niska dokładność	10 ² – 10 ⁴ CFU/mL
Metody molekularne (PCR)	- wysoka czułość i specyficzność, - wiarygodne wyniki, - możliwość analizy jakościowej i ilościowej w czasie rzeczywistym (qPCR), - detekcja kilku genów w trakcie jednej analizy (multiplex PCR)	- część etapów przygotowania próbki jest czasochłonna, - składniki próbki mogą prowadzić do inhibicji, - konieczność tworzenia krzywej wzorcowej (qPCR), - utrudniona optymalizacja procesu i projektowanie primerów (multiplex PCR), - analiza danych wymaga doświadczonego personelu,	10 ¹ – 10 ³ CFU/mL
Metody elektromigracyjne (PAGE, CE, CIEF)	- koncentracja i rozdział mikroorganizmów, i ich produktów sekrecyjnych,	- dobór warunków analizy, - agregacja i interakcja komórek	-

¹ Większość systemów wykrywa m.in. dwutlenek węgla lub pH jako wskaźnik wzrostu drobnoustrojów.

Metody immunologiczne (ELISA)	- wysoka specyficzność i wydajność, - łatwa obsługa, - możliwość analizy jakościowej i ilościowej	- wymaga zastosowania specyficznych przeciwciał, - wysoka granica wykrywalności, - fałszywie dodatnie lub fałszywie ujemne wyniki	10 ⁴ CFU/mL
Metody spektroskopowe (NIR, Raman)	- szybka analiza, - próbka nie zostaje zniszczona, - wysoka specyficzność (Raman)	- utrudniona interpretacja danych, - kłopotliwe przygotowanie próbki (Raman)	10 ⁷ CFU/mL
Metody spektrometryczne (MALDI)	- wysoka wydajność, - wysoka czułość, - szybka procedura przygotowania próbki, - łatwa obsługa oprogramowania, - stosunkowo niski koszt analiz	- zależna od wzrostu mikroorganizmu, - ograniczona baza danych,	10 ⁶ CFU/mL

W celu przeprowadzenia identyfikacji molekularnej mikroorganizmów przeprowadza się amplifikację materiału genetycznego z zastosowaniem takich metod takich jak PCR, qPCR (ilościowa PCR w czasie rzeczywistym) i RT-qPCR (PCR w czasie rzeczywistym z odwrotną transkrypcją) w oparciu o gen 16S rRNA (16S rDNA) lub 26S rRNA. Gen 16S rRNA, budujący małą podjednostką rybosomów u prokariotów, charakteryzuje się występowaniem naprzemiennych obszarów konserwatywnych i heterogenicznych. Dzięki tej zróżnicowanej budowie, gen doskonale nadaje się do sekwencjonowania i identyfikacji mikroorganizmów. Otrzymaną sekwencję porównuje się z bazą danych, która zawiera sekwencje znanych mikroorganizmów i w oparciu o występujące między nimi podobieństwo kolejności występowania par zasad nukleotydowych oblicza się stopień identyczności wyrażany wartościami procentowymi [12]. Strategia ta jest odpowiednia do identyfikacji wielu gatunków i rodzajów bakterii, aczkolwiek może nie być wystarczająca w przypadku wybranych gatunków bakterii o wysokim stopniu podobieństwa sekwencji nukleotydowej genu 16S Rrna klasyfikowanych jako niektórych blisko spokrewnione szczepy, np. *E. coli* i *Shigella* spp [13]. Wymusza to konieczność zanalizowania sekwencji dodatkowych genów, dla których dostępne bazy danych nie są już tak obszerne, jak w przypadku bazy 16S rRNA. W pracy [P6] wykorzystałam sekwencjonowanie regionu 16S rRNA kodującego cząsteczki 16S rRNA do walidacji opracowanych metod spektrometrycznych. W wyniku przeprowadzonych badań zaobserwowałam, iż w przypadku 6/111 izolatów bakteryjnych zastosowanie sekwencjonowania genu 16S rRNA nie było wystarczające do uzyskania wiarygodnej identyfikacji, co podkreśla konieczność zastosowania dodatkowych protokołów analitycznych. Na szczególną uwagę zasługuje przypadek identyfikacji szczepów należących do gatunku *Pseudomonas aeruginosa*, dla których, pomimo zastosowania dodatkowych, specyficznych dla rodzaju *Pseudomonas* primerów, nie uzyskałam wiarygodnej identyfikacji.

W wielu przypadkach pomocne jest zastosowanie techniki multiplex PCR, która dzięki jednoczesnemu użyciu kilku różnych primerów umożliwia jednoczesną amplifikację wielu różnych fragmentów DNA w czasie trwania pojedynczej reakcji. Mogą to być fragmenty specyficzne dla danych gatunków bakterii, jak i różne geny oporności na antybiotyki np. bla_{oxA},

bla_{NDM}, bla_{CTX-M} [14]. W przypadku niektórych próbek możliwe jest wykrycie kilku różnych patogenów i genów oporności bezpośrednio z próbki. Abram i wsp. stosując podejście multiplex PCR zidentyfikowali niektóre bakterie (*E. coli* i *K. pneumoniae*) i uzyskali dla nich profilowanie wrażliwości na antybiotyki bezpośrednio z próbek krwi pełnej [15]. Natomiast, Sigmund i wsp. zastosowali tę technikę do identyfikacji zakażeń i wykrycia oporności z próbek płynu sonikacyjnego i mazi stawowej [16]. Jak podkreślili autorzy mimo, iż swoistość metody była wysoka, ogólna czułość wykrywania oporności bezpośrednio z próbek klinicznych była niska. Czułość tej metody istotnie wzrasta w przypadku analizy izolatów bakteryjnych [17]. Warto zaznaczyć, że multipleksowane testy PCR charakteryzują się ograniczoną zdolnością identyfikacji docelowych genów, a ich zakres nie obejmuje w pełni wszystkich istotnych mechanizmów oporności. Ograniczenia te sprawiają, że ta technologia ta nie może być uniwersalnie stosowana do analizy wszystkich próbek [14]. Wadą analiz bezpośrednio z próbek jest również fakt, iż metody PCR umożliwiają analizę całego DNA zawartego w próbce, nie ma więc możliwości odróżnienia czy wyizolowany materiał genetyczny pochodzi od żywych, martwych komórek, czy jest pochodzenia pozakomórkowego [18]. W ramach pracy doktorskiej również zastosowałam podejście multiplex PCR w celu jednoczesnej detekcji kilku genów odpowiedzialnych za występowanie antybiotykooporności na β -laktamy wśród bakterii Gram(-), co pozwoliło mi na walidację metody spektrometrycznej [P6].

Mimo że proces samej amplifikacji i sekwencjonowania jest stosunkowo szybki i dokładny, odpowiednie przygotowanie próbek przysparza wielu problemów. Obejmuje ono wiele etapów, takich jak rozcieńczanie, filtracja, ekstrakcja, i ostatecznie oczyszczanie wyizolowanego DNA/RNA. Ważne jest przestrzeganie zasad aseptyki i dokładne postępowanie zgodnie z protokołem, aby uniknąć kontaminacji, która prowadzi do fałszywych wyników. Niewłaściwie zaprojektowane primery, źle dobrane proporcje reagentów czy warunki temperaturowe cyklu wpływają na skuteczność amplifikacji i mogą utrudniać interpretację wyników [8]. Warto zauważyć, że pomimo wysokiego potencjału, techniki te najczęściej służą do wykonania najprostszych analiz. Tylko niektóre laboratoria mikrobiologii klinicznej posiadają środki finansowe i przeszkolony personel umożliwiający dostęp do bardziej zaawansowanych możliwości tej techniki [11], [19]. Korzystanie z zewnętrznych laboratoriów zwiększa koszty analizy i wydłuża czas oczekiwania na wyniki. Z tego powodu do rutynowych analiz zaleca się stosowanie innych technik analitycznych, a techniki genomowe należy traktować jako referencyjne w sytuacjach problematycznych.

Nowoczesne techniki identyfikacji i analizy mikroorganizmów powinny cechować się wysoką czułością, krótkim czasem do uzyskania wyniku oraz obejmować możliwie jak najszerszy zakres różnych gatunków mikroorganizmów. Dużym zainteresowaniem cieszą się więc metody immunologiczne, które opierają się na zdolności układu immunologicznego do rozpoznawania i reagowania na obce antygeny, takie jak białka, polisacharydy czy inne cząstki mikroorganizmów. Techniki te są zwykle bardzo szybkie, czułe i specyficzne ponieważ

wykorzystują reakcję przeciwciało-antygen. Najpopularniejszy test, ELISA (ang. *enzyme-linked immuno sorbent assay*), pozwala wykryć antygeny obecne na powierzchni komórki lub toksyny wytwarzane przez patogen, dzięki czemu możliwa jest identyfikacja m.in. *E. coli*, *Salmonella* spp., *L. monocytogenes*, *Bacillus cereus* i enterotoksyn produkowanych przez gronkowce [8]. W celu detekcji zajścia reakcji, metody immunologiczne często łączy się z innymi technikami takimi jak: rozdzielanie elektroforetyczne (tzw. Western Blot), mikroskop fluorescencyjny oraz cytometr przepływowy [20], [21]. Mimo że techniki immunologiczne są niezwykle przydatne i szeroko stosowane w diagnostyce medycznej i mikrobiologii, to mają także pewne wady. W wielu przypadkach wymagane jest przygotowanie wstępne próbek, ponieważ wszelkie zanieczyszczenia: sole, kwasy, jony metali lub inne związki mogą istotnie wpływać na zajście reakcji i jej wynik. Przeciwciała mogą również (i) wykazywać pewną krzyżową reaktywność, tj. wiązać się niespecyficznym z antygenami, co może prowadzić do fałszywie dodatnich lub fałszywie ujemnych wyników i (ii) nie być dostępne na rynku w przypadku wszystkich drobnoustrojów będących przedmiotem zainteresowania, co zwiększa koszty i czas analizy [8], [21].

Możliwości zastosowania metod elektromigracyjnych w analizach mikroorganizmów szerzej omówiłam w publikacji przeglądowej [P1] wchodzącej w zbiór artykułów stanowiących podstawę niniejszej pracy doktorskiej. Skłonność komórek mikroorganizmów do agregacji w trakcie procesu elektromigracji jest często uważana za niekorzystne zjawisko, które znacznie utrudnia rozdzielenie poszczególnych gatunków mikroorganizmów. Z drugiej strony zjawisko to można wykorzystać w przypadku analizy pojedynczych izolatów do pre-koncentracji komórek. Uzyskując pojedynczy sygnał możliwe jest określenie wrażliwości bakterii na antybiotyki, których obecność często przyczynia się do zmiany ładunku powierzchniowego komórek, co bezpośrednio wpływa na czas elektromigracji zagregowanych komórek. Podejście to umożliwiło mi wyznaczenie równania, które w oparciu o czas elektromigracji komórek pozwala oszacować odsetek komórek żywych i martwych [22]. Równanie zastosowałam w publikacji [P2] do detekcji antybiotykooporności bakteryjnych izolatów klinicznych – *E. coli* ESβL, *S. aureus* MRSA oraz *S. aureus* wrażliwego na metycylinę (MSSA, ang. *methicillin-sensitive S. aureus*). Uzyskane wyniki zwalidowałam w oparciu o pomiary z użyciem cytometrii przepływowej (FC, ang. *flow cytometry*) oraz gęstości optycznej (OD, ang. *optical density*). Uzyskane wyniki wskazują na szeroki potencjał aplikacyjny tych metod w analizach antybiotykooporności.

Metody spektroskopowe tj. spektroskopia Ramana [23] oraz spektroskopia w podczerwieni z transformacją Fouriera (FTIR, ang. *fourier-transform infrared spectroscopy*) [24] dają możliwość zarejestrowania profili molekularnych charakterystycznych dla danych mikroorganizmów. Otrzymane widma są specyficzne i odzwierciedlają charakterystykę wszystkich składników komórkowych tj. kwasy tłuszczowe, białka, polisacharydy i kwasy nukleinowe. W naszym zespole, Złoch i wsp. zastosowali z powodzeniem metodę FTIR

do rozróżnienia dwóch blisko spokrewnionych szczepów bakterii ślinowych – *Streptococcus salivarius* i *Streptococcus vestibularis* [25]. Dużą zaletą tych technik jest przede wszystkim szybkość, nieinwazyjność (nie wymagają lizy komórek w celu uwolnienia badanych biomolekuł), oraz fakt, iż nie wymagają zastosowania dodatkowych odczynników. Jednakże, ze względu na złożoność biomolekuł obecnych w komórce bakteryjnej na widmach obserwujemy nakładanie się na siebie pasm, co utrudnia interpretację i analizę widm. Aby osiągnąć wysoką dokładność identyfikacji wymagane jest znaczne wydłużenie czasu pomiaru (Raman) oraz odpowiednie przetworzenie widma m.in. normalizacja widma, transformacja pochodnej (FTIR). Ponadto, aby techniki te mogły znaleźć zastosowanie w rutynowych analizach zarówno do identyfikacji mikroorganizmów jak i oceniania ich wrażliwości na antybiotyki niezbędne jest znaczne rozbudowanie bazy widm referencyjnych [23], [24].

Przełom w analizie mikroorganizmów nastąpił pod koniec ubiegłego stulecia wraz z rozwojem technik spektrometrii mas, zwłaszcza techniki laserowej desorpcji/ionizacji wspomaganej matrycą (MALDI, ang. *matrix-assisted laser desorption/ionization*). Umożliwia ona w łatwy sposób szybką i precyzyjną identyfikację wielu rodzajów mikroorganizmów na podstawie charakterystycznych profili białkowych (podejście proteomiczne) porównywanych z widmami referencyjnymi [26]. W ostatnich latach pojawiły się nowe protokoły umożliwiające bezpośrednią identyfikację szczepów chorobotwórczych z niektórych próbek klinicznych, głównie krew, mocz, płyn mózgowo-rdzeniowy, co skraca czas oczekiwania na wynik identyfikacji [27]. Koszt zużycia odczynników podczas pojedynczej analizy jest stosunkowo niski (około 0,5\$ [28]), co sprawia, iż technika ta stanowi konkurencję dla metod biologii molekularnej. Zastosowanie MALDI w identyfikacji mikroorganizmów jest coraz częściej wprowadzane w rutynowych analizach diagnostycznych, co stanowi przełom w dziedzinie medycyny laboratoryjnej. Jednakże ze względu na wysoki koszt aparatury dostęp do tej technologii w krajach rozwijających się jest ograniczony. W ubiegłym roku ponad 100 szpitali i placówek medycznych w Polsce zostało wyposażonych w ten sprzęt w ramach akcji WOŚP, co znacznie przyczyni się do podniesienia standardów opieki medycznej w naszym kraju.

Technika MALDI nie tylko umożliwia identyfikację mikroorganizmów, ale również reprezentuje wszechstronne narzędzie o szerokim spektrum zastosowań. Poza identyfikacją poszczególnych gatunków bakterii czy grzybów, technika ta umożliwia precyzyjne różnicowanie między poszczególnymi, blisko spokrewnionymi szczepami. Ponadto, umożliwia identyfikację konkretnych białek lub peptydów, co jest kluczowe w analizie czynników wirulencji czy toksyn bakteryjnych. Umożliwia również analizę różnorodnych związków chemicznych obecnych w komórkach mikroorganizmów, takich jak kwasy nukleinowe, aminokwasy czy lipidy, co powoduje, iż technika MALDI stanowi wszechstronne narzędzie w badaniach nad metabolizmem i składem chemicznym komórek mikroorganizmów. Dzięki

temu możliwe jest jej zastosowanie w badaniach nad opornością bakterii na niektóre klasy antybiotyków [29].

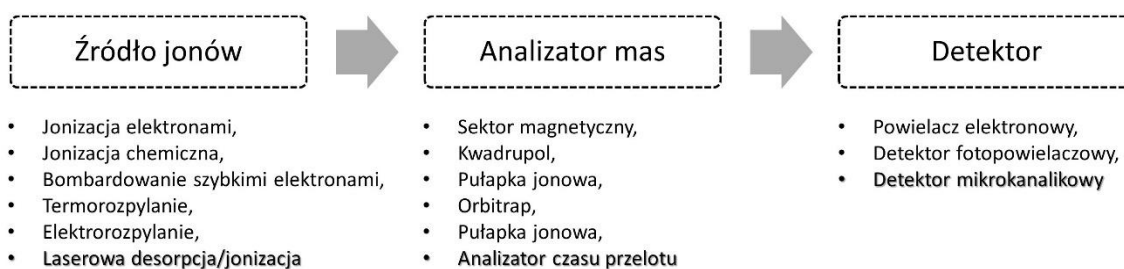
Wszechstronne możliwości techniki MALDI sprawiają, że znajduje ona zastosowanie również w mikrobiologicznych laboratoriach środowiskowych, przemysłowych i innych obejmujących szeroko rozumiany obszar life science. Podobnie jak każda technika analityczna, metoda ta nie jest pozbawiona pewnych ograniczeń, niemniej jednak, wysoki potencjał i możliwości tej technologii stanowią cenne źródło inspiracji dla wielu badaczy, motywując do ciągłego jej doskonalenia. W kolejnym rozdziale niniejszej pracy skoncentrowałam się na szczegółowym omówieniu możliwości i ograniczeń technologii laserowej desorpcji jonizacji w kontekście analizy mikroorganizmów.

2. Techniki LDI w analizie mikroorganizmów

2.1. Budowa urządzenia oraz podstawy jego działania

Spektrometria mas to obszerna dziedzina technik analitycznych, której działanie opiera się na pomiarze stosunku masy do ładunku (m/z) jonów na podstawie ich ruchu w polu elektrycznym lub magnetycznym. Warunkiem pomiaru jest przekształcenie cząsteczek analizowanej próbki w jony w fazie gazowej. Rozdzielanie jonów według ich stosunku m/z pozwala na uzyskanie charakterystycznego widma masowego, będącego wykresem abundancji w funkcji wartości m/z . Każdy spektrometr mas zawiera następujące elementy: układ wprowadzenia próbki, źródło jonów, które jest odpowiedzialne za przekształcenie cząsteczek próbki w formę jonową, analizator mas, który dokonuje rozdzielania jonów na podstawie wartości m/z , oraz detektor, który rejestruje ilość występujących jonów [30].

Spektrometria mas znajduje zastosowanie w różnych dziedzinach nauki, w tym w chemii organicznej, biochemii, biologii molekularnej, farmakologii oraz analizie środowiskowej. Dzięki tej technologii możliwe jest precyzyjne określanie mas cząsteczkowych, identyfikacja nieznanymi substancji, analiza struktury białek, a także badanie interakcji molekularnych. Spektrometria mas umożliwia również badanie zmienności izotopowej, co pozwala na odkrywanie różnic w składzie chemicznym izotopów danego pierwiastka [30]. Skuteczność i zakres analiz, jakie może przeprowadzić dany spektrometr mas, zależą od budowy źródła jonów i analizatora (rodzaje przedstawiono na Rysunku 2) [30], [31], oraz innych parametrów technicznych urządzenia.



Rysunek 2. Najważniejsze elementy budowy spektrometru mas wraz z przykładami stosowanych technik.

Laserowa desorpcja/ionizacja (LDI) jest skuteczną metodą wytwarzania jonów gazowych. Impulsy lasera powodują desorpcję substancji stałej z powierzchni i tworzą mikroplazmę jonów i obojętnych cząsteczek, które mogą reagować między sobą w gęstej fazie gazowej w pobliżu próbki. Wykorzystanie LDI jest jednak ograniczone do związków o niskich masach cząsteczkowych (<3000 Da) ze względu na zależność procesu jonizacji od właściwości fizycznych analitu, tj. termodyfuzja, absorpcja promieniowania UV, lotność oraz stabilność termiczna. Sytuacja ta uległa radykalnej zmianie wraz z rozwojem desorpcji/ionizacji laserowej wspomaganą matrycą (MALDI). W 1988 roku Karas i Hillenkamp zastosowali kwas nikotynowy jako matrycę ułatwiającą jonizację białek o wysokich masach cząsteczkowych (>

10 000 Da): lizozym, β -laktoglobulina A, albumina, trypsyna [32], β -D- galaktozydaza (wyizolowana ze szczepu *E. coli*), katalaza [33]. W tym samym roku Tanaka i wsp. do analizy polimerów i białek o wysokich masach cząsteczkowych jako matrycę zastosowali nanocząstki kobaltu o średnicy 30 nm [34].

Najpowszechniejsze w technice MALDI ze względu na łatwość eksploatacji (krótki impuls i wysoka energia lasera) oraz niską cenę są lasery emitujące światło w zakresie ultrafioletu, takie jak: N_2 $\lambda = 337$ nm, Nd:YAG $\lambda = 266$ lub 355 nm. Najważniejszym kryterium jest określenie mocy impulsu lasera, która powoduje desorpcję matrycy, ponieważ zbyt duża może prowadzić do rozległej fragmentacji i powodować utratę rozdzielczości. Czasem spotyka się również wykorzystanie laserów z zakresu podczerwieni, takich jak Er:YAG $\lambda = 2,94$ μ m, CO_2 $\lambda = 10,6$ μ m, które mając niższą energię zmniejszają zjawisko fragmentacji, jednakże w ich przypadku obserwuje się niższą czułość [30]. Ze względu na pulsacyjny charakter jonizacji w technice MALDI, najczęściej stosuje się analizator czasu przelotu (TOF, ang. *time-of-flight*), który umożliwia natychmiastową detekcję w szerokim zakresie mas. Rozdział jonów następuje w próżni (zazwyczaj) i opiera się na fundamentalnej zależności między czasem przelotu a masą naładowanego jonu – lżejsze jony docierają do detektora szybciej. Niektóre aparaty MALDI-TOF poza trybem liniowym pracy analizatora, wyposażone są również w tryb reflektromowy, który wydłużając drogę rozdziału powoduje zwiększenie rozdzielczości [35]. Dodatkowo, w przypadku gdy pojedynczy analizator masy typu TOF nie wystarcza do pełnej analizy docelowych analitów w skomplikowanej mieszaninie, połączenie z drugim analizatorem mas TOF umożliwia przeprowadzenie tandemowej analizy MS. Pierwszy analizator masy TOF umożliwia selekcję jonów prekursorowych, które są następnie fragmentowane w komórce zderzeniowej. Uzyskane fragmenty są następnie analizowane przez drugi analizator masy TOF, co pozwala uzyskać tandemowe widmo masowe [29].

W trakcie prowadzenia badań korzystałam z trzech spektrometrów mas typu LDI tj. (1) UltrafleXtreme MALDI-TOF MS wyposażony w laser Nd:YAG o $\lambda = 355$ nm (Bruker Daltonik GmbH, Bremen, Brema, Niemcy), (2) Microflex LT MALDI-TOF MS wyposażony w laser N_2 o $\lambda = 337$ nm (Bruker Daltonik GmbH, Bremen, Brema, Niemcy), (3) EXS2600 MALDI-TOF MS wyposażony w laser N_2 o $\lambda = 337$ nm (Zybio Inc., Chongqing, Chiny). Jako jedna z pierwszych zweryfikowałam skuteczność aparatu EXS2600 do identyfikacji bakterii o znaczeniu klinicznym [P5]. W chwili publikacji, moja praca była drugą na świecie, która dotyczyła walidacji spektrometru Zybio. W oparciu o uzyskane wyniki i poczynione przeze mnie obserwacje producenci aparatu EXS2600 dokonali drobnych zmian w systemie.

2.2. Matryca i jej skuteczność

W technice MALDI jonizacja próbki zachodzi dzięki zastosowaniu matrycy, która pochłania energię emitowaną przez laser, pośredniczy w przekazywaniu energii do analitu i jego jonizacji. Dzięki temu możliwa jest jonizacja substancji nielotnych, o dużej masie cząsteczkowej

i związków polarnych. W trakcie procesu ablacji, cząsteczki analitu ulegają zazwyczaj jonizacji poprzez protonację (w trybie jonów dodatnich $[M + H]^+$) lub deprotonację (w trybie jonów ujemnych $[M-H]^-$) w wyniku oddziaływań z sąsiednimi cząstkami matrycy. Podczas tego procesu analit może również oddziaływać z innymi jonami obecnymi w próbce np. Na^+ , K^+ tworząc addukty, zwane również jonami quasi-molekularnymi². MALDI jest uważane za metodę miękkiej jonizacji, ponieważ obecność nadmiaru matrycy minimalizuje fragmentację analitów [36]. W trakcie prowadzonych badań stosowałam jonizację w trybie dodatnim [P3-P8].

Klasyczne matryce stosowane w technice MALDI są zazwyczaj organicznymi związkami chemicznymi, często kwasami karboksylowymi, które zawierają w swojej budowie układ sprzężonych wiązań typu π i/lub pierścień aromatyczny, dzięki którym wykazują silną absorpcję promieniowania w zakresie UV. Dodatkowo, związki używane jako matryce powinny cechować się niską reaktywnością chemiczną, łatwą sublimacją, stabilnością w warunkach wysokiej próżni, jednorodnym krystalizowaniem, oraz po desorpcji powinny dostarczać znaczne ilości jonów (protonów) niezbędnych do jonizacji analitu [30], [37].

Dobór właściwej matrycy stanowi jeden z kluczowych etapów w protokole przygotowywania próbki do analizy. Matryca wpływa na uzyskanie satysfakcjonującego stosunku sygnał/szum (S/N), powtarzalność sygnałów i jakość widm masowych. Matryce różnią się w swoich właściwościach chemicznych i fizycznych, co wpływa na skuteczność i selektywność procesu jonizacji. Mimo potencjału wielu związków, jedynie niewiele z nich znajduje rutynowe zastosowanie w technice MALDI. W tabeli 3 przedstawiono przykłady najczęściej stosowanych związków chemicznych wraz z ich zastosowaniem.

Większość komercyjnych matryc umożliwia jonizację analitów tylko w jednym trybie (dodatnim lub ujemnym), co wynika z ich budowy chemicznej. Kwas 2,5-dihydroksybenzoesowy (DHB) i kwas α -cyjano-4-hydroksybenzoesowy (HCCA) są najczęściej stosowane w detekcji jonów dodatnich, ponieważ ich grupa karboksylowa działa jako donator protonów, podczas gdy 9-aminoakrydyna (9-AA) jest preferowana w wykrywaniu jonów ujemnych, ponieważ jego grupa aminowa działa jako akceptor protonów [38]. Polarność matrycy ma również istotny wpływ na zdolność jonizacji różnych analitów. Ogólnie przyjmuje się, że matryce bardziej polarne ułatwiają desorpcję i jonizację cząsteczek o charakterze polarnym, takich jak białka. Z kolei matryce o niższej polarności, na przykład DHB, często są stosowane w analizach substancji o mniejszej polarności, takich jak lipidy [39]. Aby zwiększyć ich skuteczność, czasami stosuje się mieszaniny dwóch lub trzech związków. Powszechnie używana matryca Super-DHB jest mieszaniną DHB i kwasu 2-hydroksy-5-metoksybenzoesowego (stosunek 9:1), co zwiększa wszechstronność jej zastosowania. Jest to matryca dedykowana do analizy związków mniej polarnych, tak więc Khor i wsp. przy jej użyciu otrzymali bogatszy profil lipidowy, co pozwoliło im rozróżnić blisko spokrewnione szczepy

² IUPAC zaleca używanie terminu jony quasi-molekularne (zamiast jony molekularne) wskazując, że obserwowana masa jest równa jonowi cząsteczkowemu zwiększona o masę jonu kationizującego (a nie o masę protonu jak w jonie molekularnym).

Mycobacterium [40]. Jednakże, w przypadku rozróżniania szczepów w oparciu o profil białkowy jej zastosowanie nie jest już tak istotne. W ramach pracy magisterskiej uczestniczyłam w badaniach, które wykazały, że mimo iż matryca Super-DHB generowała nieznacznie więcej sygnałów w porównaniu do HCCA, ich intensywność była niższa, a zdolność rozróżnienia szczepów *S. aureus* mniejsza [41]. Badania te wskazują, że wybór konkretnej matrycy ma wpływ na ogólny kształt profili bakteryjnych w ramach tego samego szczepu. W ramach pracy doktorskiej stosowałam matrycę HCCA [P3, P5, P6, P7] oraz DHB [P4, P7] w celu walidacji i opracowania metod przygotowania spotów, stanowiących know-how niniejszej dysertacji.

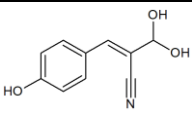
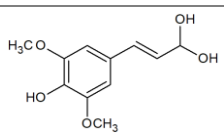
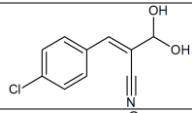
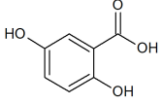
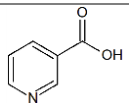
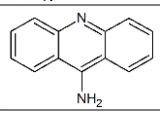
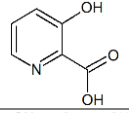
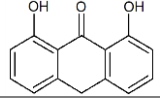
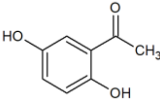
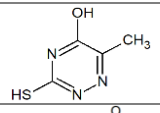
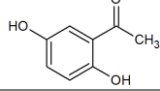
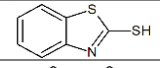
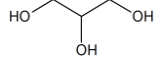
Duże znaczenie odgrywa również sposób krystalizacji matrycy. Obecność małych kryształów (<10 µm) zwiększa czułości detekcji. Natomiast, niejednorodny rozkład kryształów matrycy przyczynia się do powstawania tzw. „sweet spotów” w których to obserwowana jest zwiększona ilość analitu, co wpływa na niską powtarzalność wyników [31]. Obserwuje się to np. w przypadku DHB, którego kryształy przypominają igielki. Aby uniknąć wystąpienia tego zjawiska stosuje się matryce ciekłe, lub ich mieszaniny z matrycą stałą (np. DHB + anilina/pirydyna). Jednakże ich zastosowanie ze względu na niższą wydajność jonizacji, wysokie tło chemiczne (S/N) i zwiększone ryzyko zanieczyszczenia źródła jonów jest ograniczone [39].

Większość stosowanych matryc to związki o niskiej masie cząsteczkowej (LMW, ang. *low-molecular weight compounds*), zazwyczaj < 300 Da. W trakcie analizy MALDI na widmach widoczne są sygnały użytej matrycy, co znacznie utrudnia analizę innych związków LMW występujących w próbce w zakresie m/z poniżej 1000, takich jak metabolity czy lipidy. Matryca silnie absorbuje promieniowanie UV, co przyczynia się do tego, iż na widmach masowych obserwujemy nie tylko jony molekularne ($[MH]^+$), ale również jony powstałe w wyniku jej fragmentacji (np. w wyniku oderwania cząsteczki CO_2 , H_2O), addukty z innymi jonami (głównie Na^+ , K^+), klastry składające się z kilku cząsteczek matrycy ($[2MH]^+$, $[3MH]^+$, $[4MH]^+$) oraz jony będące kombinacjami wyżej wymienionych możliwości [39], [42]. Ilość sygnałów i zakres mas w jakich występują zależy od budowy matrycy i jej właściwości oraz zastosowanego trybu analizy i mocy lasera. Rutynowo stosowana w wielu analizach matryca HCCA, generuje znacznie więcej sygnałów (około 300) w porównaniu do DHB czy SuperDHB (obie < 100) [43].

Jednym z rozwiązań tego problemu jest zastosowanie buforów zawierających związki amonowe (np. bromek cetylotrimetyloamoniowy), których dodatek do matrycy tłumi sygnały pochodzące od matrycy. Rozwiązanie to nie jest idealne, ponieważ związki amonu mogą również tłumić sygnały analitu, jeśli stosunek matrycy/związku amonu/analitu nie zostanie starannie zoptymalizowany [39], [42]. Alternatywne podejście polega na uniknięciu problemu tła matrycy w niskim zakresie m/z poprzez zastosowanie matrycy o wysokiej masie cząsteczkowej (np. związki na bazie porfiryn, tiofenu). Ich zastosowanie natomiast obniża precyzję pomiaru i utrudnia kalibrację widm w niskich zakresach m/z . Dla niektórych LMW

analitów można przeprowadzić również proces derywatyzacji (np. sterole w odpowiadające im estry pikolinyłowe), jednakże wymaga to przeprowadzenia dodatkowych etapów przygotowania, oczyszczania, co oznacza, że jedna z mocnych stron MALDI, jaką jest ograniczona manipulacja próbką, zostaje utracona [39].

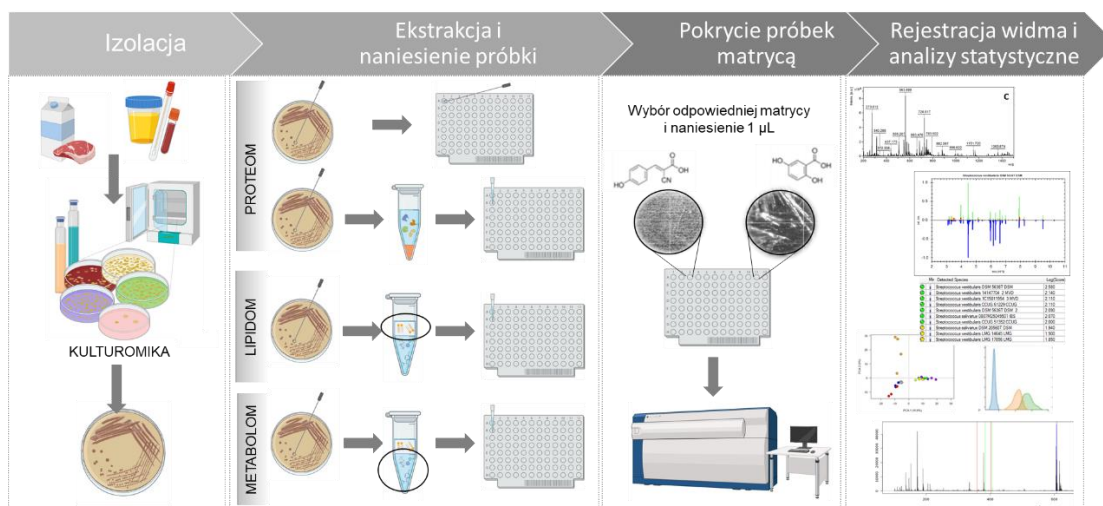
Tabela 3. Przykłady matryc stosowanych w technice MALDI-TOF wraz z zastosowaniem i długością fali przy której wykazują maksimum absorpcji (λ_{max}) i preferowanym trybem jonizacji (+ tryb dodatni; - tryb ujemny) [27], [30], [31], [37], [39].

Związek chemiczny (skrót)	Wzór strukturalny	λ_{max} [nm]	Zastosowanie	Tryb jonizacji
kwasy α -cyjano-4-hydroksycynamonowy (HCCA)		337 353	białka, peptydy, fragmentacja	+
kwasy 4-hydroksy-3,5-dimetoksycynamonowy (kwasy synapinowy, SA)		337 353	duże polipeptydy i białka >10 kDa	+
kwasy 4-chloro- α -cyjanocynamonowy (CICCA)		337 353	lipidy, peptydy, fosfolipidy, fosfopeptydy	+
kwasy 2,5-dihydroksy-benzoesowy (DHB)		337 353	lipidy, peptydy, cząsteczki organiczne, białka, węglowodany	+
kwasy nikotynowy (NA)		266	białka, peptydy	+
9-aminoakrydyna (9-AA)		388	lipidy	—
kwasy 3-hydroksypikolinowy (3-HPA)		337 353	kwasy nukleonowe, oligosacharydy	+
Ditranol (DIT)		337	polimery	+
2,5-dihydroksyacetofenon (2,5-DHAP)		337 353	białka, peptydy	+
6-Aza-2-tiotymina (6-ATT)		337 353	białka, peptydy	—
2,4,6-trihydroksyacetofenon (THAP)		337	oligonukleotydy < 3 kDa	+
2-merkaptobenzotiazol (MBT)		330	lipidy, białka, peptydy	—
glicerol		2,94 μm 2,79 μm	matryca ciekła białka, peptydy	+

Rozwiązaniem, cieszącym się coraz większą popularnością, jest zastąpienie klasycznych matryc nanocząstkami (NP, ang. *nanoparticle*) m.in. metalicznymi, niemetalicznymi (węgiel, krzem), oraz tlenków metali. Te materiały zapewniają bardziej wyraźne tło z ograniczonymi sygnałami interferencyjnymi, co sprawia, że są szczególnie skuteczne w analizie związków o niskiej masie cząsteczkowej. Z tego powodu w pracach [P7, P8] zastosowałam nanocząstki srebra do jonizacji LMW (rozdział 2.6).

2.3. Przygotowanie próbek

Przygotowanie próbek mikroorganizmów do analizy z zastosowaniem techniki MALDI może różnić się w zależności od rodzaju próbki oraz celu analizy (Rysunek 3). W przypadku próbek klinicznych (np. wymaz, mocz, ślina) i środowiskowych (np. woda, żywność, gleba) w pierwszym kroku należy przeprowadzić izolację mikroorganizmów tak aby uzyskać czyste kultury. W większości przypadków do identyfikacji potrzeba około $10^4 - 10^8$ komórek. Następnie, w zależności od celu analizy, przeprowadza się odpowiednie ekstrakcje, takie jak ekstrakcja białek, lipidów, metabolitów itd., w celu wydobycia analitu z komórki mikroorganizmów. Gotową próbkę nanosi się na płytkę MALDI i pokrywa roztworem matrycy, a po wyschnięciu umieszcza w spektrometrze i rejestruje widmo. W przypadku klasycznej identyfikacji mikroorganizmów zarejestrowane widma białkowe są porównywane do widm referencyjnych i na tej podstawie identyfikowane. Poniżej opisano wpływ poszczególnych etapów przygotowania próbki na analizę mikroorganizmów. W trakcie badań opracowałam autorskie rozwiązania mające na celu m.in. przygotowanie próbek klinicznych, dobór warunków hodowlanych, przygotowanie ekstraktów i sposoby naniesienia próbek na płytkę MALDI.



Rysunek 3. Uproszczony proces przygotowania próbek do przeprowadzenia analiz mikroorganizmów z wykorzystaniem techniki MALDI.

Hodowla i izolacja

Pierwszym krokiem umożliwiającym analizę mikrobiomu próbki z wykorzystaniem techniki MALDI jest hodowla i izolacja mikroorganizmów. Od tego etapu zależy czy końcowy wynik będzie odzwierciedlał stan faktyczny. Stwarza to trudności, szczególnie w przypadku próbek o skomplikowanym mikrobiomie, gdzie istnieje duże zróżnicowanie gatunkowe i ilościowe. Mikroorganizmy wymagają bowiem różnych warunków hodowlanych ze względu na ich zróżnicowaną fizjologię, metaboliczną aktywność i preferencje środowiskowe. Stosując jednakowe warunki hodowli w praktyce możliwa jest hodowla tylko tych gatunków, które rosną szybko i są mniej wymagające. W wielu przypadkach klinicznych wybór warunków hodowli nieznanych bakterii często zależy od klinicznych objawów choroby i nie jest łatwy do standaryzacji.

Uwzględniając stwierdzenie Bilena, iż wszystkie mikroorganizmy nadają się do hodowli pod warunkiem zapewnienia odpowiednich warunków, zastosowanie wysokowydajnej izolacji (tzw. kulturomika) umożliwia uzyskanie bogatszej i bardziej reprezentatywnej populacji mikroorganizmów z danego środowiska [44]. Podejście to polega na zwielokrotnieniu warunków hodowlanych poprzez: (i) zastosowanie podłoża o odmiennym pH i różnych składnikach odżywczych, (ii) zmianę temperatury inkubacji, (iii) zmianę wilgotności, (iv) stężenie tlenu lub dwutlenku węgla, (v) czas inkubacji. W praktyce umiejętne zastosowanie kulturomiki stwarza możliwość pełniejszego zrozumienia składu mikrobiomu w badanej próbce, co ma istotne znaczenie w dziedzinie mikrobiologii środowiskowej, medycznej czy przemysłowej.

Wraz z rozwojem techniki MALDI, stosowanie podejścia kulturomicznego do analizy mikrobiomu nabrało sensu i jest coraz częściej stosowane w celu uzyskania szybkiej i skutecznej identyfikacji mikroorganizmów. Ze względu na kluczową rolę mikroflory jelitowej człowieka w zdrowiu i równowadze organizmu, większość badań skupia się na tym obszarze [45]. W publikacji [P3] przedstawiłam skuteczność połączenia kulturomiki i MALDI w przypadku identyfikacji mikrobiomu towarzyszącemu infekcjom stopy cukrzycowej. Zastosowanie tego podejścia pozwoliło mi zidentyfikować nie tylko gatunki najczęściej spotykane w tego typu infekcjach (m.in. *S. aureus*, *P. aeruginosa*), ale również szczepy wolno rosnące o wysokich wymaganiach pokarmowych, np. *Helcococcus kunzii*. Niniejsza praca dowodzi, iż wykorzystanie kulturomiki może stanowić użyteczne narzędzie do standaryzacji procesów hodowli i izolacji mikroorganizmów w laboratoriach mikrobiologicznych w zależności od pochodzenia próbki. Na podstawie wyników eksperymentu, w kolejnych badaniach zmodyfikowałam warunki hodowli mikroorganizmów, co pozwoliło na wykrycie większej różnorodności gatunków w analizowanych próbkach [P6].

Niektóre warunki zastosowane w trakcie hodowli mikroorganizmów mogą znacząco wpływać na profil białkowy/lipidowy co może utrudniać analizy i powodować rozbieżności

w wynikach. W kontekście podłoży, niektóre składniki podłoża mogą indukować lub tłumić syntezę określonych białek/lipidów przez mikroorganizmy np. hemoglobina obecna w pożywkach wzbogacanych krwią przyczynia się do występowania dodatkowych sygnałów białkowych na widmach masowych. Zastosowanie podłoży nieselektywnych znacząco wpływa na jakość otrzymywanych widm masowych i umożliwia dokładniejszą identyfikację (w oparciu o profil białkowy) [46]. Warunki wzrostu wpływają również na możliwość rozróżniania szczepów blisko spokrewnionych. Złoch i wsp. uzyskali wyższą zdolność dyskryminacji szczepów *S. aureus* uzyskali przy zastosowaniu podłoża wzbogacanego krwią owczą w stosunku do jego wersji podstawowej [41]. Warunki te najprawdopodobniej uruchomiły u badanych szczepów bakterii nowe szlaki metaboliczne, prowadząc do pojawienia się dodatkowych różnicujących sygnałów. Co więcej, bakterie hodowane na podłożach stałych są bardziej heterogeniczne niż na podłożach płynnych. Niejednorodność związana jest z faktem, że kolonie składają się z komórek o różnym wieku (starsze w środku, młodsze na obwodzie kolonii). I odwrotnie, pożywki płynne mają bardziej jednorodne populacje komórek, zsynchronizowane pod względem wzrostu [47]. W trakcie wzrostu bakterie wykazują zmiany morfologiczne i zmiany w składzie ściany komórkowej, które mogą przekładać się na uzyskane profile. Zazwyczaj wraz ze wzrostem czasu hodowli obserwuje się obniżenie jakości widma i mniejszą odtwarzalność sygnałów. Długość czasu hodowli odgrywa ważną rolę w przypadku identyfikacji bakterii sporujących, tj. *Bacillus*, *Clostridium*, których obecność w żywności stanowi zagrożenie dla zdrowia. Janiszewska i wsp. wykazali, że w przypadku szczepów *Bacillus* poprawna identyfikacja była możliwa do 5 godzin inkubacji [48]. Po upływie tego czasu na widmach masowych pojawiły się sygnały pochodzące od wytworzonych sporów, co obniżyło zdolność do poprawnej identyfikacji gatunkowej bakterii. Istotnym czynnikiem jest również temperatura w której prowadzona była hodowla. Pod tym względem bakterie można podzielić na trzy rodzaje: psychrofilne (optymalna temperatura wzrostu $< 20^{\circ}\text{C}$), mezofilne ($20 - 45^{\circ}\text{C}$) i termofilne ($> 45^{\circ}\text{C}$). Reakcją bakterii na zmieniające się warunki środowiskowe wywołane stresem temperaturowym jest dostosowanie płynności błon komórkowych poprzez zmianę składu fosfolipidów, co również wpływa na rejestrowane profile białkowe/lipidowe [47].

Analiza białek

Metoda klasycznej identyfikacji MALDI-TOF MS opiera się na analizie profili białkowych mikroorganizmów, zazwyczaj rejestrowanych w zakresie 2 000 – 20 000 Da. W skład tych profili wchodzi występujące w dużych ilościach białka rybosomalne oraz białka metabolizmu podstawowego. Ponieważ skład białek różni się w zależności od rodzaju i gatunku bakterii, powstają unikalne widma, które można porównać z profilami białkowymi zawartymi w bibliotece widm referencyjnych. Dzięki temu możliwe jest ustalenie taksonomicznej przynależności badanego mikroorganizmu do rodzaju i gatunku, a w niektórych przypadkach nawet szczepu [49].

W celu uzyskania jak najwyższego prawdopodobieństwa dopasowania widma istotne jest aby stosowana procedura ekstrakcji była zbliżona do tej, która była stosowana w trakcie tworzenia bazy referencyjnej. Najczęściej producenci udostępniają użytkownikom różne metody ekstrakcji, wśród których można wymienić: bezpośrednie naniesienie próbki, ekstrakcję na płytce i pełną procedurę ekstrakcji. Pierwszy sposób jest najprostszy i najszybszy. Polega na rozsmarowaniu pojedynczej kolonii z płytki agarowej bezpośrednio na spot na płytce i pokrycie matrycą. Metoda ta umożliwia identyfikację 90 – 95% mikroorganizmów i ze względu na prostotę jest najczęściej stosowana. W drugiej metodzie po nałożeniu na spot biomasy bakteryjnej pokrywa się ją 70% kwasem mrówkowym (FA), który prowadzi do zniszczenia ściany komórkowej co ułatwia ekstrakcję białek. Podejście to pozwala na uzyskanie lepszej jakości widm masowych, szczególnie w przypadku gatunków Gram-dodatnich oraz drożdży. Ostatnia metoda polega na ekstrakcji białek z komórek bakteryjnych przy użyciu różnego rodzaju rozpuszczalników i jest zalecana, gdy wymagane jest uzyskanie wyższej jakości widm. W tym celu najczęściej (wg zaleceń producentów) stosuje się wodę, etanol, FA i acetonitryl. Po przygotowaniu ekstrakt nakłada się na spot i pokrywa matrycą [47]. Jak wspomniano wcześniej matryca wpływa na jonizację, tak więc w celu identyfikacji mikroorganizmów standardowo zaleca się stosować HCCA. Wybór odpowiedniej metody ekstrakcji zależy od budowy analizowanych mikroorganizmów. Bakterie Gram-ujemne są zazwyczaj identyfikowane z dużym prawdopodobieństwem za pomocą metody bezpośredniej. Z kolei, grubsza ściana komórkowa u bakterii Gram-dodatnich nadaje im zwiększoną oporność na lizę. Z tego powodu w ich przypadku zaleca się przeprowadzanie pełnej procedury ekstrakcji.

W trakcie badań posługiwałam się podejściem proteomicznym w celu identyfikacji mikroorganizmów (głównie bakterii) z próbek klinicznych: wymazy stopy cukrzycowej [P3, P6] i mocz pacjentów cierpiących na raka prostaty [P5].

Analiza lipidów

Lipidy są kluczowymi składnikami komórek bakteryjnych, pełniąc istotną rolę w metabolizmie, przechowywaniu energii oraz sygnalizacji komórkowej. Są one głównym składnikiem dwuwarstwy fosfolipidowej, która stanowi strukturę błony komórkowej bakterii. Wszechstronność funkcji lipidów w komórkach wynika z ich specyficznej budowy i obejmuje m.in. różnice w długości i rodzaju wiązań w budujących je kwasach tłuszczowych oraz rodzaju związku tworzącego tzw. głowę m.in. glicerol, glicerol + grupa fosforanowa, reszty cukrowe [50]. Ta zróżnicowana struktura lipidów sprawia, że bakterie posiadają charakterystyczne dla danego rodzaju klasy lipidów. Analiza profili lipidowych mikroorganizmów przez długi czas była możliwa przy zastosowaniu chromatografii gazowej, jednakże konieczność derywatyzacji lipidów była uciążliwa. Wraz z rozwojem techniki MALDI możliwe stało się szybkie i precyzyjne określanie profili lipidowych. Oprócz lipidów występujących powszechnie we wszystkich komórkach bakteryjnych, istotne jest zdefiniowanie lipidów specyficznych dla

konkretnego gatunku, które wykazują znaczną zmienność, co może ułatwić identyfikację. Bakterie Gram-ujemne charakteryzują się obecnością lipopolisacharydu, który składa się z lipidu A, rdzenia oligosacharydowego i antygeny O. Struktura lipidu A lub antygeny O może być stosowana jako biomarker do identyfikacji bakterii. Z kolei bakterie Gram-dodatnie zawierają charakterystyczne glikolipidy, glukolipidy i kwas lipotejchowy [51]. Lipid A może być również obiecującym narzędziem do wykrywania oporności na kolistynę (patrz rozdział 2.5).

Dzięki temu zróżnicowaniu drobnoustrojów lipidomika stanowi komplementarną metodę identyfikacji drobnoustrojów w technice MALDI. W publikacji [P4] przeprowadziłam szczegółową charakterystykę profili lipidowych 39 izolatów bakterii uzyskanych z próbek ran osób cierpiących na infekcje stopy cukrzycowej. Wyniki badań wykazały istotne różnice w profilach lipidowych analizowanych gatunków. Różnice te stanowią podstawę dla przyszłych badań mających na celu rozwinięcie bibliotek lipidów mikroorganizmów, co przyczyni się do doskonalenia procesów identyfikacji bakteryjnej.

Istnieją trzy zwalidowane metody ekstrakcji lipidów z hodowli komórkowych, w tym mikroorganizmów: metoda Folcha, metoda Bligha-Dyera, oraz metoda Matyasha. Najpowszechniejszą metodą jest metoda Folcha, która wykorzystuje dwufazowy układ rozpuszczalników składający się z chloroformu/metanolu/wody w stosunku objętościowym 8:4:3 (v/v/v). Metodę tę cechuje duża czułość w ekstrakcji fosfolipidów i lipidów obojętnych, m.in. fosfatydylocholiny, sfingomieliny, fosfatydyloetanolamina i triacylogliceroli. W przypadku metody Bligha-Dyera stosuje się ten sam układ rozpuszczalników, ale w stosunku 2:2:1,8 (v/v/v), co redukuje ilość wykorzystywanego chloroformu. Konieczność zastosowania chloroformu, będącego związkiem wysoce toksycznym i kancerogennym, czyni te metody nieodpowiednimi do stosowania na dużą skalę. Zastosowanie w metodzie Matyasha nietoksycznego eteru metylo-tert-butyloвого (MTBE) stanowi alternatywę dla chloroformu. Stosunek objętościowy MTBE/metanol/woda wynosi odpowiednio: 10:3:2,5 (v/v/v). Ponadto, MTBE jest bardziej polarny, przez co w większym stopniu miesza się z wodą bez separacji faz. W konsekwencji faza organiczna zawiera więcej wody i tym samym poprawia skuteczność ekstrakcji dla poszczególnych klas lipidów o charakterze kwasowym. W każdej z tych metod uzyskana faza wodna zawiera związki rozpuszczalne w wodzie tj. cukry, aminokwasy, witaminy, które mogą być użyte do analizy metabolomu [50], [52]. Zastosowanie tych samych odczynników w metodzie Folcha i Bligha-Dyera sprawia, iż ekstrakty lipidowe mają zbliżony skład. Wraz z Arendowskim i współ., porównaliśmy zastosowanie wymienionych powyżej trzech metod do ekstrakcji lipidów z komórek bakteryjnych czterech gatunków bakterii (izolaty kliniczne: *S. epidermidis*, *E. faecalis*, *E. coli*, *P. mirabilis*) [53]. Wyniki badań wykazały, iż najwięcej sygnałów na widmach lipidowych uzyskaliśmy dla ekstraktów otrzymanych metodą Bligha-Dyera, zarówno w trybie jonów dodatnich jak i ujemnych.

W analizach lipidomicznych wchodzących w skład niniejszej dysertacji stosowałam metodę Folcha [P4,P7] do ekstrakcji lipidów z komórek izolatów bakteryjnych ze stopy cukrzycowej.

2.4. Referencyjne bazy danych i ich ograniczenia

Na światowym rynku dostępnych jest coraz więcej producentów, którzy wraz ze sprzętem dostarczają swoje oprogramowanie oraz bazy danych do identyfikacji mikroorganizmów w oparciu o profile białkowe, m.in.: BioTyper (Bruker Daltonics, Niemcy) [54], [55], Vitek MS (bioMérieux, Francja) [56], [57], Axima (Shimadzu, Japonia) [58], Andromas (Andromas SAS, Francja) [59], MicroIDSys (ASTA, Korea Południowa) [60], MicrobeLynx (Waters Corporation, USA) [61], EXS (Zybio, Chiny) [62]. Ich skuteczność w identyfikacji bakterii może różnić się w zależności od specyfikacji sprzętu, oprogramowania, algorytmów identyfikacji oraz zasobów baz danych i częstotliwości ich aktualizacji. Wszystkie systemy są przeznaczone do celów badawczych (RUO, *Research Use Only*), a jedynie dwa pierwsze, BioTyper i Vitek, posiadają certyfikat IVD (*In Vitro Diagnostic*) i mogą być stosowane do celów diagnostycznych. Oba systemy cieszą się znacznym zainteresowaniem wśród użytkowników, co przekłada się na obfitość dostępnych prac porównawczych [54], [57], [63].

Porównanie widm następuje w oparciu o wartości m/z oraz intensywności sygnałów, a każdy producent ma własny algorytm tworzenia profilu białkowego. W przypadku systemu BioTyper widma w bazie danych tworzone są poprzez wielokrotny pomiar profilu danego szczepu i określenie sygnałów wchodzących w skład tzw. głównych profili widmowych (MSP, ang. *main spectrum profile*) [64]. Program wyraża podobieństwo widm jako wartość $\log(score)$ w zakresie 0,000 – 3,000, gdzie wartość wskaźnika: >2,300 wskazuje na wiarygodną identyfikację do poziomu gatunku; 2,000 – 2,299 wskazuje na wiarygodną identyfikację do poziomu rodzaju i prawdopodobną identyfikację do gatunku; 1,700 – 1,999 wskazuje na prawdopodobną identyfikację do poziomu rodzaju; <1,700 brak identyfikacji. W przypadku niektórych szczepów (np. *Mycobacteria* [65]) możliwe jest stosowanie niższych progów identyfikacji. Natomiast system Vitek, tworząc bazę widm, wykorzystuje algorytm oparty na podziale widma na 13 000 zdefiniowanych przedziałów, dla których określana jest ważność. Nieznane widmo próbki poddawane jest temu samemu procesowi i na tej podstawie dopasowywane do tzw. SuperSpectra, uzyskiwanego z co najmniej 15 widm referencyjnych. Wynik identyfikacji wyrażany jest jako wartość ufności w zakresie od 0 - 100%, gdzie: 99,9% – wskazuje na dopasowanie idealne, 60% - 99,8% – dopasowanie dobre, natomiast wartości < 60% uważa się za brak identyfikacji [64], [66].

Tworzenie bazy danych determinuje możliwości jej późniejszego zastosowania oraz skuteczność. Kluczowym czynnikiem jest obszerność bazy danych nie tylko pod kątem ilości szczepów, ale również ich różnorodności. Choć większość systemów umożliwia uzyskanie wysokiego prawdopodobieństwa dopasowania dla szerokiego zakresu mikroorganizmów, niektóre z nich mogą być bardziej precyzyjne dla konkretnych gatunków. Literatura wskazuje,

iz BioTyper z powodu dużej ilości różnych gatunków w bazie lepiej sprawdza się w przypadku większości bakterii i drożdży, a Vitek w przypadku *Mycobacteria*, *Actinomycetes* i grzybów strzępkowych [67]. Baza Vitek zbudowana jest w oparciu o kliniczne szczepy pochodzące z różnych regionów świata i hodowanych w różnych warunkach (tj. pożywki, temperatura), co może w niektórych przypadkach przekładać się na jej korzyść.

Zastosowanie proteomicznego podejścia w technice MALDI początkowo skupiało się na stworzeniu gotowego rozwiązania mającego usprawnić prace laboratoriów klinicznych. Z tego powodu w skład baz wchodzi głównie szczepy kliniczne, które najczęściej wywołują choroby zakaźne. Sprawia to, iż wiele bakterii środowiskowych (gatunki należące do m.in. *Bacillus*, *Brachybacterium*, *Legionella*, *Pseudomonas*, *Vibrio*) jest błędnie lub niepoprawnie identyfikowanych z powodu braku lub niewystarczającej ilości referencyjnych widm w bazie [68]. Ponadto, bakterie występujące w odmiennych środowiskach charakteryzują się większą różnorodnością i mogą posiadać różne cechy fenotypowe, które mogą wpływać na ich identyfikację. Niestepski i in. zauważyli znaczny wpływ środowiska na poprawność identyfikacji bakterii z grupy *Bacteroides fragilis* [69]. 100% poprawność identyfikacji uzyskali tylko dla szczepów wyizolowanych z kału ludzkiego i szczurzego. Szczepy wyizolowane ze ścieków oczyszczonych i szpitalnych uzyskały niższą poprawność, odpowiednio 40% i 20%. Wiele systemów umożliwia użytkownikom tworzenie własnych baz danych, co pozwala częściowo przezwyciężyć problem braku odpowiedniego dopasowania, jednakże stworzenie własnej bazy danych wiąże się z nakładem czasu i środków finansowych. Pinar-Mendez i wsp. utworzyli bazę do analizy wody pitnej, zawierającą 319 różnych szczepów bakterii [70]. Zastosowanie tej biblioteki znacząco zwiększyło skuteczność identyfikacji bakterii środowiskowych w wodzie do 76,2%, w porównaniu z bazą BioTyper, która osiągnęła wynik 54,8%. Fergusson i wsp. również tworząc własną bazę zwiększyli zdolność identyfikacji szczepów *Burkholderia*, *Caballeronia* i *Paraburkholderia* [71]. Niektóre bazy danych tworzone przez użytkowników są ogólnodostępne np. BacteriaMS³, Spectra bank⁴ co znacząco podnosi dokładność identyfikacji szczególnie szczepów środowiskowych.

W przypadku braku prawidłowego widma system albo nie zidentyfikuje organizmu, albo wskaże błędny wynik. Dokładna, chociaż w rzeczywistości niepoprawna identyfikacja, może stanowić wyzwanie, zwłaszcza w kontekście blisko spokrewnionych organizmów, które mogą charakteryzować się różnymi poziomami ryzyka oraz zróżnicowanymi protokołami bezpieczeństwa w warunkach laboratoryjnych i diagnostycznych. Problem ten dotyczy także gatunków pomiędzy którymi występuje wysokie podobieństwo proteomiczne tj. *Escherichia coli* i *Shigella spp.* [72], wielu gatunków w obrębie paciorkowców (m. in. *Streptococcus anginosus*, *S. bovis*, *S. mitis*, *S. mutans*, *S. salivarius*, *S. sanguinis*, *S. pneumoniae*) [73], *Enterobacter cloacae* complex [74] oraz grupy *Bacillus pumillis* [75]. Producenci baz danych

³ <http://www.bacteriams.net>

⁴ <http://www.spectrabank.org>

często umożliwiają użytkownikom wykorzystywanie zaawansowanych narzędzi, które służą do dogłębnej analizy i porównywania widm masowych. Takie narzędzia obejmują między innymi tworzenie dendrogramów, które pozwalają na wizualizację podobieństw lub różnic pomiędzy badanymi próbkami, generowanie macierzy korelacji, dzięki którym można ocenić wzajemne zależności między różnymi zmiennymi, przeprowadzenie analizy skupień, która umożliwia grupowanie danych na podstawie ich podobieństwa, oraz realizowanie analiz wielowymiarowych, pozwalających na badanie złożonych wzorców i zależności w danych wielowymiarowych. Korzystanie z tych zaawansowanych funkcji, oferowanych przez firmę Bruker, stanowiło ważny element mojej pracy podczas prowadzenia badań naukowych.

Bruker posiada również dodatkowy program ClinProTools, który zwiększa precyzję identyfikacji niektórych gatunków należących do *Streptococcus* [67]. Niemniej jednak, te rozwiązania często wymagają doświadczenia od użytkowników i są specyficzne dla określonych systemów. W literaturze możemy znaleźć doniesienia o możliwość rozróżnienia np. *Shigella spp.* od *E. coli* przy użyciu klasycznej techniki MALDI ze specyficzną biblioteką referencyjną [76] lub algorytmem interpretacji sygnałów [77], ale metody te nigdy nie zostały wdrożone w rutynowych analizach. Ponadto, wiele prac wskazuje, iż zastosowanie algorytmów opartych o uczenie maszynowe (ang. *machine learning*) ma duży potencjał w poprawie dokładności i efektywności identyfikacji szczepów charakteryzujących się wysokim stopniem podobieństwa [72], [78].

Wysokie pokrewieństwo proteomiczne jest wynikiem wysokiego podobieństwa genomowego, co oznacza, że zastosowanie dodatkowych metod, takich jak techniki biologii molekularnej, w tych przypadkach jest również ograniczone. Analiza lipidów, które nie są bezpośrednio determinowane przez sekwencje DNA, lecz są produkowane w procesach metabolicznych organizmu, przedstawia się jako obiecujące podejście w badaniach. Produkty te, będące wynikiem złożonych szlaków metabolicznych, charakteryzują się bogatą różnorodnością funkcjonalną i strukturalną. Ta właściwość sprawia, że lipidy mogą wykazywać znacząco większą zmienność między poszczególnymi osobnikami tego samego gatunku bakterii w porównaniu do białek, które są ściślej kontrolowane przez geny. Taka zmienność lipidów może dostarczać unikalnych informacji o stanach fizjologicznych, adaptacjach do środowiska czy nawet interakcjach międzygatunkowych, czyniąc je kluczowymi wskaźnikami w mikrobiologicznych badaniach diagnostycznych i ewolucyjnych. Właśnie z tego względu rozwijałam w swojej pracy doktorskiej badania lipidomiczne, mając na celu głębsze zrozumienie mechanizmów regulacji i funkcji lipidów w różnych kontekstach biologicznych. Wiele prac wskazuje, iż zastosowanie podejścia lipidomicznego w analizach MALDI umożliwia nawet rozróżnienie bliskospokrewnionych bakterii. Złoch i wsp., z naszej grupy badawczej w ICNT, rozróżnili paciorkowce ślinowe (*S. salivarius* i *S. vestibularis*) [25], Pizzato i wsp. szczepy *E. coli* i *Shigella* [78], a AlMasoud i wsp. szczepy *Bacillus* i *Brevibacillus* [79]. Aktualnie jest brak lipidowych bazy danych służących do identyfikacji mikroorganizmów porównywalnych pod względem wielkości i różnorodności z bibliotekami

białkowymi. Autorzy prac tworzą bazy danych, które wykorzystują tylko do własnych potrzeb. Profile lipidowe są znacznie bardziej zależne od warunków środowiska mikrobiologicznego niż białkowe, dlatego ich zastosowanie bez ujednolicenia procesu hodowli, przygotowywania próbek i warunków analizy jest niemożliwe. Najpopularniejszą bazą danych wykorzystywaną w analizach lipidomicznych mikroorganizmów jest LIPID MAPS⁵, który dostarcza kompleksowych informacji na temat struktury i funkcji pojedynczych lipidów, w tym ich klasyfikacji, właściwości fizykochemicznych i szlaków metabolicznych.

Przedstawione dane podkreślają istotne znaczenie rozwijania przez producentów systemów identyfikacyjnych w celu doskonalenia technologii MALDI. Nasz zespół współpracuje z przedstawicielami firmy Bruker (Brema, Niemcy) oraz Zybio (Chiny) przeprowadzając analizy, których wyniki umożliwiają rozwój baz danych i poprawę sprawności systemów. W publikacji [P5] porównałam skuteczność dwóch różnych systemów MALDI (Bruker BioTyper i Zybio EXS2600) w szybkiej identyfikacji izolatów bakteryjnych wyizolowanych z próbek moczu pacjentów cierpiących na raka prostaty. Uzyskane wyniki potwierdziły, że obydwie platformy są równie dobrym wyborem pod względem wydajności i szybkości analitycznej w rutynowych procedurach diagnostyki mikrobiologicznej. Dzięki zastosowaniu obu systemów zwiększyłam skuteczność identyfikacji izolatów – tylko 34 z 1979 nie zostały zidentyfikowane przez oba systemy. 8,5% analizowanych próbek zidentyfikowałam tylko przez jeden system, co podkreśla konieczność ciągłego rozwoju baz danych i ich wzbogacania, szczególnie o szczepy rzadziej izolowane w próbkach klinicznych należące do rodziny *Actinomycetia* oraz *Bacilli* tj. *Bacillus*, *Brevibacillus*, *Brachybacterium*, *Lactococcus*. Dzięki temu możliwe będzie wykorzystanie techniki MALDI również do rutynowych analiz próbek środowiskowych, które wykazują większą różnorodność. Badania tego rodzaju próbek są istotne m.in. ze względu na potencjał probiotyczny wykazywany przez wiele szczepów bakteryjnych, który jest pożądanym ze względu na korzyści zdrowotne i ekologiczne, jakie mogą przynieść dla ludzi i środowiska. Podczas badań doktoranckich aktywnie uczestniczyłam w poszukiwaniach szczepów o potencjale probiotycznym [80], [81]. Identyfikacja tych szczepów za pomocą techniki MALDI stanowiła wyzwanie ze względu na ograniczoną różnorodność dostępnych danych w bazach.

2.5. Analiza antybiotykooporności

Uniwersalność MALDI sprzyja rozwojowi coraz większej liczby aplikacji opartych na tej technologii, z których jednymi z najbardziej obiecujących do zastosowania w praktyce klinicznej są metody oceny wrażliwości na środki przeciwdrobnoustrojowe (AST, ang. *antimicrobial susceptibility testing*). Opracowano różne metody analizy AST, jednakże

⁵ <https://www.lipidmaps.org/>

większość z nich wymaga standaryzacji i dodatkowych badań przed wprowadzeniem ich do rutynowego zastosowania w praktyce klinicznej. Najistotniejsze omówiono poniżej.

Identyfikacja biomarkerów związanych z opornością

Jednym z pierwszych doniesień w literaturze o możliwości zastosowania techniki MALDI w analizie antybiotykooporności była praca Edwards-Jonesa z 2000 roku, w której to porównał widma masowe całych komórek siedmiu szczepów referencyjnych MSSA i siedmiu izolatów klinicznych MRSA w zakresie m/z 500 – 10 000 [82]. W dolnym zakresie mas autorzy zidentyfikowali specyficzne grupy biomarkerów, co pozwoliło im prawidłowo odróżnić szczepy wrażliwe na metycylinę od opornych. Kolejne badania wykazały, iż szczepy MRSA wytwarzają toksynę białkową (PSM-mec), która jest wykrywalna metodą MALDI jako sygnał m/z $2415 \pm 2,00$. Peptyd ten nie powoduje oporności na metycylinę, ale jego ekspresja jest powiązana z opornością, co pozwala przewidzieć nosicielstwo genu *mecA*⁶ u szczepów *S. aureus* [83]. Bruker opracował oprogramowanie (MBT subtyping module), które bazując na tej zależności dostarcza pośredniego dowodu oporności na metycylinę. Chociaż obecność PSM-mec wiąże się z opornością na metycylinę również u gronkowców koagulazo-ujemnych (np. *S. epidermidis*, *S. haemolyticus*), większość z nich go nie wytwarza, co sprawia, że użyteczność tego systemu jest ograniczona głównie do szczepów *S. aureus* [84], [85].

Podejście polegające na poszukiwaniu na widmach sygnałów (m/z) pochodzących od biomarkerów oporności można wykorzystać również do identyfikacji plazmidów niosących różne geny (głównie β -laktamaz). Dla szczepów *K. pneumoniae* wytwarzających karbapenemazy (KPC) obserwuje się sygnał przy m/z 11 109 [86], a dla *A. baumannii* produkujących cefalosporiny przy m/z ~40 279 [87]. Z kolei szczepy *Bacteroides fragilis* oporne na karbapenemy od wrażliwych można rozróżnić w oparciu o przesunięcia sygnałów w przedziale 4000 – 5500 Da [88]. Nakano i wsp. wyznaczyli na widmach masowych pięć sygnałów (m/z : 3184, 5702, 7415, 7445, 12 662 Da) umożliwiających rozróżnienie szczepów *Enterococcus faecium* opornych od wrażliwych na wankomycynę (VRE) [89].

Zaletą podejścia opierającego się na poszukiwaniu biomarkerów jest brak konieczności przygotowywania dodatkowych próbek. Próbkę przygotowaną do identyfikacji mikroorganizmów (ekstrakty białkowe) można wykorzystać do detekcji biomarkerów szczepów opornych na antybiotyki. Jednakże, opisane wyżej metody są specyficzne dla konkretnych mechanizmów oporności i zależne od gatunku bakterii.

Analiza lipidu A

Lipid A wchodzi w skład lipopolisacharydu (LPS), który jest kluczowym elementem błon zewnętrznych bakterii Gram-ujemnych. Struktura lipidu A jest skomplikowana i zróżnicowana, a jej skład może się różnić w zależności od gatunku bakterii. U większości bakterii Gram-

⁶ koduje białko PBP2a (białko wiążące penicylinę), które zapewnia szczepom *S. aureus* oporności na antybiotyki β -laktamowe, takie jak penicyliny i cefalosporyny.

ujemnych oporność na kolistynę⁷ wynika z modyfikacji chemicznych lipidu A, za pośrednictwem mutacji kodowanych chromosomowo lub aktywności enzymów oporności na kolistynę. W wyniku tego do natywnej formy lipidu A dołącza się ugrupowanie 4-amino-4-deoksy-L-arabinozy (L-Ara4N) i/lub fosfoetanolaminy (PEtN), co powoduje wzrost masy lipidu o 131 Da dla L-Ara4N i 123 Da dla PEtN. Widma zarejestrowane dla szczepów wrażliwych posiadają sygnał pochodzący jedynie od natywnej formy lipidu A, a dla szczepów opornych obserwuje się również sygnały o zwiększonej masie świadczące o zajściu modyfikacji lipidu. Na podstawie tych sygnałów i ich intensywności oprogramowanie oblicza współczynnik oporności, który wskazuje czy badany szczep wykazuje oporność [90].

Metoda ta jest szybka i dokładna, jednakże jej zastosowanie ogranicza się do analizy tylko tych gatunków bakterii dla których budowa i masa cząsteczkowa lipidu została dokładnie scharakteryzowana m.in. *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *M. morganii*, *A. baumannii*, *Salmonella enterica*. Kolejne ograniczenie stanowią wymagania aparaturowe. Do przeprowadzenia analiz wymagane jest zastosowanie trybu jonizacji ujemnej, który do niedawna nie był dostępny w instrumentach dedykowanych dla rutynowych laboratoriów klinicznych [90], [91].

Wykrywanie hydrolizy antybiotyków β -laktamowych

Jednym z najbardziej powszechnych zastosowań techniki MALDI w detekcji antybiotykooporności jest wykrywanie aktywności β -laktamaz, na podstawie pomiaru produktów hydrolizy cząsteczek antybiotyku w supernatancie z nad płynnego podłoża hodowlanego zawierającego szczepy bakterii inkubowane w obecności badanego antybiotyku. Hydrolizie pierścienia β -laktamowego za pośrednictwem β -laktamazy towarzyszy przyłączenie cząsteczki wody i reakcja ta prowadzi do zmiany masy antybiotyku o 18 Da. Zhydrolizowana cząsteczka antybiotyku może ulec dalszym reakcjom nieenzymatycznym, do których głównie należy odłączenie grupy karboksylowej prowadzące w efekcie do zmiany masy cząsteczkowej o -44 Da. Wszystkie produkty zachodzących reakcji można łatwo wykryć metodą MALDI. Procedura przygotowania próbki wymaga zawieszenia komórek bakteryjnych w buforze z antybiotykiem i przeprowadzenia inkubacji w temperaturze 37°C. Czas inkubacji zależny jest od zastosowanego antybiotyku i waha się od 30 minut dla ceftriaksonu i imipenemu do nawet 6 godzin dla ceftazydymu. Po inkubacji supernatant nakłada się na płytkę i pokrywa matrycą. W ten sam sposób należy przygotować kontrole dodatnią (wzorcowy szczep oporny) i ujemną (wzorcowy szczep wrażliwy). Ocena oporności następuje w oparciu o porównanie intensywności sygnałów pochodzących od formy natywnej i form zhydrolizowanych antybiotyku oraz ich znormalizowanie względem różnicy zmierzonej dla kontroli pozytywnej i negatywnej – parametr logRQ [92]. Ze względu na małe natężenia sygnału w przypadku zhydrolizowanych cząsteczek, analiza z wykorzystaniem zestawów IVD komercyjnie

⁷ Kolistyna jest antybiotykiem ostatniej szansy, stosowanym w leczeniu trudnych infekcji bakteryjnych charakteryzujących się wielolekoopornością.

dostępnych i stosowanych w rutynowych laboratoriach diagnostycznych wymaga zastosowania standardu matrycy dostarczanego przez producenta wraz z matrycą. Wówczas podczas wyznaczania parametru logRQ zamiast zsumowanego natężenia cząsteczek zhydrolizowanych wykorzystuje się intensywność standardu wewnętrznego.

Metodologię tę można zastosować do badania wrażliwości bakterii Gram-ujemnych (m.in. *Enterobacteriaceae*, *Pseudomonas* spp., *Acinetobacter* spp.) na różne klinicznie stosowane karbapenemy (tj. meropenem, imipenem, ertapenem) i cefalosporyny (tj. cefotaksym, ceftazydym, ceftriakson). Czułość metody wynosi 90 – 95%, przy czym najwyższą obserwuje się dla antybiotyków, które wymagają krótkiego czasu inkubacji. Antybiotyki te należą do związków LMW, co oznacza, że interpretacja ich sygnałów przy zastosowaniu HCCA jako matrycy może być kłopotliwa. Obserwuje się to szczególnie w przypadku meropenemu i imipenemu, dla których wymagane jest zastosowanie odmiennych procedur. W przypadku meropenemu w celu ograniczenia supresji sygnałów zaleca się stosowanie jako matrycy DHB. Jednakże niejednorodna krystalizacji DHB utrudnia automatyzację procesu i wdrożenie w rutynowych zastosowaniach. W przypadku imipenemu częstym zjawiskiem jest brak detekcji sygnałów pochodzących od cząsteczek zhydrolizowanych. Rozwiązaniem tego problemu jest dodanie do matrycy wewnętrznego standardu, jak to zostało opisane we wcześniejszym paragrafie [93].

Zgodnie z raportem opublikowanym w 2022 roku przez Światową Organizację Zdrowia, w Polsce odnotowuje się wzrost liczby zakażeń szczepami wykazującymi lekooporność, głównie na penicyliny i antybiotyki β -laktamowe, zwłaszcza karbapenemy i cefalosporyny trzeciej generacji (takie jak cefotaksym, ceftazydym, ceftriakson) [94]. W oparciu o te informacje w publikacji [P6] technikę MALDI zastosowałam do szybkiej detekcji oporności na wyżej wymienione klasy antybiotyków. Przedmiot badań stanowiły szczepy, które wyizolowałam z wymazów z ran osób cierpiących na infekcje stopy cukrzycowej m.in. *E. coli*, *P. aeruginosa*, *P. mirabilis*. Walidację metody wykonałam w oparciu o wyniki uzyskane z analiz molekularnych (PCR) i metod konwencjonalnych (testy paskowe). Badania te udowodniły, iż zastosowanie techniki MALDI w laboratoriach mikrobiologicznych do detekcji oporności może znacznie skrócić czas oczekiwania lekarzy na wyniki antybiogramu i przyczynić się do implementacji skuteczniejszej antybiotykoterapii.

2.6. Zastosowanie nanostruktur

Bezmatrycowe platformy LDI, stanowiące nowoczesną alternatywę dla tradycyjnej techniki MALDI, zyskały uznanie w kontekście analizy metabolitów, które charakteryzują się niskimi masami cząsteczkowymi, przede wszystkim ze względu na ich zdolność do generowania niskiego sygnału pochodzącego od tła w zakresie m/z poniżej 1000 Da, co w konsekwencji daje większą liczbę sygnałów o wysokim stosunku S/N (sygnał do szumu). Kluczowym aspektem

tych platform jest zastąpienie konwencjonalnej matrycy przez różnorodne nanomateriały, które są aplikowane na powierzchni podłoża. Dzięki temu innowacyjnemu podejściu możliwe jest osiągnięcie wysokiej efektywności procesu desorpcji i jonizacji związków niskocząsteczkowych znajdujących się w próbkach, co jest kluczowe dla dokładnej analizy chemicznej. Takie metody, znane pod nazwą wspomagana powierzchniowo laserowa desorpcja jonizacja (SALDI, ang. *surface-assisted laser desorption ionization*) oraz laserowa desorpcja jonizacja wspomagana nanostrukturami (NALDI, ang. *nanostructure assisted laser desorption ionization*), otwierają nowe możliwości w zakresie precyzyjnego badania składu chemicznego próbek, oferując jednocześnie znaczną redukcję efektów tłumiących sygnał, co jest często obserwowanym ograniczeniem w przypadku technik opartych na tradycyjnych matrycach MALDI. Akronim NALDI głównie odnosi się do metody opracowanej przez firmę Bruker Daltonics wykorzystującej powierzchnie zawierające nanodruki krzemowe.

Ich powstanie zainspirowane było wspomnianą wcześniej pracą Tanaki i wsp., w której zastosowano nanocząstki kobaltu do jonizacji białek i polimerów [34]. Sunner i Chen zastosowali zawieszone w glicerolu cząstki grafitu o wielkości od 2 do 150 μm do jonizacji małych molekuł [95]. Proponowana terminologia SALDI miała na celu zaakcentowanie kluczowej roli, jaką powierzchnia oraz jej struktura odgrywają w procesie desorpcji i jonizacji. Jednakże, początkowe badania nie odzwierciedlały w pełni rzeczywistych warunków pomiarowych, gdyż ablacja nie następowała bezpośrednio z modyfikowanej powierzchni, lecz z matrycy nieorganicznej zanurzonej w glicerolu. Użycie glicerolu miało również wpływ na ograniczenie zastosowań do systemów pomiarowych, w których płytki umieszczane są w pozycji pionowej. Wobec tych ograniczeń, w dalszych badaniach pojęcie SALDI zaczęto stosować z odniesieniem do nanostruktur zintegrowanych z powierzchnią płytki lub do powierzchni, które zostały specjalnie zmodyfikowane przed aplikacją analizowanej próbki. Takie podejście pozwala na efektywniejsze wykorzystanie procesu desorpcji i jonizacji, co przyczynia się do zwiększenia dokładności i wiarygodności wyników analizy [96].

W kontekście technologii SALDI, różnorodne powierzchnie są testowane pod kątem ich skuteczności jako substraty, z różnym powodzeniem. Analizując skład pierwiastkowy, można zaklasyfikować większość podłoży SALDI do trzech głównych kategorii: podłoża na bazie węgla (takie jak fulereny i grafen), podłoża na bazie półprzewodników (np. krzem) oraz podłoża na bazie metalu. Każda z tych kategorii charakteryzuje się specyficznymi właściwościami fizykochemicznymi, które mogą wpływać na efektywność procesu desorpcji i jonizacji, a tym samym na jakość i precyzję wyników analizy. Wybór odpowiedniego typu podłoża zależy od specyfiki badanej próbki oraz oczekiwanych wyników analizy, co podkreśla znaczenie dogłębnej wiedzy na temat właściwości różnych materiałów wykorzystywanych jako substraty w metodzie SALDI [97].

Szczególным zainteresowaniem w dziedzinie biologii cieszą się metody oparte na nanocząstkach (NP) metali, szczególnie metali szlachetnych. Właściwości fizykochemiczne NP metalicznych zależą od ich rozmiarów i/lub morfologii, co wpływa na wydajność

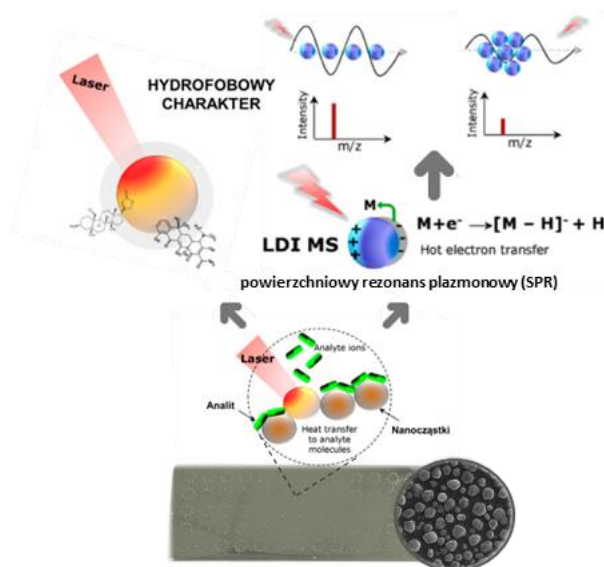
desorpcji/ionizacji i wewnętrzny transfer energii w procesie SALDI. McLean i wsp. stosując nanocząstki złota (AuNP) w technice LDI wykazali, iż AuNP o średnicy 10 nm charakteryzowały się 10-krotnie wyższą wydajnością w wykrywaniu białek niż ich odpowiedniki o średnicy 2 nm [98]. Gámez i wsp. stosując sferyczne AuNP (śr. 30 nm) i w kształcie nanoprętów AuNP (śr. 18 nm, dł. 50 nm) w technice LDI odnotowali 2,8-krotnie niższy próg fluencji lasera przy 355 nm w przypadku sferycznych AuNP [99]. Właściwości fizykochemiczne substratów wykorzystywanych w technologii SALDI mają znaczący wpływ na efektywność procesów desorpcji oraz jonizacji indukowanych przez laser. Chociaż jasne jest, że właściwości te odgrywają kluczową rolę, jednakże względny wpływ poszczególnych właściwości na ogólną wydajność metody wciąż nie jest w pełni wyjaśniony. W tym kontekście jednym z głównych wyzwań jest brak standaryzacji procesów syntezy nanomateriałów, co jest konieczne do osiągnięcia większej jednorodności strukturalnej i rozmiarowej produktu końcowego. Osiągnięcie takiej standaryzacji byłoby kluczowe dla zapewnienia powtarzalności i wiarygodności wyników uzyskiwanych za pomocą technologii SALDI, poprzez minimalizację zmienności wewnętrznej związanej z heterogenicznością substratów. Dążenie do większej jednorodności w produkcji nanomateriałów może zatem znacząco przyczynić się do lepszego zrozumienia wpływu poszczególnych cech fizykochemicznych na proces desorpcji/ionizacji, co z kolei może doprowadzić do optymalizacji i ulepszenia metodologii SALDI. Literatura wskazuje, że najczęściej stosowane metody syntezy nanocząstek (m.in. srebra, złota) do LDI opierają się na chemicznej redukcji soli [100], [101] lub elektrochemicznym wzroście na powierzchni katody [102]. Synteza tymi metodami, mimo iż prosta i tania często prowadzi do uzyskania niejednorodnych struktur i niekontrolowanej agregacji.

Nanocząstki metali można otrzymać metodami chemicznymi, fizycznymi i biologicznymi, które można ogólnie podzielić na dwa podejścia: odgórne (metoda niszcząca) i oddolne (metoda konstrukcyjna). Podejście oddolne jest bardziej preferowane, ponieważ zapewnia lepszą kontrolę właściwości fizykochemicznych produktu końcowego i jego składu chemicznego [103]. W trakcie realizacji mojej rozprawy doktorskiej zastosowałam i rozwijałam metodę chemicznego osadzania z fazy gazowej (CVD, ang. *chemical vapour deposition*). Ta technika umożliwia syntezę nanocząsteczek (NP) bezpośrednio na podłożu poprzez proces sublimacji prekursora. W metodzie tej prekursor w formie gazowej jest transportowany do powierzchni podłoża, gdzie ulega serii reakcji chemicznych (w przypadku metali głównie rozkładowi termicznemu). Proces ten prowadzi do ostatecznego utworzenia cienkiej warstwy nanocząsteczek. Takie podejście charakteryzuje się wysoką kontrolą nad morfologią i składem chemicznym powstałej warstwy, co jest kluczowe dla uzyskania materiałów o pożądanych właściwościach fizykochemicznych [104]. Zsyntetyzowane tą metodą AgNP wykazują potencjał do zastosowania w technice LDI do analizy LMW, co przedstawiłam na przykładzie lipidów wyizolowanych z komórek bakteryjnych w pracy [P7] oraz standardach lipidów i sacharydów [P8].

Zastąpienie cząsteczek matrycy nanocząstkami srebra znacznie zmniejsza ilość sygnałów tła chemicznego, co zapewnia łatwiejszą analizę danych. Sygnały związane z jonami srebra są wysoce powtarzalne i mogą służyć jako wewnętrzny kalibrant. Dodatkowo właściwości antibakteryjne i przeciwgrzybicze AgNP pomagają w konserwacji analizowanej próbki, co ma szczególne znaczenie w przypadku obrazowania MS [105]. Sprawia to, iż AgNP są często stosowane w technice LDI w kontekście analiz (jakościowych i ilościowych) związków biologicznych tj. kwasy nukleinowe [106], kwasy karboksylowe [107], cukry i kwasy tłuszczowe [108], lipidy [109], oraz leki i ich metabolity [110].

W literaturze wykorzystanie techniki SALDI w kontekście analizy mikroorganizmów nie cieszy się wielką popularnością, a powstałe prace głównie koncentrują się na analizie całych komórek bakteryjnych. Gopal zastosował niemodyfikowane AgNP jako sondy powinowactwa do wiązania całych komórek *E. coli* i *S. marcescens* w celu zwiększenia czułości LDI w ich detekcji bezpośrednio z produktów żywnościowych tj. jogurty [111]. Lukowski i wsp. zastosował AuNP do analizy metabolitów (w tym toksyn) obecnych w biofilmie tworzonemu przez *B. subtilis* [112]. W publikacji [P7] przedstawiłam możliwość rejestrowania profili lipidowych bakterii przy użyciu techniki LDI wspomaganej AgNP. Otrzymane profile lipidowe umożliwiły rozróżnienie dwóch bliskospokrewnionych szczepów *E. coli* (szczepy wykazywały odmienną wrażliwość na β -laktamy), co nie było możliwe w oparciu o profile zarejestrowane za pomocą MALDI.

Mechanizm desorpcji i jonizacji w układach z nanostrukturami jest skomplikowany i nie w pełni poznany. Zakłada się, że mechanizm desorpcji/jonizacji dla analitu znajdującego się na lub w pobliżu nanocząstek polega na absorpcji impulsów lasera przez nanocząstki, czego skutkiem jest niemal natychmiastowe, gwałtowne ogrzewanie analitu i gwałtowne przejście fazowe [96]. W przypadku nanocząstek metali istotne znaczenie przypisuje się również silnie hydrofobowej powierzchni oraz występowaniu powierzchniowego rezonansu plazmonowego (SPR, ang. *surface plasmon resonance*) (patrz Rysunek 4).



Rysunek 4. Mechanizm laserowej desorpcji/jonizacji w układach wspomaganych nanocząstkami metali.

Interakcja promieniowania laserowego z nanocząstkami metali inicjuje szereg złożonych zjawisk fizycznych i chemicznych. W procesie tym obserwuje się dystrybucję energii promieniowania, która częściowo rozprasza się poprzez mechanizmy takie jak emisja fotonów, czyli rozpraszanie światła. Jest to zjawisko wykorzystywane między innymi w technice spektroskopii Ramana ze wzmocnieniem powierzchniowym (ang. *Surface Enhanced Raman Spectroscopy*), która pozwala na detekcję molekuł z wyjątkowo wysoką czułością dzięki lokalnemu wzmocnieniu pola elektromagnetycznego wokół nanocząstek. Ponadto, znaczna część energii jest absorbowana przez nanocząstki, co może skutkować wzrostem ich temperatury oraz występowaniem zjawiska SPR. Absorpcja energii promieniowania prowadzi do zwiększenia energii kinetycznej elektronów w nanocząstce, co ma kluczowe znaczenie dla generowania lokalnie wzmocnionego pola elektromagnetycznego. Wzmocnienie to ma bezpośredni wpływ na zwiększenie efektywności procesów desorpcji oraz jonizacji analitów, co jest istotne w kontekście zastosowań analitycznych, takich jak masowa spektrometria z laserową desorpcją/ionizacją. Takie zjawiska podkreślają znaczenie nanocząstek metali jako mediatorów w przekazywaniu energii promieniowania lasera, umożliwiając nie tylko bardziej efektywne wykorzystanie promieniowania w celach analitycznych, ale również otwierając nowe możliwości w badaniach nad materiałami na poziomie nano. Dzięki temu, zrozumienie mechanizmów oddziaływania promieniowania lasera z nanocząstkami i ich wpływu na procesy analityczne staje się kluczowe dla rozwoju nowoczesnych metod spektroskopowych i spektrometrycznych [36].

W ramach badań opisanych w publikacji [P8], po raz pierwszy zastosowałam powierzchnię pokrytą nanocząstkami srebra uzyskanymi metodą chemicznego osadzania z fazy gazowej (CVD), aby przeprowadzić analizę jakościową standardów lipidów oraz węglowodanów. Proces ten umożliwił zbadanie mechanizmów jonizacji oraz fragmentacji poszczególnych związków, co zaowocowało ważnymi obserwacjami dotyczącymi efektywności jonizacji. Jonizacja standardów zdeterminowana była również przez strukturę analitów. Dla wszystkich związków zaobserwowałam tworzenie adduktów z jonami Na^+ , podczas gdy tylko niektóre związki tworzyły stabilne addukty z jonami Ag^+ . Otrzymane nanostruktury wykazały wysoką selektywność w stosunku do trójglicerydów. Na podstawie uzyskanych wyników sformułowano hipotezę, sugerującą, że ilość energii absorbowanej przez nanocząstki ma bezpośredni wpływ na procesy jonizacji i fragmentacji analitów.

Dodatkowo, nanocząstki srebra pełniły funkcję fotokatalizatora, co miało kluczowe znaczenie dla zwiększenia efektywności procesu jonizacji analitów. To odkrycie otwiera nowe perspektywy dla dalszych badań nad mechanizmami fotokatalizy i jej zastosowaniem w analizie chemicznej. Ważnym wnioskiem płynącym z pracy jest również stwierdzenie, że technika laserowej desorpcji/ionizacji wspomaganej nanocząstkami (nano-LDI) oferuje możliwość przeprowadzenia ilościowych analiz związków o niskiej masie cząsteczkowej (LMW), co odróżnia ją od tradycyjnej techniki MALDI, często ograniczonej przez efekty tłumienia sygnału i niską efektywność jonizacji dla tych związków. Dzięki zastosowaniu nano-

LDI, możliwe jest osiągnięcie wyższej czułości i specyficzności w analizie chemicznej, co ma istotne znaczenie dla rozwoju metod analitycznych i zwiększenia ich zastosowań w różnorodnych dziedzinach naukowych. Wyniki przedstawione w publikacji **[P8]** stanowią istotny wkład w rozwój technik spektrometrycznych, demonstrując potencjał zastosowania nanocząstek jako fotokatalizatorów w procesach jonizacji oraz otwierając nowe możliwości dla ilościowej analizy związków o niskiej masie cząsteczkowej.

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Cel pracy

Celem zasadniczym przedstawionej rozprawy doktorskiej jest opracowanie i walidacja nowoczesnych metod analitycznych, ze szczególnym uwzględnieniem spektrometrii mas z laserową desorpcją/ionizacją oraz technik elektromigracyjnych, które zostaną zastosowane do precyzyjnej identyfikacji mikroorganizmów patogennych oraz oceny ich antybiotykooporności. W ramach tej pracy dąży się do osiągnięcia głębszego zrozumienia mechanizmów molekularnych, które leżą u podstaw interakcji pomiędzy patogenami a środkami przeciwbakteryjnymi, co ma kluczowe znaczenie w kontekście rosnącego problemu występowania oporności na antybiotyki. Rozprawa ma na celu nie tylko rozwój metodologiczny, ale również weryfikację skuteczności tych metod w kontekście realnych próbek klinicznych, co umożliwi ocenę ich przydatności w rutynowej diagnostyce mikrobiologicznej. Integralną częścią pracy będzie dogłębna analiza porównawcza nowo opracowanych metod z tradycyjnymi podejściami, co pozwoli na ustalenie ich zalet, ograniczeń oraz potencjalnych obszarów zastosowania. Opracowanie takich metod wymaga zrozumienia złożonych interakcji biochemicznych i fizykochemicznych zachodzących podczas procesów desorpcji/ionizacji oraz elektromigracji, co będzie realizowane przez eksperymentalne badania próbek zawierających różnorodne mikroorganizmy patogene. Dodatkowo, szczególny nacisk zostanie położony na wykorzystanie tych metod do identyfikacji mechanizmów antybiotykooporności, co ma istotne znaczenie dla opracowania nowych strategii terapeutycznych i przeciwdziałania rozprzestrzenianiu się opornych szczepów bakteryjnych. W konsekwencji, przedstawiona rozprawa doktorska ma na celu nie tylko przyczynienie się do interdyscyplinarnego rozwoju nauk analitycznych i mikrobiologicznych, ale również dostarczenie praktycznych narzędzi diagnostycznych, które mogą znacząco poprawić skuteczność wykrywania i identyfikacji patogenów, a tym samym przyczynić się do lepszego zarządzania terapią antybiotykową w środowisku klinicznym.

Realizacja celu rozprawy doktorskiej obejmuje następujące cele szczegółowe:

- Istotność zastosowania nowoczesnych technik w analizie mikroorganizmów [P1]
- Ocena wrażliwości szczepów klinicznych na antybiotyki z zastosowaniem elektroforezy kapilarnej [P2]
- Zastosowanie podejścia kulturomicznego w hodowli mikroorganizmów i jego wpływ na wydajność procesu identyfikacji w technice MALDI [P3]
- Charakterystyka profili lipidowych bakterii i ich wpływ na możliwość identyfikacji w technice MALDI [P4]

- Ocena skuteczności dwóch systemów MALDI w szybkiej identyfikacji izolatów bakteryjnych [P5]
- Zastosowanie techniki MALDI do szybkiej identyfikacji i detekcji antybiotykooporności bakterii z próbek klinicznych oraz porównanie uzyskanych wyników z innymi metodami [P6]
- Zastosowanie techniki CVD do pokrycia powierzchni płytki nanocząstkami srebra oraz charakterystyka uzyskanej warstwy [P7, P8]
- Wykorzystanie AgNP w technice LDI do rozróżniania klinicznych szczepów bliskospokrewnionych bakterii w oparciu o ich profile lipidowe, porównanie z techniką MALDI [P7]
- Ocena zastosowania techniki LDI wspomaganej AgNP w analizach jakościowych i ilościowych LMW [P8]

W celu realizacji celów szczegółowych zastosowano następujące instrumenty:

- Elektroforeza kapilarna 3DCE z detektorem UV-VIS DAD o zakresie λ od 200 do 600 nm (Hewlett Packard, USA)
- Spektrometr mas UltrafleXtreme MALDI-TOF MS wyposażony w laser Nd:YAG o $\lambda = 355$ nm, tryb jonizacji dodatni/ujemny (Bruker Daltonik GmbH, Bremen, Niemcy)
- Spektrometr mas Microflex LT MALDI-TOF MS wyposażony w laser N₂ o $\lambda = 337$ nm, tryb jonizacji dodatni (Bruker Daltonik GmbH, Bremen, Niemcy)
- Spektrometr mas EXS2600 MALDI-TOF MS wyposażony w laser N₂ o $\lambda = 337$ nm, tryb jonizacji dodatni/ujemny (Zybio Inc., Chongqing, Chiny)
- Spektrofotometr UV-Vis NanoDrop 2000 (Thermo Fisher Scientific, USA)
- Czytnik mikropłytek UV-VIS (Multiskan FC microplate photometer (Thermo Fisher Scientific, USA)
- Cytometr przepływowy MACSQuant VYB (Miltenyi Biotec, USA)
- Mastercycler PRO S thermocycler (Eppendorf AG, Hamburg, Niemcy)
- Zestaw do elektroforezy żelowej poziomej
- Aparatura do przeprowadzenia chemicznego osadzania z fazy gazowej (konstrukcja własna dr hab. P. Piszczka, prof. UMK)
- Skaningowa mikroskopia elektronowa (SEM) w w połączeniu ze spektroskopią rentgenowską z dyspersją energii (EDX) (Quanta 3D FEG, USA)
- Aparat do dyfrakcji rentgenowskiej (XRD) (Quantax 200 XFlash 4010, Bruker, Niemcy)
- Mikroskop sił elektronowych (AFM) wyposażony w sondę SPM (Nanoscope III, Veeco Digital Instruments, USA)

W celu realizacji celów szczegółowych wykorzystałam metody badawcze obejmujące zastosowanie:

- Podejścia kulturomicznego,
- Zestawu primerów: 16S rDNA (27F, 1492R), genów E β BL (MultiTSO-T, MultiTSO-O, MultiTSO-S, MultiCTXMGp1), genów karbapenemaz (KPC, GES, GIM, VIM, OXA, NDM, IMP), genów AmpC o rozszerzonym spektrum (CMY-1, CMY-2)
- Zestawu MBT STAR-BL IVD (Bruker Daltonik GmbH, Bremen, Niemcy)
- Zestawu paskowej detekcji oporności bakterii na różne klasy antybiotyków –E-test (Biomerieux, Francja)

Metodyki badań zostały szczegółowo opisane w poszczególnych pracach [P2 – P8] stanowiących całość niniejszej dysertacji.

Publikacja [P1]

A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques

B. Buszewski, **E. Maślak**, M. Złoch, V. Railean-Plugaru, E. Kłodzińska, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* **2021**, 39:116250



A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques



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ABSTRACT

Fast determination, identification and characterization of pathogens is a significant challenge in many fields, from industry to medicine. Standard approaches (e.g., culture media and biochemical tests) are known to be very time-consuming and labor-intensive. Conversely, screening techniques demand a quick and low-cost grouping of microbial isolates, and current analysis call for broad reports of pathogens, involving the application of molecular, microscopy, and electromigration techniques, DNA fingerprinting and also MALDI-TOF methods. The present COVID-19 pandemic is a crisis that affects rich and poor countries alike. Detection of SARS-CoV-2 in patient samples is a critical tool for monitoring disease spread, guiding therapeutic decisions and devising social distancing protocols. The goal of this review is to present an innovative methodology based on preparative separation of pathogens by electromigration techniques in combination with simultaneous analysis of the proteome, lipidome, and genome using laser desorption/ionization analysis.

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1. Introduction

Microorganisms have always been extremely important for human life and they are known for both positive and negative reasons. They are particularly associated with food sciences [1], genetic engineering, medicine [2], biotechnology as well as other areas of life sciences [3]. They are used for their unique features which enable production of various therapeutic compounds [2], and food and food-related products [1], as well as decomposition of such components as lignocellulosic biomass for second-generation ethanol [3]. On the other hand, the genetic features and biochemical abilities of microorganisms make them dangerous for industry (e.g. food spoilage) as well as for human health. Approximately 1400 pathogens can cause human diseases [4], with pathogenic bacterial strains and viruses posing a particular threat.

The first group of pathogens is currently a real problem. This is mainly due to the growing number of strains resistant to commercially available antibiotics widely used in therapies. Mass production of these substances in recent years and the lack of discipline in their use in many sectors (e.g. in medicine, animal husbandry and agriculture) have contributed to microbes developing a number of defense mechanisms [5]. Bacteria show two types of antibiotic resistance. The first one is congenital resistance, characteristic of the species, and the second one is acquired resistance, characteristic of a particular strain. Congenital (innate) resistance may be connected with a lack of characteristic binding sites (e.g. porin deficiency) or presence of specific structures that prevent the drug from entering the cell (e.g. a layer of exopolysaccharide or alginate polysaccharide). This type of resistance is not the result of implementing specific therapeutic agents and usually does not involve medical consequences. More dangerous is the acquired resistance of bacteria which were originally sensitive to the drug. In addition, the latter type of resistance can be divided into primary and secondary resistance. Primary resistance is related to spontaneous mutation that can occur without contact with the

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drug and cannot be passed on to other bacteria species. In contrast, secondary (induced) resistance occurs as a result of pathogen coming into contact with antibiotics; genes with information about the drug resistance mechanism can be transferred to other bacterial cells. Moreover, the growing problem is that microorganisms may exhibit multiple resistance mechanisms at the same time. Therefore, depending on the range of resistance, they can be classified into three groups: extensively drug-resistant (XDR), sensitive to up to two groups of antibiotics; multidrug-resistant (MDR), resistant to three or more groups of antibiotics; and drug-resistant (PDR), resistant to all groups of antibiotics. Currently, antibiotic resistance of bacteria is a global problem. It is estimated that the drug-resistant strains cause about 2.8 million infections and approximately 25,000 deaths per year only in the United States [5]. In Europe antibiotic-resistant pathogens cause about 25,000 deaths a year as well. It is believed that already in 2050 resistant strains will cause more deaths than cancers [6].

Next to the pathogenic drug-resistant bacteria, viruses are another group of pathogens highly dangerous to people. Viruses can be defined as infectious agents, which propagate themselves via multiplication in parasitized living cells [7]. The form of virus existence and replication is at the same time virtue and disadvantage. Their multiplication cannot occur without appropriate host cells. However, after the integration viruses become a part of the cell and therefore their complete destruction cannot be achieved without cell death. Another problem which stems from the intracellular existence of the viruses is that it is hard or even impossible to produce effective medicine to control them [8]. Moreover, the submicroscopic size of the viruses (the size of the majority of animal virus's ranges from 30 to 300 nm) enables very fast spread of viral units [7]. It is even more relevant for viruses whose target cells are exposed to environmental influence. Microbial agents such as bacteria, fungi or viruses can move around the environment by air via air pollutants. Recent reports suggest that atmospheric particulate matter (PM) may increase the effectiveness of pathogen spread because it creates a microenvironment suitable for their persistence. These particles can act as carriers for microorganisms and, what is more, they can serve as nutrition for the latter and make them more toxic. Inhalation transports particles (especially these smaller than 2.5 μm) deep into the lungs. This allows the pathogens to develop within the respiratory tract and cause infections [9].

The respiratory tract infections caused by viruses, such as the influenza virus, are one of the most frequent infections in the world. Annually influenza virus causes infections in 5–15% of the population [10]. Coronaviruses (CoVs) also cause respiratory tract infections. There are seven known human coronaviruses: HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, SARS-CoV, MERS-CoV and SARS-CoV-2. In 2003, SARS was the cause of the rapidly spreading respiratory disease with a mortality rate of nearly 10%. In 2012, MERS-CoV caused severe acute respiratory infection with a fatality rate close to 40% [11]. Currently in 2020, the world is struggling with another epidemic caused by a virus from this group, SARS-CoV-2. Studies from many countries indicate the existence of positive correlations between high levels of air pollution (PM_{2.5}, PM₁₀, nitrogen dioxide, and ozone) and SARS-CoV-2 infections [9].

The situation with SARS-CoV and SARS-CoV-2 has revealed that fast and relevant detection of the infection is one of the most effective ways to prevent its distribution and reduce the ravages caused by the epidemic [12]. Rapid identification of bacterial and viral infections is a strategic stage in the fight for the patient's life because only a thorough understanding of the source of the disease allows implementation of a proper therapy. Unfortunately, despite their high accuracy, the methods currently used for this purpose (based on molecular biology) are expensive, time-consuming, and

can be performed only in well-equipped laboratories with a high biosafety level (BSL 2 or 3) and qualified staff, access to which is limited in many regions of the world [13]. The accurate identification of pathogenic microorganisms is essential for scientists involved in many areas of applied research and industry, from clinical microbiology to food production. There are a lot of criteria for the classification of the numerous methods used in identification and characterization of microorganisms; however, generally they can be divided into direct and indirect techniques. Direct methods include conventional isolation of pure cultures of microorganisms and their various phenotypic analyses, while indirect methods are culture independent and include microscopic techniques which are the powerful tools in the identification of microbes by visualization of the characteristic structures. It also makes it possible to detect microbes in a viable but not culturable state.

Identification of pathogens based on the traditional methods – which includes determining their morphology, physiology, chemistry and biochemical characterization – is estimated to take at least 2–5 days. In addition, most phenotypic methods used in microbiological laboratories are time and material consuming. Importantly, phenotypic methods are not always suitable to identify microorganisms at the species level, or much more often at the strain level. One of the strategies to reduce time needed for microbial identification is the use of molecular biology techniques, which may also be supplemented with numerous molecular fingerprinting techniques [13]. Each of these methods has their strengths and weaknesses, and the most recent research approach involves the use of a compilation of multivariate techniques. Such implementation and application of hyphenated techniques seems to have a great potential for future development. In order to obtain the most precise identification, classification and characterization of pathogens, it is extremely important to choose appropriate techniques, as well as have a thorough understanding of the mechanisms of their action (Fig. 1).

Taking the above into consideration, today's world of science faces an enormous challenge: how to develop new pathogen identification methods which are fast, cheap and easy to use, as well as accurate and repeatable.

2. Analytical challenges and strategies in determination of pathogens

Due to the constantly growing number of infections caused by pathogenic microorganisms, the development of fast and cheap methods of microbial identification that are also effective and unambiguous is an extremely important aspect and the challenge of modern science. Reliable and efficient identification of pathogens is a key tool enabling implementation of appropriate therapies, and thus saving lives. Unfortunately, conventional techniques used in diagnostic laboratories to identify pathogens based on biochemical tests that differentiate bacteria phenotypically are time consuming, work intensive and often inadequate to differentiate between phenotypically similar species [13].

2.1. Molecular biology approach

The rapid development of molecular biology has enabled the use of a more precise diagnostic tool, which is polymerase chain reaction (PCR), followed by sequencing of the conserved fragment of the 16S rRNA gene. This method was developed in the early 1980s. It is based on in-situ DNA replication that allows exponential amplification of target DNA in the presence of synthetic oligonucleotide primers and thermostable DNA polymerase [14]. A new version of the PCR technique is real time-PCR (RT-PCR), which

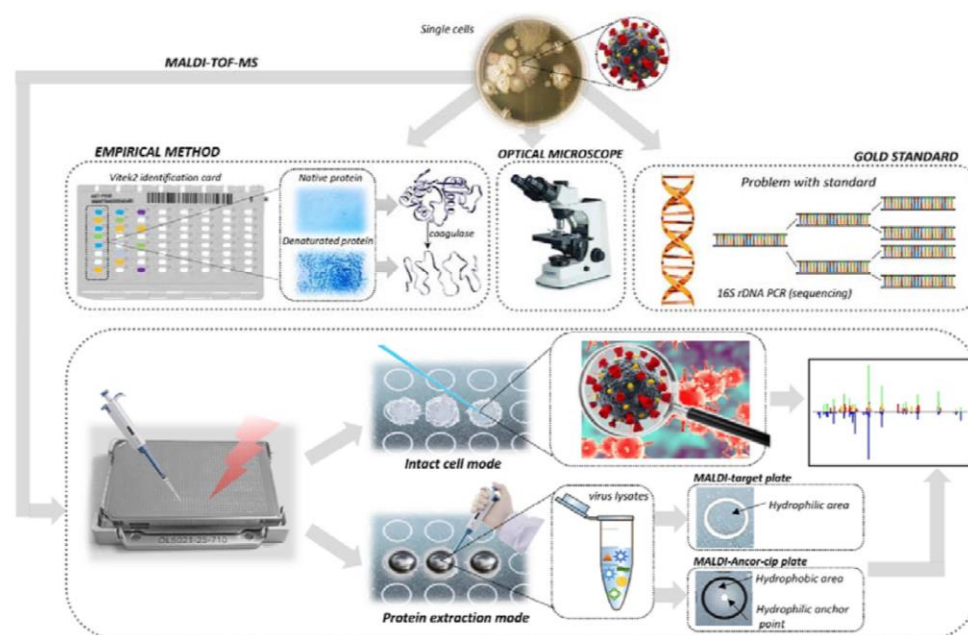


Fig. 1. Pathogen identification approaches with particular regard to viruses.

allows tracking the obtained amplicons in real time after each cycle of the amplification reaction. The application of RT-PCR reduces the risk of sample contamination and shortens the analysis time from about 5 h in the case of classic PCR to about 40 min [13]. The sensitivity and efficiency of PCR techniques may depend not only on the presence of enriching components in the culture medium but also on DNA extraction solution, temperature and cyclic conditions as well as the concentration of primers or matrices [14]. Products obtained from the PCR reaction are usually subjected to sequencing – a technology that allows determining the order of nucleotide bases in a DNA molecule. Nowadays, 16S rRNA sequencing technique is considered as a “gold standard” in micro-organism identification [13].

Molecular biology methods are characterized by high sensitivity, accuracy, discriminatory power and reproducibility. However, next to the numerous advantages there are many limitations. The main problems include a long analysis time of up to 3 days. This is mainly related to the fact that such research is usually carried out by specialized external institutions with qualified staff, access to commercial databases and dedicated equipment [14]. In addition, the costs of analysis are so high that this technology cannot be used in routine diagnostics. At the same time, the problem is the limited number of gene sequences available in databases and the lack of strict criteria for interpreting the results [15]. Analysis of the 16S rRNA gene also does not allow differentiation of many closely related species, particularly if they overlap in more than 98% of the sequence. Such a phenomenon was observed, among others, for the *Burkholderia* and *Staphylococcus* species [16].

Another group of microbes that are dangerous to human life are viruses. The structure of the viruses is relatively simple in comparison to other living forms. Viral particles mainly consist of genetic material and proteins. Genetic material can be represented by RNA ((+) single-stranded, (–) single-stranded and double-stranded) or DNA (linear double- or single-stranded and circular double-stranded). Viral proteins are divided into two main groups:

structural and non-structural proteins. Structural proteins form the viral unit and comprise the proteins of the capsid, which protect nucleic acids; the envelope proteins, which take part in the viral-in-cell fusion; matrix proteins, coating the inner leaf of the viral envelope and taking part in envelope assembly. Viral envelope also consists of a lipid bilayer derived from the host cell membrane. The non-structural viral proteins, such as RNA/DNA polymerases and proteases, are coded by the viral genome, but are absent in virus particles [17]. Such a simple structure enables identification of viruses through two main ways, by determining the genetic material or by profiling proteins. For this reason, as in the case of bacteria, viruses are mainly identified through PCR analysis.

In the laboratory practice, quantitative PCR analysis is usually used for virus identification. The detection of amplified viral nucleic acids in quantitative PCR is performed with specific fluorescent probes comprising a small nucleotide sequence attached to the fluorescent indicator. The technique is highly sensitive but suffers from numerous drawbacks. Firstly, PCR analysis requires isolation of genetic material from the investigated microorganism with very high quality, as any impurities derived from the clinical sample (RNase or DNase, EDTA, phenol, urea, denatured albumin, prosthetic groups of hemoglobin, polysaccharides, etc.) can inhibit Taq polymerase and lead to false-negative results [18]. It is even more essential for the RNA-containing viruses, where identification begins with transformation of RNA to DNA by reverse transcription. It was shown that high concentration of viral RNA in the sample can inhibit reverse-transcription-PCR [19]. Even though it was possible to overcome the problem by simple sample dilution, this factor may not be taken into account by laboratory staff, especially in routine analysis. This could be the reason for the false-negative results of the SARS-CoV-2 in the patients with clinical symptoms of COVID-19 as it was shown by Li et al. [20]. The authors also suggest that the presence of a coinfection as well as improper specimen collection and transportation might have burdened the analysis. The statistics gathered in Beijing Haidian Section of Peking University Third

Hospital from 21 to 31 January 2020 shows that approximately 20% of tests were false negative [20]. The second important issue in PCR analysis is the selection of appropriate primers for the reaction. DNA polymerases can only add new nucleotides, which are called primers, to a DNA fragment that already exists. What is usually used as primers are the highly conserved regions of DNA. The design of appropriate primers is a painstaking work that often requires full genome sequencing with subsequent data processing, which may take several months [21]. Moreover, viruses – particularly the RNA-containing ones – can easily mutate, which may lead to changes in the matrix or even in conserved genome areas. Changes in the matrix sequence lead to mismatches with probe sequence and therefore to false-negative results in quantitative PCR [22], which often requires continuous probe adjustment [23]. The changes in conserved regions can reduce the template-primer binding affinity that contributes to low efficiency of the reaction [21]. Additionally, the high adaptive properties of viruses are a reason for their great genetic diversity, which may burden the analysis due to possible cross-reactivity among viruses with a close genome sequence. The sensitivity of the PCR analysis also makes it very vulnerable to cross-contamination, which can lead to false-positive results [21]. Sample contamination may occur at each stage of the investigation: during sample collection and transportation, at the stages of genetic material isolation, and post-PCR isolation by aerosol of a purified genome or PCR products. Finally, some virus infections may exhibit very similar symptoms (e.g. influenza and SARS), so for correct identification of the infectious agent, several parallel tests for each possible virus should be done.

PCR analysis can thus be considered as a labor-extensive and high-cost technique, which restricts its usefulness. For instance, identification of HIV infection with a PCR test is done only for newborns, as in these cases the alternative method detecting particular antibodies cannot be used due to the presence of maternal antibodies in an infant up to 18 months after birth. However, as with other RNA viruses, the quantitative PCR analysis for HIV produces many false-positive and false-negative results because of high genetic variability of the analyte and difficulties in designing good primers.

2.2. Immunochemistry techniques

Viral protein detection is the basis of such immunoassay tests as ELISA, immunoblotting, and immunofluorescence assay. It is based on the interaction of specific antibodies with virus proteins [24]. Antibodies are proteins produced by higher life forms as the response to the presence of foreign material (antigen), which can be viruses or their parts (e.g. proteins). A distinctive feature of an antibody is a specific binding to a particular antigen, which enables the former to identify the presence of antigen in the sample. The virtue of immunoassay analysis is that the specimen can be checked without any pretreatment, which makes it possible to perform such tests automatically and continuously. However, immunoassay analysis suffers from cross-reactivity, leading to false-positive results. Cross-reactivity is a possibility of antibody binding to molecules with similar epitopes (binding sites). The genetic modifications of epitopes, often observed in viruses, are the reason for false-negative results [25]. Although serological tests are considered easier to perform than PCR, in general they are less specific and their usage may be limited by the fact that antibodies appear later during the disease course. Moreover, the production of antibodies is a complex process, which among others involves immunization of the animals at the initial stage of assay, which excludes quick response to changes in the viral proteome. Additionally, high concentration of bilirubin or hemoglobin in the sample can also interfere with the analysis, as their absorbance

interferes with the optical detection system of some assays [25]. Finally, the immunoassay antibody needs to be prepared individually for each antigen (proteins in particular) [24].

2.3. LDI approaches

What has proven to be an alternative to molecular biology techniques is matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a relatively new but very promising method of microorganism identification. The use of soft ionization makes it possible to record the characteristic molecular profiles of a microorganism (the so-called “fingerprint”), which can be compared with the spectra deposited in the database, thus enabling identification of the microorganism [26]. The main advantages of this technology include low cost, speed and simplicity of analysis (it requires minimal sample preparation) as well as the accuracy of the obtained results.

With MALDI-TOF MS, bacteria can be identified at the genus and species level, and sometimes down to the subspecies level. This technique enables the analysis of whole bacterial cells and their individual components [27]. Most often, identification of microbes occurs as a result of comparing their protein profiles with those recorded in a database, e.g. Bruker BioTyper, bioMérieux VITEK MS, and Andromas [13,27]. The simplest identification of bacteria using MALDI can be performed for intact microbial cells. However, protein extraction with formic acid is often carried out as well, which results in increased identification rates [28]. The development of easy-to-use, commercially available systems for bacterial identification by MALDI-TOF MS has become the standard of practice in many clinical microbiological laboratories around the world [27].

In addition, this technique also allows tracking changes in bacterial metabolism and detecting resistance to antibacterial agents such as antibiotics – e.g. a novel method of phenotypic antimicrobial susceptibility testing (AST) called direct-on-target microdroplet growth assay (DOT-MGA) [29]. The MALDI-TOF MS technique was also shown to be able to distinguish e.g. *Sindbis virus* [30] and influenza virus [31] by proteotyping. The proteotyping of all the mentioned viruses was performed with detection of peptide fingerprints of tryptic digests of the whole virus. In the case of influenza virus it was possible to differentiate the H1N1 strain unique for the vaccine from seasonal type A (H1N1) strains, showing high sensitivity of the method in virus typing and subtyping [31]. The study of *Sindbis virus* showed that 5-min tryptic digestion is enough to obtain specific peptide fingerprint spectra [30]. However, what is even more interesting is the possibility to apply the existing systems used in clinical practice for pathogen identification in viral discrimination. Musaji et al. demonstrated that the influenza virus can be detected by subjecting the intact virus to MALDI-TOF MS [32]. The authors used the spectra of corresponding neuraminidase and hemagglutinin in proteins as well as the entire influenza virus to make a comparison. The advantage of MALDI-TOF MS in virus identification may also be that laboratories can quickly adjust to the appearance of new viral strains, as the reagents are the same for all investigations. Moreover, changes in databases can be made on-line without any delay. In contrast, the PCR as well as immunoassay analyses require the presence of specific reagents (primers and antibodies), which prevents a quick reaction to the appearance of new viruses or new virus mutations. However, MALDI-TOF MS is successfully utilized in MassARRAY analysis, which is the substitution of fluorescent probes in genotyping, and makes it possible to overcome the problem of mismatch. MassARRAY can also be used for monitoring mutations. Still, utilization of MALDI-TOF MS for virus investigation is less reported in the literature, so this matter requires further investigation. Unfortunately, this method also has many limitations. One

of the greatest problems is the insufficient number of spectra deposited in commercial databases as the quality of reference databases is a basic condition for correct identification of microbial isolates. Currently, numerous works are underway to supplement the existing databases with new spectra obtained for pathogens accurately identified by other techniques, in particular rare bacterial species [33]. Many research groups also create their own databases that ensure quick and accurate identification of microbial species of interest [34]. Other disadvantages of MALDI-TOF MS include the fact that in order to obtain a good quality spectrum it is necessary to have clean or well isolated colonies and a relatively high concentration of cells in the sample (10^5 – 10^9 cells per spot), which lengthens the whole process due to the necessity of including the culturing step [35]. In addition, the composition of the culture medium and the type of the matrix used during the analysis may affect the identification efficiency [35]. Like molecular biology methods, MALDI may not be able to properly distinguish the spectra of some closely related bacterial species, which can lead to their misidentification. Such a problem concerns e.g. differentiation of *Shigella* species from *Escherichia coli* [36]. Regarding identification of viruses, the crucial limiting factors – besides poor spectral libraries – are the relatively low protein content and higher molecular weight of viral proteins [37]. Moreover, viruses are also known to be difficult to multiply even under ideal conditions.

Nevertheless, scientists predict that MALDI-TOF technique will quickly become a standard tool for routine identification in virology labs – as soon as researchers are able to overcome the major obstacles related to inconsistencies surrounding the extraction protocols or to low reproducibility of the results due to the matrix effect in the direct detection mode [38]. In the face of the current COVID-19 pandemic, pathogen identification via MALDI-TOF technique is proposed as an easy-to-use and robust approach for large-scale SARS-CoV-2 testing in developing countries which lack the necessary laboratory resources and access to PCR kits. Nachtigall et al. proved that MALDI-MS can be used to reliably detect SARS-CoV-2 in nasal swab samples if appropriate machine learning analysis (e.g. decision tree, DT; k-nearest neighbors, KNN; or support vector machine with a radial kernel, SVM-R) is applied [39]. The authors found a concordance level acceptable in clinical diagnostics between results obtained using routine RT-PCR and the proposed MALDI-ML approach (>80%). The study used nasal swab samples without prior purification; therefore, the proposed approach could be used in a follow-up confirmation test using the RT-PCR gold standard. In turn, Gould et al. suggested that MALDI-TOF MS technique also shows high potential in the identification of viral strains within breath samples since its coupling with electrospray ionization (ESI) allows analysis of such big molecules as DNA or RNA [37]. Since it is possible to detect viral RNA in tidal breath samples [40], ESI-MALDI-MS technique offers an ideal solution to rapid and non-invasive detection of viral infections, potentially also SARS-CoV-2.

In addition to identifying bacteria based on specific protein profiles, the MALDI technique also offers identification based on the analysis of profiles of other cellular components such as lipids or nucleic acids. Currently, a large increase in interest can be observed over the use of bacterial cell lipid profile analysis for taxonomic identification [41]. This approach allowed the differentiation of *Bacillus* and *Brevibacillus*, with the average correct identification rate of 62.23% [42]. Similarly, lipid fingerprinting enabled differentiation of the bacteria of *Bacillus* spp., difficult to distinguish by conventional techniques [43]. There have also been several reports on the use of the MALDI technique for bacterial DNA and RNA analysis [44]. MALDI mass spectrometry was used to detect PCR products obtained from *Legionella* after rapid purification to

remove salts and unreacted primers [45]. In addition, MALDI technique allowed the analysis of posttranscriptional modifications of bacterial RNA.

An innovative strategy able to improve the analysis of pathogens may be application of nanotechnology-assisted laser desorption/ionization time-of flight mass spectrometry (NALDI-TOF MS). This technique replaces traditional organic matrices with a nanostructured silicon-based target plate [46]. The main role of nanoparticles in this method is to enrich analyte particles and enable their efficient desorption and ionization [47]. For this purpose, nanomaterials with porous structure, large surface area, easy functionalization, high heat transfer efficiency and photo-absorption properties are highly preferred. Metal nanoparticles as well as metal oxide nanoparticles were found to efficiently transfer heat to the analyte molecules under laser irradiation, which favors more effective ionization. Other nanomaterials useful in NALDI are silicon based ones, particularly porous structure materials, which are very useful in enriching analyte molecules from the sample [47]. The virtue of NALDI systems is their strong hydrophobic surface, which may enhance the ionization of hydrophobic peptides and proteins from the sample [48]. Current reports indicate that this method can be successfully used to analyze a wide range of organic particles such as lipids or proteins, while the lack of need for organic matrices eliminates uneven distribution and different sizes of crystals as well as background peaks in the low mass range; it also has a positive impact on the reproducibility of analyses [46,47]. Structural proteins in viruses often contain hydrophobic regions, which help maintain protein-protein association [49]. Thus the NALDI approach may be helpful in their investigation.

2.4. Electromigration approach

Another group of analytical techniques which can find an application in microbial diagnostics are electromigration techniques. In recent years the use of capillary electrophoretic (CE) techniques such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), or isotachopheresis (ITP) for separation, characterization and identification of the wide range of microorganism types has been the focus of growing attention. In comparison with GC and LC, electromigration techniques demonstrate better separation efficiencies, the need for very low volumes of sample and reagents, as well as the ability to separate cations, anions and uncharged molecules in a single run. The main advantage of electromigration techniques is the possibility to exploit several microbial parameters – size, shape and charge – which are very advantageous to their separation, characterization and identification. This set of analytical techniques also offers many advantages, such as very short analysis time or the possibility of direct analysis of biological samples [50,51]. The literature provides several examples of CE used for determination of bacterial pathogens [52,53], yeast cells [54], various types of human and plant viruses [55] or even bacteriophages [56]. The separation of pathogens is possible on the basis of the difference in their electrophoretic mobilities or isoelectric points (pI).

The first report on the use of capillary electrophoresis for pathogen determination was published by Hjerten et al., in 1987; they described the migration of *Tobacco mosaic virus* and *Lactobacillus casei* bacteria in 20 mM Tris-HCl buffer [55]. In 1993, Ebersole and McCormick separated four bacterial species in TBE buffer and proved by collecting individual fractions after the electrophoresis process that most bacteria (80%) are alive [57]. A year later, Torimura published a paper on the electrophoretic behavior of nine bacterial species, determining their electrophoretic mobility [58]. In the 1990s, Pfetsch and Welsch [59] as well as Glynn [60] determined the electrophoretic mobility of bacteria in various buffer

solutions. The pioneering work of Buszewski and Kłodzińska showed that capillary zone electrophoresis is an effective tool for identification of *Staphylococcus aureus* and *Escherichia coli* as well as *Proteus vulgaris* [52]. Another paper of Buszewski and coworkers presented the clinical use of a fast screening test based on CZE analysis for identification of *E. coli* infection in biological samples such as infected wounds and ulcerations [53]. Kłodzińska et al. applied CZE for identification of *E. coli* and *Helicobacter pylori* in urine samples [61].

Although this analytical method provides many solutions in the field of pathogen identification, it is important to remember its limitations and drawbacks such as uncontrolled aggregation (clumping) of bacterial cells and their adhesion to the capillary surface [62,63]. This can also be a source of capillary clogging if aggregation occurs to a large extent. Moreover, factors such as pH, osmolarity or high electric fields can also affect the analysis of bacterial cells by CE [64]. According to the electrokinetic theory, microorganisms are often considered as biocolloids, mainly due to the complex structure of their cell wall and protonation equilibria formed at the interface; for such a system, understanding the electrophoretic process is more complicated. The cell wall composition is specific for individual types of bacterial species – they have different content of proteins, phospholipids, polysaccharides or another organic components [65]. The wall structure also determines the division of bacteria into two main types: Gram(+) and Gram(–). All compounds present in the bacterial cell wall structure strongly affect the surface charge of microorganisms. This can be explained by the presence of many functional groups undergoing the protonation process. Therefore, it is important to develop a method that minimizes the uncontrolled aggregation and adhesion problem. Some research groups proposed the addition of poly(ethylene oxide) (PEO) to the buffer solution [63,66] which functioned as a focusing agent. Kłodzińska et al. identified the *E. coli* and *H. pylori* in human urine by using PEO in the CE analysis [61]. Another approach is capillary surface modification e.g. by divinylbenzene (DVB) or preparation of new types of materials for bio-separation [52,66]. Moreover, Yu and Li emphasized the importance of proper preparation of bacteria samples – the vortex and sonication processes seem to be crucial in decreasing the aggregation level of microorganisms [67]. However, over past years a new method based on microbial surface modification by divalent metal ions was suggested [54,62]. Application of this method may result in controlled aggregation of microorganism's cells. Rogowska et al. investigated the impact of the cell surface modification by Ca^{2+} ions on the control clumping of *Saccharomyces cerevisiae*, and the results of this study pointed out that during the CE analysis of cells modified by 5 mM Ca^{2+} , and with the increasing value of medium pH, the sharpening of the peaks and the reduction in the number of *S. cerevisiae* aggregates were observed [54]. This phenomenon was correlated with the role played by calcium ions in the controlled clumping of yeast cells by binding surface-carboxyl groups or lectin-like mechanisms of flocculation, in which Ca^{2+} enables the lectins to acquire a suitable active conformation and thus interact with carbohydrate residues of α -mannans present on the cell surface [68]. Moreover, results obtained by Rogowska et al. indicate that the proposed new sample preparation approach for wild microorganism strains may become in the future a foundation for the application of capillary electrophoresis in diagnostic laboratories [54]. Different pH and modification of the cells by Ca^{2+} influence the molecular profiles of yeast cells but do not affect the identification quality of MALDI-TOF MS equipped with the BioTyper database. Such data may provide a foundation for coupling capillary electrophoresis and the MALDI-TOF MS analysis. Another example can be evaluation of the Zn^{2+} aggregation activity on probiotic strains. Studies conducted by

Buszewski et al. described the electrophoretic determination of *Lactococcus lactis* modified by zinc ions at different concentration levels [69]. The referenced study shows the formation of microbial aggregates after the surface modification and, what is more, proposes the possible mechanism of this process.

2.5. The use of CE in modern hyphenated techniques approach

Due to very high demand for SARS-Cov-2 identification, alternatives to the standard PCR tests are potentially useful for increasing the number of samples screened. Gómez et al. describe a technical approach to SARS-Cov-2 testing by amplifying fragments of the viral genome with 5'-fluorescent primers followed by capillary electrophoresis [70]. The proposed method allowed the analysis of 96 samples in approximately 5 h. The two SARS-Cov-2 fragments were successfully amplified in the positive samples, while the negative samples did not render fluorescent peaks. The researchers validated 20 virus positive and 10 virus negative samples. The aim of that study was not to substitute the gold PCR technique, but to develop an alternative to facilitate the analysis when the demand for COVID-19 testing exceeds the capacity of the PCR of any lab that can implement the CE technique [70]. Already in 2008, Buszewski et al. proposed using capillary electrophoresis, PCR and physiological assays in differentiation of clinical strains of bacteria. The electrophoretic measurements involved the differential mobility of bacteria in a fused silica capillary under the direct current electric field. To perform coagulase gene typing, the repeated units encoding hypervariable regions of the *S. aureus* gene are amplified using the PCR technique, by restriction enzyme digestion, then followed by the analysis of restriction fragment length polymorphism patterns as well as sequencing. Finally, the results of electrophoretic measurements were compared with molecular analysis [71]. The same group utilized CE, zeta potential and coagulase gene polymorphism as a very specific combination for differentiation of the same species of clinical bacterial strains. The data presented in this contribution suggested that electrophoretic behavior and the values of zeta potential should be very useful in distinguishing between closely related strains which exhibited coagulase gene/protein polymorphism [72]. Coupling standard PCR or molecular biology methods with capillary electrophoresis seems to be a very powerful tool to overcome the limits imposed on many labs by the PCR requirements and thus increase the testing capacity.

Nowadays, also the lab-on-a-chip (LOC) technology has demonstrated its superior capability of rapid determination of pathogens. This type of technique combination is characterized by short analysis time, low sample and power consumption, high integration, high throughput and portability; thus it shows great potential in the areas of genetics, proteomic and cellular research. For example, it has been proven that a number of genetic analysis steps such as sample purification, nucleic acid amplification and amplicon separation can be done on microfluidic devices. Polymerase chain reaction–capillary electrophoresis microdevice for pathogen detection is another method which can be used for sample separation and purification. The combination of the polymerase chain reaction with microcapillary electrophoresis (mCE) has shown excellent performance in terms of sensitivity, speed and specificity. A fast static PCR reaction was performed in a nanoliter scale chamber and the resultant PCR amplicons were subsequently separated in the CE microchannel. Kim and coworkers utilized a capillary electrophoresis-based multiplex PCR (CEMP) panel to enable the detection of viral and bacterial pathogens associated with community-acquired pneumonia (CAP) [73]. They simultaneously determined 13 common unculturable CAP viral and bacterial pathogens within 4 h. They also evaluated the performance of

a commercially available panel with 314 samples collected from CAP patients. Finally, the authors compared the results to those obtained with the liquid chip-based Luminex xTAG Respiratory Viral Panel (RVP) Fast Kit (for viruses) and the agarose gel-based Seegene PneumoBacter ACE Detection Kit (for atypical bacteria). All positive samples were further verified by the Sanger sequencing method. The proposed procedure provides a rapid and accurate method for high-throughput detection of pathogens in patients with CAP [74].

Another significant and useful analytical solution is capillary electrophoresis coupled to MALDI mass spectrometry. While CE-MALDI interface has been explored by numerous groups in a variety of contexts, the coupling with MSI is a relatively recent and innovative technique. The increased number of identifications in biological samples is largely attributed to improved separation resolution. Other methods often couple CE with MALDI using fraction collection; however, by continuously collecting liquid (containing bacterial and/or viral cells) from the capillary onto the plate and performing MS imaging, the resolution can be dramatically expanded. The research presented by Delaney used larger injection volumes and enhanced separation resolution with a positively-charged capillary coating that reduces peptide adsorption and reverses electroosmotic flow (EOF) [75]. With this method, an increase in the number of detected neuropeptides in two tissue sample types was obtained with good reproducibility. Other studies presented by Horká et al. involved nano-etched fused-silica capillary used for on-line preconcentration and electrophoretic separation of bacteriophages from large blood sample volumes with off-line MALDI-TOF mass spectrometry [56]. This is very important because bacteriophages have a great potential for developing specific diagnostic and therapeutic tools against bacterial infections. The phages that exhibit a selective lytic effect towards bacterial cells can be applied directly into living tissues. The authors monitored staphylococcal phages using capillary electrophoretic methods on fused-silica capillaries with different morphology of surface roughness. After electrophoretic analysis, viability of the detected phages was verified by the modified “double-layer drop assay” method, and collected phage fractions were simultaneously analyzed off-line by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Small viruses have diameters in the range of tens of nanometers. Bacteria are generally larger, some by a factor of 100, and the larger size leads to increased complexity. The microbial surface charge originates from the ionization of surface molecules and the adsorption of ions from solution. Bacterial cell walls and membranes contain numerous proteins, lipid molecules, teichoic acids, and lipopolysaccharides that contribute to this characteristic charge. The undesired effects occurring during electrophoretic analysis such as aggregation and/or adhesion of the cells have a strong effect on the separation process. For a better understanding of this phenomenon, Buszewski and coworkers decided to apply a fluorescence stereomicroscope as an in-line detection unit for observation of electrophoresis of microorganisms. It was the first such in-line combination of CE and fluorescence stereomicroscope which allowed direct observation of migrating zones of bacterial cells and determining the viability of these cells [76]. Taking into account the mechanism of bacterial migration in the direct current electric field the knowledge of the electrical properties of microorganisms is of benefit for the biofilms electrokinetic formation – aggregates with a defined structure. It also plays a very important function in detection of physical changes of bacterial cells and is emerging as a modern diagnostic tool to determine their phenotypic features. One of such changes is bacterial viability. It is widely known that when the cell dies, the membrane potential breaks down and the viable cells are more voluminous. These

changes are directly associated with alteration in zeta potential for particular bacterial species, strains or even viable or dead bacteria. In the case of medical diagnosis and pharmaceutical application, an analytical method capable of detecting viable or dead cells with a simple sample preparation procedure is also highly desirable. Zeta potential measurements are helpful in understanding the electrophoretic separation behavior and provide more information about the charge which is present on the cell surface of the examined bacteria. The electrophoretic data, the zeta potential measurements and microscope observations were compared and found to be consistent. Furthermore, the use of unique and very precise CE-NMR coupling helped to estimate which characteristic functional groups present on the surface of microbial cells play a key role in electrophoretic separation. The NMR spectrum represents all functional groups in a sample, and functional groups on the surface of the particle are not identified separately. On the other hand, the FTIR and XPS spectra obtained for microorganisms identified the impact of functional groups on their properties [77].

The separation of bacterial cells might be a basis of fast capillary electrophoretic cell sorting (fractionation). Here, any practical applicability of such a method would be directly connected with the viability of the cells which, after being electrophoretically sorted, could be further cultivated or directly examined by detection methods of choice. Capillary electrophoresis was used, for example, for the determination of bacteria viability in pharmaceutical products or as sterility test in food samples. The bacteria were stained with live/dead fluorescent dye test solutions before the electrophoretic process. Then with a fluorescence detector it was possible to assess the ratio of living and dead cells in the preparation, which corresponded to its quality. However, a much more sensitive method involving a flow cytometer (FC) to determine the viability of microbial cells can be used as well. Salzano et al. performed analysis by cytometry and capillary electrophoresis in ethanol-stressed *Oenococcus oeni* strains and assessed changes of membrane fatty acids composition. As malolactic bacteria play an essential role in wine production methods that use malolactic fermentation (MLF), efficient control of these microbiological processes requires increased knowledge of bacterial behavior under stress conditions that can affect bacterial viability and activities. Salzano et al. assessed the different physiological states of *O. oeni* strains by nucleic-acid double-staining flow cytometry assay, which allowed the researchers to differentiate between viable and damaged/membrane-compromised (dead) cells of *O. oeni* strains, and to study ethanol-induced variations of cell membrane fluidity and permeability with the application of capillary electrophoresis to evaluate the changes in the charging rates on cell surface [78].

3. Sample preparation techniques for pathogen determination

Another scientific challenge is pre-separation and pre-concentration of samples, particularly when pathogens are uncultured and their concentration in the matrix is low. According to the literature data [79], electrophoretic approach might be promising for both pre-concentration and subsequent separation of pathogens according to their properties. Furthermore, adhesion of bio-colloids such as microorganisms onto the inner capillary surface can also be used for their concentration and separation from large sample volumes of high conductivity matrices. Thus techniques such as capillary zone electrophoresis (CZE) [80], isoelectric focusing (CIEF) [81] and isotachopheresis (CITP) [82] deserve researchers' attention. For instance, CIEF is a great tool for separation of amphoteric analytes due to differences in their isoelectric points (pI); it also provides an opportunity to increase sample concentration in a single step [79]. Horká et al. used isoelectric focusing for

analysis of three microbial strains (*Micrococcus luteus*, *Dietzia* sp., and *Rhodotorula mucilaginosa*) [79]. The pre-separation and pre-concentration of microbial cells was possible due to cellulose-based separation medium. Then the collected fractions of cells were used for the MALDI-TOF MS analysis. Another work of Horká et al. presents the combination of capillary electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the analysis of methicillin resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa* [83]. Bacterial strains were pre-concentrated from the rough part of the capillary surface with the use of isotachophoretic stacking, collected from the capillary, and then off-line analyzed by MALDI-TOF-MS. Thus the coupling of electrophoretic methods with matrix-assisted laser desorption/ionization mass spectrometry seems to be pivotal for sample preparation and microbial analysis (Fig. 2).

Electromigration techniques can also be used when it comes to viruses. In 1987 Hjertén et al. published the first paper demonstrating the possibility of analysing virus samples with capillary zone electrophoresis [55]. They recorded a single, sharp peak of *Tobacco mosaic virus*. Schnabel et al. were the first to apply CIEF to determine the pI value of human rhinovirus serotype 2 – HRV2 [84]. Both of these techniques can be also used in pre-separation and pre-concentration of virus samples from culture media. Horká et al. examined the conditions for simultaneous separation and detection of phage K1/420 and *S. aureus* by CZE and CIEF [85]. First, they purified and pre-concentrated the bacteriophage from the matrix using preparative IEF – the harvested fraction of the phage was pre-concentrated approximately ten times. In the next step the authors detected separated phage particles in the presence of its host, *S. aureus*, with the use of CZE and MALDI-TOF MS.

Pre-separation and pre-concentration is particularly important in the case of biological samples, which are usually available in small volumes and have low concentration of the analyte in biological matrices. The team led by Horká conducted an experiment in a real environment represented by whole human blood and human plasma [56]. In the test they used five different staphylococcal phages, which were separated and pre-concentrated with

the use of CE techniques. CIEF allowed determination of pI values of the examined phages while micellar electrokinetic chromatography (MEKC) made it possible to choose the proper type of capillaries for separation of the phage mixture. The researchers then used off-line combination of MEKC in an FS capillary with a roughened part, and MALDI-TOF MS analysis of the collected fractions. A plasma sample was spiked with *S. aureus* and phage K1/420. The MALDI results showed that the bacteriophage was effectively separated from the remaining components of the sample. The authors also amplified phage K1/420 on the host cell directly in the MEKC capillary, which allowed them to pre-concentrate bacteriophages from very low initial concentrations. It is possible to increase concentration of the phages even more by performing two-step propagation [86]. In this process, both the phages adhered on the capillary and the phages generated by the propagation interacted again with the new host cells.

Field-flow fractionation (FFF) is another method which can be used for sample separation and purification. It is particularly suited to samples of biotechnological interest, where analytes range from macromolecules such as proteins or nucleic acids to micron-sized particles such as bacteria, viruses and whole cells. This technique is similar to LC but has no stationary phase, which makes it possible to avoid unwanted interactions of analytes with the phase. FFF relies on the interaction of analytes with an externally generated field, which is applied perpendicularly to the direction of the mobile phase flow. Due to differences in their density, size or surface properties, which result in different retention times, the field drives the analytes into different laminar flows [13,87]. The separation of viruses has been successfully achieved by the use of e.g. sedimentation FFF (which uses a sedimentation field generated by a centrifuge) and asymmetrical flow FFF.

After pre-concentration or separation, a sample can be identified by laser desorption/ionization techniques (LDI), such as the MALDI, NALDI, SELDI (surface enhanced laser desorption/ionization) and DIOS (desorption/ionization on silicon) (Fig. 3). LDI methods allow analyzing many biomolecules, but they are rarely applied to the detection of viruses in complex biological samples.

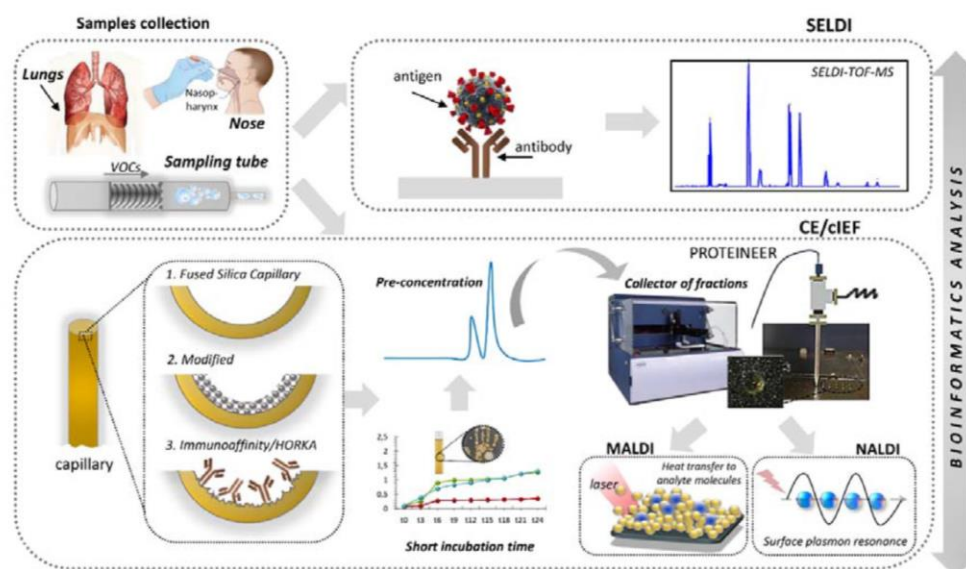


Fig. 2. Coupling electromigration techniques with laser desorption/ionization approach in virus analysis.

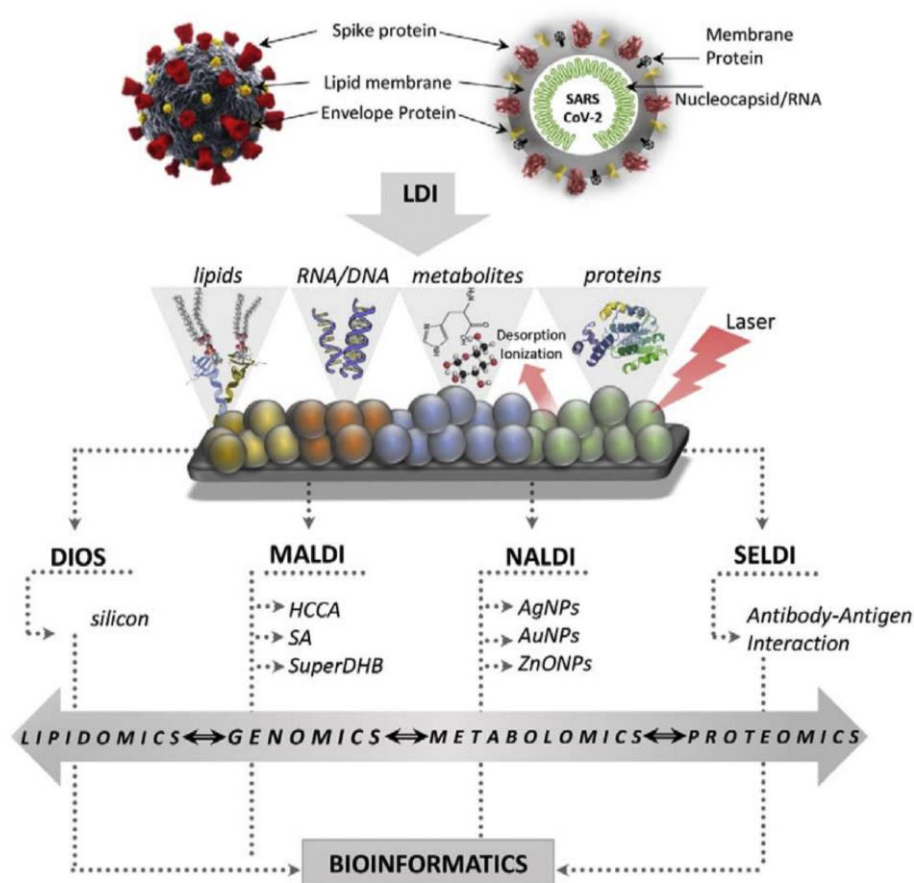


Fig. 3. Laser desorption/ionization analysis of virus molecular compounds.

MALDI is the most common of LDI methods and has been already used to investigate a wide variety of viruses, also from clinical samples. This method was applied to identify for instance polioviruses [88], viruses isolated from respiratory tract samples [89], human *Papillomavirus* [90], and SARS-CoV-2 [39]. A similar technique is SELDI, which was used by Golizeh et al. to compare serum proteome profiles at different stages of fibrosis in HCV and HIV/HCV infected patients [91]. Table 1 summarizes virus analyses by approach to pathogen identification.

4. Future directions in identification of microbial pathogens

The last decades saw the emergence of many modern techniques for determination and identification of microbial samples. Despite their limitations, culture and microscopy are still two of the staple techniques. PCR and other genetic approaches are particularly significant in case of non-culturable pathogens. MS has been shown to be a useful, quick and easy approach to identification of microbial samples and detection of microbial threats; however, it is reserved for pure isolates and cannot be used for complex samples since they may promote background interference. This may be circumvented through the use of separation-based methods, such as chromatography – HPLC, LC-MS or field flow fractionation (FFF). In the future, determining limits of detection for pathogens will

continue to be a key goal of clinical microbiology. Combination of the above (and possibly other) methodologies and instrumentation will surely improve the pathogen detection capability.

One of the most promising options is the lab-on-a-chip technology that can perform various laboratory operations on a miniaturized scale. Miniaturization of the devices such as A4F separation canal or LDI targets allows reduction of reagents, which results in reduction of analysis costs. Another important advantage is speed – by using chips it is possible to obtain simultaneously a multiplicity of parameters. What is more, this technology allows automation and high-throughput screening. However, it is mainly used for research purposes. Its usefulness for routine diagnostics is limited due to relatively high costs of equipment and insufficient standardization, which results in low reproducibility of the results. In the case of microarrays, the type of molecules immobilized in the array affects their application – DNA microarrays can be applied to testing for DNA from pathogenic organisms or to resequencing of a pathogen, antibody microarrays can detect pathogen proteins or antigens present in clinical or environmental samples, and small-molecule microarrays offer novel approaches for differentiating between pathogens. There are several texts in the literature describing the application of microarrays to detection of pathogens, such as viruses. Fig. 4 presents future perspectives of the lab-on-a-chip.

Table 1
Summary of virus analyses by approach to pathogen identification (n/a - not available).

Virus	Sample source	Analytical Method	No. of samples	Reference
HBV, HCV	serum	PCR	730	[92]
Enteric viruses	stool samples	monoplex or multiplex RT-PCR	227	[93]
HRSV	nasopharyngeal and oropharyngeal swab	real-time RT-PCR	334	[94]
DENV, ZIKV	serum	RT-qPCR	46	[95]
Respiratory viruses	throat swabs and sputum samples	RT-qPCR	408	[96]
Respiratory viruses	nasopharyngeal aspirates, nasopharyngeal and oral pharyngeal swab	rRT-PCR	8173	[97]
SARS-CoV-2	sputum, nose and throat swabs	real-time RT-PCR	297	[98]
LASV	serum, lung and spleen tissue, blood	RT-qPCR	82	[99]
Respiratory viruses	respiratory tract swabs and aspirates	MALDI-TOF MS	58	[89]
HR HPV	uterine cervix cytology	MALDI-TOF MS	356	[90]
Poliovirus	cerebrospinal fluid, throat swab, stool samples	MALDI-TOF MS	5	[88]
SARS-CoV-2	nasal swab	MALDI-MS + machine learning analysis	362	[39]
Enterovirus EV71	serum	LDI-MS with cellulose acetate membrane and Ag or Au NPs	n/a	[100]
HIV, HCV	serum	SELDI MS	151	[91]
Bacteriophages				
Triaviruses, Phieta viruses, Biseptimaviruses, Kayviruses, Twortvirus, P68virus	reference and isolates	MALDI-TOF MS	n/a	[101]
vB-SdyS-ISF003	wastewater and sewage	PCR and sequencing DNA	n/a	[102]
Kayvirus K1/420	medicament isolate	CZE, MALDI-TOF MS	n/a	[85]
Staphylococcal phages (K1/420, 11, P68)	physiological saline solution, human serum	MALDI-TOF MS	n/a	[86]
Staphylococcal phages (K1/420, 11, P68, 3A, 77)	blood, serum	MALDI-TOF MS	n/a	[56]

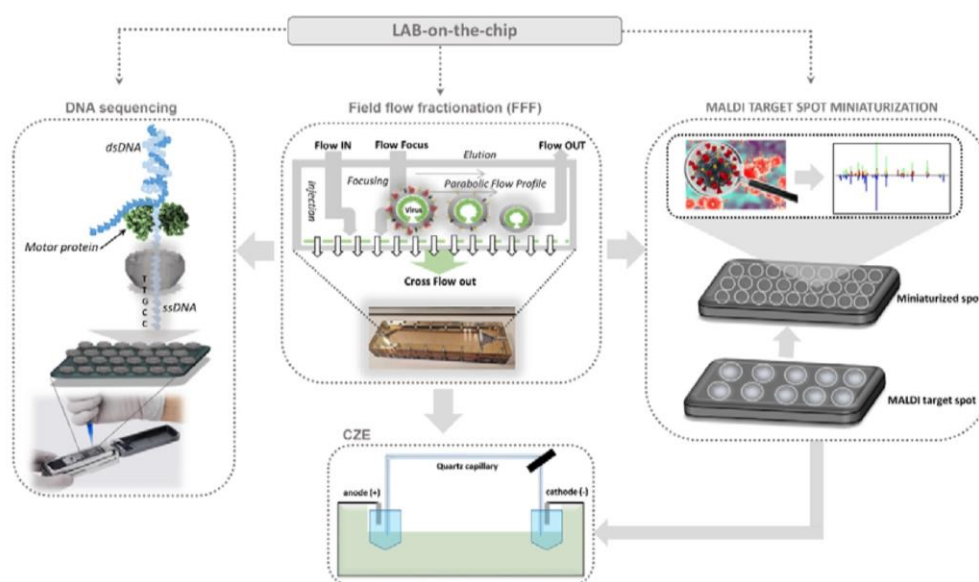


Fig. 4. Future perspectives of lab-on-a-chip.

The development of new, effective methods of microbial identification should be based on a modern interdisciplinary approach. In order to improve microbial identification, pathogens must be characterized comprehensively. A very important step in microorganism identification is the selection of culture conditions for clinically relevant pathogens as well as virus samples (e.g. *tobacco mosaic virus*, *hepatitis virus* or clinical isolates of CoVID-19). The optimization of virus growing should be performed on the models of viruses, e.g. *Tobacco mosaic virus* cultivated on the plant leaves or *hepatitis virus* cultivated on commercially available hepatocyte cells. After these procedures, the isolated pathogens can be pre-

concentrated by using electromigration techniques. For this step capillary zone electrophoresis (CZE) and capillary isoelectric focusing (CIEF) can be used in different capillary coatings modes. Another possibility is the use of optical and fluorescence microscopy and flow cytometry to verify the effectiveness of the method in the case of bacteria. Matrix-assisted laser desorption/ionization technique is the latest generation tool used for rapid identification and classification of microorganisms; it is characterized by high precision, low sample consumption, short analysis time and relatively low unit cost. Results obtained in this way will be compared with the results of sequencing of the conservative 16S rDNA gene,

which is now considered to be the gold standard in microorganism identification. Nowadays the nanostructure-assisted laser desorption/ionization (NALDI) approach complements the classic MALDI in the field of modern bioanalytics.

Coupling sample treatment devices to commercial CE systems is of special interest with regard to developing in vivo analysis, facilitated by the ability of electrophoretic systems to use small volumes of sample and provide rapid analyses. For example, a microdialysis needle on-line coupled to a commercial CE system enabled in vivo monitoring of the evolution of a drug during brain tissue analysis. Miniaturization of sample treatment devices will undoubtedly expand the potential of this combination further. As to interfacing non-integrated detection modes with commercially available CE equipment, the properties of a specific analyte dictate which method is best suited to its detection. For example, with analytes containing fluorophore/electroactive groups, fluorescence/electrochemical detections can be an appropriate choice, made with a view not only to enhancing sensitivity but also to improving the apparent selectivity through specificity. CE-PCR, CE-MALDI TOF MS, CE-fluorescence stereomicroscope, CE combination with flow cytometry, as well as very specific CE-NMR combinations seem to be very promising tools that can supplement the standard methods used in medical diagnostics, especially in the face of the present epidemiological threat. Additionally, these unique combinations make it possible to explain the phenomena occurring during electrophoresis of microorganisms and elucidate its full mechanism. It should be taken into account that the use of MALDI MS coupled with electromigration techniques is a very selective method of detection and constitutes a type of imaging. It is also very important to fully learn the mechanism of the separation process itself as well as the mutual interactions between the cells. Moreover, the 2020 pandemic has very clearly demonstrated the need for further developments in this field of analytics.

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.

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Publikacja [P2]

Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approach

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RESEARCH ARTICLE

Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches

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Abstract

One of the challenges medicine faces is the constantly growing resistance of pathogens to various classes of antibiotics. In this study, we investigated the use of capillary electrophoresis (CE) to characterize and assess the physiological states of three clinical bacterial strains—methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA), and *Escherichia coli* extended-spectrum β -lactamases (ES β L)—exposed to different antibiotics. All chosen bacteria are the leading causes of healthcare-associated and hospital-acquired invasive infections in adults. In the first part of the research, it was determined the optimal incubation time of the tested strains with antibiotics, represented as an optimal time of 24 h. In the second part, we have compared two approaches: flow cytometry (FC) as a standard method and CE as a proposed alternative approach. The viability of clinical strains treated with different class antibiotics calculated in CE measurements was strongly correlated (>0.83 for MSSA, >0.92 for ES β L and MRSA) with the viability obtained on the basis of FC measurements. As a result, CE has a chance to become a modern diagnostic method used in clinical practice. The CE cutoff was found to be 50%; above this value, the strain shows resistance to the action of the antibiotic.

KEYWORDS

bacteria, capillary electrophoresis, clinical strain, flow cytometry, viability

Abbreviations: AMOX, amoxicillin; ASA, antibiotic susceptibility assay; CEF, cefotaxime; CEFL, cefazolin; CL_{IP}, imipenem with cilastatin; CLIN, clindamycin; CTR, ceftriaxone; ES β L, extended-spectrum β -lactamases; FC, flow cytometry; L_{eff} , effective capillary length; L_{tot} , total capillary length; MET, metronidazole; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; PBP, penicillin-binding proteins; TD-ASA, time-dependent antibiotic susceptibility assay.

1 | INTRODUCTION

Knowing the structure of a bacterial cell, we are able to understand how antibiotics affect it. There are four basic mechanisms of action of antibiotics, causing changes in the permeability of the cell membrane, blocking the process of cell wall synthesis, disrupting the process of protein production, and inhibiting the synthesis of nucleic acids. Antibiotics can be classified according to their mechanism of action [1]. They can also be classified according to their chemical structure into: β -lactam antibiotics, macrolides, aminoglycoside, and tetracycline antibiotics. β -Lactam antibiotics include penicillins, five generations of cephalosporins, carbapenems, and monobactams and are the most frequently used commercial antibiotics to treat infectious diseases caused by various bacteria strains [2]. β -Lactam antibiotics are considered drugs of last resort, which means that they are often used in the most severe infections. The mechanism of their action is based on changing the pathway of synthesis of peptidoglycans included in the cell wall, which leads to cell lysis. In turn, bacterial resistance to β -lactams is controlled by a variety of mechanisms, which are classified into four basic strategies: (i) reducing the permeability of cell membranes, (ii) removing drugs from inside the cell, (iii) modifying natural PBPs (penicillin-binding proteins) so that they have no affinity for β -lactams, (iv) synthesis of enzymes that hydrolyze drug molecules [3].

Years of human abuse of antibiotics in various industrial and food sectors, poor hygiene, and ineffective infection prevention in healthcare significantly impacted the development of antibiotic resistance. Currently, no single β -lactam is free from resistance. A report published by World Health Organization in 2017 highlighted a list of 12 species of bacteria that are considered global priority pathogens—with medium-to-critical resistance to antibiotics [4]. The critical strains on this list include Enterobacteriaceae, which are more and more often showing resistance to β -lactam antibiotics. The greatest threat is posed by strains that have become resistant to many antibiotics, like some *Escherichia coli* strains, which provide themselves resistance through the production of ES β L (extended-spectrum β -lactamases) enzymes, which are responsible for resistance to most β -lactam antibiotics. Worldwide, ES β L-producing *E. coli* isolates are prevalent among animal (average 63%), human (42%), and environmental (>30%) samples [5]. *E. coli* is a leading cause of intestinal, cardiovascular, urinary, and nervous system infections. The toxins they produce can cause diarrhea, ulceration, or inflammation [6]. Among the high-priority bacterial pathogens are methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant

S. aureus strains [4]. *S. aureus* is responsible for multiple human infections, including bacteremia, skin and soft tissue infections (e.g., diabetic foot, bedsores, folliculitis, and others), infective endocarditis, osteomyelitis, arthritis, and prostheses, and inflammation of the lungs, meninges, stomach, intestines, and urinary tract infections [7]. To overcome the ever-growing problem of antibiotic resistance, new types of anti-infective drugs with different mechanisms of action are being synthesized. One of the types is antimicrobial peptides that are mainly obtained from various natural sources—animals, plants, and other organisms. Depending on the structure of these peptides (the type of amino acid residues), these agents can affect the viability of the bacteria by disrupting the integrity of the membrane or moving to specific intracellular elements [8, 9].

Microbial susceptibility testing is based on determining the concentration of an antibiotic that is able to effectively reduce the viability of the bacteria, which will stop the development of the infection [10]. However, they are dependent on many environmental factors, which can lead to incorrect conclusions [11]. RNA/DNA-based detection methods make it possible not only to determine the resistance gene but also to determine the viability by using additional DNA intercalating dyes [12]. However, flow cytometry (FC) remains the most popular diagnostic technique that enables viability analyses of bacterial cells treated with antibiotics. It requires labeling the cells with one or more dyes. Fluorescent dyes that emit light at different wavelengths are the most commonly used ones [13]. In our previous work, we presented the possibility of using capillary electrophoresis (CE) to assess the viability of bacterial ATTC strains treated with various antibiotics [14]. The obtained results showed a high correlation between the viability obtained by FC and the CE study, proposed as an alternative method. Until now, electrophoresis in the analysis of bacterial cell viability required additional connections. Armstrong used a CE system equipped with a laser-induced fluorescence detector [15]. Buszewski examined the viability by measuring the zeta potential and using the CE fluorescence stereomicroscope setup [16, 17].

In turn, in the present study, we employed this method to the clinical strains: MRSA and MSSA (methicillin-sensitive *S. aureus*), and *E. coli* ES β L—all three strains are considered pathogens and two of them—resistance. For each strain, we selected several antibiotics based on EUCAST (European Committee for Antimicrobial Susceptibility Testing) and CLSI (Clinical Laboratory Standards Institute) guidelines. Using the complementary approach, two studies were conducted in which the viability of the bacteria was assessed. Analyses were first performed by

optical density (OD) measurements followed by CE analysis and confirmed by standard FC technique. The first part of the research was carried out in order to determine the optimal time of incubation of bacteria with antibiotic solutions, which would enable the reduction of viability. The second part was aimed at the possibility of using CE in the assessment of the viability of the tested pathogens treated with different antibiotics.

2 | MATERIALS AND METHODS

2.1 | Samples preparation

In the present study, three clinical bacterial strains were used: MRSA, MSSA, and ES β L *E. coli*. The strains came from the collection of the Provincial Polyclinical Hospital in Toruń. The strains were aerobically revitalized on plates with Tryptic Soy Agar (Sigma Aldrich, Germany) at 37°C for 24 h. Then the bacterial biomass was transferred to the liquid Mueller Hinton Broth medium (Sigma Aldrich, Germany), and an inoculum = 1×10^6 CFU/ml (colony forming unit) was prepared for each strain.

Based on the guidelines (EUCAST and CLSI) for each bacterium, a set of antibiotics and their concentrations was selected. The following antibiotics were used: clindamycin (CLIN), imipenem with cilastatin (CL_IP), cefotaxime (CEF), metronidazole (MET), amoxicillin (AMOX), ceftriaxone (CTR), and cefazolin (CEFL) (all products purchased from Sigma Aldrich, Steinheim, Germany). The antibiotics were dissolved in deionized water obtained from the Milli-Q apparatus (Millipore Intertech, MA, USA)—the final concentration was between 0.1 and 8 mg/ml.

In the first step to select the appropriate incubation time for bacterial strains with antibiotics, time-dependent antibiotic susceptibility assay (TD-ASA) was performed. One antibiotic, which EUCAST and CLSI recommend, was used for each strain—for *S. aureus*, it was CLIN, and for *E. coli*, it was CL_IP. Bacterial cells were mixed (1:1) with previously mentioned antibiotic drugs at a constant concentration of 8 mg/L. The TD-ASA study was performed by determining the bacterial viability after 1, 12, and 24 h of incubation with the selected antibiotics. After this time, the samples were triplicate subjected to the OD measurement, FC, and CE for the viability investigation. In the next step, ASA with the remaining antibiotics was performed for the tested strains in various concentrations. Bacterial cells were mixed (1:1) with antibiotic's solutions and incubated for 24 h. After this time, the bacterial viability was also measured three times using OD, FC, and CE (see Figure 1).

2.2 | Optical density measurements

Measurements of the samples (both TD-ASA and ASA) were performed at the wavelength of 600 nm (λ) using a UV-Vis spectroscope (Multiskan FC Microplate Photometer). The effect of the background's own absorbance of the pure medium was taken into account. Controls were bacterial suspensions in the medium.

2.3 | Flow cytometric measurements

To each sample, 1 μ l of propidium iodide solution (Sigma Aldrich, Poznań, Poland) was added at a concentration of 100 μ g/ml. The samples were then shaken and incubated at room temperature in the dark for around 30 min at room temperature. For their analysis, the MACSQuant VYB cytometer (Miltenyi Biotec, Clopper Road, USA) was used. A blue laser (488 nm) at channel pairs of 655–730 and 585/40 nm was used to differentiate between live and dead cells. Each sample was measured three times. MACSQuantify was used for the gating procedure, and standard spreadsheet software (Excel 2016) was used for further processing.

2.4 | Capillary electrophoresis measurements

All analyzed samples (TD-ASA and ASA) were rinsed twice with deionized water, and then the surface of the bacterial cells was modified with 0.005-M Ca(NO $_3$) $_2$. Purified from excess calcium ions, the bacterial pellet was suspended in a TB buffer (Tris C_{Tris} = 4.5 mM, boric acid C_{B} = 50 mM, pH 7.98–8.3) and subjected to electrophoretic analysis by means of CE using the apparatus HP3DCE with spectrophotometric diode array detector and fused silica capillaries of a 75- μ m inner diameter with a total capillary length (L_{tot}) of 33.5 cm, and an effective length (L_{eff}) of 25 cm (Composite Metal Services, Shipley, UK). The viability of the bacteria incubated with the antibiotic solutions was calculated based on their electromigration times of viable cells (without antibiotic—control samples) and dead cells (viable cells inactivated with 70% ethanol). For this purpose, we used the formula we proposed (Equations 1 and 2), where X [% , m/m]—percentage of live cells, Y [% , m/m]—percentage of dead cells, t_{A} —electromigration time of dead cells (MRSA— 2.03 ± 0.08 , MSSA— 1.89 ± 0.09 , *E. coli* ES β L— 2.11 ± 0.08), t_{B} —electromigration time of live cells (MRSA— 2.66 ± 0.09 , MSSA— 2.65 ± 0.08 , *E. coli* ES β L— 3.22 ± 0.05), t_{i} —electromigration time of cells treated with an antibiotic

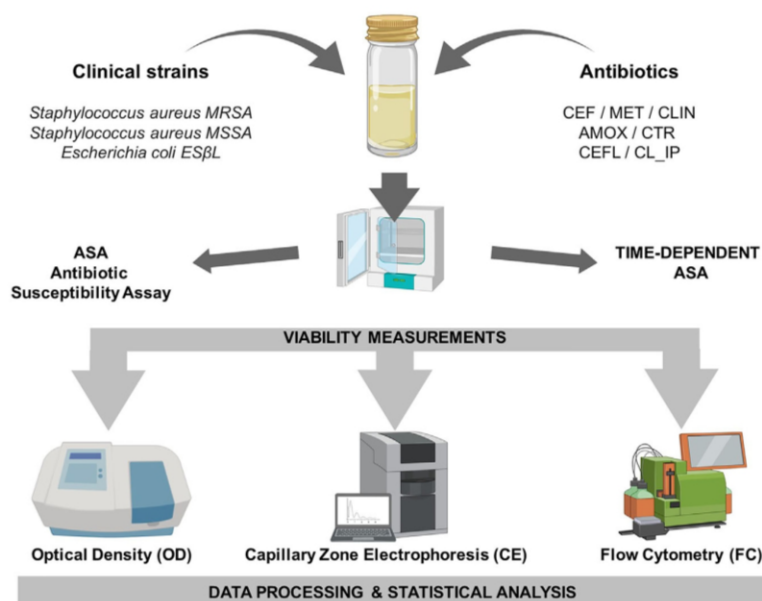


FIGURE 1 Scheme of workflow

(Figure S1). More information on the CE analysis conditions and the calculation of bacterial viability can be found in our previous work [14]. Peak area (P) in function of OD ($P = f(\text{OD})$) was used for quantification of live bacterial cells. The obtained calibration curves for studied clinical bacterial strains were presented in Figure S2.

$$X [\%] = \frac{t_i - t_A}{t_B - t_A} 100\% \quad (1)$$

$$Y [\%] = 100\% - X [\%] \quad (2)$$

2.5 | Statistical analyses

All data were processed using a complimentary approach. For both processing of raw data and for performing the Pearson correlation coefficient test, the Microsoft Excel 2016 was used. The correlation analysis of the obtained data was performed on the basis of Student's t -test for $p = 0.05$. Two-dimensional scatterplots with the regression lines were used to determine the relationship between the variables CE and FC using STATISTICAL Release 7 software.

3 | RESULTS

ASA: First, TD-ASA has been performed in order to find the optimal incubation time of three clinical strains (MRSA, MSSA, and *E. coli* ESβL); the bacteria cells were

exposed up to 48 h individually for all selected antibiotics (Section 2.1). Based on the viability determined by FC technique, a Pearson's test and dendrograms were plotted (Figure 2A), which show the similarity between the measurements after 1, 24, and 48 h of incubation. There was no significant difference between the incubation of 1 and 48 h for MSSA (Figure 2A), and all correlations were >0.99 . Only for this strain, a greater similarity was observed between the 1- and 24-h measurements on dendrograms. In turn, in the case of MRSA and ESβL strains, the strongest correlation observed between the measurements performed after 24 and 48 h was 0.9983 and 0.9994, respectively, which corresponds to the results represented by the dendrograms. Therefore, once 24 h was the common and optimal time for all strains, the ASA experiments were performed with 24 h of incubation.

For this step, seven antibiotics were used that had different mechanisms of action on the bacteria: five β -lactam antibiotics belonging to carbapenems (CL_IP), penicillins (AMOX), and cephalosporin (CEF, CTR, and CEFL), and two others (MET and CLIN). The *in vitro* efficacy of antibiotics against three clinical strains, MRSA, MSSA, and *E. coli* ESβL, was assessed by measuring the viability of bacteria through OD, CE, and FC measurements. In order to test the usefulness and correctness of the proposed CE model, a separate correlation matrix was prepared for each bacterium in the Pearson correlation tests (see Figure 2B). The correlation values significant at a $p = 0.05$ level ranged from -0.9999 to 0.9846 , which pointed from strong negative to strong positive correlation. For all bacteria, a high negative correlation (around -0.9) was

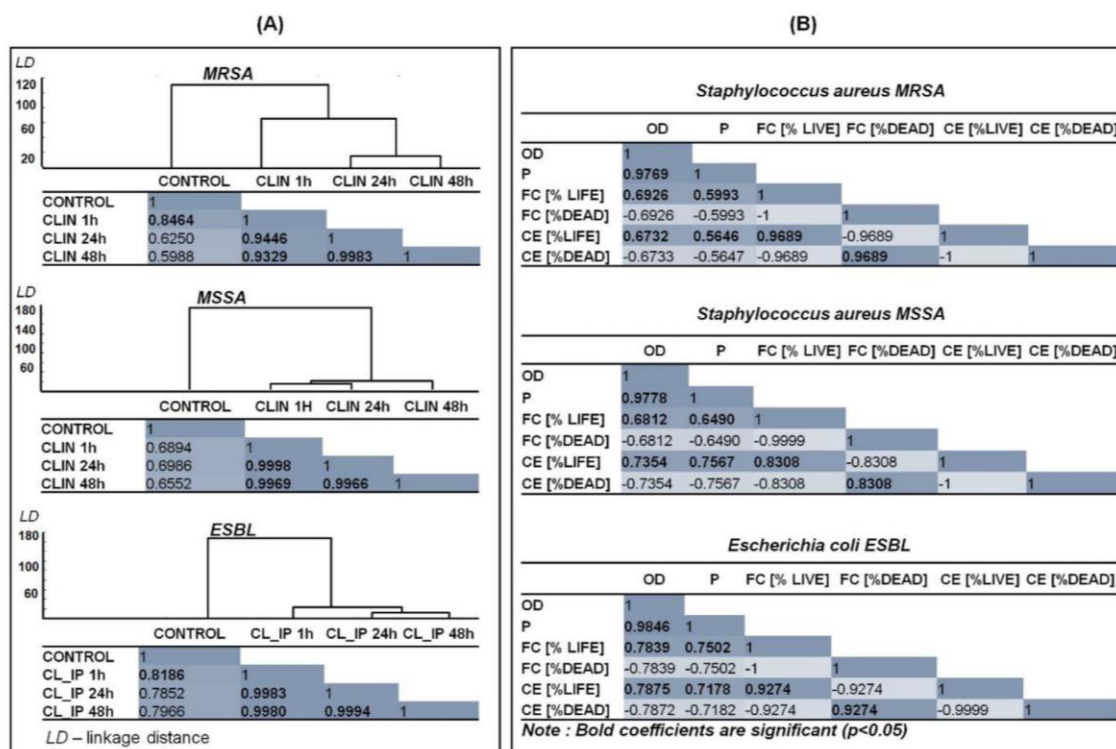


FIGURE 2 (A) Dendrograms and Pearson's test performed between different incubation times in time-dependent antibiotic susceptibility assay (TD-ASA). (B) Correlation matrix showing the significance between used methods: CF, capillary electrophoresis (CE), optical density (OD), and peak area (P) in the antibiotic susceptibility assay

observed between the percentage of viable cells obtained by the FC and the percentage of dead cells by CE (and vice versa). A smaller negative correlation (around -0.7) was also observed between the OD or peak area values and the percentage of dead cells measured with both FC and CE. For the remaining parameters, a strong positive correlation was noticed (>0.56). The highest values (>0.9) were identified between the FC and CE methods (both when comparing live and dead cells) for the MRSA and ESBL. In the case of the MSSA strain, the correlation coefficient was slightly lower ≈ 0.83 , however, still positive correlated, closed to 1. A strong correlation was also obtained between the OD and peak area—for all strains >0.97 .

OD measurement was used in order to screening study of the incubation of bacteria with antibiotics that affects their viability. All samples treated with antibiotics had lower OD values compared to the control samples (control samples OD > 0.9 —Figure 3). In the case of the MRSA strain, the use of MET decreased the OD value to the smallest extent (down to 0.45). For the remaining antibiotics, the OD values were about 0.10. In the case of the MSSA strain, after incubation with antibiotics, the OD ranged around 0.4–0.5, the exception was the use of CLIN at a concen-

tration of 4 and 8 mg/ml, which lowered the OD value below this value (<0.4). For the ESBL strain, the lowest values were observed after the use of CL_IP—the OD values ranged between 0.16 and 0.06 depending on the concentration of the antibiotic. For the remaining antibiotics, the measured OD values were found to increase more than 0.25.

To confirm the obtained results for the same samples, analyses were performed using the standard FC technique. Measurements made with FC were in most cases close to the values obtained using CE. The percentage of dead and live bacteria obtained by FC is also presented in Figure 3. For the MRSA strain, the viability of bacteria after incubation with β -lactam antibiotics was very similar to that obtained with CE—the percentage of viable cells was $<50\%$. The use of the same antibiotics for the MSSA strain also gave similar values to the CE measurements—live cells accounted for $>85\%$. MET was an antibiotic for which the use of FC resulted in lower values of the viability of *S. aureus* strains than by means of CE—the difference between the methods was $>16\%$. On the other hand, for the ESBL strain, the largest difference between the measurements was noted with the use of AMOX—the percentage

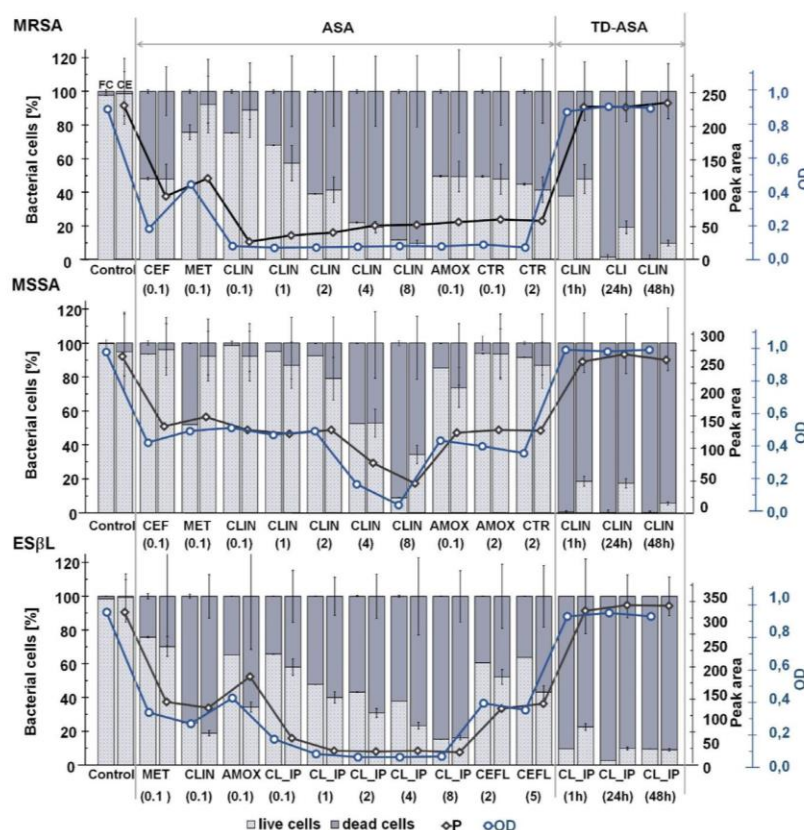


FIGURE 3 Optical density (OD), peak area (P), and bacterial cells percentage (% m/m) of live and dead bacteria incubated with various antibiotics calculated on the basis of flow cytometry (FC) and capillary electrophoresis (CE) measurements. The values in brackets refer to the concentration for the antibiotic susceptibility assay (ASA) test or to the time for the time-dependent antibiotic susceptibility assay (TD-ASA) test. Light gray represents the percentage of live cells and dark gray represents the percentage of dead cells. AMOX, amoxicillin; CEF, cefotaxime; CEFL, cefazolin; CL_IP, imipenem with cilastatin; CLIN, clindamycin; CTR, ceftriaxone; MET, metronidazole

of viable cells was 30% higher with the FC application. The remaining antibiotics used for ESβL gave similar values of viability to CE measurements.

Another approach performed in the present study was CE technique using for the determination of viability of the cells. Such a method is proposed as an alternative method. In this context has been proposed an algorithm: The percentage of live and dead bacterial cells in CE was calculated according to Equations (1) and (2), and these values are presented in the form of a graph in Figure 3. The addition of the antibiotic in each case lowered the percentage of live bacterial cells while increasing the rate of dead cells. The incubation of *S. aureus* strains with β-lactam antibiotics similarly decreased the viability of the bacteria, with MRSA percent viable bacteria being approximately 50% and with MSSA about 90%. The use of MET for both strains of *S. aureus* slightly reduced the viability—the percentage of viable cells in both cases was 92%. CLIN, which is recommended (according to EUCAST and CLSI)

in infections caused by *S. aureus* strains, has been used in the broader concentration ranging from 0.1 to 8 mg/ml (in the ASA study). In the case of both strains, a decrease in viability was observed with increasing concentration. However, for the MRSA strain, a decrease below 50% was achieved for the concentration of 2 mg/ml, whereas, for the MSSA strain, such a decrease was observed for concentrations above 4 mg/ml. β-Lactam antibiotics incubated with the *E. coli* ESβL strain were CEFL, AMOX, and imipenem. In the case of using 2 mg/ml of CEFL, the viability of bacteria was noticed 52%. With the use of the higher concentration (5 mg/ml) of the bacterial cells, it was reduced to 43%. Better results were obtained after incubation with AMOX—despite its low concentration, the viability decreased to 34%. Imipenem was used over a broader range of concentrations. The decrease in the viability of the bacteria was concentration dependent, and for the concentration of 1 mg/ml and higher, the viability was lower than 50%. The use of CLIN also significantly reduced

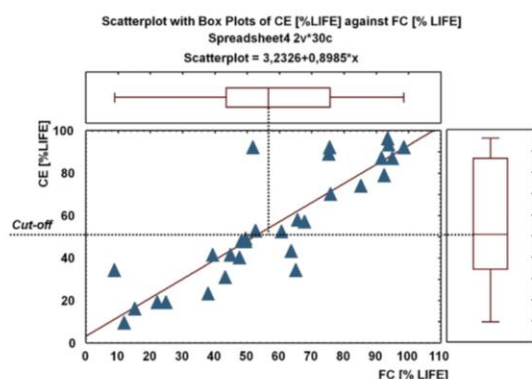


FIGURE 4 The scatterplot and box plots show a cut-off point based on capillary electrophoresis (CE) and flow cytometry (FC) data (% , m/m)

the viability (up to 19%) of the ES β L strain. In turn, MET contributed the least to the increase in the percentage of dead cells—over 70% of cells remained alive.

3.1 | Cutoff

Based on the obtained viability data, a cut-off point was established for the FC and CE methods in order to correlate these methods (Figure 4). To illustrate the relationship between the calculated viability using the proposed formula in the CE and the corresponding bacterial viability effect obtained by FC, a scatterplot with box plots was generated. On this basis, it was determined that the cut-off point in CE is 50%. Hence, in this context, the cut-off point will be assessed only for viability and not as the inhibitory consequence. Inhibitory effects of effective antibiotics decrease the total amounts of bacterial cells compared to the not-treated (control) bacterium.

4 | DISCUSSION

Determining sensitivity to antibiotics is inextricably linked with the ability of individual bacterial strains to defend themselves against the adverse effects of the drug through the use of natural resistance mechanisms. In this study, we investigated the use of CE to characterize and assess the physiological states of three clinical bacterial strains (MRSA, MSSA, and *E. coli* ES β L) exposed to different antibiotics. Based on the TD-ASA, it was determined that the optimal incubation time of the tested strains with antibiotics was 24 h. The use of shorter incubation, especially in the case of strains showing resistance to a broad spectrum of compounds—MRSA and ES β L, does

not allow one to achieve an equilibrium state between the value of living and dead cells. Considering this view, the ASA study then was carried out on a large spectrum of antibiotics and taking into account several concentrations. In the present study, we have compared the most common method for estimating cell counting: two considered as standard OD and FC, and one proposed as an alternative CE. A drawback of OD measurements is that they do not actually measure the number of cells directly. This is due to the fact that the cells of individual strains of microorganisms differ in sizes and abilities to aggregate [18]. In the case of CE, the aggregation of cells and their adhesion on the capillary walls is also observed, which has an influence on the broadening of the signals. Horká et al. used a capillary modified with (3-glycidyloxypropyl)trimethoxysilane to distinguish between *S. aureus* strains [19, 20]. Thanks to this, they obtained two signals—from MRSA and MSSA, which were differentiated from each other. In our research, we wanted to obtain a single signal from all cells, which we achieved by modifying the surface of microorganisms with calcium ions. The addition of divalent ions ($\text{Ca}^{2+}/\text{Mg}^{2+}$) enables the formation of a cationic bridge between the surfaces of the cell walls, where negatively charged surface functional groups are present. This type of complexation neutralizes the electrical double layer of the bacterial surface, which ensures controlled aggregation and concentration of bacteria into one cluster [21, 22].

In the study, we obtained a correlation of 0.68–0.78 between the OD value and cell viability in the other methods (FC and CE). A much stronger correlation (>0.97) was obtained between the OD values and the peak areas on the electropherograms. Similarly, high correlation was obtained by our group in the previous work by testing bacterial ATTC strains [14]. Moreover, Crispo et al. also obtained strong correlation analyzing *Saccharomyces cerevisiae* cells [23]. However, it should be remembered that on the basis of these two values, it is not possible to conclude about the viability of bacterial cells or their exact number. A strong correlation between the peak area in capillary isoelectric focusing and the number of cells was obtained by Ruzicka et al., but this relationship was only linear to a limited extent [24]. In the case of the electrophoretic discrimination of living and damaged cell subpopulations, not the peak area but the migration time seems to be the key for this differentiation [14]. The viability of clinical strains treated with antibiotics, calculated on this basis, was strongly correlated (>0.83 for MSSA, >0.92 for ES β L and MRSA) with the viability obtained on the basis of FC measurements. As the FC technique is widely used for determining viability, it appears to be the powerful and optimal for comparing the results obtained with the novel proposed CE approach.

The antibiotics used in the study showed various effects on the viability of the clinical strains as they have different mechanisms of action. β -Lactam antibiotics disrupt the final stage of peptidoglycan synthesis, due to which the structure of the cell wall is defective and bacteria die [25]. Macrolides and lincosamides (e.g., CLIN) are examples of antibiotics that interfere with protein synthesis by binding to the bacterial 50S ribosomal subunit. In general, they are active mainly against Gram-positive bacteria and have only limited activity against Gram-negative bacteria [1, 26]. Inhibition of DNA replication is possible thanks to the use of, for example, nitroimidazole antibiotics (e.g., MET) that break down DNA or quinolone and fluoroquinolone antibiotics that interfere with DNA induction, preventing the unfolding and duplication of bacterial DNA [1, 27]. The effect of antibiotics on the clinical strains was related to the minimum inhibitory concentration (MIC₅₀) determined by EUCAST and CLSI. The antibiotic to which all strains were resistant (percentage of viable bacteria remained >50% after incubation) was MET. The EUCAST and CLSI guidelines do not provide values for the minimum inhibitory concentrations of MET for the tested strains. The low effectiveness of MET (viability of the tested strains was >70%) is probably due to the fact that this antibiotic has been used in the treatment of infections for almost 60 years and, as a consequence, can generate a drug resistance [28]. Numerous studies have demonstrated the incidence of MET resistance in recent years for both *E. coli* and *S. aureus* strains and others [29, 30]. The greatest sensitivity to CLIN was observed for the *E. coli* strain, which proves that the strain did not develop resistance to the respective antibiotic. This is a surprising result, as in the literature most reports indicate that *E. coli* strains (isolated from environmental and clinical samples) are characterized by high resistance to CLIN [31, 32]. In the case of *S. aureus* strains, both were sensitive to CLIN (according CLSI); however, the lower concentration of the antibiotic to a greater extent reduced the viability of the MRSA strain compared to the MSSA. Research shows that CLIN is effective in treating invasive MRSA and MSSA infections [33]. All used β -lactam antibiotics decreased the viability of the MRSA strain only (less than 50%—which complies with CLSI standards). In the case of MSSA, the viability of bacteria after incubation with β -lactams remained high at around 80%. Among the β -lactam antibiotics used for *E. coli* ES β L, imipenem showed the strongest bactericidal effect (the strain was found to be susceptible using both the EUCAST and CLSI standards). Carbapenems are the treatment of choice against ES β L-producer in various infections. The characteristic feature of *E. coli* ES β L is the insensitivity to AMOX and CEFL, which proves that false-positive results were obtained for the CE method [34, 35].

Summing up, CE in a system coupled with FC seems to be an effective method in relation to recommenda-

tions for the treatment of infections with the selected pathogens. Moreover, the correlation coefficient between electrophoresis and cytometric testing for the analyzed bacterial strains (both Gram-positive and Gram-negative) remains high. As a result, CE has a chance to become a modern diagnostic method used in clinical practice. The creation of the CE/FC system database in the future may also allow for “one-step” diagnostics using only the electrophoretic test. Thanks to this, the waiting time for the result will be shortened from several hours to a few minutes and the doctors will be able to verify the effectiveness of the prescribed therapy almost immediately. However, this requires further research on a more extensive number of antibiotics, and bacterial strains, especially the most pathogenic strains such as *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and many others.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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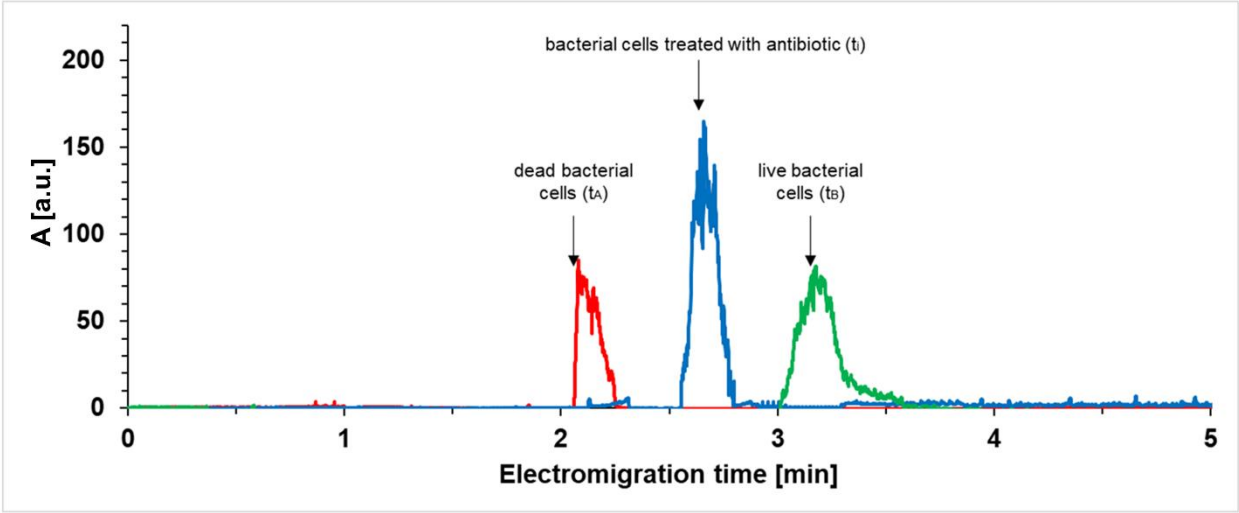
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SUPPORTING INFORMATION

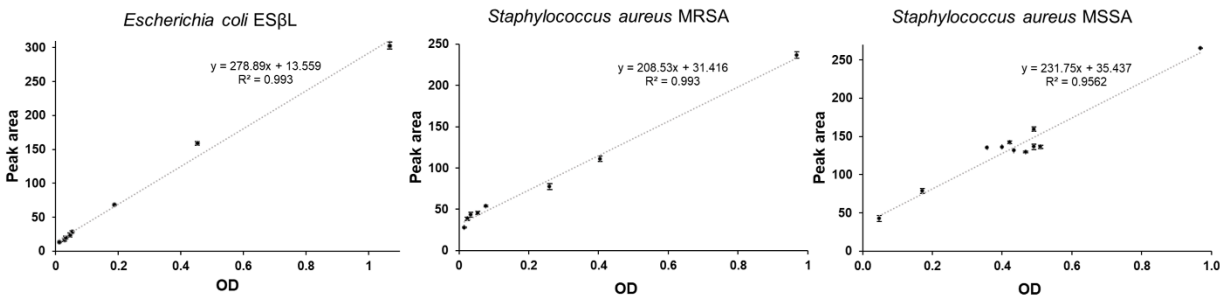
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Publikacja [P2]
Supplementary materials



Supplementary Figure 1. Illustrative drawing showing electrophoreograms of three types of tested *E. coli* bacterial cells: live cells, dead cells and cells incubated with an antibiotic.



Supplementary Figure 2. Dependence of the peak area on absorbance (OD) for tested clinical strains.

Publikacja [P3]

Culturomics approach to identify diabetic foot infection bacteria

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Article

Culturomics Approach to Identify Diabetic Foot Infection Bacteria

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Abstract: The main goal of the study was to evaluate the usefulness of the culturomics approach in the reflection of diabetic foot infections (DFIs) microbial compositions in Poland. Superficial swab samples of 16 diabetic foot infection patients (Provincial Polyclinical Hospital in Toruń, Poland) were subjected to culturing using 10 different types of media followed by the identification via the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) and Biotyper platform. Identified 204 bacterial isolates representing 18 different species—mostly *Enterococcus faecalis* (63%) and *Staphylococcus aureus* (44%). Most of the infections (81%) demonstrated a polymicrobial character. Great differences in the species coverage, the number of isolated Gram-positive and Gram-negative bacteria, and the efficiency of the microbial composition reflection between the investigated media were revealed. The use of commonly recommended blood agar allowed to reveal only 53% of the entire microbial composition of the diabetic foot infection samples, which considerably improved when the chromagar orientation and vancomycin-resistant enterococci agar were applied. In general, efficiency increased in the following order: selective < universal < enriched < differential media. Performed analysis also revealed the impact of the culture media composition on the molecular profiles of some bacterial species, such as *Corynebacterium striatum*, *Proteus mirabilis* or *Morganella morganii* that contributed to the differences in the identification quality. Our results indicated that the culturomics approach can significantly improve the accuracy of the reflection of the diabetic foot infections microbial compositions as long as an appropriate media set is selected. The chromagar orientation and vancomycin-resistant enterococci agar media which were used for the first time to study diabetic foot infection microbial profiles demonstrate the highest utility in the culturomics approach and should be included in further studies directed to find a faster and more reliable diabetic foot infection diagnostic tool.

Keywords: diabetic foot infection; culturomics; MALDI-TOF MS; bacteria

1. Introduction

The DFI is becoming a serious global life threat due to the increasing population of diabetes among which nearly 15–20% will suffer from a diabetic foot ulcer (DFU) during their lifetime [1,2]. A key factor in the effective management of the DFIs is a comprehensive empirical antimicrobial therapy that covers the most probable causative agents. Although international guidelines developed by e.g., the IDSA (Infectious Diseases Society of America, Arlington, VA, USA) and the IWDGF (the International Working Group on the Diabetic Foot) have become a vital instrument in choosing such treatments, there

is a growing need for reports on the DFI microbial compositions in specific geographical regions to provide local treatment guidelines since microbiological studies of the DFIs conducted so far have yielded inconsistent results [3,4].

The accurate bacteria identification and diagnosing DFI in diabetic patients is still challenging due to usually a polymicrobial nature of the infection and the confounding effect of neuropathy and ischemia on the local and systemic inflammatory response [5]. Moreover, as with other skin wound infections, there are difficulties to define the pathogenic character of the microbial species involved due to the presence of a great number of commensal ones which, however, might become pathogenic when the opportunity arises [6].

Currently, several different approaches are used to identify and define the human microbiota. Mostly, the diagnosis in clinical microbiology relies on the phenotypic identification that consists in culturing microorganisms to grow and isolate their colonies [7]. However, the traditional culture method does not allow for the identification of new bacteria and those presented atypical phenotypic profiles. Such limitations can be overcome using the 16S rDNA gene sequencing which paved the way to identify rare, fastidious, and new microorganisms [8,9]. Recent studies indicated that the application of molecular techniques, such as the 16S rDNA PCR amplification, enabled to identify a greater diversity of the DFI microbiota including fastidious anaerobes and Gram-negative species than the standard culture methods [5]. Due to significant advances in the DNA sequencing techniques such as metagenomics which enable to investigate the microbial composition based on the genetic material directly recovered from the sample, many scientists started to believe that culture would no longer be needed [7,10]. Nevertheless, the microbial identification via molecular techniques faces several obstacles such as difficulties to define specific phenotypic characteristics for new species due to the limited number of available biochemical tests, overlooking minority species, the inability to distinguish between live/dead cells or the inability to assess antibiotic susceptibility [7,11]. Moreover, in the case of metagenomics, pure cultures of microorganisms are not provided and further strain characterization including host-interactions is not achievable [12].

As many studies have proven, culturing is still essential to describe new prokaryotic species and filling metagenomics gaps [13]. It is possible due to the use of fast and cost-effective bacterial identification by MALDI-TOF MS along with the multiplication of culture conditions called the culturomics approach [14]. Such a complementary strategy to metagenomics and the 16S rDNA analysis allows for a very rapid bacterial identification and description of the microbiological background of the disease; it also led to the discovery of hundreds of new human-associated bacterial species [15,16]. Culturomics was introduced in order to optimize culture conditions and revealed the potential to dramatically increase the number of bacteria identified by culture methods causing scientists to revise the term “unculturable organisms” since all microorganisms, following the statements of Bilen and his colleagues, are cultivable providing that the right conditions and tools are supplied [6,13]. The establishment of different culture conditions dedicated to a specific clinical specimen, including those that suppress the culture of majority populations and improve the growth of fastidious microorganisms, which could be further subjected to the rapid identification via the MALDI analysis (in less than one hour) is the crucial step of this approach [17,18]. The ability to isolate rather than simply obtain sequences of bacterial species is a big advantage of culturomics since it allows the further investigation of biological significance, features, and therapeutic potentials including antimicrobial susceptibility [13,18].

The growth conditions applied in the laboratory which lie at the heart of the culturomics approach, are the most important aspect of isolating and cultivating microorganisms, therefore, it is believed that the transition from the undefined to the defined set of media is crucial to enable interlaboratory comparisons and obtain reproducible results [19]. In most of the studies hitherto conducted on the DFI microbiota, culture conditions are not under debate and their selection is limited to the commonly accepted recommendations. There is a large variation in the type of the culture media used by researchers—from a

single medium (e.g., Luria Bertani [20]) to several ones (e.g., Columbia blood, Trypticase soya, Chapman, M17, Eosin methylene blue, Hektoen and Cetrimide agars [21]) which may contribute to significant discrepancies in the identification results noted. Therefore, the main goal of the study was to evaluate the relative abilities of different media to recover bacterial isolates from DFI patients in Poland using the culturomics approach. Moreover, the influence of the type of medium on the composition of bacterial molecular profiles, and thus on the reliability of identification results, was discussed. In the research, commercially available culture media were used, which may facilitate the implementation of the established method in routine clinical laboratories in the future.

2. Results

2.1. Microbiological Background of Diabetic Foot Infection Samples

The applied MALDI analysis parameters allowed to obtain MS spectra of protein extracts of all tested bacterial isolates (exemplary spectra are presented in Figure 1), which were then used for identification via the MALDI Biotyper Compass Explorer 4.1 platform and BDAL database—updated version 1.0.16.0 with 6,903 Main Spectra (MSP) entries (27 June 2017). Detailed information about obtained score values depending on the culture media used are presented in Table S1.

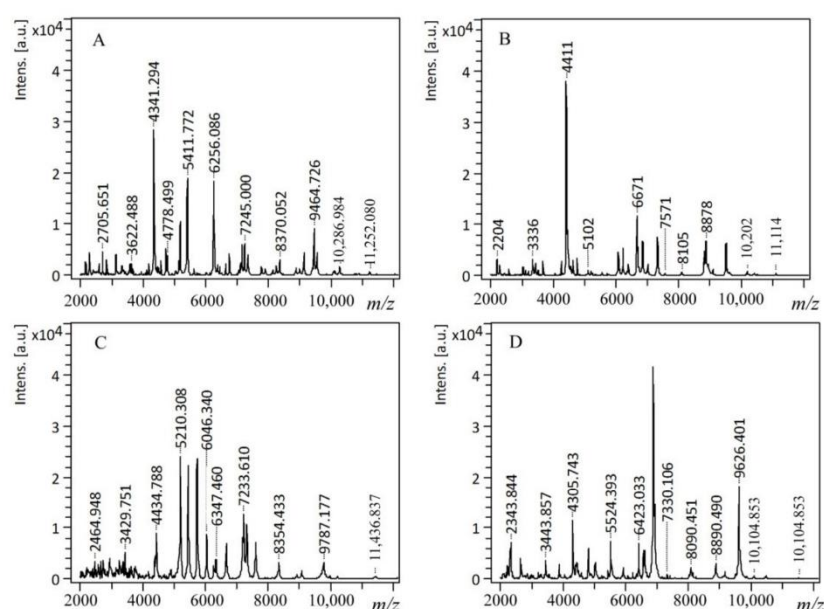


Figure 1. Exemplary mass spectra of bacterial protein extracts (A)—*Enterococcus faecalis*, (B)—*Klebsiella oxytoca*, (C)—*Pseudomonas aeruginosa*, (D)—*Staphylococcus aureus* obtained during MALDI TOF/MS analysis using positive ion mode. Intens. [a.u.]: signals (peaks) intensities given in arbitrary units; m/z : mass-to-charge ratio, the ratio of an ion's mass (m) in atomic mass units (amu) to its formal charge (z)—in the case of our study $z = +1$ due to the use of positive ionization mode.

As a result of the microbiological composition analysis of the superficial swab samples obtained from the 16 DFI patients received 204 bacterial isolates representing 18 different species—8 Gram-negative and 10 Gram-positive—belonging to 3 types of bacteria: Firmicutes (50.0%), Proteobacteria (44.4%), and Actinobacteria (5.6%) (Figure 2).

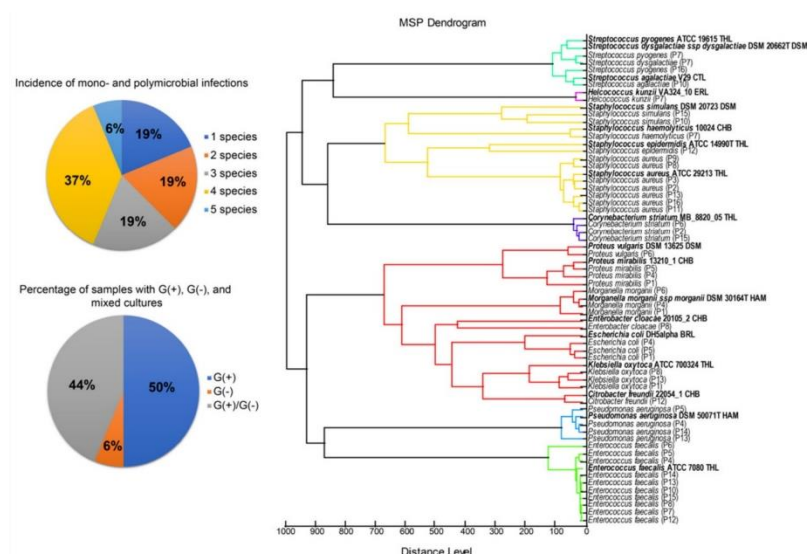


Figure 2. Phyloproteomic relationship between identified DFI bacterial species (MSP dendrogram) with diagrams showing percentage of mono- and polymicrobial infections as well as incidence of Gram-positive, Gram-negative, and mixed cultures among investigated samples.

Streptococci and staphylococci were the highly dominant group among all isolated bacterial strains. Most of the identified bacterial species represented facultative anaerobes, of which more than 80% are part of the microbiome of the gastrointestinal tract, skin, or respiratory tract. Nine of all identified bacterial species (50%) occurred in at least two patients, while another 9 were characteristic for individual patients. Most patients were affected by polymicrobial infections (81%)—from 2 to 5 different species at the same time (Figure 2). Most often, 4 different bacterial species were present in diabetic foot wounds (in the case of ca. 37% patients). The highest number of species was observed in patient P4, while samples derived from three patients were classified as monobacterial—only *Staphylococcus aureus* presence in each case.

Gram-positive bacteria were the most commonly isolated type of bacteria (15 out of 16 patients) and in the case of 50% samples were the only type of bacteria presented in the samples. Gram-negative bacteria were much less common—among 8 examined patients. The most common Gram-positive species were *Enterococcus faecalis* (10 cases, 63%), *Staphylococcus aureus* (7 cases, 44%) and *Corynebacterium striatum* (3 cases, 19%). Among the Gram-negative bacteria, *Pseudomonas aeruginosa* (4 cases, 25%) along with *Escherichia coli*, *Morganella morganii*, and *Proteus mirabilis* were the most frequently isolated—each strain was presented in the samples of 3 patients (19%).

2.2. DFI Species Coverage by Culture Media

All Gram-positive and Gram-negative species were isolated on all media except AZI. In 9 out of 10 media Gram-positive isolates constituted the majority—from 58% (COL) to 100% (AZI) (Table 1).

On one medium—CHRA—the percentage of Gram-negative bacteria was slightly higher than Gram-positive—52%. Considering the most dominant Gram-positive species, *S. aureus* demonstrated the highest percentage on MHA, BHI, and MAN medium, *E. faecalis* on CHRA, AZI, and VRE, while *C. striatum* on BLA medium. Regarding dominant Gram-negative species, *P. aeruginosa* were mostly isolated on TSA, *E. coli* and *M. morgani* on CHRA, while *P. mirabilis* on MAN, BLA, and BHI.

Table 1. Species coverage depending on culture media type used shown as percentage of identified isolates obtained using individual culture media.

Bacteria Species		Percentage of Identified Isolates Obtained Using Individual Culture Media									
		COL	MHA	TSA	BHI	BLA	CHRA	AZI	BCP	MAN	VRE
Gram-positive	<i>Corynebacterium striatum</i>	6%	5%	9%	5%	13%	11%	-	9%	-	8%
	<i>Enterococcus faecalis</i>	16%	16%	19%	19%	9%	18%	50%	18%	10%	28%
	<i>Streptococcus agalactiae</i>	5%	5%	5%	4%	-	3%	6%	5%	-	4%
	<i>Streptococcus dysgalactiae</i>	5%	5%	5%	5%	4%	-	-	4%	-	4%
	<i>Streptococcus pyogenes</i>	5%	5%	5%	5%	4%	3%	6%	5%	-	-
	<i>Staphylococcus aureus</i>	16%	21%	19%	24%	13%	7%	32%	18%	50%	20%
	<i>Staphylococcus epidermidis</i>	-	-	-	-	-	-	-	-	10%	-
	<i>Staphylococcus haemolyticus</i>	5%	5%	5%	5%	4%	3%	6%	4%	10%	4%
	<i>Staphylococcus simulans</i>	5%	-	-	-	4%	3%	-	-	-	4%
	<i>Helcococcus kunzii</i>	-	-	-	-	4%	-	-	-	-	4%
Gram-negative	<i>Escherichia coli</i>	-	-	-	-	-	11%	-	4%	-	-
	<i>Klebsiella oxytoca</i>	11%	11%	-	9%	8%	11%	-	9%	-	-
	<i>Citrobacter freundii</i>	-	5%	5%	5%	4%	4%	-	4%	-	4%
	<i>Enterobacter cloacae</i>	5%	-	5%	-	-	4%	-	-	-	-
	<i>Morganella morganii</i>	-	-	-	5%	4%	7%	-	4%	-	-
	<i>Proteus mirabilis</i>	11%	11%	9%	14%	13%	4%	-	9%	20%	8%
	<i>Proteus vulgaris</i>	-	-	-	-	4%	4%	-	4%	-	-
	<i>Pseudomonas aeruginosa</i>	5%	11%	14%	-	8%	7%	-	4%	-	8%

2.3. Evaluation of the Culture Media Usefulness for the Investigation of DFI Microbial Compositions

The analysis revealed great differences between tested culture media in terms of the number of isolated Gram-positive and -negative bacterial species (Table 2).

Table 2. Number of Gram-positive and -negative species and isolates obtained depending on the culture medium used.

Medium	Species			Isolates		
	G(+)	G(−)	Σ	G(+)	G(−)	Σ
COL	8	5	13	12	7	19
MHA	7	4	11	12	7	19
TSA	7	4	11	14	7	21
BHI	7	4	11	14	7	21
BLA	9	6	15	14	10	24
CHRA	7	8	15	14	14	28
AZI	5	0	5	16	0	16
BCP	7	7	14	14	9	23
MAN	4	1	5	8	2	10
VRE	8	4	12	19	6	25

The largest number of different Gram-positive species was isolated on BLA medium (9 out of 10 species), universal COL medium, and selective VRE medium- 8 species for both. The lowest number of Gram-positive species were noted for AZI (5) and MAN (4) media. In the case of Gram-negative, the use of the CHRA medium enabled to detect all identified Gram-negative species (8). A slightly lower number was observed for BCP (7) and BLA medium (6), while the use of AZI medium completely inhibited the growth of this bacteria type according to its specification. In total, the most species were isolated on BLA and CHRA media (15 out of all 18 species), and the least on selective media: MAN and AZI (5 species each).

Considering the total number of different isolates derived from all samples tested, the lowest number of Gram-positive isolates was noted for MAN (8), while the highest for VRE (19). In the case of Gram-negative bacteria, the most isolates were obtained using

CHRA medium (14), and the least on MAN (2) and AZI (0). In total, the largest number of bacterial isolates were obtained on the medium CHRA (28), VRE (25), and BLA (24), and the least on AZI (16) and MAN (10).

Taking into account the percentage of the reflected microbial profiles of the all tested DFI samples, the use of a single medium allowed to reveal up to 59% of the whole microbial compositions; however, in the case of half of the media, it did not exceed the value of 50% (Table 3).

Table 3. The usefulness of the culture media to reflect the microbial compositions of the tested DFI samples using the MALDI technique expressed as the percentage of all identified isolates in the individual patients. Total—percentage of identified isolates for all investigated patients; In 2 media set—average percentage of reflected microbial composition when the individual medium is used in combination with another one and calculated from values obtained for all 9 possible combinations (mean $\pm$ SD); In 3 media set—average percentage of reflected microbial composition when the individual medium is used in combination with two other media and calculated from values obtained for all possible combinations—36 (mean $\pm$ SD).

Patient	Reflected Microbiota Pattern [%]									
	COL	MHA	TSA	BHI	BLA	CHRA	BCP	AZI	MAN	VRE
P1	25	25	25	25	25	75	0	0	0	0
P2	50	50	50	100	50	50	50	0	0	50
P3	100	0	0	100	0	0	100	100	100	100
P4	20	20	0	20	20	60	40	20	20	60
P5	0	50	50	25	50	75	50	25	25	50
P6	0	25	25	25	75	75	50	25	0	0
P7	75	75	75	75	100	50	75	50	25	100
P8	75	50	75	50	75	75	50	25	25	50
P9	0	100	100	100	100	0	100	100	100	100
P10	100	67	67	67	33	67	67	67	33	67
P11	100	100	100	100	100	100	100	100	100	100
P12	33	33	33	33	33	33	33	33	33	67
P13	50	25	50	50	25	50	50	50	25	50
P14	50	50	50	50	50	50	50	50	0	50
P15	0	0	0	0	67	67	33	0	0	100
P16	50	50	100	50	50	50	50	50	0	0
Total	46	45	50	54	53	55	56	43	30	59
In 2 media set	63 $\pm$ 6	62 $\pm$ 5	66 $\pm$ 5	65 $\pm$ 6	67 $\pm$ 5	73 $\pm$ 4	66 $\pm$ 5	65 $\pm$ 8	60 $\pm$ 7	71 $\pm$ 5
In 3 media set	73 $\pm$ 4	73 $\pm$ 4	75 $\pm$ 4	74 $\pm$ 4	75 $\pm$ 3	81 $\pm$ 2	74 $\pm$ 3	76 $\pm$ 3	72 $\pm$ 4	78 $\pm$ 3

The highest percentage was revealed for CHRA (55%), BCP (56%), and VRE (59%) medium. The use of a set of two media improved the reconstruction of the infection species composition by 17% on average and ranged from 60% to 73%. The highest values were reached for the media set containing CHRA or VRE—73% and 71% on average, respectively. When 3 media were simultaneously applied, the reliability of the reflection of the infections compositions improved by another 9% and ranged 72–81%. Similarly, CHRA and VRE media demonstrated the highest usefulness—81% and 78%, respectively.

2.4. Selection of the Most Effective Sets of Culture Media

The use of 10 different culture media resulted in 45 various two-component sets of culture media (Figure 3).

Considering individual media types, the average efficiency of the infections background reflection increased in the following order: selective (59%) < universal (61%) < enriched (68%) < differential (74%). The worst results were obtained for the set composed of two highly selective media—MAN and AZI—where the percentage of the reflected microbial composition of the investigated DFI samples did not even reach 50%. In contrast, the most useful media appeared to be CHRA and VRE in case of which their simultaneous

use gave 82% coverage, which increased up to 88% when universal tryptic soy agar was additionally included.

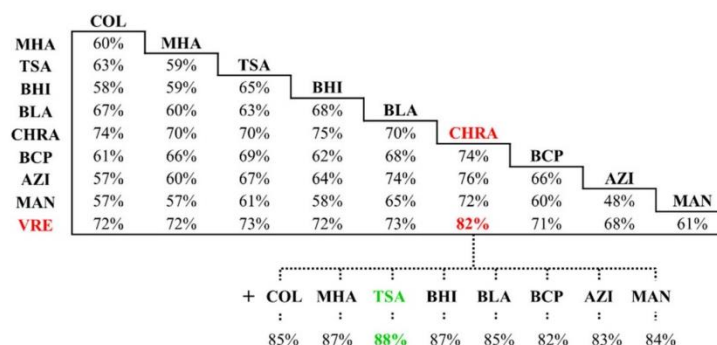


Figure 3. Selection of the most effective two-component and three-component sets of culture media based on the effectiveness of reflecting the microbial composition of the samples (expressed as a percentage of all identified bacterial species for the certain patient) using individual combinations of media. The most effective two-component set of media—CHRA + VRE (highlighted with red color), was selected for searching the most efficient three-component set (marked with the green color).

3. Discussion

Applied culture conditions allowed to reveal the predominance of the Gram-positive species over the Gram-negative ones (59.6% to 40.4%) in the investigated samples. It confirms the common statement that Gram-positive species are most often isolated from DFI wounds in the developed European countries [22]. Similar observations were made for example in the work Jneid et al. [11] (France, 54.7%) or Dang et al. [23] (United Kingdom, 56.7% and 63.4% in 1998 and 2001, respectively). Besides the geographical location, the stage of infection has also an important impact on the microbial composition of the DFI wounds, since for patients with mild or early-stage infections Gram-positive bacteria were characterized as the predominate type [22]. Most of the investigated infections (81%) demonstrated the polymicrobial character (from 2 to 5 species at the same time). The nature of the DFI could be either mono- or polymicrobial, however, earlier studies have reported a higher frequency of polymicrobial ones [24,25] which may arise from the severity of the infections since severe ones exhibit a mostly higher percentage of polymicrobial patterns [26]. Nevertheless, the DFI samples investigated in our studies derived from patients affected by early stage or mild infections, therefore, the dominance of the polymicrobial infections could be explained by the notable participation of commensal skin microbiome rather than the severity of infections. This issue has been repeatedly shown for the DFI or other skin wound infections and still constitutes a key challenge in making a reliable diagnosis to apply an effective treatment [27].

Regarding identified bacterial species, all were previously reported in the literature as present during DFI development. Two species—*E. faecalis* and *S. aureus*—constituted the vast majority of all the identified isolates. *S. aureus* is considered to be the most common causative agent of the DFI among patients from Europe and North America, especially in the initial stages of the infection [28,29]. Moreover, *S. aureus* and other aerobic Gram-positive cocci are often reported as predominant pathogens in the monomicrobial DFIs [30], which was indicated in our study where among all monobacterial infections only *S. aureus* was detected. Such a phenomenon emerged as a substantial problem in view of the increasing appearance of methicillin-resistant *S. aureus* (MRSA) in DFIs observed in recent years [30]. Surprisingly, *E. faecalis* turned out to be the most dominant species. *E. faecalis* is found to be one of the most frequently isolated bacterial species in diabetic foot ulcer [31], however, the available literature fails to present studies proving this strain to be dominant except the work of Shettigar et al. [32] which reported similar frequency of

the *E. faecalis* occurrence within the DFI samples—65%. It is believed that the presence of this bacteria in the wound contributes to the worsening of diabetic foot ulcers, especially during the polymicrobial infection, due to its capability to produce prolific amounts of biofilm and a high frequency of carrying drug-resistance genes which in the case of *E. faecalis* can be easily transferred to other bacterial species such as *S. aureus* [33,34].

Although the obtained results are in line with the previous European studies. One of the recent works by Michalek et al. [35]—also carried out in Poland—presented completely different observations. Among the 81 positive samples derived from 54 patients, the authors noted that the majority of the samples were monomicrobial (58.0%). Moreover, the share of Gram-positive species was only 22.9% while Gram-negative ones constituted a highly dominant group—77.1%. Additionally, significant differences in the species composition between our studies and the above-mentioned studies were observed. The most dominant species in our studies—*E. faecalis* (63%), accounted for only 2.5% identified isolates in the work by Michalek et colleagues [35]. Similarly, *P. aeruginosa* presented in a quarter of our samples constituted only 8.5% isolates in the above-mentioned work. Interestingly, despite considerable variations between the results of both studies, the percentage of *P. mirabilis* was very close—19% and 19.5% in our work and the work by Michalek et al., respectively, which has not yet been reported at such a level. This phenomenon may indicate that the results of the DFI microbial composition testing can vary significantly depending on the culture conditions used, even when hospitals are separated by a short geographic distance (~270 km in a straight line).

Our studies revealed big differences between the efficiency of culture media to reflect the DFI microbial composition resulted from their various species range coverage (see Tables 1 and 3). Interestingly, the use of the single Columbia blood agar (BLA), which along with other blood enriched agars such as chocolate agar belongs to the most often used media for isolation of DFI microbiota [36–39], was sufficient to reflect only 53% of the entire microbiological profiles of the studied clinical cases and was lower compared to either differential media (CHRA and BCP) and selective VRE medium. However, the reflection of the DFI microbial compositions using BLA medium significantly improved when additionally CHRA and VRE media were applied—by 32%. Nowadays differential chromogenic agars (e.g., CHROMagar *Staph aureus*–CASA, MRSA ID) are becoming more popular than historical routine agars such as blood agar or mannitol salt agar in terms of *S. aureus* and MRSA detection within diabetic foot wounds [40]. One of the last studies on the Chromagar orientation was used to boost the discriminatory power of routine phenotypic identification protocols for UTI (Urinary tract infections) bacteria among diabetic patients attending clinics in Bushenyi district of Uganda [41], however, best of our knowledge there is a lack of studies about their application in the investigation of the entire DFI microbiota. The results obtained by us indicate the high utility of the Chromagar orientation in the investigation of DFI microbiota via the culturomics approach and fulfill a recent conclusion of Perry [42] that the combination of differential chromogenic media and MALDI-TOF MS analysis may compensate for any lack of specificity and may contribute to obtain results in an even shorter time. The same applied to selective VRE medium, which in combination with the CHRA gave almost the same efficiency in the reflection of the DFI microbial compositions as in the three-component media set including also the commonly used Columbia blood agar.

Microbial identification via the MALDI-TOF MS technique mainly relies on the comparison of mass spectra representing specific molecular fingerprints of microorganisms, mostly proteins. It is known that the composition of the growth medium may lead to changes in proteins expression reflected in the variation of molecular profiles what may affect the result of identification [43]. MSP spectra used for identification are a set of signals consisting mainly of conserved ribosomal proteins, which constitute about 50% of the sum of all signals [44]. The high proportion of ribosomal proteins implied high repeatability and reliability of bacterial species identification expressed by the Score values observed in our studies (Table S1). This is clearly demonstrated by the examples of bacteria for which the

highest Score values have been obtained, e.g., *M. morganii* cultured on VRE (Score value— 2.48 ± 0.05), where signals 5382.4 *m/z*, 6353.4 *m/z*, 7275.3 *m/z*, that represented different ribosomal proteins (50S ribosomal protein L34, L32, and L29, respectively—according to the UniProt database), were detected in large amounts (Figure S1C). Contrary to this, the composition of the medium significantly influences the expression of non-ribosomal bacterial proteins that are more or less metabolic status dependent, e.g., those responsible for drug resistance such as carbapenemases [45]. In our research, the greatest differences in identification levels were observed for species *C. striatum*, *M. morganii*, and *P. mirabilis* cultured on the BLA and VRE medium (Figure S1). For all mentioned species, the use of VRE medium gave significantly higher Scores values compared to the BLA—from 0.4 (*C. striatum*) to 0.7 (*M. morganii*) (Table S1). The proteomic analysis of the mass spectra composition via UniProt database revealed many differences in the presence/absence of specific signals in mentioned bacterial species depending on the applied medium (Table S3). Considering only characterized proteins, analysis disclosed from 11 to 59 signals that differentiate investigated bacteria regarding BLA and VRE media used. These changes included signals from both the lower mass range, e.g., 3094.3 *m/z* (Potassium-transporting ATPase subunit F), 5333.4 *m/z* (Heat shock protein J) or 6232.5 *m/z* (Lysophospholipase)—*M. morganii*, as well as those of higher *m/z*, such as 11684.1 *m/z* (Protein translocase subunit SecE) and 11707.4 *m/z* (DNA topoisomerase (ATP-hydrolyzing))—*C. striatum*. The noted phenomenon may result from the considerably different media compositions, such as the presence of defibrinated sheep blood and starch in the case of BLA as well as yeast extract, aesculin, ferric ammonium citrate, and sodium azide in VRE. A similar observation was noted in our previous work Złoch et al. 2020 [43], where the choice of the media types significantly influenced molecular profiles of different *S. aureus* strains leading to large variation in the efficiency of their strain-typing. It may result from the induction of various metabolic pathways in bacteria in response to specific media components. Additionally, some researchers pointed out that the use of blood-containing media poses a risk of contamination of the MS profiles with blood-related proteins [46]. Our findings suggest that although the use of all investigated culture media allowed to reliable bacteria identification at the species level, nevertheless, in the case of some bacterial species, the media selection may significantly improve the quality of the identification what was proved for *C. striatum*, *P. mirabilis*, and *M. morganii* cultured on the VRE medium. Therefore, during the evaluation of the usefulness of culture media for recovery microbial background of the DFI samples, besides selective properties of the culture media, also their influence on the molecular profiles of the bacteria should be concerned.

4. Materials and Methods

4.1. Clinical Samples

The superficial swabs were collected from infected diabetic foot wounds from 16 patients from Provincial Polyclinical Hospital in Toruń (Poland) using flocked swab (ESwab Collection System, Copan, Murrieta, CA, USA) according to the local guidelines by a specialist nurse applying the Levine technique. After wound debridement, samples for bacterial culture were obtained by swabbing the wound (the swab was rotated over a 1 cm² area of the viable non-necrotic wound tissue). To avoid contaminating bacterial flora, wounds were debrided from necrotic or non-viable tissue or slough and rinsed with saline before swabbing. The samples were immediately placed into a liquid transport medium (Amies δswab, Deltalab, Nemours, France) and transported to Centre for Modern Interdisciplinary Technologies, where they were stored at -80°C . The Ethical Committee approved the studies (Bioethical Commission's permission no. 68/2019). Information about gender and age of the investigated DFI patients are provided in Table S2.

4.2. Bacteria Isolation and Culturing Technique

After defrosting, samples were thoroughly vortexed and a series of 10-fold serial dilution (10^{-1} to 10^{-3}) were prepared from them. For this purpose, 0.5 mL of the clinical

sample was added to a test tube containing 4.5 mL of sterile peptone water (Sigma Aldrich, Steinheim, Germany) and vortexed (first dilution— 10^{-1}). 100 μ L of each dilution was plated onto 10 different types of culture media: Tryptic Soy Agar (TSA; Sigma Aldrich, Germany), Mueller Hinton Agar (MHA; Sigma Aldrich, Germany), Columbia Agar Base (COL; Oxoid, UK), Columbia Blood Agar (BLA; Oxoid, UK), Brain Heart Infusion Agar (BHI; Sigma Aldrich, Germany), CHROMagar Orientation (CHRA; GRASO Biotech, Poland), Azide Blood Agar (AZI; Oxoid, UK), Glucose Bromocresol Purple Agar (BCP; Sigma Aldrich, Germany), Mannitol Salt Agar (MAN; Oxoid, UK), Vancomycin-Resistant Enterococci Agar (VRE; Oxoid, UK). All media were in the form of ready-to-use powders except for BLA, which was prepared by adding defibrinated sheep blood (GRASO Biotech, Poland) to the sterilized and dissolved Columbia blood agar base to the final concentration 5% (v/v). After incubation at 37 °C for 18–24 h on the basis of morphological differences, single colonies of bacteria were selected, from which reduction cultures were prepared on the same media in order to obtain pure cultures (incubation at 37 °C for 18–24h).

4.3. Identification of Bacterial Isolates Using MALDI-TOF MS Technique

The common formic acid/acetonitrile method of protein extraction was used according to the protocol of the producer of the MALDI Biotyper system—the company Bruker Daltonik (Bremen, Germany). 1 inoculation loop (10 μ L) of biomass was added to an Eppendorf tube containing 300 μ L of sterile deionized water and mixed. Then, in order to inactivate viable bacterial cells, 900 μ L of 96% ethyl alcohol was added to the suspension and mixed again followed common procedure recommended by manufacturer. The suspension containing rendered non-viable bacterial cells was centrifuged (1300 rev/min, 5 min), the supernatant was discarded and the remaining cell pellet was dried using a vacuum centrifuge at room temperature. Then 10 μ L formic acid (FA) and 10 μ L acetonitrile (ACN) was added to dried cell pellet. After mixing, the sample extract was centrifuged (13,000 rev/min, 5 min) and 1 μ L of supernatant was transferred onto a MALDI MTP 384 ground steel target sample spot (Bruker Daltonik GmbH, Germany). After air-drying, the sample spot was overlaid with 1 μ L of respective matrix solution α -cyano-4-hydroxycinnamic acid (HCCA; Sigma Aldrich, Switzerland): 10 mg/mL in standard solvent solution (50% ACN, 47.5% water and 2.5% trifluoroacetic acid).

Samples were analyzed using a ultrafleXtreme MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with the smartbeam-II laser—positive mode. Spectra were collected manually using manufacturer software, flexControl version 3.4 build 135 (parameters: 500 shots in-one-single spectra to frequency 2500, m/z range = 2000–20,000, acceleration voltage = 25 kV, global attenuator offset = 20% and attenuator offset = 34% and its range = 34%, laser power = 40%), and subjected to smoothing using the Savitsky–Golay method (width 2 m/z , cycles 10) and baseline corrections using the TopHat algorithm (signal to noise threshold 2; peak detection algorithm—centroid) followed by calibration using the Bruker's Bacterial Test Standard (BTS; Bruker Daltonik, Bremen, Germany) in quadratic mode via manufacturer software, flexAnalysis version 3.4 build 76. Each sample was measured in quadruplicate (two spots per samples measured twice). Validated mass spectra were used for bacterial identification via MALDI Biotyper 4.1 Platform (Bruker Daltonik GmbH, Bremen, Germany) based on both raw spectra (RAW) and main spectra (MSP).

5. Conclusions

The introduction of the MALDI-TOF MS technique into the clinical laboratories caused the application of the culturomics approach for the fast and reliable analysis of infections to become meaningful. Still, the selection of the most suitable growth conditions is challenging since it requires extending the research to various geographic regions and a wider range of media. Performed studies proved that the choice of the suitable culture media for culturomics approach demonstrates considerable influence on both efficiency of reflecting the microbial composition of the DFI samples (selective properties of the media) as well as on the quality of the identification in the cases of some bacterial species (impact on the

composition of bacterial molecular profiles). Our studies indicated that especially two media—chromagar orientation and vancomycin-resistant enterococci agar, which have not yet been widely used for revealing the DFI microbial composition, demonstrated high utility in studying the microbial composition of the DFI in patients in Poland by means of culturomics approach. Further studies involving samples derived from other parts of Poland, Europe, and the rest of the world may help to justify whether its application for routine analysis will be beneficial.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz (KB 68/2019, 29 January 2019).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Publikacja [P3]

Supplementary materials

		COL	MHA	TSA	BHI	BLA	CHRA	BCP	AZI	MAN	VRE
Gram-positive	<i>Corynebacterium striatum</i>	2.42 ± 0.07	2.30 ± 0.01	2.38 ± 0.07	2.36 ± 0.05	1.96 ± 0.20	2.20 ± 0.15	2.34 ± 0.07	-	-	2.33 ± 0.17
	<i>Enterococcus faecalis</i>	2.16 ± 0.10	2.19 ± 0.13	2.01 ± 0.22	2.09 ± 0.11	2.19 ± 0.11	2.12 ± 0.12	2.14 ± 0.12	2.10 ± 0.15	2.21 ± 0.06	2.12 ± 0.16
	<i>Streptococcus agalactiae</i>	2.27 ± 0.12	2.32 ± 0.02	2.15 ± 0.18	2.25 ± 0.13	2.32 ± 0.09	2.26 ± 0.11	2.28 ± 0.06	2.26 ± 0.02	-	2.14 ± 0.10
	<i>Streptococcus pyogenes</i>	2.18 ± 0.11	2.22 ± 0.10	2.30 ± 0.11	2.20 ± 0.07	2.32 ± 0.14	2.39 ± 0.06	2.36 ± 0.05	2.11 ± 0.18	-	-
	<i>Staphylococcus aureus</i>	2.27 ± 0.15	2.04 ± 0.17	2.22 ± 0.12	2.12 ± 0.19	2.15 ± 0.13	2.10 ± 0.19	2.04 ± 0.13	2.24 ± 0.09	2.06 ± 0.17	2.11 ± 0.21
	<i>Streptococcus dysgalactiae</i>	2.14 ± 0.15	2.27 ± 0.07	2.30 ± 0.08	2.25 ± 0.05	2.09 ± 0.07	-	2.33 ± 0.06	-	-	2.22 ± 0.06
	<i>Staphylococcus epidermidis</i>	-	-	-	-	-	-	-	-	1.78 ± 0.07	-
	<i>Staphylococcus haemolyticus</i>	2.04 ± 0.08	2.14 ± 0.15	2.17 ± 0.13	1.99 ± 0.11	2.11 ± 0.12	1.93 ± 0.12	2.05 ± 0.07	2.46 ± 0.14	1.86 ± 0.12	1.98 ± 0.17
	<i>Staphylococcus simulans</i>	1.81 ± 0.01	-	-	-	1.77 ± 0.02	1.85 ± 0.08	-	-	-	2.01 ± 0.01
	<i>Helcococcus kunzii</i>	-	-	-	-	2.24 ± 0.08	-	-	-	-	2.41 ± 0.03
Gram-negative	<i>Escherichia coli</i>	-	-	-	-	-	2.07 ± 0.11	2.00 ± 0.18	-	-	-
	<i>Klebsiella oxytoca</i>	1.87 ± 0.14	1.86 ± 0.07	-	1.95 ± 0.14	2.00 ± 0.11	1.94 ± 0.13	1.96 ± 0.12	-	-	-
	<i>Citrobacter freundii</i>	2.24 ± 0.10	2.08 ± 0.08	2.08 ± 0.17	2.01 ± 0.15	2.18 ± 0.14	2.03 ± 0.10	1.89 ± 0.07	-	-	2.33 ± 0.09
	<i>Enterobacter cloacae</i>	1.97 ± 0.17	-	1.79 ± 0.05	-	-	2.05 ± 0.17	-	-	-	-
	<i>Morganella morganii</i>	-	-	-	-	1.79 ± 0.05	2.17 ± 0.17	2.37 ± 0.07	-	-	2.48 ± 0.05
	<i>Proteus mirabilis</i>	2.13 ± 0.23	2.06 ± 0.09	2.17 ± 0.15	2.22 ± 0.11	1.89 ± 0.14	2.10 ± 0.11	2.13 ± 0.21	-	-	2.35 ± 0.04
	<i>Proteus vulgaris</i>	-	-	-	-	1.78 ± 0.09	1.92 ± 0.04	2.05 ± 0.09	-	2.26 ± 0.07	-
	<i>Pseudomonas aeruginosa</i>	2.05 ± 0.14	2.19 ± 0.14	2.07 ± 0.12	2.02 ± 0.20	1.96 ± 0.13	1.99 ± 0.16	2.00 ± 0.17	-	-	2.04 ± 0.16

Table S1 Impact of the culture media composition on the identification level of bacteria expressed as score value (mean + SD) obtained using MALDI Biotyper 3.0 Platform.

Patient	Gender	Age
P1	Male	80 yrs.
P2	Female	63 yrs.
P3	Male	69 yrs.
P4	Female	85 yrs.
P5	Female	87 yrs.
P6	Male	74 yrs.
P7	Male	57 yrs.
P8	Male	73 yrs.
P9	Female	68 yrs.
P10	Male	62 yrs.
P11	Male	54 yrs.
P12	Male	53 yrs.
P13	Male	81 yrs.
P14	Female	65 yrs.
P15	Male	71 yrs.
P16	Male	65 yrs.

Table S2 List of investigated DFI patients with information about gender and age.

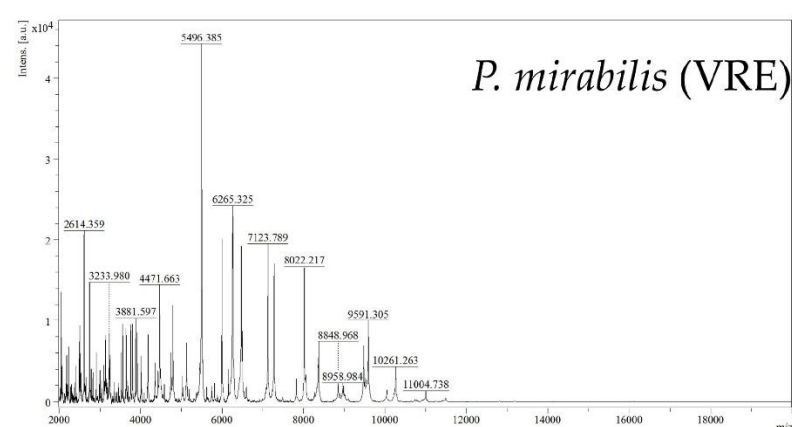
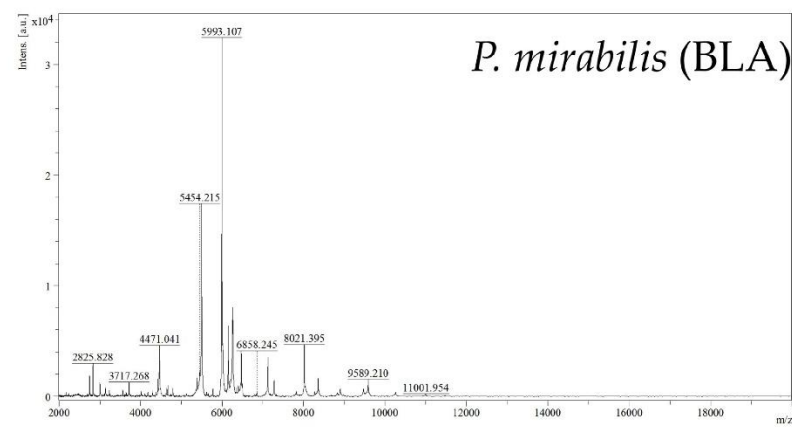
<i>C. striatum</i>			
<i>m/z</i>	VRE	BLA	Protein
3215.6	+	-	Transposase
4688.4	-	+	50S ribosomal protein L36
6232.2	-	+	Amino acid dehydrogenase
6880.9	-	+	Transposase-like protein/Two-component system response regulator
7231.6	-	+	50S ribosomal protein L35
7245.9	-	+	50S ribosomal protein L35
10316.7	+	-	30S ribosomal protein S15
10595.7	+	-	Plasmid stabilization protein
11191.8	+	-	50S ribosomal protein L24
11684.1	-	+	Protein translocase subunit SecE
11707.4	-	+	DNA topoisomerase (ATP-hydrolyzing)
<i>P. mirabilis</i>			
<i>m/z</i>	VRE	BLA	Protein
4372.6	-	+	Glucose-6-phosphate isomerase
4484.9	-	+	50S ribosomal protein L36
4668.4	-	+	RNase E inhibitor protein
4757.0	+	-	3-deoxy-D-manno-octulosonate 8-phosphate phosphatase
5026.6	+	-	Protein of uncharacterized function (DUF2857)
5110.4	+	-	Arylsulfatase
5120.3	+	-	Cytoplasmic protein
5365.8	-	+	Peroxidase
5382.0	-	+	Rho-binding antiterminator
5456.1	+	-	Integrase
5496.4	+	-	IrpP
5778.0	-	+	Single-stranded DNA-binding protein
6007.6	-	+	ATP-dependent helicase HepA
6032.7	-	+	TdRPase
6052.8	-	+	LysR-family transcriptional regulator
6096.3	-	+	Spermidine/putrescine ABC transporter membrane protein
6105.6	-	+	Isocitrate dehydrogenase (NADP(+))
6122.2	-	+	YtxH domain-containing protein
6255.1	-	+	Fimbrial subunit
6281.1	+	-	ABC transporter substrate-binding protein
6468.2	+	-	N-acetylmuramoyl-L-alanine amidase AmiC
6492.9	-	+	Ribosome modulation factor
6858.2	-	+	UPF0434 protein PMI0721
7138.8	+	-	Phage protein
7142.7	-	+	Fimbrial protein
7178.4	-	+	Holin
7233.4	+	-	Plasmid-like protein
7291.9	+	-	IS element transposase
7295.3	-	+	Fimbrial chaperone
7829.2	-	+	50S ribosomal protein L31
7830.1	+	-	Lipoprotein

7979.3	-	+	Aminomethyl transferase family protein
8021.4	-	+	KTSC domain-containing protein
8043.1	-	+	Inner membrane transport protein Yha
8329.1	+	-	Glutaredoxin-like protein NrdH
8356.6	-	+	Protein SlyX
8358.6	+	-	Coproporphyrinogen III oxidase
8393.6	-	+	Deoxyribose-phosphate aldolase
8826.0	-	+	Adenosine-3'(2'),5'-bisphosphate nucleotidase
8846.8	-	+	Trehalose repressor
8906.5	-	+	Ribosome modulation factor
8927.0	-	+	Acyl-CoA thioesterase/SirA-like protein
8999.0	+	-	Lipoprotein
9018.7	+	-	Fimbrial chaperone protein
9448.0	-	+	Uncharacterized N-acetyltransferase YjaB
9471.8	-	+	Cyclic di-GMP-binding protein
9474.2	+	-	Pyocin activator protein PrtN
9509.3	-	+	Ig-like domain-containing protein
9512.7	+	-	Putative phage protein
9553.1	+	-	Carbon dioxide concentrating mechanism protein CcmL
9574.5	-	+	Spermidine export protein MdtJ
9589.2	-	+	Putative membrane protein insertion efficiency factor
9591.3	+	-	Sec-independent protein translocase protein TatA
9608.0	-	+	Peptide permease
10219.2	+	-	Putative DNA-binding transcriptional regulator
10286.9	+	-	Na(+)-translocating NADH-quinone reductase subunit D
10988.5	+	-	DNA topoisomerase (ATP-hydrolyzing)
11002.0	-	+	7,8-dihydroneopterin aldolase
11004.7	+	-	Insulinase family protein
<i>M. morganii</i>			
<i>m/z</i>	VRE	BLA	Protein
3094.3	+	-	Potassium-transporting ATPase subunit F
3373.0	+	-	Phage tail protein
3744.2	-	+	Diacylglycerol kinase
5333.4	+	-	Heat shock protein J
5395.9	+	-	50S ribosomal protein L34
5497.8	-	+	IrpP
6202.6	+	-	CAAX protease
6232.5	+	-	Lysophospholipase
6386.4	+	-	Ribosome modulation factor
6536.9	-	+	TetR_C_1 domain-containing protein
6614.2	+	-	50S ribosomal protein L30
6919.8	+	-	DUF2526 protein
6929.7	-	+	Transposase
7067.8	-	+	Lipoprotein/?
7108.6	-	+	Sulfur carrier protein ThiS
7132.9	-	+	Holin

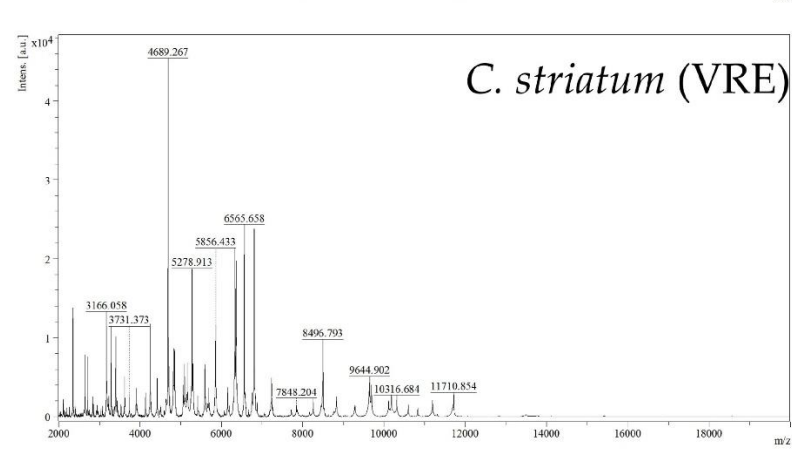
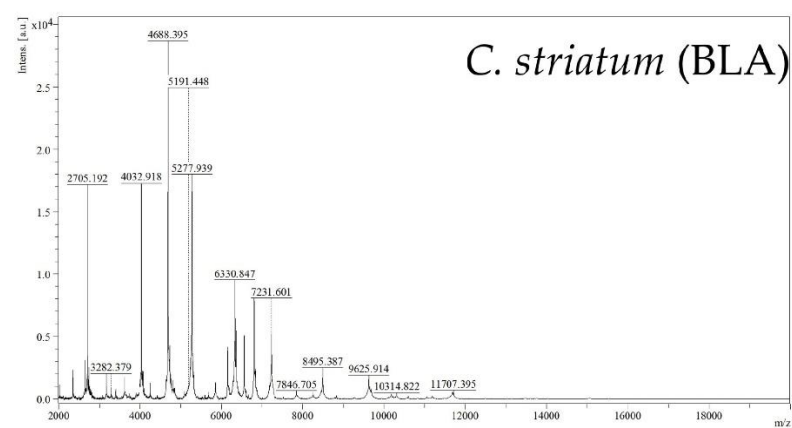
7148.3	-	+	DUF3761 domain-containing protein
7197.5	+	-	Holin
7208.4	+	-	Erythromycin esterase/Hemolysin expression modulating protein
7300.9	+	-	TetR family transcriptional regulator
7328.4	+	-	DNA polymerase I
7742.2	-	+	DUF4222 domain-containing protein
7928.0	-	+	DNA-binding protein/XRE family transcriptional regulator
8298.7	+	-	Probable [Fe-S]-dependent transcriptional repressor
8326.4	-	+	DUF1471 domain-containing protein
8340.2	+	-	Putative cI repressor protein
8410.8	-	+	Protein SlyX
8669.8	+	-	Acyl carrier protein
8702.0	-	+	TIGR03758 family integrating conjugative element protein
8814.6	+	-	Acyl-CoA dehydrogenase
8829.5	+	-	2Fe-2S ferredoxin-like protein
8887.0	-	+	YajA protein
8979.3	+	-	zinc_ribbon_2 domain-containing protein
9268.3	+	-	DNA-binding protein
9279.7	-	+	Putative zinc finger/helix-turn-helix protein, YgiT family
9282.4	+	-	DNA-binding protein HU-beta
9449.6	-	+	DNA-binding protein HU-alpha
9465.6	+	-	DNA-binding protein HU-alpha
9492.1	-	+	Outer membrane protein W
9589.9	-	+	Phage tail assembly protein T
9924.0	+	-	DUF2798 domain-containing protein
10258.4	-	+	HTH cro/C1-type domain-containing protein
10271.3	-	+	Acetolactate synthase 2 regulatory subunit
10664.7	+	-	N-acetyltransferase/XRE family transcriptional regulator
11056.9	-	+	DUF2190 family protein

Table S3 Summary of signals observed on MALDI-TOF MS spectra with characterized m/z values that differed between VRE and BLA culture medium, according to the database of Universal Protein (UniProt). Table does not include signals from uncharacterized proteins

A



B



C

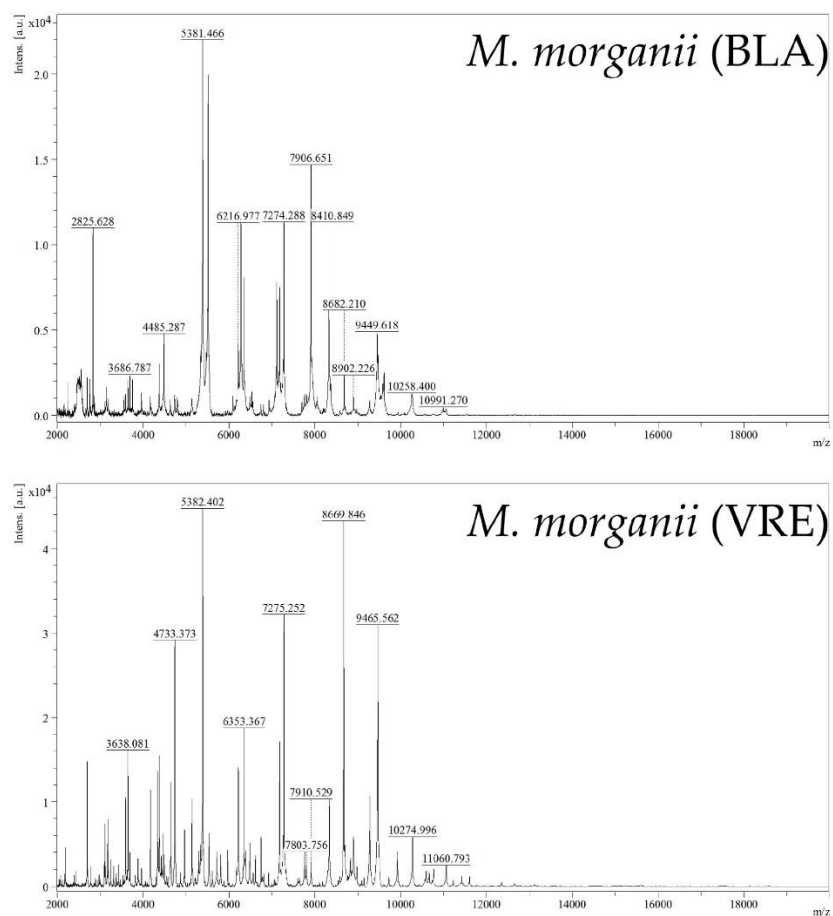


Figure S1 Comparison of the molecular profiles of the (A) *P. mirabilis*, (B) *C. striatum*, and (C) *M. morganii* cultured on the Columbia blood agar (BLA) and vancomycin resistant enterococci agar (VRE) generated during MALDI-TOF MS analysis and analyzed using FlexAnalysis software.

Publikacja [P4]

Lipidomic characterization of the microbiome in people with diabetic foot infection using MALDI-TOF MS

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Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS

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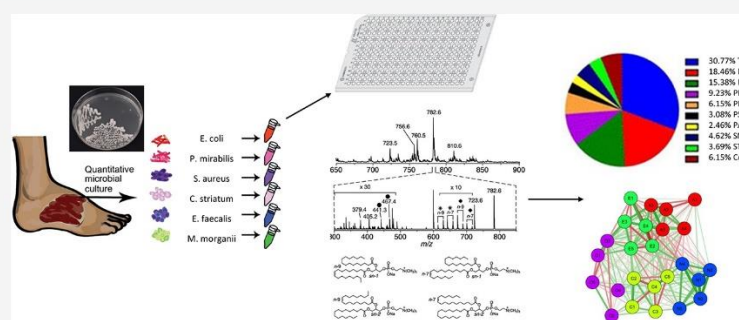
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ABSTRACT: Lipidomic profiling has emerged as a powerful tool for the comprehensive characterization of bacterial species, particularly in the context of clinical diagnostics. Utilizing matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), this study aims to elucidate the lipidomic landscapes of bacterial strains isolated from diabetic foot infections (DFI). Our analysis successfully identified a diverse array of lipids in the cellular membranes of both Gram-positive and Gram-negative bacteria, revealing a total of 108 unique fatty acid combinations. Specifically, we identified 26 LPG, 33 LPE, 43 PE, 114 PG, 89 TAG, and 120 CLP in Gram-positive bacteria and 10 LPG, 14 LPE, 124 PE, 37 PG, 13 TAG, and 22 CLP in Gram-negative strains. Key fatty acids, such as palmitic acid, palmitoleic acid, stearic acid, and oleic acid, were prominently featured. Univariate analysis further highlighted distinct lipidomic signatures among the bacterial strains, revealing elevated levels of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) in Gram-negative bacteria associated with DFI. In contrast, Gram-positive strains demonstrated increased or uniquely fluctuating levels of triglyceride (TAG) and cardiolipin (CLP). These findings not only underscore the utility of MALDI-TOF MS in bacterial lipidomics but also provide valuable insights into the lipidomic adaptations of bacteria in diabetic foot infections, thereby laying the groundwork for future studies aimed at constructing microbial lipid libraries for enhanced bacterial identification.

INTRODUCTION

Diabetes is classified as a civilization disease that has a significant impact on the entire society. It is a chronic metabolic disease that results from impaired insulin secretion or action. One of the major complications of diabetes is not only hyperglycemia but also the diabetic foot syndrome (DFS).¹ According to the World Health Organization (WHO), diabetic foot syndrome is defined as “foot ulceration associated with neuropathy and various degrees of ischemia and infections”.² Diabetic foot ulcer (DFU) and diabetic foot infection (DFI) are among the most common complications of diabetic foot syndrome. DFU and DFI cause high morbidity, which is manifested by systemic toxicity, gangrene formation, and amputation of the lower limbs.^{3,4} DFI can appear as a result of a minor scratch, through scrapes, and blisters that often lead to diabetic foot ulcers. In contrast, diabetic ulcers often lead to infections.³ DFI is an infection of the soft tissues

or bones that can lead to hospitalization and even lower limb amputation.^{5,6}

A common phenomenon in chronic wounds is the presence of various species of microorganisms on their surface. Whether a wound heals or deteriorates depends on the microbiome.⁷ Due to the disturbed or impeded wound healing process in diabetics, damage to the skin on the feet is an easy target for pathogenic microorganisms and a convenient place for their development. Monitoring microorganisms from wounds plays an important role in treating diabetic foot infections and is

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challenging due to usually the polymicrobial nature of the infection, rapid changes in species composition occurring as the disease progresses, and coexistence of commensal bacteria as well as true and opportunistic pathogens.⁸ Both aerobic and anaerobic bacteria are involved in the pathogenesis of foot wound and ulcer infections. Among them, we can distinguish both Gram-positive bacteria (especially *Staphylococcus aureus* and beta-hemolytic streptococci) and Gram-negative bacteria (*Enterobacteriaceae* and *Pseudomonas* bacteria).^{9–11} Moreover, correlations between the intestinal microflora and bacteria with DFI have been noticed.^{12,13} Intestinal bacteria participate in digestive processes and contribute to the synthesis of nutrients beneficial for the body, support the absorption of electrolytes and minerals, play an important role in destroying toxins, prevent the development of harmful pathogens, support the immune system, and can affect inflammation. A study by Wang et al. showed a positive correlation between the intestinal microflora of mice and the concentration of lipopolysaccharides (LPS) in the plasma. LPS is a building block of the bacterial cell wall and is a component of the intestinal microflora, and thus may contribute to the inflammation of diabetes.¹⁴

Among the methods that have significantly contributed to the transformation of the field of microbial diagnostics are mass spectrometry platforms targeting microbial products, primarily proteins.^{15,16} Given this, pathogens identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis of high abundance proteins (ribosomal, among others) is emerging as the dominant technology in many clinical laboratories.¹⁷ Nevertheless, the application of such an approach meets some constraints like a commonly encountered failure to differentiate some closely related species that demonstrate high genotypic similarities (e.g., *Escherichia coli* vs *Shigella*, *Mycobacterium tuberculosis* complex, *Enterobacter cloacae* complex and so on) or different phenotypes (e.g., antibiotic resistant/sensitive), laborious protein extraction procedures required for microorganisms encased in the complex, thick cell walls, or poor efficiency in detecting pathogens directly in the clinical specimens.^{18,19} Due to the limitations mentioned above associated with MALDI profiling of bacterial proteins, the attention of researchers is directed to the search for new molecular targets that may allow overcoming the prevailing limitations, among which the most promising approach seems to be a lipidomics study.²⁰ Lipids perform many important and unique biological functions that aim to maintain cell homeostasis. Lipids are the main structural component of cell membranes, so they influence such properties of the cell membrane as its fluidity and curvature as well as all types of interactions taking place in the membrane. Moreover, lipids are involved in the processes of energy transport and storage as well as signal transmission in the cell. Maintaining the concentration of individual lipids building cellular structures or participating in the cell signaling pathways is crucial for maintaining cellular homeostasis and is strictly regulated by lipid metabolism.^{21–23} Therefore, changes or defects in lipid metabolism have been associated with the pathogenesis of diabetes.²⁴ The qualitative and quantitative composition of cell lipids may also be related to the presence of various phenotypic features of microorganisms.²⁵ Therefore, the structure and functioning of the cell membrane are related to the qualitative and quantitative composition of the membrane lipids.

Microbial lipidomics has soared as a novel method of lipid analysis using MALDI-TOF MS that has the potential to address some of the challenges encountered by the proteomic approach and thereby complement them regarding the identification and classification of bacteria.^{26,27} It is known that some lipids show species-specific characteristics; therefore, they can be used as a bacteria chemical barcode during MS analysis and complement common protein-based microbial identification platforms.²⁸ For example, Leung et al.²⁹ found out that glycolipids can be successfully used for identification and distinguishing clinically important ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) when MALDI-TOF MS analysis in negative ion mode is applied. Despite the significant progress that has been made in lipid analysis using MS, there is still a great need for their further development, including sample preparation and analysis conditions optimization (lipids are far more dependent on microbial environmental conditions than proteins) as well as bioinformatics resources for the sake of building of robust and accurate databases comparable in size and variety to the protein mass spectra libraries currently provided by commercial systems.^{30,31}

The main and foremost objective of the conducted study was to accurately identify and provide detailed characterization of the lipidomic profiles of bacteria obtained from wound samples of individuals suffering from diabetic foot infections. The lipidomic analysis aimed to determine qualitative differences in the composition of cellular lipids among different strains of Gram-positive and Gram-negative bacteria. For this purpose, lipid fingerprints obtained from lipidomic analysis of lipid extracts, utilizing matrix-assisted laser desorption/ionization (MALDI) techniques and advanced chemometric techniques, were compared. It should be emphasized that there are existing studies in the scientific literature concerning lipidomic analysis conducted in individuals with diabetic foot ulcers.^{32,33} Additionally, a comparison of the lipid profiles of Gram-positive and Gram-negative bacteria was performed. The information obtained from these studies will be extremely valuable for future research endeavors focused on the construction of microbial lipid libraries for identification purposes.

MATERIALS AND METHODS

Clinical Samples. The superficial swab samples from 15 patients from Provincial Polyclinical Hospital in Toruń (Poland) from infected diabetic foot wounds were collected using a flocked swab (ESwab Collection System, Copan, Murrieta, CA, USA) by applying the Levine technique. In order to avoid contamination of the bacterial flora, the wounds were cleared of necrotic or nonviable tissue and rinsed with physiological saline before the swab was taken. After wound debridement, samples for bacterial culture were obtained by swabbing the wound (the swab was rotated over a 1 cm² area of the viable non-necrotic wound tissue). The samples were immediately placed into a liquid transport medium (Amies Øswab, Deltalab, Nemours, France) and transported to the Centre for Modern Interdisciplinary Technologies, where they were stored at –80 °C. The Ethical Committee approved the studies (Bioethical Commission's permission no. 68/2019). Information about gender and age of the investigated DFI patients is provided in Table S1.

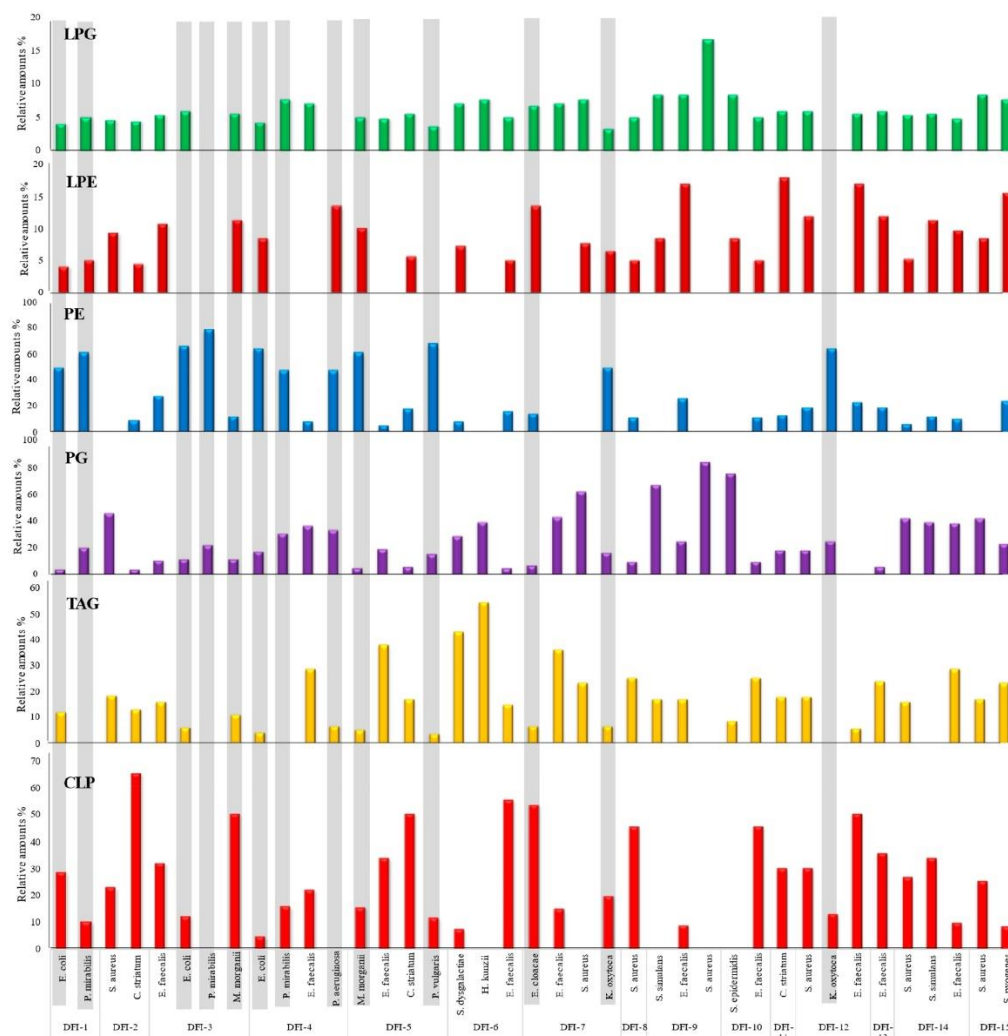


Figure 1. Abundance of different lipid classes in Gram positive and Gram-negative bacteria. Each color represents another lipid class: LPG – lyso-phosphatidylglycerol, LPE – lyso-phosphatidylethanolamine, PG – phosphatidylglycerols, PE – phosphatidylethanolamine, TAG – triglyceride, CLP – cardiolipin. Gray shade – Gram-negative bacteria.

Investigated Strains. 39 bacterial isolates representing 15 different species were obtained from diabetic foot infections: *E. faecalis* (10 strains), *S. aureus* (7 strains), *C. striatum* (3 strains), *S. simulans* (2 strains), *S. dysgalactiae*, *H. kunzii*, *S. epidermidis*, and *S. pyogenes* – 1 strain each (all Gram positive bacteria), and *P. mirabilis*, *E. coli* – 3 strains each, *M. morganii*, and *K. oxytoca* – 2 strains each, *P. aeruginosa*, *P. vulgaris*, *P. cloacae* – 1 strain each (all Gram-negative bacteria) were used in research.

Chemicals. Methanol, acetonitrile, trifluoroacetic acid (TFA), chloroform, sodium chloride, and water (all of high purity grade) were purchased from Sigma-Aldrich (Steinheim, Germany). The applied matrix 2,5-dihydroxybenzoic acid (DHB) and mass standards kit for calibration were from Sigma-Aldrich.

Lipid Extraction from Bacteria. The extraction of the lipid fraction from bacteria was carried out according to the Folch et al. procedure with modifications.³⁴ Bacterial pellets (approximately 50 mg of each DFI strain) were dissolved in 2

mL of chloroform/methanol (2:1, vol/vol) mixture and 0.5 mL of sodium chloride (0.05 M NaCl), respectively. The solutions were ultrasonicated for 10 min at room temperature, shaken for 10 min on a rotary shaker (200 rpm), and centrifuged at 5000 rpm for 20 min. After centrifugation, the lower layer was removed, and the process was repeated adding 0.5 mL of chloroform to the upper phase. The lower phases were combined and evaporated on a Labconco CentriVap DNA concentrator (Kansas City, USA). The extracted lipids were stored at -20°C for future analysis.

MALDI-TOF MS Analysis. Mass spectrometric measurements were performed by using a MALDI-TOF/TOF MS instrument (Bruker Daltonics, Bremen, Germany). The instrument was equipped with a modified neodymium-doped yttrium aluminum garnet (Nd: YAG) laser (1-kHz Smartbeam-II, Bruker Daltonik) operating at the wavelength of 355 nm which was used for all measurements. The extraction voltage was 25 kV, and gated matrix suppression was applied to prevent the saturation of the detector by matrix ions. All

spectra were acquired in reflector positive mode within an m/z range of 200–1600 at 80% of laser power and global attenuator of 50%. All mass spectra were acquired and processed by using flexControl and flexAnalysis software, respectively (both from Bruker Daltonik).

The extracted lipids were dissolved in 0.5 mL of methanol. A 10 mg amount of DHB matrix was dissolved in 1 mL of the mixed solution (30:70, acetonitrile/0.1% TFA in water). AnchorChip MALDI target plate (anchor diameter of 800 μm ; Bruker Daltonik GmbH, Bremen, Germany) was used for sample deposition. The mass spectra were calibrated by using the cesium triiodide cluster. Each sample was analyzed 3 times.

All of the lipid species were identified by using the LIPID MAPS online database (<http://www.lipidmaps.org>).

Nomenclature of Lipid Species. Lipid nomenclature involves a systematic framework for identifying and characterizing lipid species. These species are assigned symbols like PL $x:y$, which carry crucial insights into their overall fatty acid (FA) composition. Within this system, the abbreviation “PL” indicates the lipid class, such as phosphatidylcholine or triglycerides. The value ‘ x ’ denotes the total count of carbon atoms in the lipid’s structure, while ‘ y ’ signifies the cumulative count of double bonds present in the fatty acid residues within that specific lipid species. To provide a more comprehensive breakdown of individual fatty acid constituents within the lipid structure, distinct positions are specified. The underscore notation (PL $x_1:y_1_x_2:y_2$) is for positions that are unspecified along the glycerol backbone. This labeling allows for finer elucidation of the lipid’s makeup.

Statistical Analysis. The Shapiro–Wilk test was performed to investigate data distribution and therefore selected appropriate methods for comparisons between lipid means in different bacteria. The Mann–Whitney U test was applied to verify statistically relevant differences between lipid responses among isolated bacterial species. The aforementioned methods were conducted on IBM SPSS v.23 software (IBM Corp., Armonk, NY, USA). A significance criterion of $p < 0.05$ was considered. The following methods were performed in R environment, using RStudio v.1.2.1335 console (PBC, Boston, MA, USA). Obtained p values were adjusted for multiple comparisons using the Benjamini–Hochberg method (`p.adjust` function). Boxplots aiming to graphically show differences between lipid content in Gram-positive and Gram-negative bacteria were built using “ggpubr”. Network analysis employing the “sna” package was used to reveal connections between bacterial species and DFI cases—in this case, input was a binary matrix where 1 and 0 referred to species presence and absence, respectively. The package “igraph” was employed to build a lipid pathway analysis network, displaying hierarchical relationships between the detected lipids in most DFI-related bacteria. The input consisted of a list of edges linking lipid molecules to its subspecies and nodes associated with total ion intensity.

■ RESULTS AND DISCUSSION

Lipids Profiles of Gram-Positive and Gram-Negative Bacteria. The lipidome of Gram-positive and Gram-negative bacteria are mainly: phosphatidylglycerols (PGs), lysophosphatidylglycerols (Lys-PGs), phosphatidylethanolamines (PEs), lysophosphatidylethanolamines (Lys-PEs), triglycerides (TAGs) and cardiolipins (CLPs). Particular classes of lipids found in Gram-positive and Gram-negative bacterial cells extracted from people with DFI, is presented in Figure 1.

These lipids are primarily building components of the cell membrane; therefore, the structure and functioning of the cell membrane are related to the qualitative and quantitative composition of membrane lipids. The balance in the lipid composition of the cell membrane also affects the proper distribution of membrane proteins and, consequently, membrane transport, DNA replication and cell division.³⁵ Changes in the permeability of the cell membrane resulting from the modification of its structure may be related to the reduced penetration of antibiotics into the cell.³⁶

The study used 39 bacterial isolates, which were obtained from superficial swab from 15 patients; 8 species were Gram-positive and 7 were Gram-negative bacteria. Figure 1 shows relative amounts of all of the identified obtained lipids from Gram-positive and Gram-negative bacteria. As a result of the studies (Figure 1), a large variability in the qualitative composition of lipids was observed among Gram-positive and Gram-negative bacteria. In Gram-positive bacteria: 26 LPGs, 33 LPEs, 43 PEs, 114 PGs, 89 TAGs and 120 CLPs molecules of lipids were identified, and in Gram-negative bacteria, 10 LPGs, 14 LPEs, 124 PEs, 37 PGs, 13 TAGs and 22 CLPs molecules of lipids were identified, respectively (Table S2). Differences in the lipid profile patterns between Gram-positive and–negative bacteria have been previously demonstrated in the literature. Zhang et al.³¹ using desorption electrospray ionization mass spectrometry (DESI-MS) to measure lipids directly from 16 different bacterial species pointed out that it is easy to differentiate Gram-positive and Gram-negative bacteria since spectra of Gram-negative bacteria were more abundant in fatty acids, LPG, LPE, PE, and PG, while spectra of Gram-positive bacteria contain lipopeptides and very limited abundance of PG, PE, and LPG. Similar findings noted Lellman and Cramer,³² using the liquid atmospheric pressure (AP) MALDI MS technique to lipid profiling of multiple bacterial strains including *E. coli*, *Klebsiella pneumoniae*, *Campylobacter jejuni*, *S. aureus*, *S. epidermidis*, *S. pyogenes*, *Lactobacillus brevis*, *E. faecalis*, *Enterococcus hirae*, and *P. aeruginosa* where PEs and PGs were more abundant among Gram-negative species while cardiolipins and lyso-phospholipids were dominant signals within MS spectra of Gram-positive ones. Results of our studies proved that Gram-negative bacterial cells consist of a much higher number of PEs compared to the Gram-positive ones, which makes PEs a potentially good biomarker for the indication of a Gram type of bacteria present in the sample. Nevertheless, the revealed much higher number of LPGs and LPEs in the Gram-positive bacteria is inconsistent with the results of the above-mentioned works as well as with the statement that Gram-positive bacteria demonstrated much lower phospholipid signals resulting from the structural characteristics of their cell membrane. One possible explanation of such phenomena may be related to differences in the process of the sample preparation and ionization techniques since Zhang et al.³¹ used ESI techniques while in the case of a second cited work³² authors used atmospheric pressure conditions as well as a simple ethanol/formic acid extraction protocol commonly used for proteins detection and liquid support matrix (LSM), formed of matrix chromophore molecules and the addition of a viscous support liquid such as glycerol which use resulted in the production of ‘electrospray ionization-like’ multiply charged ions. Indeed, Lellman and Cramer revealed great differences in lipids MS profiles between liquid and solid AP-MALDI-MS conditions.³² It is known that the analysis of lipid mixtures is challenging for

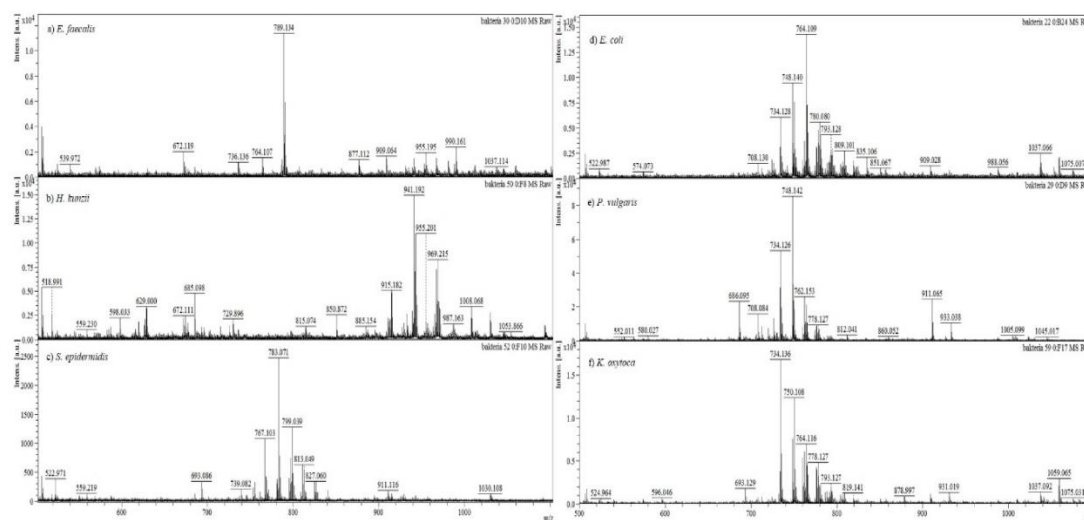


Figure 2. MALDI-TOF MS mass spectra recorded in positive mode of Gram-positive: (a) *E. faecalis*; (b) *H. kunzii*; (c) *S. epidermidis*, and Gram negative bacteria: (d) *E. coli*; (e) *P. vulgaris*; (f) *K. oxytoca*.

many reasons. Different classes of lipids affect the intensity of others, so MALDI mass spectra can change if they are recorded at different analyte concentrations. Moreover, the choice of the extraction protocol may also significantly influence the result of the lipid profiling since techniques based on the use of chloroform favor detection bulk lipids such as PEs, while more polar compounds like lysophosphatidic acid or (poly)-phosphoinositides are incompletely extracted. Furthermore, some lipids may stick to the precipitated proteins present in the form of the white ring at the interface between the aqueous and the organic phase during lipids extraction via the Folch method and therefore may be lost since only the organic layer is used for further analysis. Matrix selection also matters since for example DHB, which is the most commonly used matrix in lipid analysis, tends to ionize phospholipids with quaternary ammonium groups such as lyso(monoacyl)-phosphatidylcholine or sphingomyelin, whereas negatively charged phosphatidylinositol and phosphatidylserine are detected with very low sensitivity when the positive ion detection mode is used.³³ All together this may explain the differences in the lipidomic profiles of the Gram-positive and -negative species revealed in our and cited works and emphasize the need for looking for optimal and standardized lipid extractions and analysis protocols to facilitate interlaboratory trials comparison.

In addition, variability in the lipid composition within one group of strains was also observed. For example, the *E. coli* strain that was identified in patient DFI-1 (1-LPG, 1-LPE, 12-PE, 1-PG, 3-TAG, and 7-CLP), DFI-3 (1-LPG, 11-PE, 2-PG, 1-TAG, and 2-CLP), and DFI-4 (1-LPG, 2-LPE, 15-PE, 4-PG, 1-TAG, and 1-CLP) has different lipid compositions. The highest number of phosphatidylethanolamines lipid molecules (18 molecules of PE) was identified for the *P. vulgaris* strain in the DFI-5 patient, 10 phosphatidylglycerols for the *S. aureus* strain in the DFI-2 patient, 8 triglycerides for the *E. faecalis* strain in the DFI-5 patient, and 15 cardiolipin for *C. striatum* in patient DFI-2, respectively. PG molecules have been identified in every bacterium, except *E. faecalis* (DFI-12).

On the example of *E. coli* (−) and *S. aureus* (+), a large difference in the lipid composition of bacterial cytoplasmic

membranes can be observed. Major lipids of *E. coli* (DFI-1) glycerol phosphate based lipids include zwitterionic phosphatidylethanolamine (PE, 48% of membrane), anionic phosphatidylglycerol (PG, 4%), cardiolipin (CLP, 28%) and triglycerides (TAG, 12%). In contrast, in *S. aureus* (DFI-2), PE is 0%, PG is 45%, and CLP is 23%; the remaining 14% are zwitterionic lipids: lyso-phosphatidylglycerol 5% and lyso-phosphatidylethanolamine 9%. The observed differences in lipid content may reflect differences in lipid metabolism and membrane structure between the strains studied. The cell wall of Gram-positive bacteria is thick due to the combination of murein with another polysaccharide polymer—teichoic acid or glycerol polymer, which is a component of phosphatidylglycerol. Murein can also combine with existing proteins and create a multilayer structure itself.⁴⁰ In Gram-negative bacteria, there is an additional layer of the outer membrane.⁴¹ The outer membrane is composed of proteins and lipopolysaccharide (LPS).⁴² Lipopolysaccharide consists of lipid A, a core part, and a polysaccharide chain (referred to as the O antigen). Lipid A has a sugar core, which consists of two molecules of glucosamine. Then, arabinose is attached to one end of the disaccharide and phosphate residues and ethanolamine (which is part of phosphatidylethanolamine) to the other.

Lipid-Profiling by MALDI-TOF for Microbial Identification. The lipid profile of Gram-positive and Gram-negative bacteria was recorded using MALDI-TOF MS. The scanning range was set to m/z 400–2000. Figure 2 shows mass spectra detected in positive ionization for selected bacterial strains. It can be seen from the spectrum that the dominant ions detected are mostly intact lipids distributed in the m/z range of 600–1000. This is consistent with previous work by Lellman and Cramer, where the largest distribution of lipid ion signals for Gram-negative species was detected in the m/z 650–800 range (mainly associated with an abundance of PEs), and that for Gram-positives in the m/z 900–1000 range was due to the presence of LPG and cardiolipins.³⁸ In addition, it was observed that the lipid signals detected in Gram-negative bacteria were much stronger than those in Gram-positive bacteria. Probably, the thick layer of peptidoglycan in Gram-

positive bacteria may hinder the partial release of lipids from the bacterial cell membranes.⁴³

108 combinations of fatty acids in lipid molecules were identified for bacteria isolated from the diabetic foot infection (Table S2). The identified acids contained from 12 to 22 carbon atoms in the acyl chain and from 0 to 6 double bonds. The most common fatty acids for all lipid molecule-building DFI bacterial strains are C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), and C18:1 (oleic acid). Omega-3 (ω -3) and omega-6 (ω -6) fatty acids such as C18:2 ω -6 (linoleic acid), C18:3 ω -3 (α -linoleic acid), C20:4 ω -6 (arachidonic acid), and C22:6 ω -3 (docosahexaenoic acid) were also identified in the tested bacterial strains (Table S2). The content of particular classes of lipids, as well as the type of fatty acids constituting the side chains of lipids (number of carbon atoms, degree of unsaturation, presence or absence of branching) in DFI bacterial cells, depends on many factors, e.g. growth phase of bacterial cells and culture conditions.⁴⁴

Escherichia coli was for many years a model organism in the description of cell membrane lipids.⁴⁴ Bacterial proteins are the subject of intensive research, and the state of knowledge about the role of phospholipids in bacterial strains is scarce. The arrangement and composition of membrane lipids and modifications of the “polar heads” of phospholipids affect the interactions between other molecules and the environment. The structure and dynamics of plasma membrane phospholipids play an important role in the penetration of antibiotics into the cell.⁴⁵ Therefore, an important goal of the research was to determine the importance of lipid structures in bacteria isolated from DFI. In the conducted studies, the lipid profile was characterized including fatty acids that build specific molecules of phospholipids and triglycerides. Lipid molecules species such as PE 12:0_12:0, PE 16:1_16:1, PG 16:1_15:0, PG 16:0_16:1, PE 17:0_17:0 have only been identified for *P. vulgaris* (DFI-5) bacteria. It can be considered that the phospholipids identified as differentiators in this study may be potential biomarkers of DFI in the case of *P. vulgaris* bacteria. In addition, all identified lipids are major components of the cell membranes. PG 15:1_15:0 was only identified for *H. kunzii* (DFI-6), TAG 18:0_18:0_20:2 for *S. dysgalactiae* (DFI-6), and PE 18:2_18:3, PE 20:1_20:1, TAG 16:1_18:3_18:3 for *P. aeruginosa* (DFI-4), respectively. It was also observed that for the *E. coli* strain that was isolated from patients 1 and 3, two different potential biomarkers TAG 20:2_20:2_21:2 and PG 16:0_15:0 were identified, respectively. Differences in the lipid composition within the same strain result from the high phenotypic variability of the bacteria and variable fat metabolism. Many studies have shown that temperature, pressure, pH, and nutrients determine the level of fats, including the composition of fatty acids and lipid groups found in bacterial membranes.^{44,46} Differences in the composition of fatty acids contained in lipids are their important chemotaxonomic features and may be of practical importance in the diagnosis of this group of bacteria at the species level. A similar correlation was observed for the *C. striatum* strain isolated in patients 2 and 5. These two species differed in the composition of the fatty acids in the cardiolipin class: CLP 16:0_18:1_16:1_17:0, CLP 16:1_18:1_17:0_18:0, CLP 16:1_18:1_18:0_18:0, CLP 22:0_22:0_22:0_21:1 (DFI-2) and CLP 18:0_18:0_18:0_18:0, CLP 18:1_19:1_18:1_19:1, CLP 18:1_19:1_18:1_19:0, and CLP 20:1_21:0_21:0_21:0 (DFI-5). Combinations of fatty acids in cardiolipin molecules

such as CLP 16:1_16:1_16:1_16:1, CLP 18:1_19:0_18:1_19:0, and CLP 20:1_20:1_21:0_21:0, CLP 20:1_20:0_21:0_21:0 occur only in the strain: *P. mirabilis* (DFI-4), *S. aureus* (DFI-15), and *E. faecalis* (DFI-3), respectively. The dominant lipid molecules for all bacterial strains were LPG 12:0 (92%), PE 18:2_20:4 (69%), and CLP 18:1_19:0_19:1_19:0 (72%).

As a result of the conducted research, it was observed that the lipid profile for each bacterial strain is unique and nonrepeatable. The analysis of fatty acids in lipid molecules reveals diverse lipid composition. The classes of lipids in the cell membranes of Gram-positive and Gram-negative bacteria differ. Moreover, the identified lipids are recognized as components of cell membranes. Lipid diversity also arises from disruptions in phospholipid and triglyceride metabolism during the infection process, leading to cellular dysfunction and death. All identified and characterized lipid molecules serve as differentiators and can be regarded as potential biomarkers for DFI. Noticed observations appeared to prove the concept by Solntceva et al. that MALDI analysis of the lipids can provide subspecies-level classification supporting microbial identification at a species level yielded by protein fingerprinting, thus helping accomplish several diagnostic goals simultaneously.²⁰ Furthermore, recognition of microbial species-specific lipids could be a game changer in rapid microbial diagnostics since the detection of the distinct microbial lipids directly from body fluids could allow avoiding the need for culture on agar plates or in liquid medium providing that a suitable method of their enrichment from the samples is given.¹⁷ Depending on the length of the acyl chain and the degree of unsaturation, fatty acids in lipid molecules can have either a positive or negative effect on systemic insulin sensitivity.⁴⁷ It is likely that a different insulin sensitivity is the cause of changes in fatty acid composition. Clinical studies have demonstrated that reduced concentrations of polyunsaturated fatty acids (PUFA) in phospholipids lead to decreased insulin sensitivity.⁴⁸ Additionally, abnormal fatty acid metabolism results in decreased glucose uptake, leading to excessive intramuscular triglyceride (IMTG) accumulation and insulin resistance.⁴⁹

Obtained results prove the concept that lipid signals obtained during MALDI analysis are well suited to act as a chemical fingerprint for differentiating microorganisms.²⁰ The significant advantage of the proposed MALDI approach is that information about fatty acids composition is also provided; however, without the need of using a multistep protocol required when gas chromatography (the most traditional and old technique) is applied, which involves saponification of bacterial cells in sodium hydroxide, methylation with hydrochloric acid, lipid extraction, and a wash at a basic pH which typically takes several hours.⁵⁰ The powerful impact of the MALDI approach in the differentiation of the bacteria based on their lipidomic profiles, even at the strain level, has been recently proven for *E. coli* strains isolated from DFI wounds that differed in beta-lactams antibiotic resistance level.⁵¹

Statistical Evaluation of Obtained Lipid Profiles. The Figure 3 is a network showing the interrelations between DFI cases and detected bacteria, giving a graphical representation of the bacterial prevalence in DFI samples. The most recurrent simplified microbiome in DFI consisted of *E. faecalis* coexisting with *S. aureus*. The bacterial variety ranged from 2 to 4 species coexisting in the same sample site—each of these configurations were present in equal proportions, representing 80%

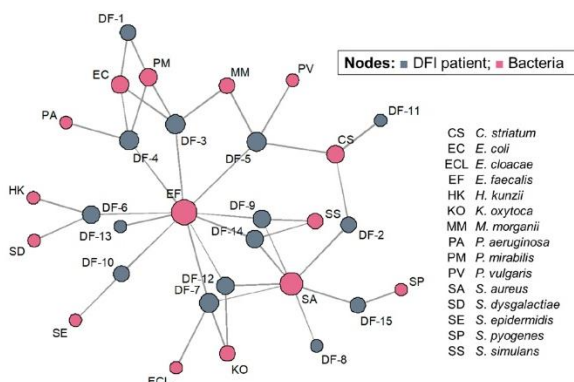


Figure 3. Network analysis showing the relationships between DFI samples and identified bacteria species. DF – diabetic foot infection case.

of sampled DFI, whereas colonization by only one species was less frequent (20% of DFI cases). From assessed bacterial mixtures, only one of them was composed exclusively of Gram-negative bacteria, with most of them consisting of the

homogeneous presence of Gram-positive bacteria (40% of the cases). Mixtures containing both Gram-negative and Gram-positive species represented 33.4% of DFI samples. Such a trend is confirmed by other bacteriological studies on DFI, which report that Gram positive cocci are the most frequently isolated species.⁵²

Among the bacterial species identified in DFI samples, those with a higher incidence were *E. faecalis* (10, 25.6%), *S. aureus* (7, 17.9%), *E. coli* (3, 7.7%), *C. striatum* (3, 7.7%), and *P. mirabilis* (3, 7.7%). First, lipids characteristic for each of the most predominant DFI bacteria were investigated. For this purpose, a statistical comparison of lipid composition was performed considering a single species against all others. The Figure 4 shows a features plot of the obtained statistical significance (expressed as the negative common logarithm of adjusted *p* value) versus the magnitude of the change (expressed as the binary logarithm of the fold-change in relation to lipid intensities in other species). In this manner, lipids plotted toward the top part of the graph displayed a greater relevance (lower *p* values). Lipids plotted at the right part of the graph presented an increased response in relation to the remaining bacteria (blue dots), and those located across the *y*-axis were incident only in the given bacteria (purple

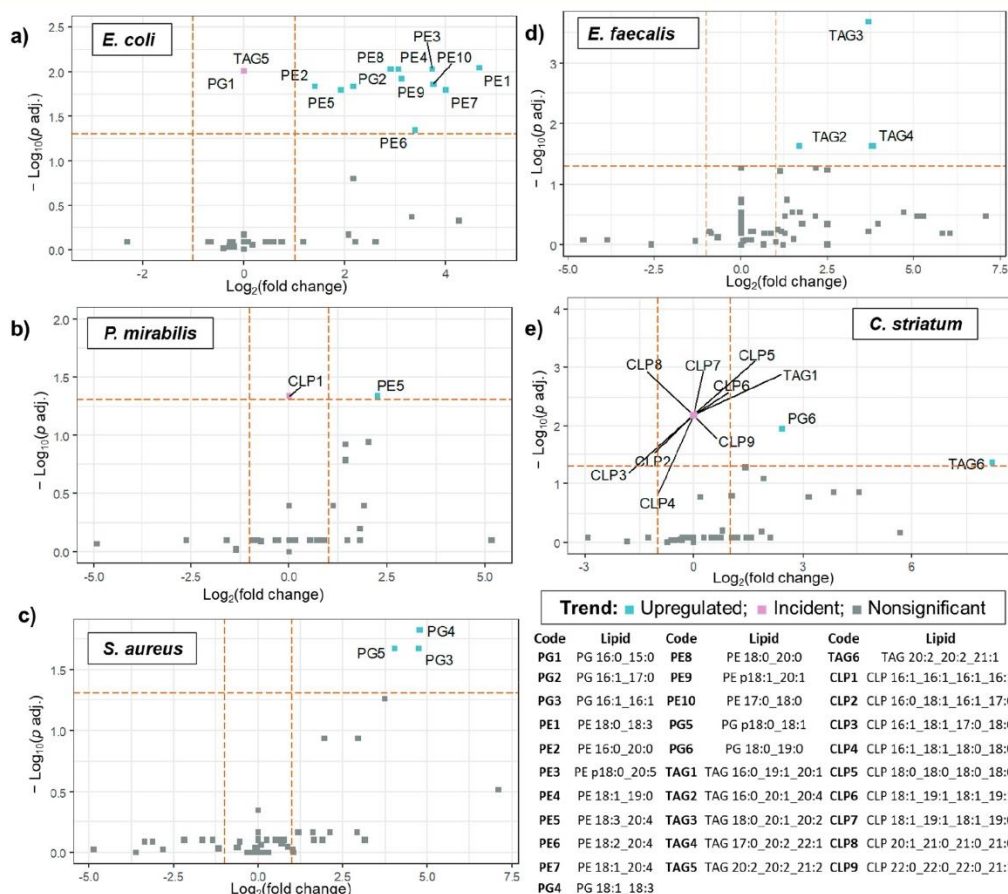


Figure 4. Feature relevance plot, showing discriminant lipids found for (a) *E. coli*, (b) *P. mirabilis*, (c) *S. aureus*, (d) *E. faecalis*, and (e) *C. striatum*. The horizontal threshold line shows where *p* adjusted < 0.05. PG – phosphatidylglycerols, PE – phosphatidylethanolamine, TAG – triglyceride, CLP – cardiolipin.

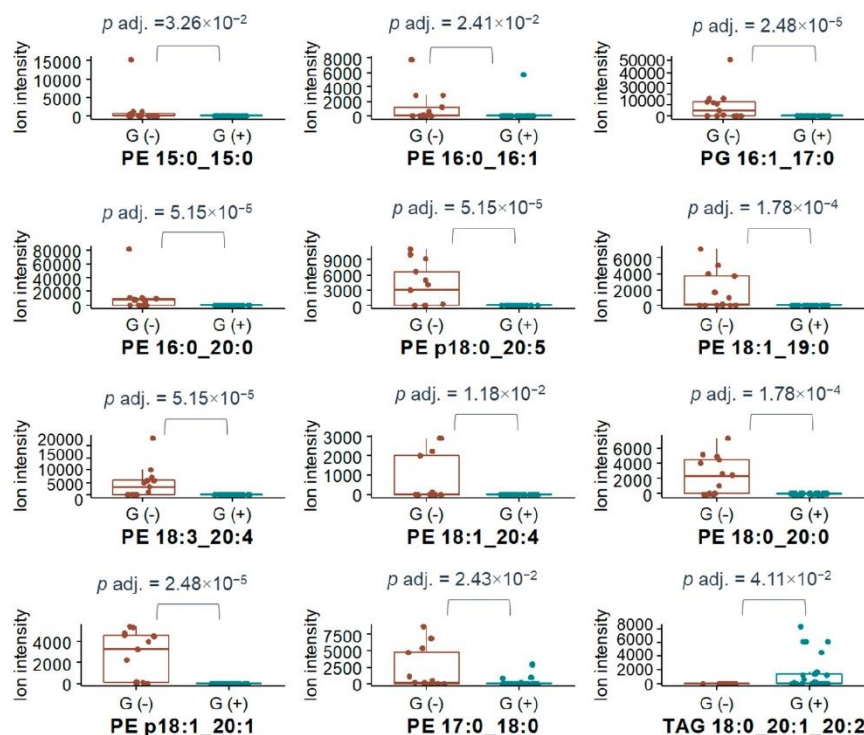


Figure 5. Statistically significant changes in lipid intensities for Gram-negative and Gram-positive bacteria. PG – phosphatidylglycerols, PE – phosphatidylethanolamine, TAG – triglyceride.

dots). Since these are the features significantly altered in comparison to the whole set of bacterial strains isolated from DFI samples, they can serve as potential biomarkers of the given bacterial species. Such lipid molecules may be further considered in predictive models for reliable bacterial identification based on lipidomics.

This approach showed that *E. coli* species (Figure 4a) were mainly characterized by a greater concentration of several PE variants as well as distinguished incidences of TAG 20:2_20:2_21:2 and PG 16:0_15:0. Another Gram-negative bacteria, *P. mirabilis* (Figure 4b), also displayed increased levels of a PE derivate (PE 18:3_20:4). Besides that, CLP 16:1_16:1_16:1_16:1 was differentially present in their lipid composition. In the case of *S. aureus* (Figure 4c), these species were distinguished by the augmented levels of PG. Conversely, *E. faecalis* (Figure 4d) displayed remarkably increased concentrations of TAG variants in the lipid content. Finally, the lipid composition of *C. striatum* (Figure 4e) was mainly marked by the presence of unique CLP species. Additionally, for these bacteria, other differentiated lipids comprised elevated TAG derivatives (TAG 16:0_19:1_20:1—exclusively incident in these species—and TAG 20:2_20:2_21:1), as well as PG 18:0_19:0.

Indeed, PE is reported as the most abundant lipid in *E. coli*, accounting for 70–80% of total membrane lipids. In these bacterial species, PE is particularly necessary for the functioning of lactose permease—a membrane associated protein responsible for the transport of β -galactosides into the cell, for which PE deficiency results in unsuccessful coupling of substrate uptake. Moreover, PE also plays an important part in *E. coli* adhesion, possibly due to its influence

on lipopolysaccharide biosynthesis.^{53,54} Discriminating elevated levels of a PE variant in *P. mirabilis* is also expected, as PE content is generally superior in Gram-negative bacteria when compared to Gram-positive species.⁵⁵ Studies have shown that in actively growing *S. aureus*, PG is the most predominant phospholipid, which agrees with the present findings. During the stationary phase, PG content tends to decrease as CLP is augmented. PG to CLP in *S. aureus* membranes also appears to be induced by phagocytosis promoted by human neutrophils. Such alterations in phospholipid metabolism are probably coordinated by the regulation of CLP synthase.⁵⁶ A few TAG species were elevated only in *E. faecalis*. Such lipids were also detected during the exponential growth phase of these bacterial species by previous research. The role of TAG lipids in bacteria is still not extensively studied. TAGs mainly serve as a reservoir of fatty acids, also contributing for the regulation of membrane fluidity and working as an electron sink.⁵⁷ TAG accumulation tends to occur for some bacterial species when the cells develop in nonrelated carbon sources, like glucose.⁵⁸ In addition, a differential lipid profile has been observed for daptomycin-resistant *E. faecalis*, marked by reduced levels of PG and LPG in comparison to agent-sensitive strains. This indicates an increased consumption of PG substrate toward Glycerophospho-Diglucoyl-Diacylglycerol biosynthesis in resistant *E. faecalis*, which can serve as an intermediate for the *de novo* production of TAG.⁵⁷ Finally, specific CLP species were found only in *C. striatum*. A previous investigation on extracts of *Corynebacterium glutamicum* reported that after PG, CLP species were the most abundant species. However, a higher CLP content was found tightly associated with the

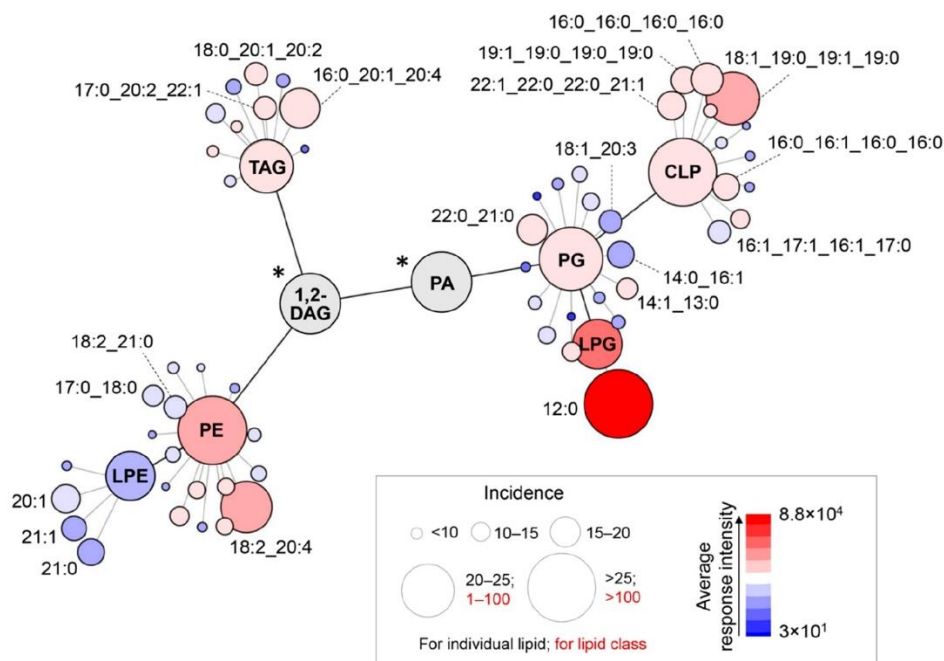


Figure 6. Lipid pathway network based on the DFI-bacteria lipidome. LPG – lyso-phosphatidylglycerol, LPE – lyso-phosphatidylethanolamine, PG – phosphatidylglycerols, PE – phosphatidylethanolamine, TAG – triglyceride, CLP – cardiolipin. * – Not measured metabolite: 1,2-DAG – 1,2-diacylglycerol, PA – phosphatidic acid.

peptidoglycan–arabinogalactan complex of the bacterial cell wall.⁵⁹ Such a lipid configuration of *Corynebacterium* species may explain why the CLP variety was the most discriminating aspect of DFI-derived *C. striatum*.

Figure 5 presents boxplots showing the lipids that displayed the greatest statistically relevant differences between their responses in Gram-positive and Gram-negative bacteria. As expected, due to their major lipid composition, Gram-negative bacteria were described by a significantly higher content of several PE species. PE 16:1_17:0 and PE p18:1_20:1 were the most unique lipid species (presenting the lowest *p* values), differentiated between the two bacteria groups. For this reason, such species can be considered as molecular indicators, characteristic for Gram-negative bacteria. On the other hand, TAG 18:0_20:1_20:2 displayed significantly greater responses for Gram-positive bacteria.

Lipid Pathway Considerations Regarding DFI Bacteria. A representation of the configuration of main lipid pathways in DFI-related bacteria is shown in Figure 6. In this, only lipids incident more than 10 times in DFI-lipidome are depicted. The connections in black show the link between lipid classes, while connections in gray refer to the relation between the individual lipids and their main classes. The size of the nodes is proportional to the prevalence of detected lipids (how many times they were detected within the samples), whereas the color code refers to the average intensity of the lipid ion. This approach clearly shows that PG, CLP, and PE are the main lipid classes identified in the total lipidome of DFI bacteria. Among them, some specific variants displayed consistently high responses. It is the case of LPG 12:0. LPG is a commonly referred membrane lipid in several Gram-positive species, however scarcely discussed regarding Gram-negative bacteria.⁶⁰ In the present study, LPG 12:0 was

detected with considerably high intensity, also for Gram-negative species. Other studies indicate that this lipid class is potentially involved in bacterial resistance mechanisms. Daptomycin-resistant strains of *S. aureus* often display an augmented LPG membrane content together with an enhanced surface positive charge. Such a lipid configuration possibly stabilizes membrane integrity, avoiding structural defects and an increased vulnerability to cationic antimicrobial agents.⁶¹ Low pH-inducible genes (*lpiA*) have been detected in Gram-negative α -proteobacteria like *Rhizobium tropici*, encoding homologues of MprF—the protein gene responsible for LPG synthesis in *S. aureus*. Deletion of *lpiA* turned *R. tropici* susceptible to cationic antimicrobial peptides, revealing that LPG confers increased resistance in acidic growth conditions.⁶⁰ Therefore, LPG detection also among Gram-negative DFI bacteria can suggest such an adaptation mechanism in the species isolated in the present study. Among CLP, as previously mentioned, CLP 18:1_19:0_19:0 was the most preponderant variant. It has been hypothesized that an increased cardiolipin content may contribute to bacterial resistance. Molecular dynamics simulations have showed that negative membrane curvature promoted by CLP can contribute to cell protection against antimicrobial peptides, by counteracting their proneness to induce a positive curvature in target membranes.⁶² Moreover, previous research demonstrated that clinical *S. aureus* strains performed metabolic adaptation. In this case were observed point mutations in the phospholipid biosynthesis gene (*cls2*) which encodes the synthesis of CLP, leading to an alteration in anionic membrane phospholipid composition. The described configuration resulted in a phenotype characterized by increased membrane CLP and reduced PG.⁶³ Indeed, PG variants displayed intensities much lower than those of CLP variants in the

present study. PG is referred to as a potential chemoattractant, stimulating the recruitment of neutrophils to the infection site.⁵⁵ For this reason, a shift toward increased synthesis of LPG and CLP, leading to reduced PG levels, may indicate a greater resistance of DFI-bacteria.

PE was the lipid more frequently detected among bacterial species, presented also in greater amounts of total lipid content, with the PE 18:2_20:4 variant configured as the most common PE molecule observed among DFI-bacteria. It appears that only a small fraction of PE is converted into LPE once such species are detected with lower intensities. As discussed previously, PE may play an important role in bacterial adhesion to substrates, enabling efficient colonization. Additionally, other experiments with PE-deficient *E. coli* have demonstrated possible functions in cell division and stress response activation.⁵⁴

The study of bacterial membrane lipids has long been recognized as a critical avenue for understanding bacterial physiology, stress adaptation, and host–pathogen interactions. For instance, alterations in bacterial lipid profiles have been implicated in various physiological processes, such as cell shape maintenance, envelope integrity, and adaptability to environmental stress. These lipidomic changes are not merely structural but functional and serve as potential targets for novel antimicrobials. This traditional understanding of bacterial lipidomics is now being significantly enriched by the advent of advanced analytical techniques, among which MALDI stands out as a complementary and versatile tool.⁶⁴

In the current landscape of lipidomics, MALDI serves as a robust adjunct to other established methods like RPLC-MS. Its utility extends from clinical settings, as demonstrated by Buszewska-Forajta et al.⁶⁵ in their analysis of lipids in prostate tissue, to cardiovascular research where Moerman et al.⁶⁶ employed it for lipid analysis in carotid atherosclerotic plaques. Beyond its role as a complementary technique, MALDI has shown its mettle in identifying a wide array of lipid classes across diverse biological systems. For example, Walczak-Skierska et al.⁶⁷ employed MALDI-TOF/MS for lipidomic analysis of lactic acid bacteria strains, while Bhandari et al.⁶⁸ used it for lipid profiling in insect models. These applications underscore MALDI's versatility and its growing role in lipidomic research, a point further emphasized by Nikitina et al.,⁶⁹ who described lipid profile changes in induced pluripotent stem cells.

Moreover, MALDI's applicability is not confined to academic research, but extends to practical and clinical settings. M. Hajjar et al.⁷⁰ utilized MALDI TOF in negative-ion MALDI-TOF for the analysis of LPS or lipid A preparations to identify *Francisella tularensis* strains. In the food industry, the technique has been employed for evaluating dietary supplements containing live bacteria, as shown by Lorbeg et al.⁷¹ In plant research, Wang et al.⁷² used it for studying lipid distribution in peanut seeds. Importantly, in the realm of microbial identification, particularly for closely related or drug-resistant strains, MALDI's role is being increasingly recognized. This is corroborated by emerging trends in the development of lipid-based databases for microbial identification, as demonstrated by the work of Maria-Theresia Gekenidis et al.⁷³ and P. Lasch et al.,⁷⁴ who have shown that MALDI-TOF MS can achieve deeper taxonomic identification and is particularly useful in clinical settings. Our approach of employing MALDI for building lipid libraries is not an isolated endeavor but is well-aligned with current scientific advance-

ments. It offers a promising avenue for bacterial identification, particularly when considering the growing interest in lipid-based databases and the technique's broad applicability across various research and clinical settings.

The network presented by Figure 6 may also depict an enrichment pattern useful for spotting a microbiome associated with DFI through a lipidomics approach. In order to confirm the usefulness of such lipid enrichment profile in identifying a phenotype derived from DFI bacteria, further studies would be necessary to compare paired bacterial species colonizing non-DFI wounds. Recent studies showed that 2 h of incubation in the presence of antibiotic (cefotaxime in that case) is enough to detect distinctive lipid patterns of beta-lactam-resistant and -sensitive *E. coli* strains.⁵¹

CONCLUSIONS

An analytical methodology has been developed that enables lipid analysis of Gram-positive and Gram-negative bacteria in people with diabetic foot disease. This approach makes it possible to understand the lipid composition of the cells of these bacteria and determine the lipidomic differences among strains with different phenotypes. The proposed procedure includes sample preparation and lipid identification using the MALDI-TOF MS technique. In addition, the use of a high-resolution TOF mass spectrometer and the created lipid database of such bacteria as *E. faecalis*, *S. aureus*, *C. striatum*, *S. simulans*, *S. dysgalactiae*, *H. kunzii*, *S. epidermidis*, *S. pyogenes*, *P. mirabilis*, *E. coli*, *M. morgani*, *K. oxytoca*, *P. aeruginosa*, *P. vulgaris*, *P. cloacae* enables the identification and proposition of the structures of the main cell lipids of these bacteria: phosphatidylglycerols, lyso-phosphatidylglycerols, phosphatidylethanolamines, lyso-phosphatidylethanolamines, triglycerides, and cardiolipins. The use of the MS/MS technique also enables determination of the length of the acyl substituents in the detected lipids.

By combining the MALDI-TOF MS technique with biostatistical and chemometric data analysis, the developed methodology can be successfully applied to lipidomics studies in various biological contexts—e.g. building lipid reference libraries for species identification, selection potential microbial-specific biomarkers for direct detection in clinical specimens, and subspecies differentiation. In addition, large variability in the qualitative composition of lipids within one group of strains was observed, which indicates a high phenotypic variability of bacterial cells and the need to analyze lipid extracts from many strains, cultured in several replicates, in order to extract information on lipidomic differences between bacterial strains. The observed differences in lipid content may reflect differences in lipid metabolism and membrane structure between the tested strains of Gram-positive and Gram-negative bacteria.

The achievements of the presented work indicate the high potential of the MALDI technique as a relatively simple and rapid method of classifying bacteria based on lipidomic profiles and respond to the recent appeal of the research community that, so far, routine identification of microorganisms by MALDI-TOF MS based on the identity and abundance of lipids has not been explored extensively to differentiate species and deserves more investigation.²⁰

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.3c03071>.

Table S1: Details information about gender and age of the investigated DFI patients (PDF)

Table S2: All identified combinations of fatty acids in lipid molecules species (XLSX)

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Publikacja [P4]
Supplementary materials

Table S1. List of investigated DFI patients with information about gender and age.

Patient	Gender	Age
P1	Male	80 yrs.
P2	Female	63 yrs.
P3	Female	85 yrs.
P4	Female	87 yrs.
P5	Male	74 yrs.
P6	Male	57 yrs.
P7	Male	73 yrs.
P8	Female	68 yrs.
P9	Male	62 yrs.
P10	Male	54 yrs.
P11	Male	53 yrs.
P12	Male	81 yrs.
P13	Female	65 yrs.
P14	Male	71 yrs.
P15	Male	65 yrs.

Table S2. All identified combinations of fatty acids in lipid molecules species.

Dane w arkuszu Excel dostępne po ściągnięciu ze strony czasopisma:

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lub po zeskanowaniu poniższego kodu QR:



Publikacja [P5]

Comparison of Bruker Microflex LT and Zybion EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms

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Comparison of the Bruker Microflex LT and Zybion EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms

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ABSTRACT

The emergence of Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI TOF MS) technology has expanded the capabilities for identifying microorganisms in clinical labs, replacing traditional biochemical testing with a proteomic approach. In the present study, we compared results between the two commercial MALDI TOF MS systems, Bruker Microflex LT Biotyper and Zybion EXS2600 Ex-Accuspec, for the identification of 1979 urinary isolates by direct extraction method. Current study found that both systems identified a high percentage of isolates to at least the genus level – Bruker 95.6 % of isolates, Zybion 92.4 %. In the case of 89.5 % of all analyzed spectra, the identification results were consistent between the used devices. The highest score values and the highest percentage of spectra identified to species were obtained for gram-negative bacteria. The results show that both systems are equally good choices in terms of analytical performance for routine microbiological diagnostic procedures.

1. Introduction

In recent years, Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI TOF MS) has been widely adopted in clinical laboratories, and it has revolutionized the field of microbiology. One key advantage of MALDI is its ability to analyze complex mixtures of biomolecules, including proteins, peptides, and nucleic acids, with high sensitivity and specificity. In clinical laboratories, MALDI is most commonly used for microbial identification, where it has largely replaced traditional biochemical tests [1]. The technique is based on proteome profiling (mainly ribosomal proteins) and involves extracting proteins from the microorganism and then using MALDI TOF mass spectrometry to record their profile [2]. The protein fingerprint is then compared to commercially available reference databases to identify the organism [3]. The ability to identify microorganisms within minutes allows clinicians to make quick decisions on antibiotic therapy, leading to better patient outcomes. In addition, the MALDI-based proteomic approach makes it possible to identify microorganisms that are difficult to culture or are resistant to antibiotics [4,5].

Nowadays, numerous companies around the world are producing or selling commercial mass spectrometry for clinical microbial

identification, such as: MALDI Biotyper (Bruker Daltonics, Germany) [6–9], VITEK MS (bioMérieux, France) [6,7,10,11], Axima (Shimadzu, Japan) [8,12] or MicroIDSys (ASTA, South Korea) [9,11]. Mentioned above systems are a popular choice in clinical microbiology laboratories. The Clinical and Laboratory Standards Institute (CLSI) has even released a guidance document to support clinical laboratories in adopting and validating commercial MALDI-TOF MS systems [13]. Recently, the variety of available MALDI TOF MS systems for microbial identification has increased, as Chinese companies such as Autobio [14], Xiamen [15] and Zybion [16] have begun distributing their products to the global market.

In this study, we compared the accuracy of species level identification of two commercially available MALDI-TOF MS systems for the identification of clinical isolates from urine samples. One of them is well known Bruker Microflex LT with Biotyper database; the second system – Zybion EXS2600 with Ex-Accuspec database, on the other hand, is not so widespread in Europe yet. Table 1 compares the practicality and the most important other technical aspects of both devices. The study focused mainly on demonstrating the capabilities of both devices in terms of evaluating identification and its level by using the shortest possible protocols (direct application of bacterial cells to the MALDI

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Table 1

Comparison of practicality and other technical aspects of Bruker Microflex LT Biotyper and Zymio EXS2600 Ex-Accuspec.

	Bruker Microflex LT	Zymio EXS2600
Technical parameters		
Laser	nitrogen, UV, 60 Hz	nitrogen, UV, 60 Hz
Mass analyzer	linear TOF	linear TOF
Mass range	up to 500,000 Da	1500–30,000 Da
Dimension (L x W x H) [mm]	520 × 690 × 1100	490 × 800 × 1215
Weight [kg]	120	116
User friendliness		
Ease of sample preparation	very good	very good
Disposable target	yes	yes
Ready-to use matrix solution	no	yes
Sample capacity per run	96 samples	96 samples
Calibration	every 96 spots	every 96 spots
Results in real-time manner	per spot	per spot
Self cleaning ion source	yes	no data
Database		
No. of species	3893	4051
No. of strains	10,834	> 15,000
Database update	chargeable	free
Customized database	available	available

plate with extraction on the plate).

2. Materials and methods

2.1. Clinical isolates

A total of 1979 clinical microbial strains from a culture collection obtained during the period 2021–2023 at the Centre for Modern Interdisciplinary Technologies in Toruń (Poland) were used in this study. All strains were routinely isolated during clinical practice from the urine of patients with diagnosed prostate cancer. Isolation of microorganisms was carried out using various culture mediums in aerobic conditions and 10 % CO₂ as we described in previous publication [17]. The isolates had been previously stored at –80 °C in Microbank (Pro-Lab Diagnostics). For microbial identification, isolates underwent sub-cultivation on 5 % sheep blood agar and were incubated at 35 °C for a duration of 24 h; extended incubation periods were allocated for species with slower growth kinetics. This study adhered to the ethical standards stipulated in the Declaration of Helsinki and received approval from the Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz (Approval No. KB/430-104/19 dated 19 December 2019).

2.2. MALDI-TOF MS analysis

All microorganisms isolates were identified by the direct colony on plate extraction method. Freshly grown overnight colony was smeared in small amounts onto 96 spot steel target plate and overlaid with 1 µL of 70 % formic acid (Sigma-Aldrich). Each spot was left to dry and then covered with 1 µL of α -cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bruker Daltonics). After drying, the target plate was loaded into the MALDI mass spectrometer (Bruker Daltonics and Zymio). Since the plates produced by both manufacturers are compatible (the same shape, size, location of spots), one plate was used during the analysis and loaded in both mass spectrometers. This made it possible to compare exactly the same samples. One spectrum was collected on each device from each spot on the plate.

2.2.1. Bruker Daltonics system

Measurements were automatically performed with Microflex LT MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany) in a positive linear mode, using FlexControl software with MBT Compass version 4.1 software (Bruker Daltonics) and a 60 Hz nitrogen laser (wavelength – 337 nm). Instrument was regularly calibrated using the bacterial test

standard (BTS, Bruker Daltonics) as recommended by the manufacturer. Mass spectra were analyzed in a mass-to-charge ratio (m/z) range of 2000 to 20,000. Each spectrum was interpreted using MALDI Biotyper automation control and the Bruker Biotyper 4.1 software and library (version rev. H/2021), with 10,834 entries (Bruker Daltonics).

2.2.2. Zymio system

Measurements were automatically performed with EXS2600 MALDI-TOF MS (Zymio Inc., Chongqing, China) in a positive linear mode, using Mass Spectrometry System software Ex-Accuspec version V1 and a 60 Hz nitrogen laser (wavelength – 337 nm). For instrument calibration, the Microbiology Calibrator (Zymio) was used. Mass spectra were analyzed in a mass-to-charge ratio (m/z) range of 2000 to 20,000. Each spectrum was interpreted using mentioned above software which contains library.

2.2.3. MALDI data interpretation

In the case of both platforms, the correctness of identification was determined based on the score value. In both cases, according to manufacturer, identification scores of <1.700 indicated no identification, scores of 1.700 to 1.999 indicated genus level identification, and scores of >2.000 indicated species level identification. The identification was considered accurate if the analysis performed by the Bruker system provided the same results as the analysis using the Zymio system. If systems provided different results, the consistency categories A, B, and C were used as additional identification indicators. Both software indicate the 10 best matching identification results. Bruker Biotyper automatically assigns the consistency categories to the first result based on subsequent results. Category A is species consistency, which means that the first and subsequent match results (score >2.00) indicate the same species and the next ones (score >1.70) indicate identifications of at least the same genus. In the case of category B – genus consistency, the first result is above >1.70 or >2.00, and subsequent matches have at least the same genus as the first one. Category C indicates no consistency of results neither between species nor genera. In the case of Zymio software, the manufacturer does not provide such information, so it was determined independently based on the above-mentioned method.

3. Results

The performance of Bruker Biotyper and Zymio EXS systems in identifying 1979 urine isolates with MALDI-TOF MS was similar, with both systems identifying a high percentage of isolates to at least the genus level. Bruker identified 95.6 % (1892/1979) of isolates, while Zymio identified 92.4 % (1829/1979). Fig. 1 illustrates the quality of the spectra identified by the MALDI systems, with Zymio identifying 83 % of the spectra down to the species level (score value >2.00) and Bruker Biotyper having a lower percentage of unidentified spectra (only 4 % with a score value <1.70).

Using this two MALDI systems, concordance in results was obtained for 1771/1979 (89.5 %) clinical isolates, either for genus or species level

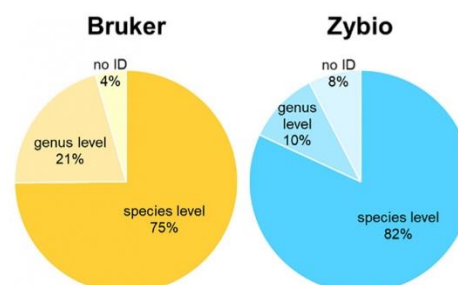


Fig. 1. Percentage of identification accuracy based on scores for the two tested MALDI systems.

Table 2
Performance of microbial identification via MALDI by Bruker versus Zybio.

Comparison of identification between Bruker and Zybio	
Agreement to the species level	1316/1979 (66.5 %)
Agreement to the genus level	455/1979 (23.0 %)
Identified only on one of the systems (to at least genus level)	169/1979 (8.5 %)
No identification	34/1979 (1.7 %)
Misidentification	5/1979 (0.3 %)

(Table 2). An additional 169 (8.5 %) samples were identified with one of the used instruments. Considering both systems 34 isolates were not identified at all despite registering the spectrum, and misidentification occurred only for five samples. Fig. 1 shows the percentage share of the quality of the spectra identified with the use of MALDI systems.

Among all the clinical strains included in this study, the MALDI technique identified (score value >2.00 and species consistency at least on one system) 4 fungal species belonging to *Candida* and *Aspergillus* genera and 123 bacterial species. Some isolates have only been correctly identified down to the genus level, however, all bacterial isolates belonged to 58 genera, 24 families, and 5 classes, most of which are known to cause human infections (particularly urinary tract infections). This shows the great diversity of genera and species that have been analyzed. Among the bacteria, 20 strains of gram-negative and 103 strains of gram-positive bacteria were identified. The majority of the identified isolates belonged to the Bacilli class (Fig. 2), comprising over

half of the total identified strains. Among them, strains belonging to the *Staphylococcus* family were most frequently identified, especially *S. epidermidis* (356 samples), *S. hominis* (245), and *S. haemolyticus* (199). Among the other classes (*Acinomycetia* and *Gammaproteobacteria*), the isolates of *Micrococcus luteus* (162) and *Enterococcus faecalis* (126) definitely prevailed, respectively.

Upon analyzing individual MALDI systems Bruker and Zybio identified a comparable number of species of microorganisms. Bruker identified a total of 104 different species of bacteria and one species of *Candida* (*C. albicans*) with consistent species identification. Meanwhile, Zybio identified 4 species of fungi and 103 species of bacteria, with a comparable level of species identification as Bruker. Among the 123 identified strains, 17 strains (categorized as A) were solely identified by the Bruker system, and 9 strains were solely identified by the Zybio system. Both systems identified 3 additional bacterial strains (*Streptococcus gallolyticus*, *Staphylococcus succinus* and *Brevibacterium luteolum*), however in both systems the identification result was < 2.00 (indicating consistency category B), therefore the identification was considered correct to the genus level. Moreover, for several other species, where the registered spectra found a match only in one of the systems, identification was only possible down to the genus level (8 species for Bruker and 5 for Zybio).

Table 3 presents a comparison of the identification of the tested isolates based on the identification result, which was divided into identified genera using both systems (details of specific species are

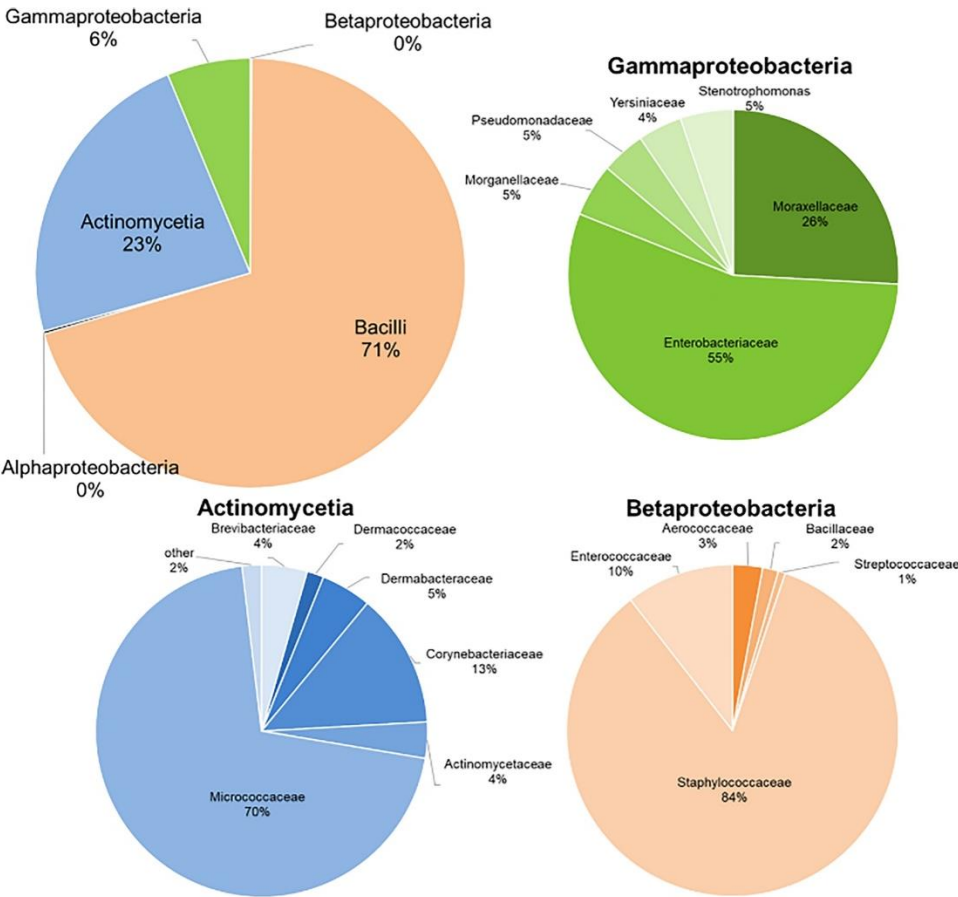


Fig. 2. Percentage of individual classes and families of bacterial strains identified by both MALDI systems.

Table 3

Comparison of the identification of the tested isolates based on the identification result using Bruker and Zybio systems. Only ID values above 1.70 were included in the average (and standard deviation).

Organisms genera (No. of isolates tested) MALDI system	Correct identification				Not reliable <1.70		Average score of ID	
	Species level >2.00		Genus level 1.70–1.99		Bruker	Zybio	Bruker	Zybio
	Bruker	Zybio	Bruker	Zybio				
Fungi (9)	6	9	2		1			
<i>Candida</i> (8)	6	8	2				2.02±0.14	2.21±0.05
<i>Aspergillus</i> (1)		1			1		–	2.11
Gram positive bacteria (1760)	1325	1448	385	199	50	113	2.12±0.17	2.20±0.17
<i>Brevibacterium</i> (19)	9	15		3	4	1	2.07±0.17	2.08±0.23
<i>Brevibacillus</i> (1)		1	1				1.81	2.36
<i>Dermacoccus</i> (6)	2		4			6	1.88±0.11	–
<i>Nesterenkonia</i> (1)	1	1					2.31	2.24
<i>Brachybacterium</i> (7)	3		4			7	1.93±0.12	–
<i>Dermabacter</i> (14)	11	4	1	6	2	4	2.16±0.11	1.96±0.20
<i>Corynebacterium</i> (55)	47	46	8	7		2	2.20±0.18	2.16±0.17
<i>Turicella</i> (1)		1	1				1.90	2.34
<i>Actinobaculum</i> (4)	4	3				1	2.45±0.06	2.49±0.04
<i>Actinomyces</i> (2)	2	2					2.27±0.17	2.45±0.07
<i>Actinotignum</i> (3)	3	3					2.12±0.10	2.34±0.08
<i>Actinomycetia</i>	6	4		2			2.26±0.11	2.08±0.19
<i>Arthrobacter</i> (1)	1					1	2.13	–
<i>Glutamicibacter</i> (2)	1		1			2	2.03±0.11	–
<i>Kocuria</i> (106)	59	73	35	24	12	9	2.06±0.17	2.13±0.22
<i>Micrococcus</i> (182)	166	146	15	29	1	7	2.23±0.15	2.22±0.18
<i>Pseudoglutamicibacter</i> (4)		4	4				1.85±0.05	2.19±0.03
<i>Rothia</i> (19)	11	14	8	2		3	2.11±0.23	2.11±0.14
<i>Schaalia</i> (2)		2	2				1.90±0.07	2.17±0.08
<i>Dietzia</i> (1)	1	1					2.33	2.28
<i>Microbacterium</i> (6)	2	4	4	2			1.87±0.08	1.88±0.11
<i>Pseudoclavibacter</i> (2)	2					2	2.20±0.15	–
<i>Bacilli</i>	17	22	8	2		1	2.10±0.17	2.22±0.17
<i>Aerococcus</i> (25)							2.28±0.14	2.25±0.18
<i>Facklamia</i> (13)	13	12		1			1.88±0.15	2.05±0.21
<i>Bacillus</i> (12)	2	8	8	4	2		1.73	–
<i>Cytobacillus</i> (1)				1	1		2.18±0.17	2.33±0.07
<i>Lactobacillus</i> (2)	2	2					2.25±0.06	2.40±0.06
<i>Leucobacter</i> (2)	2	2					–	1.78
<i>Lactococcus</i> (1)				1	1		2.24	2.00±0.41
<i>Leuconostoc</i> (2)	1	1		1	1		–	2.13±0.13
<i>Metabacillus</i> (2)		2			2		2.03	–
<i>Paenibacillus</i> (1)	1					1	2.32	2.16
<i>Pediococcus</i> (1)	1	1					2.23±0.18	2.25±0.25
<i>Streptococcus</i> (13)	11	9	2	4			1.90±0.12	–
<i>Nosocomiicoccus</i> (3)	1		2			3	2.09±0.15	2.18±0.16
<i>Staphylococcus</i> (1100)	809	929	267	108	24	63	2.29±0.12	2.37±0.11
<i>Enterococcus</i> (138)	134	136	4	2			2.21±0.20	2.30±0.15
Gram negative bacteria (123)	105	115	16	5	2	3	2.21±0.20	2.30±0.15
<i>Gammaproteobacteria</i>	15	19	4				2.20±0.18	2.32±0.11
<i>Acinetobacter</i> (19)	2	9	7	2	2		1.86±0.10	2.07±0.15
<i>Moraxella</i> (11)	1	1					2.28	2.39
<i>Psychrobacter</i> (1)	1	1					2.12	2.25
<i>Enterobacter</i> (1)							2.31±0.11	2.32±0.12
<i>Escherichia</i> (30)	30	28				2	2.24±0.16	2.30±0.13
<i>Klebsiella</i> (9)	8	9	1				2.29±0.16	2.40±0.06
<i>Proteus</i> (24)	24	24					2.45	2.45
<i>Morganella</i> (1)	1	1					2.36±0.12	2.42±0.02
<i>Providencia</i> (5)	5	5					2.15±0.20	2.16±0.28
<i>Pseudomonas</i> (6)	4	4	2	1		1	2.08±0.10	2.04±0.17
<i>Serratia</i> (5)	4	3	1	2			2.04±0.07	2.31±0.10
<i>Stenotrophomonas</i> (6)	5	6	1				2.41±0.01	2.33±0.11
<i>B*</i>	2	2					2.14±0.19	2.22±0.13
<i>A*</i>	2	2					2.35	2.41
<i>Roseomonas</i> (2)								
<i>Sphingomonas</i> (2)								
<i>Brevundimonas</i> (1)	1	1						

* A – Alphaproteobacteria, B – Betaproteobacteria

provided in supplementary Table S1). The table lists only those isolates that were accurately identified by both systems or for which one of the software allowed accurate identification to at least the genus level when the other did not. The average identification score value for the samples listed in this table was found to be higher for the Zybio system (2.20 ± 0.17) compared to the Bruker system (2.13 ± 0.17). Upon evaluation, both the Bruker and Zybio systems exhibited superior identification efficacy for gram-negative bacteria, yielding scores of 2.21 ± 0.20 and 2.30 ± 0.15 , respectively. Notably, in both analytical setups, the

maximal score values were attributed to samples identified as members of the *Enterobacteriaceae* family. Comparing the level of identification, only for the genus *Serratia* and *Roseomonas*, a higher score was obtained using the Bruker system. In other cases, the Zybio system indicated a higher identification accuracy.

Gram-positive bacteria isolates were mostly characterized by lower identification scores in comparison to gram-negative bacteria (average value for Bruker system was 2.12 ± 0.17 , and for Zybio was 2.20 ± 0.17). For 16 genera, the average identification scores were higher with

Zybio, and for 9 with Bruker. In addition, eight additional genera were identified only utilizing Bruker software. In the case of gram-positive bacteria, identification at the species level with high scores was most often observed for enterococci. What is worth emphasizing, in most cases, the differences in the score values did not significantly affect the identification result. Especially in the case of strains belonging to *Staphylococcus*, correct identification to species level was observed despite a score value below 2.00.

Table 4 presents a compilation of divergent identification results obtained from the tested MALDI systems. Overall, 53 discrepancies in results were observed, but the vast majority of them (48; 90.5 %) still enabled accurate identification of samples at the genus level. Specifically, both the Bruker and Zybio systems demonstrated accurate identification at the species level for these samples, albeit with differences in the species identified. The most frequent discrepancies in identification occurred within the following genera: *Staphylococcus*, *Streptococcus* and *Acinetobacter*. The remaining 5 samples were deemed as mis-identifications, as the two systems indicated different genera.

4. Discussion

The emergence of MALDI-TOF MS technology has expanded the capabilities for identifying microorganisms in clinical labs, replacing traditional biochemical testing with a proteomic approach. Many microbiology laboratories especially in developed countries have already adopted the new technology, which provides a simple, rapid, high throughput, and cost-effective identification method. The continuous advancements in MALDI-TOF MS technology have led to the availability of various advanced and user-friendly systems on the market, which offer quick and reliable identification of microorganisms in clinical laboratories.

In the current investigation, we embarked on a comprehensive comparison between two commercially available MALDI-TOF MS systems, with a specific focus on the identification of urinary isolates. During the assessment period, both the Bruker Microflex LT and Zybio EXS2600 systems were seamlessly integrated into our routine diagnostic workflow for an extended duration, ensuring that evaluations were carried out concurrently and without interruptions. Historically, the Bruker Biotyper pioneered the arena of MALDI-TOF MS platforms tailored for microbial identification [18]. Consequently, it has been extensively profiled and compared in the scientific literature against numerous other systems like the BioMérieux VITEK MS [6,7,10,19] and

the Shimadzu Axima [8,12,20], establishing a robust benchmark for performance standards in microbial identification. In contrast, the EXS2600 system from Zybio is relatively nascent in the market. Consequently, the available literature offering comparative analyses of its performance relative to other systems is somewhat sparse. This scarcity underscores the importance of the few available comparative studies. For instance, Li et al. ventured into an analytical comparison between the EXS3000 system and the VITEK MS [21], shedding light on its capabilities. Additionally, Dichtl et al. broadened the comparison landscape by including the Biotyper system in their analysis [22], thereby contributing valuable insights into the relative merits and limitations of each platform. Our present research not only stands as a pivotal comparison of these two distinct systems but also boasts the distinction of utilizing the most extensive collection of clinical samples for this purpose. This expansive dataset offers the potential for more robust and statistically significant conclusions, providing a clearer picture of the strengths and potential areas of improvement for each system.

The direct colony method with formic acid was chosen as the method for preparation of all samples. The direct on-spot method offers several advantages over the protein extraction method, including faster analysis time, reduced sample handling, and reduced risk of contamination. The choice between them depends on the laboratory's resources and needs, as well as the type of bacterial sample being analyzed, as the direct on-spot method may not be suitable for all types of samples and can be affected by factors such as the thickness and homogeneity of the sample spot [23,24]. We chose this method because it shortens the identification time, which is crucial for the analysis of clinical samples. Literature suggests that this method is sufficient to identify most of both gram-positive and gram-negative bacteria [25–28].

Our results indicate that both platforms successfully identified the vast majority of the tested isolates. Bruker correctly identified (at least to the genus level) 95.6 % of all isolates. In the case of Zybio, more unidentified spectra were observed, however, among the identified ones, Zybio showed a higher performance in the identification to the species level (83 % of isolates) compared to Bruker (75 %). This difference is mostly based on better identification rates within Gram-positive cocci e. g. various strains of *Staphylococcus* group (mainly *S. epidermidis*) were only identified to the genus level by Bruker Biotyper. A large part of these strains was correctly identified to the species (compared to the results of Zybio EXS2600), however, due to the score < 2.00, the results could be considered accurate only to the level of the genera. Similar findings were noted by Deak et al. [27] and Porte et al. [29]. For gram-positive bacteria, the most accurate identification at the species level on both systems was observed for *Enterococci* (above 97 % of isolates), the result of Veen et al. study on Bruker system [30], and Xiong et al. on Zybio EXS3000 system [16] also verifies this conclusion.

The applied direct extraction method revealed reliable results, mainly for the gram-negative bacteria, which was in accordance with the results of other studies [19,27]. The highest score values and the highest percentage of spectra identified to species were obtained for enterobacteria on both systems. Differences in the structure of gram-negative and gram-positive bacteria significantly affect the results of identification using the MALDI technique. The quality of mass spectra varies according to bacterial genera. The cell wall composition of gram-positive bacteria confers them increased resistance to lysis, which may lead to more frequent identification errors when using a MALDI database that was built with the use of a proteins extraction step [19, 25].

Although the study focuses on urine isolates, the results obtained cover the vast majority of clinically relevant bacteria. Together, the two systems accurate identified 127 different microorganisms, including 123 species of bacteria. Both systems identified a similar number of bacterial species, however, Zybio was much better at identifying fungal isolates (it correctly identified 4 fungi while Bruker only one). If a validation cut-off were to be lowered to at least 1.8 (currently 2.00), as suggested by some sources [31,32], the number of organisms correctly identified to the

Table 4
List of divergent identification results for the tested MALDI systems. Mis-identifications have been underlined.

Bruker		Zybio		No.
Identification result	Av. score value	Av. score value	Identification result	
<i>Acinetobacter</i>	2.41	2.04	<i>Acinetobacter junii</i>	1
<i>gyllenbergii</i>				
<i>Acinetobacter</i>	2.37	2.15	<i>Acinetobacter</i>	8
<i>gyllenbergii</i>			<i>haemolyticus</i>	
<i>Citrobacter nurlinii</i>	2.15	2.35	<i>Citrobacter freundii</i>	2
<i>Sphingomonas</i>	2.21	2.07	<i>Sphingomonas</i>	5
<i>yabuuchiae</i>			<i>paucimobilis</i>	
<i>Kocuria carniphila</i>	2.12	1.73	<i>Bacteroides caccae</i>	1
<i>Propionibacterium sp</i>	1.88	2.14	<i>Cutibacterium avidum</i>	2
<i>Streptococcus mitis</i>	2.31	2.27	<i>Streptococcus oralis/</i>	4
			<i>mitis</i>	
<i>Streptococcus urinalis</i>	2.39	2.01	<i>Streptococcus equi</i>	5
<i>Staphylococcus</i>	2.29	2.09	<i>Staphylococcus</i>	22
<i>borealis</i>			<i>haemolyticus</i>	
<i>Staphylococcus</i>	2.38	2.16	<i>Staphylococcus</i>	1
<i>borealis</i>			<i>hominis</i>	
<i>Staphylococcus capitis</i>	2.09	2.13	<i>Paenibacillus alvei</i>	1
<i>Staphylococcus</i>	1.82	2.01	<i>Meyerozyma</i>	1
<i>pasteuri</i>			<i>guilliermondii</i>	

species level would improve by several additional species. For the Bruker system, they would be by new 22 bacterial species, mainly belonging to *Staphylococcus*, *Corynebacterium*, or *Bacillus*, and two *Candida* species, while for Zybion by 14 bacterial species (similar to those mentioned above). The degree of similarity between the score values obtained from the two methods varied across different species, however, in most cases, higher score values were observed with the Zybion system. Identifying the cause of differences between MALDI identification systems is a challenging task due to the unique characteristics of each system, such as the mass spectrometer hardware, database construction, and comparison algorithms.

In our investigation, we closely scrutinized the underlying methodology employed by both systems in the creation of their respective databases. It is intriguing to note that the foundational processes of database creation in both the Bruker and Zybion systems are strikingly analogous, necessitating the accumulation of a minimum of 21 spectra. Such a methodological requirement is pivotal, as it establishes a robust framework that minimizes the possibility of erroneously classifying transient or incidental signals as being of primary significance. Further dissection of the manufacturer-specified parameters unveils nuanced differences in their approach to signal validation. In the context of the Bruker system, for a signal to attain significance and be assimilated into the database, it necessitates representation in at least 25 % of the acquired spectra. Extrapolating from this threshold, it is inferred that, on average, nearly 70 signals are consistently demarcated as paramount within the reference spectra. This, in essence, suggests a comprehensive signal portfolio within the Bruker database. On the other hand, the Zybion system adopts a more stringent criterion. A signal, to be incorporated into the Zybion database, demands presence in no less than 50 % of the harvested spectra. This heightened threshold, while ensuring signal consistency, consequently pares down the number of signals integrated into the database, making it comparatively leaner. The sheer number of significant signals nestled within a database inherently influences the resulting identification score values. This pivotal factor might be instrumental in elucidating the disparities we observed in our comparative analysis. It was discerned that the Bruker system, possibly owing to its richer array of significant signals, manifested a proclivity towards identifying a larger proportion of bacteria solely to the genus level. Moreover, it exhibited a reduced rate of complete non-identification when juxtaposed with the Zybion system. Such differential outcomes underscore the profound influence that database construction and signal criteria exert on microbial identification.

Moreover, in this study, only clinical isolates were analyzed, the Zybion database consists of the manufacturer's own strains, which are mostly clinical strains. This may also contribute to better accuracy of Zybion system. A large number of strains in the Bruker database belongs to the collection of reference strains, e.g. ATCC or DSMZ. Considering individual databases, some discrepancies in the identification between the systems can be explained. The most frequent discrepancies were *Staphylococcus borealis* and *Staphylococcus haemolyticus* and *Acinetobacter gyllenbergii* and *Acinetobacter haemolyticus*. Checking the databases showed that the *S. borealis* and *A. gyllenbergii* strains were present only in the Bruker library, which allows us to state that these are the correct identifications for these isolates.

Comparing the technical features of the mass spectrometers observed during analysis, both instruments have similar: easy-to-operate systems, time of loading the target plate, and time of achieving vacuum. Zybion showed a shorter analysis time for the whole plate (around 15 min per plate, Bruker about 20 min). Target plates dedicated to individual spectrometers are so compatible with each other that it is possible to use them in both devices. The quality control protocol for both devices included automated calibration steps using bacterial standards, in which the user is responsible for setting its frequency.

5. Conclusions

This study demonstrates the accuracy of two different MALDI-TOF MS systems, Bruker Microflex LT Biotyper and Zybion EXS2600 EX-Accuspec, in the identification of clinical strains (urine isolates). The vast majority of local isolates were correctly identified by both devices, although Zybion reached a higher diagnostic accuracy for species level identification, which mainly affected staphylococcus genera. Most likely, these differences reflected the stricter diagnostic criteria of the Bruker system. The use of protein extracts would allow to obtain higher values; however, the aim of these studies was to use the shortest possible protocols. This research shows that the development of databases in MALDI systems requires further development. In the future, a comparison of the utility of both systems in distinguishing closely related strains such as *Escherichia coli* and *Shigella* spp. or *Streptococcus salivarius* and *Streptococcus vestibularis* should be considered.

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Data availability statement

All data generated or analyzed in this study are included in this published article (and its Supplementary Information Files).

CRediT authorship contribution statement

Ewelina Sibińska: Conceptualization, Methodology, Formal analysis, Investigation, Software, Validation, Data curation, Writing – original draft. **Adrian Arendowski:** Conceptualization, Methodology, Investigation, Software, Supervision, Writing – original draft, Writing – review & editing. **Piotr Fijałkowski:** Formal analysis, Investigation, Methodology, Validation. **Dorota Gabrys:** Funding acquisition, Resources, Project administration. **Paweł Pomastowski:** Supervision, Resources, Writing – review & editing, Project administration.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2023.116150.

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Publikacja [P5]

Supplementary materials

Supplementary table S1. Comparison of the identification of the tested isolates based on the identification result (species level) using Bruker and Zybion systems. Only ID values above 1.70 were included in the average (and standard deviation).

Correct identification								
Organisms genera (No. of isolates tested)		species level >2.00		genus level 1.70 - 1.99		not reliable <1.70		average score of ID
MALDI system		Bruker	Zybio	Bruker	Zybio	Bruker	Zybio	
Fungi (9)		6	9	2		1		2.02±0.14 2.20±0.06
<i>Candida albicans</i> (6)		6	6					2.09±0.02 2.23±0.01
<i>Candida parapsilosis</i> (1)			1	1				1.80 2.07
<i>Candida tropicalis</i> (1)			1	1				1.81 2.11
<i>Aspergillus</i> (1)			1			1		- 2.11
Gram positive bacteria (1760)		1325	1448	385	199	50	113	2.12±0.17 2.20±0.17
Actinomycetia	<i>Brevibacterium casei</i> (6)	4	6	1		1		2.08±0.19 2.25±0.12
	<i>Brevibacterium celere</i> (5)	2	4	2	1	1		2.10±0.25 2.10±0.14
	<i>Brevibacterium luteolum</i> (1)			1	1			1.93 1.86
	<i>Brevibacterium paucivorans</i> (1)	1	1					2.04 2.11
	<i>Brevibacterium ptyocampae</i> (1)	1					1	2.16 1.48
	<i>Brevibacterium ravensturnense</i> (4)	1	3	1	1	2		2.04±0.18 2.08±0.03
	<i>Brevundimonas diminuta</i> (1)	1	1					2.35 2.41
	<i>Brevibacillus reuszeri</i> (1)		1	1				1.81 2.36
	<i>Dermacoccus nishinomiyaensis</i> (6)	2		4			6	1.88±0.11 -
	<i>Nesterenkonia lacusekhoensis</i> (1)	1	1					2.31 2.24
	<i>Brachybacterium paraconglomeratum</i> (6)	3		3			6	1.93±0.12 -
	<i>Brachybacterium conglomeratum</i> (1)			1			1	1.89 -
	<i>Dermabacter hominis</i> (14)	11	4	1	6	2	4	2.16±0.11 1.96±0.20
	<i>Corynebacterium amycolatum</i> (7)	6	7	1				2.22±0.18 2.19±0.07
	<i>Corynebacterium aurimucosum</i> (6)	5	6	1				2.07±0.19 2.26±0.11
	<i>Corynebacterium frankenforstense</i> (1)		1	1				1.85 2.11
	<i>Corynebacterium freneyi</i> (1)	1			1			2.25 1.80
	<i>Corynebacterium coyleae</i> (5)	5	4		1			2.28±0.10 2.07±0.13
	<i>Corynebacterium glucuronolyticum</i> (14)	14	12		2			2.32±0.11 2.27±0.20
	<i>Corynebacterium imitans</i> (4)	2	2	2	2			2.12±0.25 1.98±0.14
	<i>Corynebacterium jeikeium</i> (1)	1	1					2.08 2.26
	<i>Corynebacterium mucifaciens</i> (1)			1			1	1.70 1.60
	<i>Corynebacterium minutissimum</i> (1)	1	1					2.30 2.24
	<i>Corynebacterium tuberculostearicum</i> (13)	11	12	2	1			2.15±0.12 2.11±0.11
	<i>Corynebacterium variabile</i> (1)	1					1	2.36 -
	<i>Turicella otitidis</i> (1)		1	1				1.90 2.34
	<i>Actinobaculum massiliense</i> (4)	4	3				1	2.45±0.06 2.49±0.04
	<i>Actinomyces oris</i> (1)	1	1					2.15 2.40
	<i>Actinomyces urogenitalis</i> (1)	1	1					2.39 2.50
	<i>Actinotignum schaalii</i> (3)	3	3					2.12±0.10 2.34±0.08
	<i>Winkia neuui</i> (6)	6	4		2			2.26±0.11 2.08±0.19
	<i>Arthrobacter luteolus</i> (1)	1					1	2.13 -
	<i>Glutamicibacter protophormiae</i> (2)	1		1			2	2.03±0.11 -
	<i>Kocuria marina</i> (2)	1		1	2			1.99±0.10 1.83±0.01
	<i>Kocuria palustris</i> (6)	3		3			6	1.98±0.15 -
	<i>Kocuria kristinae</i> (4)	3	3		1	1		2.03±0.01 2.07±0.21
	<i>Kocuria rhizophila</i> (93)	51	68	31	23	11	3	1.99±0.25 2.13±0.24
	<i>Kocuria salsicia</i> (1)	1	1					2.28 2.11
	<i>Micrococcus endophyticus</i> (4)	2	2	2	2			1.99±0.15 2.04±0.17
	<i>Micrococcus flavus</i> (1)	1					1	2.28 -
	<i>Micrococcus luteus</i> (175)	161	143	13	27	1	5	2.23±0.16 2.20±0.22
	<i>Micrococcus lylae</i> (2)	2	1				1	2.35±0.01 1.88
	<i>Pseudoglutamicibacter cummingsii</i> (4)		4	4				1.85±0.05 2.19±0.03
	<i>Rothia aerea</i> (3)	3	3					2.42±0.08 2.27±0.11

Bacilli	<i>Rothia amarae</i> (5)	1	2	4	1	1	2	1.98±0.17	2.04±0.18
	<i>Rothia dentocariosa</i> (1)	1	1					2.18	2.25
	<i>Rothia endophytica</i> (1)	1	1					2.02	2.01
	<i>Rothia kristinae</i> (6)	2	6	4				1.94±0.15	2.09±0.05
	<i>Rothia terrae</i> (3)	3	1		1		1	2.34±0.06	2.07±0.21
	<i>Schaalia radingae</i> (2)		2	2				1.90±0.07	2.17±0.08
	<i>Dietzia papillomatosis</i> (1)	1	1					2.33	2.28
	<i>Microbacterium aurum</i> (4)	2	4	4	2			1.87±0.08	1.83±0.12
	<i>Microbacterium testaceum</i> (2)				2	2		-	1.93±0.05
	<i>Pseudoclavibacter alba</i> (2)	2					2	2.20±0.15	-
	<i>Aerococcus sanguinicola</i> (3)	3	2		1			2.38±0.03	2.10±0.30
	<i>Aerococcus urinae</i> (17)	11	15	6	1		1	2.07±0.16	2.25±0.16
	<i>Aerococcus viridans</i> (5)	3	5	2				2.03±0.07	2.18±0.09
	<i>Facklamia hominis</i> (13)	13	12		1			2.28±0.14	2.25±0.18
	<i>Bacillus pumilus</i> (7)	2	5	3	2	2		2.02±0.18	2.07±0.03
	<i>Bacillus sp.</i> (2)			2	2			1.73±0.02	1.77±0.06
	<i>Bacillus megaterium</i> (2)		2	2				1.80±0.06	2.17±0.01
	<i>Bacillus endophyticus</i> (1)		1	1				1.98	2.14
	<i>Cytobacillus sp.</i> (1)			1			1	1.73	-
	<i>Lactobacillus gasserii</i> (2)	2	2					2.18±0.17	2.33±0.07
	<i>Leucobacter alluvii</i> (2)	2	2					2.25±0.06	2.40±0.06
	<i>Lactococcus garvieae</i> (1)				1	1		-	1.78
	<i>Leuconostoc sp.</i> (1)				1	1		-	1.71
	<i>Leuconostoc mesenteroides</i> (1)	1	1					2.24	2.29
	<i>Metabacillus niabensis</i> (1)		1			1		-	2.22
	<i>Metabacillus halosaccharovorans</i> (1)		1			1		-	2.03
	<i>Paenibacillus timonensis</i> (1)	1					1	2.03	-
	<i>Pediococcus pentosaceus</i> (1)	1	1					2.32	2.16
	<i>Streptococcus agalactiae</i> (5)	4	4	1	1			2.37±0.18	2.44±0.03
	<i>Streptococcus anginosus</i> (3)	2	2	1	1			2.22±0.11	2.44±0.02
	<i>Streptococcus constellatus</i> (3)	3	2		1			2.20±0.11	2.29±0.06
	<i>Streptococcus gallolyticus</i> (1)	1			1			2.01	1.78
	<i>Streptococcus sanguinis</i> (1)	1	1					2.26	2.08
	<i>Nosocomiicoccus ampullae</i> (3)	1		2			3	1.90±0.12	-
	<i>Staphylococcus aureus</i> (21)	18	16	3	2		2	2.27±0.18	2.21±0.23
	<i>Staphylococcus auricularis</i> (2)	2	2					2.05±0.01	2.18±0.23
	<i>Staphylococcus capitis</i> (50)	44	46	5	4	1		2.16±0.14	2.16±0.14
	<i>Staphylococcus caprae</i> (3)	3	3					2.17±0.15	2.20±0.09
	<i>Staphylococcus cohnii</i> (21)	17	17	4	3		1	2.11±0.15	2.06±0.16
	<i>Staphylococcus epidermidis</i> (359)	255	295	104	27	1	37	2.06±0.13	2.20±0.13
	<i>Staphylococcus equorum</i> (9)	8	4	1	2	1	3	2.08±0.07	2.05±0.21
	<i>Staphylococcus haemolyticus</i> (199)	151	175	39	24	9		2.09±0.19	2.18±0.16
	<i>Staphylococcus hominis</i> (246)	182	233	60	13	4		2.08±0.16	2.21±0.12
	<i>Staphylococcus lugdunensis</i> (55)	45	46	9	3	1	6	2.15±0.18	2.20±0.14
	<i>Staphylococcus pasteurii</i> (19)	12	18	7	1			2.02±0.15	2.16±0.11
	<i>Staphylococcus petrasii</i> (16)	14	4	2	7		5	2.11±0.09	1.88±0.20
	<i>Staphylococcus pettenkoferi</i> (14)	6	14	6		2		1.94±0.13	2.14±0.10
	<i>Staphylococcus pseudintermedius</i> (1)		1			1		-	2.06
	<i>Staphylococcus saprophyticus</i> (24)	22	17	1	7	1		2.11±0.16	2.05±0.19
	<i>Staphylococcus sciuri</i> (5)	3		2	3		2	2.02±0.08	1.87±0.02
	<i>Staphylococcus simulans</i> (6)	4	5	2			1	2.05±0.06	2.15±0.05
	<i>Staphylococcus sp.</i> (5)			4	2	1	3	1.86±0.04	1.81±0.02
	<i>Staphylococcus warneri</i> (41)	21	32	19	6	1	3	1.99±0.20	2.16±0.19
	<i>Staphylococcus xylosus</i> (4)	2	1	1	3	1		1.97±0.28	1.88±0.12
	<i>Enterococcus avium</i> (4)		4	1				2.15±0.14	2.17±0.07
	<i>Enterococcus faecalis</i> (126)	123	125	3	1			2.30±0.12	2.38±0.09
	<i>Enterococcus faecium</i> (6)	6	6					2.31±0.08	2.31±0.09
	<i>Enterococcus gallinarum</i> (1)	1	1					2.36	2.45
	<i>Enterococcus gilvus</i> (1)	1			1			2.18	1.75
Gram negative bacteria (123)		105	115	16	5	2	3	2.21±0.20	2.30±0.15
Gammaproteo bacteria	<i>Acinetobacter</i> (19)	15	19	4				2.20±0.18	2.32±0.11
	<i>Acinetobacter johnsonii</i> (6)	6	6					2.35±0.05	2.42±0.06
	<i>Acinetobacter lwoffii</i> (10)	7	10	3				2.09±0.13	2.29±0.07
	<i>Acinetobacter pseudolwoffii</i> (3)	2	3	1				2.29±0.27	2.21±0.19
	<i>Moraxella osloensis</i> (11)	2	9	7	2	2		1.86±0.10	2.07±0.15
	<i>Psychrobacter sanguinis</i> (1)	1	1					2.28	2.39

A*	<i>Enterobacter cloacae</i> (1)	1	1				2.12	2.25
	<i>Escherichia coli</i> (30)	30	28			2	2.31±0.11	2.32±0.12
	<i>Klebsiella oxytoca</i> (5)	4	5	1			2.25±0.19	2.27±0.17
	<i>Klebsiella pneumoniae</i> (4)	4	4				2.23±0.13	2.35±0.06
	<i>Proteus mirabilis</i> (24)	24	24				2.29±0.16	2.40±0.06
	<i>Morganella morganii</i> (1)	1	1				2.45	2.45
	<i>Providencia rettgeri</i> (5)	5	5				2.36±0.12	2.42±0.02
	<i>Pseudomonas aeruginosa</i> (1)	1	1				2.34	2.23
	<i>Pseudomonas oryzae</i> (3)	2	2	1	1		2.16±0.01	1.99±0.34
	<i>Pseudomonas monteilii</i> (1)	1	1				2.26	2.41
	<i>Pseudomonas sp.</i> (1)			1		1	1.82	
	<i>Serratia marcescens</i> (5)	4	3	1	2		2.08±0.10	2.04±0.17
	<i>Stenotrophomonas maltophilia</i> (6)	5	6	1			2.04±0.07	2.31±0.10
	<i>Roseomonas mucosa</i> (2)	2	2				2.41±0.01	2.33±0.11
	<i>Sphingomonas paucimobilis</i> (2)	2	2				2.14±0.19	2.22±0.13
	<i>Brevundimonas</i> (1)	1	1				2.35	2.41

* A – Alphaproteobacteria, B - Betaproteobacteria

Publikacja [P6]

**Multi-instrumental analysis toward exploring the diabetic foot infection
microbiota**

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Multi-Instrumental Analysis Toward Exploring the Diabetic Foot Infection Microbiota

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Abstract

The polymicrobial nature of diabetic foot infection (DFI) makes accurate identification of the DFI microbiota, including rapid detection of drug resistance, challenging. Therefore, the main objective of this study was to apply matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) technique accompanied by multiply culture conditions to determine the microbial patterns of DFIs, as well as to assess the occurrence of drug resistance among Gram-negative bacterial isolates considered a significant cause of the multidrug resistance spread. Furthermore, the results were compared with those obtained using molecular techniques (16S rDNA sequencing, multiplex PCR targeting drug resistance genes) and conventional antibiotic resistance detection methods (Etest strips). The applied MALDI-based method revealed that, by far, most of the infections were polymicrobial (97%) and involved many Gram-positive and -negative bacterial species—19 genera and 16 families in total, mostly *Enterobacteriaceae* (24.3%), *Staphylococcaceae* (20.7%), and *Enterococcaceae* (19.8%). MALDI drug-resistance assay was characterized by higher rate of extended-spectrum beta-lactamases (ESBLs) and carbapenemases producers compared to the reference methods (respectively 31% and 10% compared to 21% and 2%) and revealed that both the incidence of drug resistance and the species composition of DFI were dependent on the antibiotic therapy used. MALDI approach included antibiotic resistance assay and multiply culture conditions provides microbial identification at the level of DNA sequencing, allow isolation of both common (eg. *Enterococcus faecalis*) and rare (such as *Myroides odoratimimus*) bacterial species, and is effective in detecting antibiotic-resistance, especially those of particular interest—ESBLs and carbapenemases.

Introduction

Chronic wounds are one of the most devastating impairments related to diabetes among which diabetic foot infection (DFI) represents the most frequent and serious disorder [1]. Considering the growing number of diabetic (ca. 420 million patients so far) DFI is currently considered

as a predominant trigger for lower extremity amputations worldwide [2]. Accurate deciphering of the infection causative agent determines taking effective treatment, however, the reliable identification of the DFI microbial patterns in diabetic patients is still challenging due to usually the polymicrobial nature of the infection [3]. Moreover, there is a growing need for reports on the DFI microbial compositions in specific geographical regions to provide local treatment guidelines [4].

Up to date, the DFI diagnosis mostly relies on the traditional culture method and phenotypic identification of the grown colonies or the application of molecular techniques, such as the 16S rDNA PCR amplification and sequencing [5]. While the former is time-consuming and presents limited identification accuracy, the latter is characterized by high sensitivity, discriminatory power, and allows skip the culturing step, however, it requires a well-equipped laboratory and highly qualified staff, which increases the costs of analysis [6]. More recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI

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TOF MS) seems to be a good solution for mentioned limitations, since fast and cost-effective bacterial identification by MALDI TOF MS along with the multiplication of culture conditions provides high identification accuracy in relatively short time-to-results and opens the possibility for further investigation of biological features including antimicrobial susceptibility of isolates [7]. It was proved in the work Złoch et al. [8] where the accuracy of the reflected microbial patterns of the swab samples derived from DFI patients via MALDI technique significantly improved when the multiply culture conditions was applied.

Although clinicians should avoid antibiotic therapy that is unnecessary, nevertheless, successful treatment of DFIs calls for the administration of appropriate antibiotics [9]. Facing an increased rate of isolation of antibiotic-resistant pathogens, which can be observed in the past few decades, the selection of the effective antimicrobial therapy of DFI becomes more challenging than ever before [2]. In particular, this applies to the constantly growing number of multidrug-resistant (MDR) Gram-negative species such as extended-spectrum beta-lactamase (ESBL), carbapenemase-producing *Enterobacterales*, or MDR *Pseudomonas aeruginosa* [9]. The frequency of occurrence of MDR Gram-negative pathogens in different geographical area and treatment centers is widely variable, however, current data showing that besides developing countries, this problem is increasingly affecting European and other developed countries [2]. In view of this, reliable and fast detection of antibiotic-resistant Gram-negative pathogens becomes crucial for preventing the failure of the DFI treatment and further spreading of MDR [10].

Many different methods of detecting antibiotic resistance/susceptibility have found application in routine clinical practice. The most commonly used techniques are phenotypic tests such as disc diffusion or combined disc inhibitory tests, gradient minimal inhibitory concentration (MIC) strips (Etest), the Carba NP tests or most recently the modified carbapenem inactivation method (mCIM) [11, 12]. Despite their low costs and simplicity, their major drawbacks are the need for additional incubation which extends time-to-result and relative low specificity and selectivity [13]. Application of molecular methods such as targeted PCR assays including multiplex ones is more specific and selective as well as enables receive results faster (even < 4 h) and detect several different resistance genes in a single run [14, 15]. However, the use of the molecular approach is very often limited, especially in developing countries, due to high costs of the analysis, need for highly trained staff, or access to commercial databases and dedicated equipment [6].

In the last few years, special attention has been paid to the utilization of rapid biochemical assays based on antibiotic hydrolyzing activity detection, which opens the possibility of obtaining accurate and reliable results in a simple, fast, and cheap way [16]. In light of this, the MALDI TOF MS

technique turned out to be the most promising tool facilitating the indication of a wide range of β -lactamases including clinically relevant cephalosporinases and carbapenemases [17]. Several publications have demonstrated the feasibility of the MALDI TOF MS technique [18–20]. Moreover, in the recent work Złoch et al. [21] authors pointed out that the MALDI could be also used to partially classify the class of the carbapenemase present in the sample or as a fast surrogate of standard MIC assay in case of metallo- β -lactamases producing strains (MBL).

The main goal of this study was to apply the MALDI microbial identification technique with the previously established multiple culture conditions to decipher microbial patterns of the swab samples collected from DFI patients treated in the Provincial Polyclinical Hospital in Toruń, (Poland). The accuracy of the MALDI TOF MS identification was evaluated by referring to the sequencing results of the 16S rDNA region. Moreover, as Gram-negative bacteria are considered the primary cause of the multidrug resistance spreading among DFI-suffering patients, the analysis of the frequency of the occurring resistance against different classes of beta-lactams, including cephalosporins and carbapenems using different approaches (MBT STAR BL assay, Etest, multiplex PCR) was performed for the sake of choosing the most accurate one.

Materials and Methods

Clinical Samples

During studies analyzed clinical specimens derived from 31 patients (42–85 yrs, 25 males, 6 females, Supplementary Table S1) of the Provincial Polyclinical Hospital in Toruń (Poland) who suffered from diabetic foot infections. The superficial samples of wounds were collected using flocked swab (ESwab Collection System, Copan) after wound debridement according to the local guidelines by a specialist nurse applying the Levine technique. The samples were immediately placed into a liquid transport medium (Amies δ swab, Deltalab, Rubi Barcelona, Spain) and transported to *Centre for Modern Interdisciplinary Technologies (Nicolaus Copernicus University in Toruń)*, where they were stored at -80°C .

Bacteria Isolation and Culturing Technique

For bacteria isolation, serial dilution method (10^{-1} – 10^{-3}) in sterile peptone water (Sigma Aldrich, Steinheim, Germany) was applied. After defrosting, samples were thoroughly vortexed and then 0.5 mL was transferred into the test tube containing 4.5 mL of sterile peptone water (Sigma Aldrich, Germany) and again vortexed (first dilution— 10^{-1}). 100 μL

of each dilution was plated onto 5 different culture media previously selected as the most useful in DFI bacteria recovery [8]: Tryptic Soy Agar (TSA; Sigma Aldrich, Steinheim, Germany), Columbia Blood Agar (BLA; Oxoid, Basingstoke, Great Britain), CHROMagar Orientation (CHRA; GRASO Biotech, Starogard Gdański, Poland), Glucose Bromocresol Purple Agar (BCP; Sigma Aldrich, Steinheim, Germany), and Vancomycin Resistant Enterococci Agar (VRE; Oxoid, Basingstoke, Great Britain). All media were in the form of ready-to-use powders except for BLA, which was prepared by adding defibrinated sheep blood (GRASO Biotech, Starogard Gdański, Poland) to the sterilized and dissolved Colombia blood agar base to the final concentration 5% (v/v). Bacterial cultures were incubated at 37 °C for 24 h and then single colonies characterized by different morphological features were selected to obtain pure cultures using the streak plate method on the same media (incubation at 37 °C for 18–24 h).

Identification of Bacterial Isolates Using MALDI TOF MS Technique

For bacteria identification used protein extracts obtained applying formic acid/acetonitrile method according to the MALDI Biotyper protocol (Bruker Daltonik GmbH, Bremen, Germany). 1 microbial loop (10 µl) of fresh biomass was suspended in 300 µl of sterile deionized water, mixed, and then viable bacterial cells were inactivated by adding 900 µL of 96% ethyl alcohol. After vortexing, the resulted bacterial suspension was centrifuged (1300 rev/min, 5 min), the supernatant was discarded and the remaining cell pellet was dried using a vacuum centrifuge at room temperature. Consequently, to the cell pellet 10 µl formic acid (FA) and 10 µl acetonitrile (ACN) was added and mixed. The obtained extract was centrifuged (13,000 rev/min, 5 min.) and 1 µl of supernatant was transferred onto a MALDI MTP 384 ground steel target sample spot (Bruker Daltonik GmbH, Germany). After air-drying, the sample spot was overlaid with 1 µl of MALDI matrix solution—10 mg/mL α -cyano-4-hydroxycinnamic acid (HCCA; Sigma Aldrich, Switzerland) solution prepared in standard solvent solution (50% ACN, 47.5% water and 2.5% trifluoroacetic acid).

Bacterial protein extracts were analyzed using an ultrafleXtreme MALDI TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with the smartbeam-II laser—positive mode. Spectra were collected manually using manufacturer software, flexControl (parameters: 500 shots in-one-single spectra to frequency 2500, m/z range = 2000–20,000, acceleration voltage = 25 kV, global attenuator offset = 20% and attenuator offset = 34% and its range = 34%, laser power = 40%), and subjected to smoothing using the Savitsky-Golay method (width 2 m/z , cycles 10) and baseline corrections using the TopHat algorithm

(signal to noise threshold 2; peak detection algorithm—centroid) followed by calibration using the Bruker's Bacterial Test Standard (BTS; Bruker Daltonik GmbH, Bremen, Germany) in quadratic mode via manufacturer software, flexAnalysis. Each sample was measured in quadruplicate (two spots per samples measured in twice). Validated mass spectra were used for bacterial identification via MALDI Biotyper 3.0 Platform (Bruker Daltonik GmbH, Bremen, Germany) based on the both raw spectra (RAW) and Main Spectra (MSP).

Identification of Bacterial Isolates Using 16S rDNA Sequencing

First of all, the total bacterial genomic DNA were obtained using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Norcross, US) from overnight bacterial cultures (37 °C) following extraction protocol with Glass Beads S supplied by the manufacturer. The extracted bacterial DNA were used for amplification of the 16S rDNA region via PCR technique using universal bacterial primers 27F (5-AGAGTTTGATC-MTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACG ACTT-3), thermostable *Taq* DNA polymerase (Qiagen, Hilden, Germany), Mastercycler pro S thermocycler (Eppendorf AG, Hamburg, Germany), and PCR program established in the earlier work [22]. The efficiency of the obtained PCR products and purity were studied quantitatively by spectrophotometry as well as gel electrophoresis in 1% agarose. Subsequently, PCR products were send to Genomed (Warsaw, Poland) and were sequenced via the Sanger dideoxy method using the same primers, contigs were assembled via BioEdit Sequences Alignment Editor ver. 7.2.5 [23], and consensus sequences were compared with references sequences in rRNA/ITS databases of the National Center for Biotechnology Information via the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). The DNA sequences determined in this study were submitted to GenBank, and accession numbers are given in the Table S1. The evolutionary tree of identified bacterial strain was inferred based on the Neighbor-Joining and Maximum Composite Likelihood method using MEGA7 software [24] and was visualized using Interactive Tree of Life (iTOL) v 6.5.4 [25].

Determination of the Antibiotics Resistance Among Gram-Negative Strains Using Etest Method

Investigated Gram-negative strains were incubated on MHA medium (Mueller Hinton Agar; Sigma Aldrich, Steinheim, Germany) for 24 h at 37 °C. After incubation, bacterial suspensions at a density of 0.5 McFarland in saline (0.85%) were prepared. The suspension was applied to the plate with MHA medium using a swab soaked in it. One swab

was used per plate. Then strips containing different antibiotic gradient (Etest®, Biomerieux) were applied: (1) ESBL CT/CTL 16/1—cefotaxime (0.25–16 µg/mL)/cefotaxime (0.016–1 µg/mL) + clavulanic acid (4 µg/mL); (2) ESBL TZ/TZL 32/4—ceftazidime (0.5–32 µg/mL)/ceftazidime (0.064–4 µg/mL) + clavulanic acid (4 µg/mL); (3) ESBL PM/PML 16/4—cefepime (0.25–16 µg/mL)/cefepime (0.064–4 µg/mL) + clavulanic acid (4 µg/mL); (4) ceftriaxone TX 32 (0.002–32 µg/mL); (5) MBL IP/IPI 256/64—imipenem (4–256 µg/mL)/imipenem (1–64 µg/mL) + EDTA; (6) ampicillin AM (0.016–256 µg/mL) as well as (7) piperacillin PP (0.016–256 µg/mL). The MIC values were read from the zone of inhibition of bacterial growth after 20–24 h of incubation at 37 °C.

Detection of Antibiotic Resistance Among Gram-Negative Isolates Using MALDI TOF MS Technique

Detection of β -lactamase activity against different beta-lactam antibiotics—cephalosporins (cefotaxime, ceftriaxone, ceftazidime), carbapenems (imipenem, meropenem) as well as penicillins (ampicillin, piperacillin) via MALDI TOF MS technique was performed using MBT STAR-BL method according to guidelines provided by manufacturer protocol (Bruker Daltonik GmbH, Bremen, Germany). One inoculation loop (1 µL) containing sufficient amount of bacterial cells (1 to 5 colonies) was suspended in the antibiotic solution: (1) cefotaxime (CTX, final conc. 0.5 mg/mL), (2) ceftriaxone (CRO, 0.5 mg/mL), (3) ceftazidime (CAZ, 0.5 mg/mL), (4) imipenem (IMI, 0.5 mg/mL), (5) meropenem (MER, 1.0 mg/mL), (6) ampicillin (AMP, 3.0 mg/mL), and (7) piperacillin (PIP, 2.0 mg/mL) dissolved in 50 µL of MBT STAR Buffer. Prepared mixtures were subjected to incubation at 35 ± 2 °C for 30 (CRO, IMI), 120 (CTX, MER, AMP), 180 (PIP) or 360 (CAZ) minutes according to manufacturer guidelines with constant shaking (900 rpm) using Thermomixer (Eppendorf AG, Germany). After incubation samples were centrifuged (2 min., 13,000 rpm) and 1 µL of supernatants were deposited onto the MALDI MTP 384 ground steel target (Bruker Daltonik GmbH, Bremen, Germany) in duplicate, air dried, and subsequently overlaid with 1 µL of MBT STAR Matrix. For each targets and runs prepared one spot with 1 µL of MBT STAR-ACS (antibiotic calibration mass standard containing dedicated masses below $m/z = 1000$) as well as both negative (*Escherichia coli* ATCC 25922) and positive (*Klebsiella pneumoniae* ATCC BAA-1705) controls in duplicate. Target plates were analyzed using ultrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with the smartbeam-II laser—positive mode. The spectra were collected automatically via AutoXecute mode and flexControl software (Bruker Daltonik GmbH, Bremen,

Germany) as well as calibrated using quadratic calibration method using flexAnalysis software (Bruker Daltonik GmbH, Bremen, Germany). Finally, 4 spectra for each sample were obtained. Collected spectra were analyzed by means of MBT Biotyper Prototype software and results were expressed as normalized logRQ values calculated from division signal intensity of hydrolyzed forms of antibiotic by those derived from non-hydrolyzed. For normalization of the results used the respective negative and positive controls. Higher logRQ means higher antibiotic hydrolysis. Normalized logRQ ≤ 0.2 —negative results, ≥ 0.4 —positive results, values between thresholds—unclear hydrolyzation.

Detection of Antibiotic Resistance Genes Among Gram-Negative Isolates Using Multiplex PCR Technique

For detection of genes encoding different types of β -lactamases—ESBLs (TEM, SHV, CTX-M, OXA-1), carbapenemases (KPC, GES, OXA-48, GIM, NDM, VIM, IMP), and extended-spectrum AmpC (CMY-1, CMY-2)—used the same total bacterial genomic DNA isolated for 16S rDNA sequencing. Primer sets used for amplification of individual genes are listed in the Table 1. Amplification performed using thermostable *Taq* DNA polymerase (Qiagen, Hilden, Germany) and Mastercycler pro S thermocycler (Eppendorf AG, Hamburg, Germany). PCR conditions for individual set of primers selected according to references provided in the Supplementary Table S2. Products of amplification were analyzed using electrophoresis in a 1.5% agarose gel containing 0.05 mg/L ethidium bromide at 90 V for 1 h in $1 \times$ TAE buffer (TAE buffer (50x) Molecular biology grad, AppliChem GmbH, Darmstadt, Germany). For determination of product size used Perfect™ 100 bp DNA ladder (100–2500 bp, EURx, Gdańsk, Poland) as well as DNA ladder MR19 (750–3500 bp, DNA Gdańsk, Blirt S.A., Gdańsk, Poland) (Table 2).

Results

Microbial Pattern of the DFI Samples Obtained via MALDI TOF MS

Applied MALDI protocol revealed that far most of the DFI patients suffered from polymicrobial infections—97% (Fig. 1a). Moreover, half of the infected wounds contained four or more different microbial species simultaneously – 52%. Over two-thirds of the wound samples were occupied by both Gram-positive and -negative species (71%), while another 26% by only Gram-positive (Fig. 1b). In two cases (3%) only Gram-negative species have been found.

Table 1 List of primers used for amplification of individual genes encoding β -lactamases in single or multiplex PCR reaction

Set	β -lactamase	Primers	Sequence [5'→3']	Product size [bp]	Ref.
1	TEM	MultiTSO-T.for	CATTTCCGTGTCGCCCTTATTC	800	[14]
		MultiTSO-T.rev	CGTTCATCCATAGTTGCCTGAC		
	SHV	MultiTSO-S.for	AGCCGCTTGAGCAAATTAAC	713	
		MultiTSO-S.rev	ATCCCGCAGATAAATCACCAC		
	OXA-1	MultiTSO-O.for	GGCACCAGATTCAACTTTCAAG	564	
2	CTX-M-1	MultiTSO-O.rev	GACCCCAAGTTTCCTGTAAGTG		[15]
		MultiCTXMGp1.for	TTAGGAATGTGCGCGCTGyA	688	
	CTX-M-9	MultiCTXMGp1-2.rev	CGATATCGTTGGTGGTCCAT		
		MultiCTXMGp9.for	TCAAGCCTGCCGATCTGGT	561	
		MultiCTXMGp9.rev	TGATTCTCGCCGCTGAAG		
3	NDM	NDM-1-for	GGTTTGGCGATCTGGTTTTC	621	[15]
		NDM-1-rev	CGGAATGGCTCATCACGATC		
4	IMP	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	
		IMP-R	GGTTTAAAYAAAACAACCACC		
	VIM	VIM-F	GATGGTGTTCGGTCGCATA	390	
		VIM-R	CGAATGCGCAGCACCAG		
5	GIM	GIM-F	TCGACACACCTTGGTCTGAA	477	
		GIM-R	AACTTCCAACCTTTGCCATGC		
6	OXA-48	OXA-F	GCGTGGTTAAGGATGAACAC	438	
		OXA-R	CATCAAGTTCAACCCAACCG		
	KPC	KPC-Fm	CGTCTAGTTCTGCTGTCTTG	798	
		KPC-Rm	CTTGTCATCCTTGTTAGGCG		
7	GES	GES_For	CTGGCAGGGATCGCTCACTC	600	[26]
		GES_Rev	TTCCGATCAGCCACCTCTCA		
8	CMY-1	CMY-1_For	ATGCAACAACGACAATCCATCCTG	1560	[27]
		CMY-1_Rev	TCAACCGGCCAACTGCGCCAGGAT		
	CMY-2	CMY-2_For	ATGATGAAAAAATCGTTATGCT	3202	
		CMY-2_Rev	TTATTGCAGCTTTTCAAGAATGCG		

The result of the isolation step obtained 111 different bacterial isolates and two *Candida* species—*C. albicans* and *C. krusei* (patient DFI-22; Supplementary Table S1). Based on the MALDI identification, bacterial isolates represented 19 genera belonging to 16 different families—8 G(−) and 7 G(+) (Fig. 2a). Among the most abundant groups that comprise almost two-thirds of all identified bacteria were *Enterobacteriaceae* (24.3%), *Staphylococcaceae* (20.7%), and *Enterococcaceae* (19.8%), represented mainly by *Enterococcus faecalis* (19.8%), *Escherichia coli* (10.8%), and *Staphylococcus aureus* (9.0%). Other frequently isolated species were *Proteus mirabilis* (5.4%), *Pseudomonas aeruginosa* (3.6%) as well as *Morganella morganii*, *Enterobacter cloacae*, *Streptococcus agalactiae*, and *Corynebacterium striatum* – each 4.5%.

16S rDNA Sequencing Results

We performed sequencing of the 16S rDNA region to evaluate the correctness of the MALDI identifications. In

the results, 99 bacterial species have been identified—93% with species confidence (Supplementary Table S1). For 12 isolates, we do not get sequencing results due to strain loss during passaging (8 isolates) or the lack of a specific PCR product—the case of all *P. aeruginosa* strains. In the case of two isolates – *Klebsiella oxytoca* DFI-13 and *Citrobacter freundii* DFI-21—the analysis of the 16S rDNA region did not allow for a reliable determination of the species. In 7 cases, discrepancies between MALDI and 16S rDNA sequencing results have been noted—all of these concerned closely related species: *E. coli*/*S. dysenteriae*, *E. cloacae/hormaechei*, *C. freundii/braakii*, *E. cloacae/P. agglomerans*, *P. vulgaris/terrae*, and *Acinetobacter haemolyticus/gyllenbergii*. All in all, obtained MALDI identification was characterized by 98% genus and 93% species confidence, referring to the DNA sequencing results. Phylogenetic relationship of the identified bacterial isolates is presented on the Fig. 2b.

Table 2 List of the DFI patients subjected to the studies

Patient	Gender	Age	Antibiotic therapy	Description
DFI-1	M	80	C	progressive necrosis of the right foot
DFI-2	F	63	C	necrosis of podfoot
DFI-3	M	69	P	phlegmon with gas-forming bacteria
DFI-4	F	83	C	right foot necrosis
DFI-5	F	83	C	right foot necrosis
DFI-6	M	72	Ci	right foot necrosis
DFI-7	M	55	C + L	phlegmon of the right foot
DFI-8	M	71	No	right foot necrosis
DFI-9	F	66	V + P	progressive metatarsal necrosis
DFI-10	M	61	Ci + P	finger amputation
DFI-11	M	56	No	phlegmon of the left leg
DFI-12	M	63	No	deep ulceration of the left heel with necrotic symptoms
DFI-13	M	81	C	dry necrosis of the toes of the left foot
DFI-14	F	64	C	purulent necrosis of the right stump
DFI-15	M	70	A	right foot necrosis
DFI-16	M	63	No	right foot phlegmon
DFI-17	F	85	Ci	extensive area of diffuse skin necrosis
DFI-18	M	73	A	dry toe necrosis of the right foot
DFI-19	M	52	C	right foot phlegmon
DFI-20	M	74	No	toe necrosis of the right foot
DFI-21	M	68	No	purulent necrosis of the left stump
DFI-22	M	63	Ci + C	right foot phlegmon
DFI-23	M	62	A	right foot phlegmon
DFI-24	M	62	No	toe necrosis of the left foot
DFI-25	M	62	C	deep phlegmon of the soft tissue of the sole of the left foot
DFI-26	M	62	No	toe and metatarsal bones necrosis of the left foot
DFI-27	M	54	No	toe necrosis of the right foot
DFI-28	M	66	C + A	toe necrosis of the right foot
DFI-29	M	42	No	left foot phlegmon
DFI-30	M	59	C	left foot phlegmon
DFI-31	M	45	A	right foot phlegmon

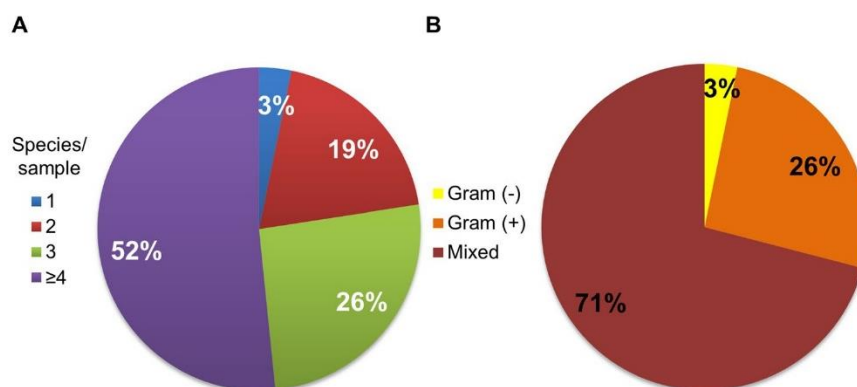
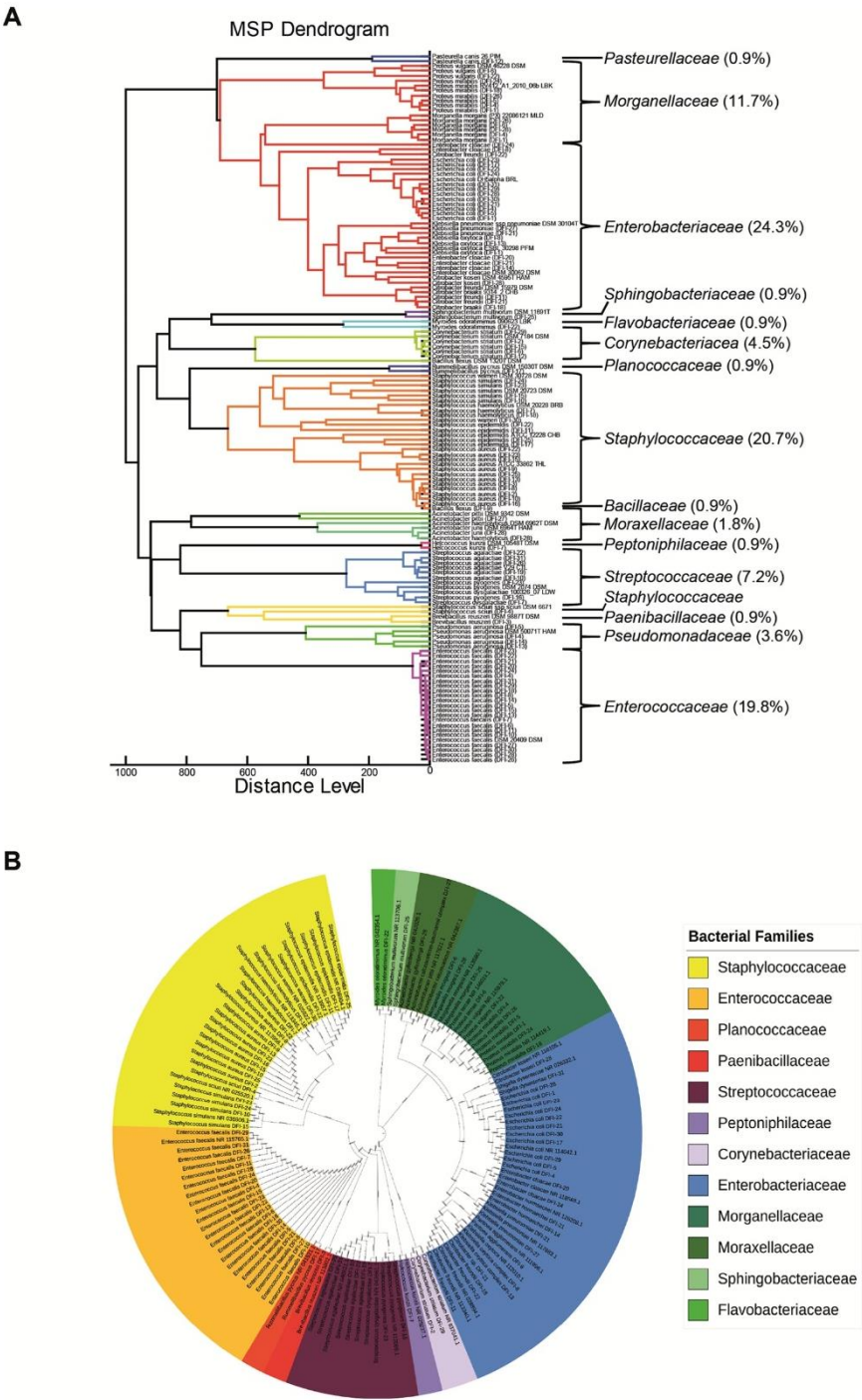
Fig. 1 Pie charts presented frequency of the mono- and polymicrobial infections (a) as well as type of the bacteria (b) within samples collected from DFI patients

Fig. 2 The results of the DFI microbiota deciphering using (a) MALDI TOF MS identification and (b) 16S rDNA sequencing technique



Antibiotic Resistance Occurrence within Gram-Negative Isolates

Applying both MBT STAR BL assay and Etest strips enabled the detection of different types of antibiotic resistance

among Gram-negative isolates examined. The examples of the Etest results and corresponding MALDI ones are presented in Fig. 3.

Regarding MALDI results, 16 out of 21 samples from DFI patients were occupied by Gram-negative bacteria that

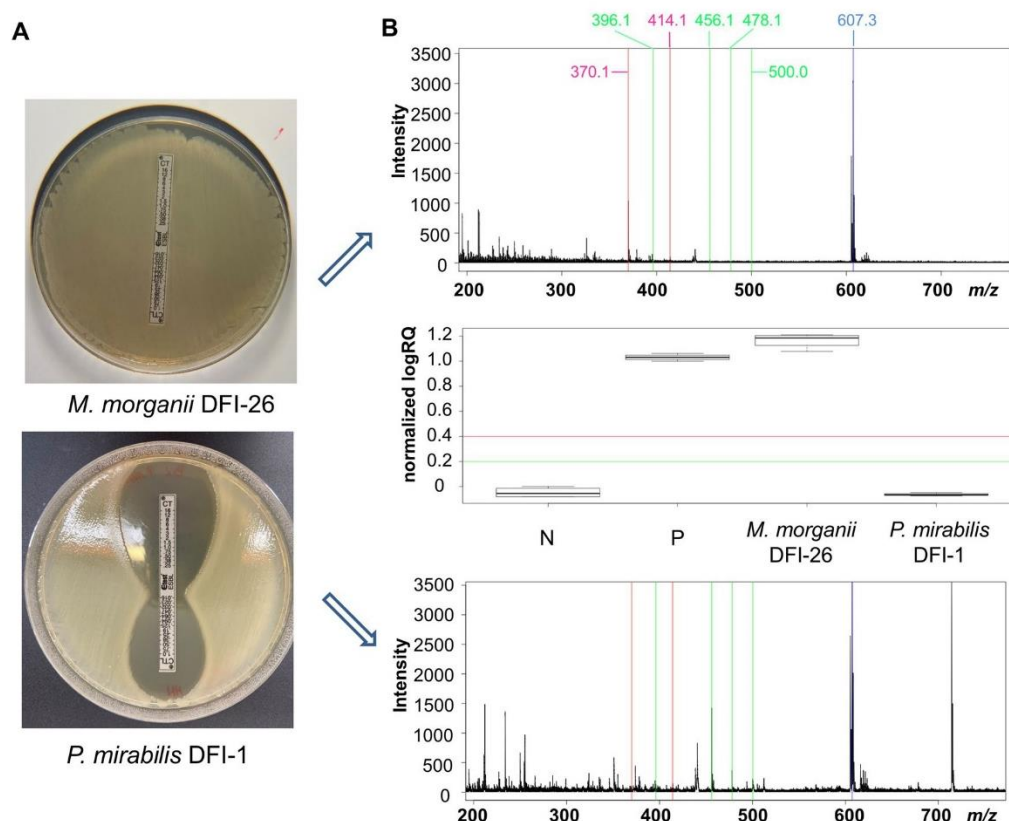


Fig. 3 Exemplary results of antibiotic resistance detection performed using Etest strips (a) and MBT STAR BL method (b) obtained for cefotaxime. N—negative control, P—positive control, normalized logRQ values ≤ 0.2 threshold—negative results, ≥ 0.4 threshold—pos-

itive results, values between these thresholds—unclear results, green lines (396.1, 456.1, 478.1, 500.0)—signals for nonhydrolyzed antibiotic (native and adducts with HCCA); red lines (370.1 and 414.1)—signals for hydrolyzed antibiotic, blue line (607.3)—standard

demonstrated resistance against at least one of the analyzed antibiotics (Table 3). The most frequent resistance against ampicillin was recorded – among 71% of patients and 52% of the isolates. Subsequently, over half of the patients (52%) showed the presence of ESBL strains covering common *Enterobacteriales* species, such as *E. coli*, *C. freundii*, *K. oxytoca*, *M. morganii*, and *P. vulgaris*, as well as rarely founded in DFI samples—*Flavobacteriaceae* (*M. odoratimimus*) and *Sphingobacteriaceae* (*S. multivorum*) members. Considering ESBLs, for all of them, hydrolytic activity against cefotaxime was detected, while in the case of ceftriaxone and ceftazidime percentage of positive results among resistant strains dropped to 38.5 and 15.4%, respectively. Only for one strain – *C. koseri* DFI-28, the performed MBT STAR BL assay gave unclear results – values between 0.2 and 0.4. Referring to other antibiotics, 29% of strains (mainly *E. coli* and *P. mirabilis*) were resistant to piperacillin, while only 10% (*P. aeruginosa* DFI-5, *K. oxytoca* DFI-8, *M. odoratimimus* DFI-22,

and *S. multivorum* DFI-25) showed enzymatic activity against carbapenems (imipenem or meropenem). The frequency of the occurrence of the specific drug resistance had been decreasing in the following order: ampicillin > ESBL > piperacillin > carbapenems. Considering results of the Etest strips, performed tests revealed higher percentage of ampicillin and piperacillin resistant strains compared to the MALDI ones – 67% and 50% Gram-negative strains, respectively. In the case of ESBL and carbapenemases-producing bacteria (CPB), the opposite observation had been noted—the share of ESBL strains was lower by 10% and for CPB by 8%. The number of unclear (undefined) results also significantly increases when Etest strips are used, which mostly refers to ESBL detection—cefotaxime 6/42 (14%) and other cephalosporins: ceftazidime 4/42 (10%), cefepime 3/42 (7%), and ceftriaxone 1/42 (2%). Unlike to MALDI results, the highest number of ESBL activity was detected using ceftriaxone which covered two-thirds of all positive strains based on

Table 3 Occurrence of the different types of antibiotic resistance among gram-negative isolates that were detected using MALDI and Etest strips approaches

Patient	Strain	MBT STAR BL results							Etest strips results						
		CTX	CRO	CAZ	IMP	MER	AMP	PIP	CTX	CRO	CAZ	CEP	IMP	AMP	PIP
DFI-1	<i>E. coli</i>														
	<i>P. mirabilis</i>														
DFI-4	<i>E. coli</i>														
	<i>P. mirabilis</i>														
	<i>P. aeruginosa</i>														
DFI-5	<i>E. coli</i>														
	<i>P. mirabilis</i>														
	<i>P. aeruginosa</i>														
DFI-6	<i>M. morganii</i>														
	<i>P. vulgaris</i>														
DFI-8	<i>K. oxytoca</i>														
	<i>E. cloacae</i>														
DFI-11	<i>C. freundii</i>														
DFI-13	<i>K. oxytoca</i>														
DFI-17	<i>E. coli</i>														
DFI-18	<i>P. mirabilis</i>														
	<i>C. braakii</i>														
DFI-20	<i>E. cloacae</i>														
DFI-21	<i>C. freundii</i>														
	<i>E. coli</i>														
	<i>E. cloacae</i>														
	<i>K. pneumoniae</i>														
DFI-22	<i>P. vulgaris</i>														
	<i>E. coli</i>														
	<i>M. odoratimimus</i>														
	<i>C. freundii</i>														
DFI-23	<i>E. coli</i>														
DFI-24	<i>E. coli</i>														
	<i>E. cloacae</i>														
	<i>P. mirabilis</i>														
DFI-25	<i>S. multivorum</i>														
DFI-26	<i>P. mirabilis</i>														
	<i>M. morganii</i>														
DFI-27	<i>K. pneumoniae</i>														
	<i>A. pittii</i>														
DFI-28	<i>C. koseri</i>														
	<i>M. morganii</i>														
	<i>E. coli</i>														
	<i>A. haemolyticus</i>														
DFI-29	<i>E. coli</i>														
DFI-30	<i>E. coli</i>														
DFI-31	<i>E. coli</i>														
		ESBL							ESBL						
		Carb.							Carb.						
		AMP							AMP						
		PIP							PIP						
Patients [%]		52%							33%						
Strains [%]		31%							21%						

Table 3 (continued)

Green—positive results, purple—negative results, blue—unclear results, CTX—cefotaxime, CRO—ceftriaxone, CAZ—ceftazidime, CEP—cefepime, IMI—imipenem, MER—meropenem, AMP—ampicillin, PIP—piperacillin, ESBL—extended spectrum β -lactamases, Carb.—carbapenemases

the Etest strips test. In the case of other cephalosporins, the percentage of positive results ranged from 33 to 42% mostly due to the high rate of undefined results.

Regarding specific antibiotics, the far most significant number of discrepancies between the two methods used were noted for cefotaxime – 16, which resulted from a higher rate of negative and undefined outcomes of Etests. Contrary to this, ampicillin hydrolysis detection was characterized by the highest complies level—~76%. Regarding bacterial species, the most different results were observed in the case of *P. aeruginosa* strains and *Citrobacter* and *Acinetobacter* genera members. Numerous discrepancies were also noted for *M. odoratimimus* and *S. multivorum*, which could be associated with their high enzymatic activity leading to undefined results in the Etests.

Screening for antibiotic-resistance genes revealed that only ten strains possessed one or more of the beta-lactamase-encoding genes tested (Table 4). They all refer to ESBLs representing Ambler class A beta-lactamases—TEM, SHV, or CTX-M. Most strains carrying resistance genes were *E. coli* strains (7) that most frequently had *TEM* (5 isolates). Regarding other genes, *SHV* occurred three times – in *K. oxytoca* DFI-8, *E. coli* DFI-17, and *K. pneumoniae* DFI-21, while *CTX-M-9* 2 times – *E. coli* DFI-4 and -5. Interestingly, only in 4 cases were ESBL genes detected for strains that demonstrated resistance according to MALDI or Etest assays – *E. coli* DFI-4 possesses *CTX-M-9*, *E. coli* DFI-5 with *TEM* + *CTX-M-9* as well as *K. oxytoca* DFI-8 and *E. coli* DFI-17 – both with *SHV* like genes. The rest strains with detected resistant genes demonstrated only activity against ampicillin or piperacillin and mostly

had *TEM* – 5 out of 6 isolates. Despite the detection of carbapenemase activity in 4 strains, PCR analyses did not reveal the presence of any of the analyzed genes encoding carbapenemases, that is, *VIM*, *IMP*, *NDM*, *GIM*, *KPC*, *GES*, or *OXA-48*.

Impact of the Antibiotic Therapy on the Microbiological Outcome of the DFI Patients

According to medical history, 10 DFI patients were not subjected to any antibiotic treatment; nine were receiving lincosamides (clindamycin), five beta-lactams, two fluoroquinolones, and five were receiving multi-antibiotic treatment (combo). Comparison of the species composition of swab samples according to the antimicrobial treatment used revealed differences in the type of microbial species detected that were considered unique to each patient group (Fig. 4a). Venn diagram analysis showed that when a particular type of antibiotic was used, the species composition of the DFI samples changed. The number of species highlighted varied depending on the type of antibiotic used. The highest number of unique microbial species (8) was found in combined antibiotic treatment, including two *Candida* species, and in patients treated with lincosamides—5. In contrast, samples from patients treated with beta-lactams and fluoroquinolones had fewer specific species, 2 and 1, respectively, but this observation may be due to the lower representation of these groups. Two bacteria, *E. coli*, and *E. faecalis*, appeared to be species common to all groups of DFI patients.

The analysis showed that the antibiotic treatment also affected the number of microbial species presents simultaneously in the sample; samples from treated patients had a higher percentage of samples with four or more microbial species—57% versus 44% (Fig. 4b). Such phenomenon was particularly evident in samples from patients on combination antibiotic therapy, where the percentage of samples containing > 3 species in the sample reached 80%. Considering the prevalence of a particular type of bacteria, antibiotic therapy generally increased Gram-positive bacteria, except for lincosamides, where the opposite trend was observed. A tremendous increase in Gram-positive bacteria was observed with beta-lactams—80% compared to 52% in the untreated group. Samples from treated patients were generally characterized by a higher ESBL share – 44% compared to 31% in the non-treated patients, but a slightly lower percentage of the ampicillin and piperacillin-resistant strains occurrence. The highest percentage of drug-resistant Gram-negative bacteria was observed in DFI patients receiving combination antibiotic therapy, with 75% resistant to ESBLs, ampicillin, and piperacillin.

Table 4 List of strains in which the tested resistance genes were detected using established multiplex PCR protocols with marked resistance according to MBT STAR BL and Etest

Patient	Strain	MALDI	Etest	Gene	
DFI-1	<i>E. coli</i>	•	•	<i>TEM</i>	
DFI-4	<i>E. coli</i>	•••	•••	<i>CTX-M-9</i>	
DFI-5	<i>E. coli</i>	•••	•••	<i>TEM, CTX-M-9</i>	
DFI-8	<i>K. oxytoca</i>	•••	•	<i>SHV</i>	
DFI-17	<i>E. coli</i>	•••	•••	<i>SHV</i>	
DFI-21	<i>E. coli</i>	•	••	<i>TEM</i>	• - ESBL
DFI-21	<i>K. pneumoniae</i>	•	•	<i>SHV</i>	• - Carbapenems
DFI-22	<i>E. coli</i>	•	••	<i>TEM</i>	• - Ampicillin
DFI-24	<i>E. coli</i>	•	••	<i>TEM</i>	• - Piperacillin
DFI-26	<i>P. mirabilis</i>	○	••	<i>TEM</i>	○ - not detected

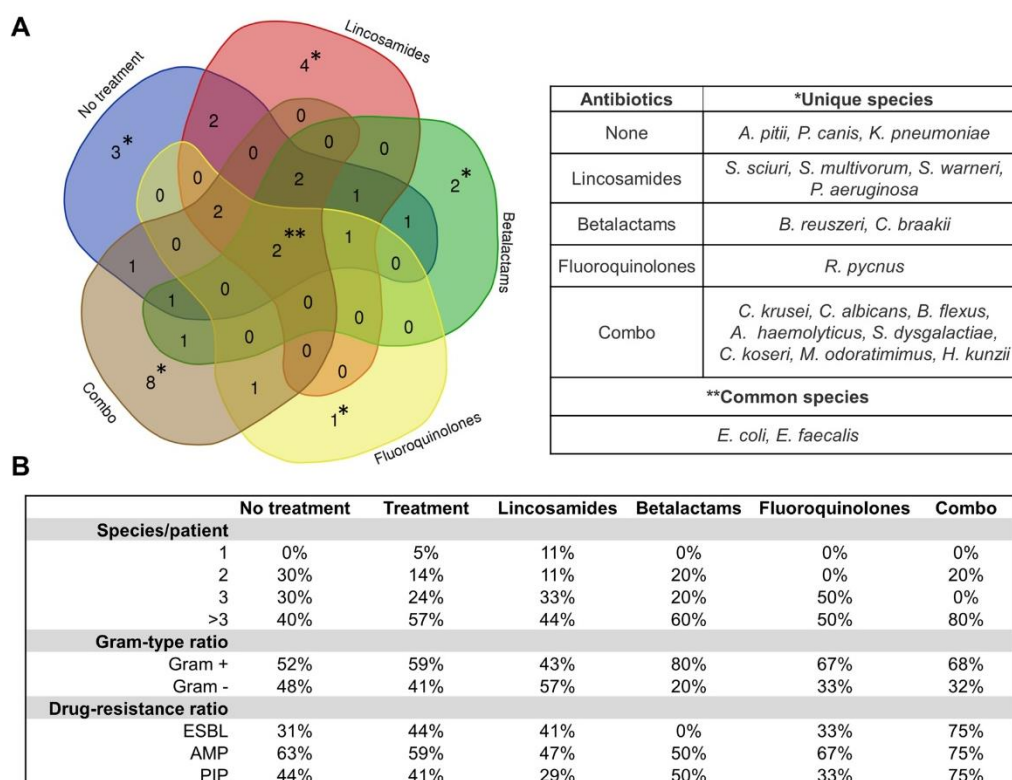


Fig. 4 Effect of the antibiotic treatment on the microbial profiles of the swab samples of the DFI patients. **a**—Venn diagram showing unique/common bacterial species depending on the antibiotic type used. **b**—influence of the treatment on the number of species per patient, Gram-type ratio as well as occurrence of the antibiotic resist-

ance (ESBL—extended-spectrum beta-lactamase, AMP—ampicillin resistance, PIP—piperacillin resistance). Resistance against carbapenems was not presented due to poor representation among investigated samples

Discussion

Optimal treatment of the infection depends on accurately identifying the microorganisms present and applying appropriate antimicrobial treatment. Failure to adequately treat infection in diabetic foot ulcers leads to progressive tissue damage, impaired wound healing, and serious complications [28]. Clinical practice of DFI diagnosis has relied chiefly on cultivation-dependent methods, which show bias towards microorganisms that thrive under isolation procedures and can grow well on laboratory culture media. Therefore, they often overlook slow-growing, fastidious, anaerobic, and unknown pathogens, which delays the appropriate treatment [29]. In the literature, we can find numerous examples of studies using the traditional culture method, where 46–85% of DFI cases were monobacterial [30, 31] with only a minority being polymicrobial infections [32].

On the other hand, there are also papers in which authors have indicated that DFIs are more often the result of polymicrobial infection with complex bacterial communities

(microbiome) that impede wound healing [33]. More recent advances in molecular biology technologies have helped to overcome obstacles accompanying traditional methods providing new insights into the bacterial diversity of DFI and have confirmed that chronic wounds, including diabetic foot ulcers, have a polymicrobial nature instead of being colonized by a single species [34, 35]. Price et al. [36] found that culture-based method revealed only nine bacterial families compared to 44 denoted using 16S rRNA sequencing which may be the reason for the high prevalence of monomicrobial infections detected by traditional culture. Nevertheless, it should be noticed that molecular approaches are limited by amplification biases, namely, by the primer choice affecting the amplification efficiency of different microbial phyla, as well as by the quality of extracted DNA which depends on the microbial taxa [37]. The optimal culture conditions selected in our previous study [8] enabled revealing a large diversity of bacterial families involved in the development of DFI including rarely detected in DFI *Pasteurellaceae*,

Sphingobacteriaceae, *Flavobacteriaceae*, *Planococaceae*, and *Peptoniphilaceae*.

Contrary to popular belief that cultures have a high false-negative rate and lack full representation of the total microbial population in wounds, especially in terms of the pathogenic burden [38, 39], culture-based methods can still play an essential role in patient management providing that modern culturomics approach with rapid microbial identification via MALDI technique is applied. As shown in our study, the simultaneous use of culture media sets of different types (universal, selective/differentiating) allows the isolation of fast-growing bacterial species as well as fastidious ones represented both Gram-positive and -negative type of bacteria in short time-to-results. Furthermore, the MALDI technique application assured high identification confidence by comparing species level with 16S rDNA sequencing – 93%. To date, only two papers have been published in this field regarding DFI research—our previous work concerning selection culture conditions [8], and work Jneid et al. [40]. In the second case, authors found a high prevalence of polymicrobial infections (88.3%) and high biodiversity (53 known and 19 unknown bacterial species). In addition, the culture conditions used allowed the isolation of species commonly found in DFI (mostly *S. aureus*, *Enterococcus faecalis*, *Enterobacter cloacae*) as well as the rarest species, such as anaerobic *Fingoldia magna*. Both studies mentioned above have proven that culturomics does work as a solution to address the limitations of conventional culturing, that is, increase the throughput of identifications and species coverage as well as play a complementary role concerning molecular methods in the exploration of complex microbiota in DFIs.

Revealed high frequency of polymicrobial infections is of utmost importance for patient management since microbial interactions may synergize the pathogenic potential of one or other microorganism, hampering their eradication and further controlling chronic wounds [38]. Liu et al. [4] hypothesized that individual bacterial species may not be able to maintain a pathogenic biofilm independently. However, pathogenic biofilm formation may occur in a symbiotic polymicrobial community in the DFU. Therefore, although most of our studies, *Staphylococcus* spp., and *Corynebacterium* spp., are considered part of healthy skin's normal microbiota, they may contribute to a pathogenic community of DFI. Several studies have highlighted the importance of CoNS and *Corynebacterium* spp. as potential pathogens of DFI and stress their importance concerning chronic wounds, especially in the case of patients with impaired immune responses such as diabetes. Our studies revealed a significant share of bacteria belonging to *Enterobacteriaceae* – 24.3% of all identified bacterial families and correlated with other culture-based studies that reported a high incidence of *Enterobacteriaceae* members in moderate to

severe diabetic foot ulcers [38, 41]. The predominance of the *Enterobacteriaceae* family has recently been reported as the largest group of aerobic Gram-negative rods in DFIs [42]. A shift towards the presence of enteric types of bacteria in the recurrent wound may be a result of self-colonization from another body site, e.g., gastrointestinal tracts, which produced a corresponding decline in wound healing since many of such Gram-negative isolates may also be multidrug-resistant which makes them very difficult to eradicate with antibiotic therapy. Indeed, drug resistance analysis among isolated Gram-negative bacterial strains showed a relatively high prevalence of ESBL (52% of isolates and 31% of patients) and carbapenemase-producing bacteria—19% of all Gram-negative isolates. Because the increasing severity of prevention and treatment of diabetic foot ulcer infections is associated with high rates of detection of multidrug-resistant bacteria, it is crucial to focus on assessing risk factors for infection with multidrug-resistant bacteria to find more effective treatments [42, 43]. Yan et al. [43] during the analysis of risk factors for multidrug-resistant organisms in diabetic foot infection among 180 patients from the Hospital of Jiangnan University (Wuxi Area), noted that 104 of all 182 isolated strains were multidrug-resistant bacteria (66 strains of Gram-negative bacteria and 38 strains of Gram-positive bacteria). In addition, the authors noted that antimicrobial use in the past 3 months was associated with multidrug-resistant bacterial infections in patients with diabetic foot ulcers ($P < 0.05$).

The microbial load, diversity, and presence of pathogenic organisms in the DFU are known to change in response to antibiotic treatment [44]. It has been noted that the wound microbiota of patients treated with antibiotics is significantly different from that of untreated patients. However, no clear distinction has been made between problematic bioburden and benign colonization, which would be clinically relevant to antibiotic treatment decisions [44, 45]. Our results showed that the use of antibiotic therapy by patients induces changes in the microbial composition and frequency of species in the wound microbiota, including the gram-type ratio and the frequency of drug resistance. The proportion of Gram-negative bacteria decreased while the antibiotic resistance rate increased. This observation of combination antibiotic therapy was remarkably accurate, indicating the highest number of unique microbial species and the highest ratio of drug-resistant strains. The resistance rate of *E. coli* was the highest among Gram-negative bacteria, consistent with previous reports. This finding may also be related to the fact that *E. coli* was also reported as the most common Gram-negative bacterium, as in many other reports, e.g. Tascini et al. [46].

Infection with MDR bacteria in DFU reduces the clinical effect of antibiotic therapy. Our study indicates that empirical antibiotic therapy for DFI should pay particular attention to the risk assessment of Gram-negative bacteria infection,

where the susceptibility pattern of Gram-negative bacteria should be regularly monitored in DFI. Nevertheless, many clinics rely on traditional culture and conventional biochemical tests, like strip test, that underestimates wound flora and may lead to inappropriate antibiotics prescribed in up to 45% of cases [47]. The excessive or inappropriate use of antibiotics not only results in ineffective treatment but also aggravates the worldwide crisis of antibiotic resistance [38]. This problem could be solved by using the latest, more adequate, and rapid drug resistance assays, such as those based on the MALDI TOF MS technique. Our study showed that using the MBT STAR BL assay increased the percentage of ESBL- and carbapenemase-producing bacteria detected compared to Etest strips. Additionally, the use of MBT STAR BL is accompanied by a significantly lower rate of unclear results. More and more researchers, including Noster et al. [48], emphasize that the MALDI technique is increasingly embraced for detecting antimicrobial resistance and will likely become an essential part of the routine laboratory soon. Although the disadvantage of this approach is that it only detects resistance conferred by hydrolysis of the target antibiotic, it has high sensitivity and specificity (98–100% and 97–100%, respectively), as well as a relatively short turnaround time—usually 30 min for typical Enterobacterales, and even shorter if an appropriate protocol modification is used, as demonstrated by Złoch et al. [21]. As our studies showed, the application of the MALDI approach could be more feasible for routine drug resistance detection among DFI isolates than molecular technique, such as multiplex PCR reactions, since the latter required expanded knowledge about the taxonomical affiliation of the isolates for suitable primers set designing.

Conclusions

Reliable deciphering of the composition of the wound microbiome in patients with DFI is crucial for subsequent effective therapy. Given the large number of microbial species that may be involved in the development of infection, especially in moderate to severe chronic wounds, practical diagnostic tools should be characterized by accurate identification, short time-to-results as well as the ability to rapidly detect drug resistance in the face of a growing global problem such as MDR bacteria. Such criteria are met by fast MALDI identification combined with multiple culture conditions and rapid detection of antibiotic resistance via MBT STAR BL assay. As demonstrated in our study, this method provides identification information at a level comparable to that obtained from DNA sequencing, allows the isolation of both common bacterial species and those considered rare, including fastidious ones, and is effective in antibiotic detection, especially for that of particular concern like ESBLs

and carbapenemases. Application of this technique may help to understand the role of the complex microbiota in the development of DFI in the context of the antibiotic therapy used by patients and its impact on the development of drug resistance. Moreover, our results indicate that culture-based methods can still be essential to routine clinical diagnosis, providing the clinician with relevant information in a reasonable time.

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Data Availability All sequence data that support the findings of this study have been deposited in GenBank and the public URL for the sequences collection is <https://www.ncbi.nlm.nih.gov/sites/myncbi/1F1i8raBplj5d/collections/61834280/public/>

Code Availability Software for DNA sequences analysis: MEGA software version 7.0.21; BioEdit Sequence Alignment Editor version 7.2.5 (12/11/2013); Interactive Tree of Life (iTOL) version 6.5.4 (01/09/2021). Software for MALDI TOF MS analysis: flexControl version 3.4 (Build 135); flexAnalysis version 3.4 (Build 76); MALDI Biotyper Compass Explorer 4.1 (Build 80), Bruker MBT STAR-BL Prototype (RUO) <https://mbtprot.bdal.de/MSBL/> (private access).

Declarations

Conflicts of interest The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Ethical Approval The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz (KB 68/2019, 29 January 2019).

Consent to Participate Informed consent was obtained from all individual participants included in the study.

Consent to Publish Not applicable.

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Supplementary materials

Supplementary Table S1. List of the identified isolates with the Score values (MALDI TOF MS) and percentage of identity with the NCBI records (16S rDNA sequencing) with obtain accession numbers.

Patient	MALDI				16S rDNA sequencing	Identity [%]	Accession no.
	RAW		MSP		Related species from NCBI*		
DFI-1	<i>Escherichia coli</i> DH5alpha BRL	2,21	<i>Escherichia coli</i> DH5alpha BRL	2,27	<i>Escherichia coli</i> NBRC 102203	99,34	MZ914687
	<i>Klebsiella oxytoca</i> VA20876_2_09 ERL	2,08	<i>Klebsiella oxytoca</i> VA20876_2_09 ERL	2,04	-		
	<i>Morganella morganii</i> 9544_1 CHB	2,01	<i>Morganella morganii</i> 9544_1 CHB	2,34	-		
	<i>Proteus mirabilis</i> DSM 788 DSM	2,09	<i>Proteus mirabilis</i> DSM 788 DSM	2,41	<i>Proteus mirabilis</i> ATCC 29906	99,86	MZ914688
DFI-2	<i>Corynebacterium striatum</i> MB_8820_05 THL	2,37	<i>Corynebacterium striatum</i> 10_G1799 ISB	2,44	<i>Corynebacterium striatum</i> ATCC 6940	99,54	MZ914698
	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,39	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,30	<i>Staphylococcus aureus</i> NBRC 100910	99,93	MZ915637
DFI-3	<i>Brevibacillus reuszeri</i> DSM 9887T DSM	1,92	<i>Brevibacillus reuszeri</i> DSM 9887T DSM	1,84	<i>Brevibacillus reuszeri</i> NBRC 15719	100	MZ918943
	<i>Staphylococcus aureus ssp aureus</i> DSM 20232 DSM	2,44	<i>Staphylococcus aureus</i> ATCC 29213 THL	2,47	-		
DFI-4	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,12	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,12	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ918944
	<i>Escherichia coli</i> ATCC 35218 CHB	2,20	<i>Escherichia coli</i> DH5alpha BRL	2,14	<i>Escherichia coli</i> NBRC 102203	99,72	MZ918945
	<i>Morganella morganii ssp morganii</i> DSM 30164T HAM	2,39	<i>Morganella morganii ssp morganii</i> DSM 30164T HAM	2,42	-		
	<i>Proteus mirabilis</i> 13210_1 CHB	2,03	<i>Proteus mirabilis</i> DSM 30115 DSM	2,19	<i>Proteus mirabilis</i> JCM 1669 / ATCC 29906	99,79	MZ918947
	<i>Staphylococcus sciuri ssp sciuri</i> DSM 20345T DSM	2,11	<i>Staphylococcus sciuri ssp sciuri</i> DSM 20345T DSM	2,18	<i>Staphylococcus sciuri</i> DSM 20345	99,86	MZ918949
	<i>Pseudomonas aeruginosa</i> 8147_2 CHB	2,31	<i>Pseudomonas aeruginosa</i> DSM 50071T HAM	2,31	?		
DFI-5	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,27	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,21	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ918965
	<i>Escherichia coli</i> MB11464_1 CHB	2,11	<i>Escherichia coli</i> MB11464_1 CHB	2,25	<i>Escherichia coli</i> NBRC 102203	99,72	MZ918988
	<i>Proteus mirabilis</i> 13210_1 CHB	2,22	<i>Proteus mirabilis</i> 13210_1 CHB	2,34	<i>Proteus mirabilis</i> ATCC 29906	99,86	MZ919118
	<i>Pseudomonas aeruginosa</i> 8147_2 CHB	2,19	<i>Pseudomonas aeruginosa</i> 8147_2 CHB	2,13	?		
DFI-6	<i>Corynebacterium striatum</i> MB_8820_05 THL	2,38	<i>Corynebacterium striatum</i> MB_8820_05 THL	2,49	-		
	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,37	<i>Enterococcus faecalis</i> DSM 6134 DSM	2,40	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ919144
	<i>Morganella morganii ssp sibonii</i> Mb19277_2 CHB	2,54	<i>Morganella morganii</i> Mb19277_2 C	2,49	<i>Morganella morganii subsp. sibonii</i> DSM 14850	99,78	MZ919317
	<i>Proteus vulgaris</i> DSM 30119 DSM	1,98	<i>Proteus vulgaris</i> DSM 46228 DSM	2,20	<i>Proteus terrae</i> N5/687	100	MZ919328
DFI-7	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,36	<i>Enterococcus faecalis</i> ATCC 29212 CHB	2,28	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ919328

	<i>Streptococcus dysgalactiae</i> ssp <i>dysgalactiae</i> DSM 20662T DSM	2,45	<i>Streptococcus dysgalactiae</i> VA20336_10 ERL	2,36	<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i> CIP 105120	99,93	MZ919346
	<i>Staphylococcus haemolyticus</i> 10024 CHB	2,57	<i>Staphylococcus haemolyticus</i> 10024 CHB	2,25	<i>Staphylococcus haemolyticus</i> JCM 2416	99,93	MZ919344
	<i>Helcococcus kunzii</i> VA324_10 ERL	2,45	<i>Helcococcus kunzii</i> VA324_10 ERL	2,42	<i>Helcococcus kunzii</i> 22	98,64	MZ919347
DFI-8	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,28	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,23	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ919357
	<i>Staphylococcus aureus</i> ATCC 29213 THL	2,38	<i>Staphylococcus aureus</i> ATCC 29213 THL	2,28	<i>Staphylococcus aureus</i> NBRC 100910	99,00	MZ919968
	<i>Klebsiella oxytoca</i> VA20879_09 ERL	2,16	<i>Klebsiella oxytoca</i> VA31877_09 ERL	2,13	<i>Klebsiella oxytoca</i> JCM 1665	99,43	MZ919988
	<i>Enterobacter cloacae</i> DSM 3264 DSM	2,20	<i>Enterobacter ludwigii</i> DSM 16688T DSM	1,66	<i>Pantoea agglomerans</i> JCM1236	99,71	MZ919989
DFI-9	<i>Staphylococcus aureus</i> ssp <i>aureus</i> DSM 20232 DSM	2,37	<i>Staphylococcus aureus</i> ATCC 33591 THL	1,80	<i>Staphylococcus aureus</i> NBRC 100910 / ATCC 12600	100	MZ920051
	<i>Bacillus flexus</i> 100331_30 USP	2,09	<i>Bacillus flexus</i> 100331_30 USP	2,15	-		
DFI-10	<i>Enterococcus faecalis</i> DSM 20371 DSM	2,29	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,20	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ921477
	<i>Streptococcus agalactiae</i> DSM 6784 DSM	2,33	<i>Streptococcus agalactiae</i> DSM 6784 DSM	2,33	<i>Streptococcus agalactiae</i> ATCC 13813	100	MZ921512
	<i>Staphylococcus simulans</i> DSM 20723 DSM	1,81	<i>Staphylococcus simulans</i> DSM 20723 DSM	1,71	<i>Staphylococcus simulans</i> MK 148	99,93	MZ921511
	<i>Staphylococcus aureus</i> ATCC 33862 THL	2,27	<i>Staphylococcus aureus</i> ssp <i>aureus</i> DSM 4910 DSM	2,33	<i>Staphylococcus aureus</i> NBRC 100910	100	MZ921946
DFI-11	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,22	<i>Enterococcus faecalis</i> DSM 20409 DSM	2,21	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ922053
	<i>Staphylococcus epidermidis</i> ATCC 14990T THL	1,66	<i>Staphylococcus epidermidis</i> ATCC 14990T THL	1,73	<i>Staphylococcus epidermidis</i> Fussel	100	MZ922259
	<i>Citrobacter freundii</i> 22054_1 CHB	2,39	<i>Citrobacter freundii</i> 22054_1 CHB	2,42	<i>Citrobacter freundii</i> LMG 3246	99,78	MZ930404
DFI-12	<i>Corynebacterium striatum</i> MB 8820_05 THL	2,48	<i>Corynebacterium striatum</i> MB 8820_05 THL	2,49	?		
	<i>Pasteurella canis</i> SO 02063_09 ERL	1,97	<i>Pasteurella canis</i> 26 PIM	2,06	-		
DFI-13	<i>Enterococcus faecalis</i> ATCC 29212 CHB	2,38	<i>Enterococcus faecalis</i> DSM 20371 DSM	2,44	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ930405
	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,28	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,41	<i>Staphylococcus aureus</i> NBRC 100910	100	MZ930410
	<i>Pseudomonas aeruginosa</i> ATCC 27853 THL	2,16	<i>Pseudomonas aeruginosa</i> ATCC 27853 THL	2,22	?		
	<i>Klebsiella oxytoca</i> VA31877_09 ERL	2,17	<i>Klebsiella oxytoca</i> 35130 PFM	2,29	<i>Klebsiella oxytoca</i> JCM 1665/ <i>Klebsiella grimontii</i> SB73	99,28/ 99,21	MZ930463
DFI-14	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,13	<i>Enterococcus faecalis</i> DSM 2570 DSM	2,29	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ930473
	<i>Pseudomonas aeruginosa</i> ATCC 27853 THL	2,09	<i>Pseudomonas aeruginosa</i> DSM 50071T HAM	2,10	?		
	<i>Enterobacter cloacae</i> DSM 46348 DSM	2,50	<i>Enterobacter cloacae</i> DSM 46348 DSM	2,39	<i>Enterobacter hormaechei</i> subsp. <i>xiangfangensis</i>	99,64	MZ930474

DFI-15	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,32	<i>Enterococcus faecalis</i> DSM 20409 DSM	2,38	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ930475
	<i>Staphylococcus simulans</i> DSM 20723 DSM	1,91	<i>Staphylococcus simulans</i> DSM 20723 DSM	2,09	<i>Staphylococcus simulans</i> MK 148	99,86	MZ930477
	<i>Corynebacterium striatum</i> MB 8820_05 THL	2,39	<i>Corynebacterium striatum</i> MB 8820_05 THL	2,44	-		
	<i>Staphylococcus aureus ssp aureus</i> DSM 11822 DSM	2,00	<i>Staphylococcus aureus</i> ATCC 29213 THL	1,95	<i>Staphylococcus aureus</i> NBRC 100910	99,93	MZ930479
DFI-16	<i>Streptococcus pyogenes</i> ATCC 19615 THL	2,42	<i>Streptococcus pyogenes</i> DSM 2072 DSM	2,47	<i>Streptococcus pyogenes</i> JCM 5674	100	MZ930480
	<i>Staphylococcus aureus</i> ATCC 29213 THL	2,36	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,34	<i>Staphylococcus aureus</i> NBRC 100910	100	MZ931308
DFI-17	<i>Escherichia coli</i> DH5alpha BRL	1,99	<i>Escherichia coli</i> DH5alpha BRL	2,20	<i>Escherichia coli</i> NBRC 102203	99,50	MZ931297
	<i>Rummeliibacillus pycnus</i> DSM 15030T DSM	2,03	<i>Rummeliibacillus pycnus</i> DSM 15030T DSM	2,17	<i>Rummeliibacillus pycnus</i> NBRC 101231	99,86	MZ931298
	<i>Staphylococcus epidermidis</i> 10547 CHB	2,18	<i>Staphylococcus epidermidis</i> 10547 CHB	2,38	<i>Staphylococcus epidermidis</i> Fussel	100	MZ931299
DFI-18	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,35	<i>Enterococcus faecalis</i> DSM 6134 DSM	2,49	<i>Enterococcus faecalis</i> ATCC 19433	99,93	MZ931305
	<i>Proteus mirabilis</i> RV412_A1_2010_06b LBK	2,46	<i>Proteus mirabilis</i> RV412_A1_2010_06b LBK	2,53	<i>Proteus mirabilis</i> ATCC 29906	99,93	MZ931326
	<i>Citrobacter braakii</i> 9314_2 CHB	2,16	<i>Citrobacter braakii</i> 20663_2 CHB	2,31	<i>Citrobacter freundii</i> LGM 3246	99,50	MZ934698
	<i>Staphylococcus haemolyticus</i> 10024 CHB	2,39	<i>Staphylococcus haemolyticus</i> 10024 CHB	2,28	<i>Staphylococcus haemolyticus</i> JCM 2416	99,86	MZ934700
DFI-19	<i>Streptococcus agalactiae</i> V29 CTL	2,50	<i>Streptococcus agalactiae</i> 03_102 CTL	2,56	<i>Streptococcus agalactiae</i> ATCC 13813	100	MZ934701
DFI-20	<i>Enterobacter cloacae</i> MB_8779_05 THL	2,11	<i>Enterobacter cloacae</i> MB_8779_05 THL	2,27	<i>Enterobacter cloacae</i> ATCC 13047	99,56	MZ934702
	<i>Enterococcus faecalis</i> 20247_4 CHB	2,26	<i>Enterococcus faecalis</i> DSM 6134 DSM	2,46	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ934703
DFI-21	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,08	<i>Enterococcus faecalis</i> DSM 2570 DSM	2,45	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ934744
	<i>Citrobacter freundii</i> 22054_1 CHB	2,27	<i>Citrobacter freundii</i> 22054_1 CHB	2,49	<i>Citrobacter sp (Citrobacter freundii</i> JCM 1657)	98,26	MZ934704
	<i>Escherichia coli</i> DH5alpha BRL	2,29	<i>Escherichia coli</i> ATCC 35218 CHB	2,33	<i>Escherichia coli</i> NBRC 102203	99,64	MZ934706
	<i>Enterobacter cloacae</i> 13159_1 CHB	2,10	<i>Enterobacter cloacae</i> DSM 46348 DSM	2,28	<i>Enterobacter hormaechei subs xiangfangensis</i> 10-17	99,79	MZ934705
	<i>Klebsiella pneumoniae ssp pneumoniae</i> 9295_1 CHB	2,16	<i>Klebsiella pneumoniae ssp pneumoniae</i> 9295_1 CHB	2,43	<i>Klebsiella pneumoniae</i> DSM 30104	99,64	MZ934707
DFI-22	<i>Candida albicans</i> ATCC 10231 THL	1,88	<i>Streptomyces badius</i> B192 UFL	1,45	-		
	<i>Candida krusei</i> ATCC 6258 VML	1,92	<i>Mycobacterium celatum</i> DSM 44243T DSM	1,22	-		
	<i>Proteus vulgaris</i> DSM 13387NT HAM	2,23	<i>Proteus vulgaris</i> DSM 13625 DSM	2,37	<i>Proteus vulgaris</i> ATCC 29905	99,35	MZ951127
	<i>Staphylococcus aureus ssp aureus</i> DSM 20232 DSM	2,06	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,47	<i>Staphylococcus aureus</i> NBRC 100910 / ATCC 12600	100	MZ951129
	<i>Escherichia coli</i> DH5alpha BRL	2,00	<i>Escherichia coli</i> DH5alpha BRL	2,31	<i>Escherichia coli</i> NBRC 102203	99,15	MZ951131

	<i>Streptococcus agalactiae</i> 04_158 CTL	2,12	<i>Streptococcus agalactiae</i> V29 CTL	2,47	<i>Streptococcus agalactiae</i> ATCC 13813	99,93	MZ951130
	<i>Myroides odoratimimus</i> LMG 4029T HAM	1,90	<i>Myroides odoratimimus</i> LMG 4029T HAM	2,27	<i>Myroides odoratimimus</i> CCUG 39352	99,85	MZ951134
	<i>Enterococcus faecalis</i>	2,20	<i>Enterococcus faecalis</i> DSM 2570 DSM	2,34	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ951133
	<i>Staphylococcus epidermidis</i> CCM 4505 CCM	1,87	<i>Staphylococcus epidermidis</i> ATCC 14990T THL	1,91	<i>Staphylococcus epidermidis</i> NBRC 100911	100	MZ951138
	<i>Citrobacter freundii</i> 13158_2 CHB	1,61	<i>Citrobacter freundii</i> 22054_1 CHB	2,07	<i>Citrobacter freundii</i> ATCC 8090 = mtcc 1658	99,79	MZ951142
DFI-23	<i>Enterococcus faecalis</i> ATCC 29212 CHB	2,23	<i>Enterococcus faecalis</i> DSM 6134 DSM	2,43	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ956264
	<i>Staphylococcus simulans</i> DSM 20323 DSM	1,96	<i>Staphylococcus simulans</i> DSM 20323 DSM	2,19	<i>Staphylococcus simulans</i> MK 148	99,86	MZ951146
	<i>Escherichia coli</i> W3350 MMG	2,06	<i>Escherichia coli</i> DH5alpha BRL	2,20	<i>Escherichia coli</i> NBRC 102203	99,42	MZ951157
	<i>Staphylococcus aureus</i> ATCC 33591 THL	1,91	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,38	<i>Staphylococcus aureus</i> NRBC 100910	100	MZ951147
	<i>Streptococcus pyogenes</i> ATCC 19615 THL	2,10	<i>Streptococcus pyogenes</i> ATCC 19615 THL	2,36	<i>Streptococcus pyogenes</i> JCM 5674	100	MZ951159
DFI-24	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,24	<i>Enterococcus faecalis</i> DSM 20409 DSM	2,44	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ951163
	<i>Escherichia coli</i> DH5alpha BRL	2,16	<i>Escherichia coli</i> DH5alpha BRL	2,51	<i>Escherichia coli</i> NRBC 102203	99,34	MZ951164
	<i>Enterobacter cloacae</i> MB_8779_05 THL	2,05	<i>Enterobacter cloacae</i> MB_8779_05 THL	2,18	<i>Enterobacter cloacae</i> ATCC 13047	99,78	MZ951167
	<i>Proteus mirabilis</i> 9482_2 CHB	2,08	<i>Proteus mirabilis</i> DSM 46227 DSM	2,34	<i>Proteus mirabilis</i> ATCC 29906	99,93	MZ951168
	<i>Staphylococcus simulans</i> DSM 20723 DSM	1,62	<i>Staphylococcus simulans</i> DSM 20723 DSM	1,93	<i>Staphylococcus simulans</i> MK 148	99,86	MZ951169
DFI-25	<i>Sphingobacterium multivorum</i> DSM 11691T HAM	2,39	<i>Sphingobacterium multivorum</i> DSM 11691T HAM	2,22	<i>Sphingobacterium multivorum</i> NBRC 14947	99,71	MZ955455
	<i>Staphylococcus aureus</i> ATCC 33591 THL	2,40	<i>Staphylococcus aureus</i> ssp aureus DSM 3463 DSM	2,37	<i>Staphylococcus aureus</i> NBRC 100910	99,93	MZ956162
	<i>Staphylococcus epidermidis</i> 10547 CHB	2,12	<i>Staphylococcus epidermidis</i> 10547 CHB	2,16	<i>Staphylococcus epidermidis</i> Fussel	100	MZ956274
DFI-26	<i>Proteus mirabilis</i> 9482_2 CHB	2,42	<i>Proteus mirabilis</i> 13210_1 CHB	2,33	<i>Proteus mirabilis</i> JCM 1669	99,93	MZ955635
	<i>Enterococcus faecalis</i> ATCC 29212 CHB	2,36	<i>Enterococcus faecalis</i> 20247_4 CHB	2,46	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ955864
	<i>Morganella morganii</i> (E) 21086317 MLD	2,48	<i>Morganella morganii</i> ssp morganii 15284_1 CHB	2,50	<i>Morganella morganii</i> LMG 7874	99,85	MZ955868
	<i>Streptococcus agalactiae</i> V29 CTL	2,44	<i>Streptococcus agalactiae</i> V29 CTL	2,51	<i>Streptococcus agalactiae</i> ATCC 13813	99,93	MZ955884
DFI-27	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,39	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,42	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ955986
	<i>Acinetobacter pittii</i> DSM 9321 DSM	2,24	<i>Acinetobacter pittii</i> DSM 9321 DSM	2,27	<i>Acinetobacter pittii</i> DSM 21653	99,79	MZ955994
	<i>Klebsiella pneumoniae</i> ssp pneumoniae 9295_1 CHB	2,29	<i>Klebsiella pneumoniae</i> ssp pneumoniae 9295_1 CHB	2,38	<i>Klebsiella pneumoniae</i> DSM 30104	99,50	MZ956116
DFI-28	<i>Enterococcus faecalis</i> ATCC 29212 CHB	2,33	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,51	<i>Enterococcus faecalis</i> ATCC 19433	100	MZ956119
	<i>Citrobacter koseri</i> DSM 4570 DSM	2,37	<i>Citrobacter koseri</i> 9553_1 CHB	2,58	<i>Citrobacter koseri</i> LMG 5519	99,36	MZ956120

	<i>Morganella morganii</i> (E) 21086317 MLD	2,38	<i>Morganella morganii</i> RV_BA_03_A L BK	2,56	<i>Morganella morganii</i> NBRC 3848	99,78	MZ956128
	<i>Escherichia coli</i> DH5alpha BRL	2,21	<i>Escherichia coli</i> DH5alpha BRL	2,43	<i>Escherichia coli</i> NBRC 102203	99,20	MZ956129
	<i>Acinetobacter haemolyticus</i> LMG 1033 HAM	2,39	<i>Acinetobacter junii</i> DSM 6964T HAM	2,12	<i>Acinetobacter</i> <i>gyllenbergii</i> RUH 422	100	MZ956131
DFI-29	<i>Escherichia coli</i> MB11464_1 CHB	2,17	<i>Escherichia coli</i> ATCC 25922 CHB	2,23	<i>Escherichia coli</i> NBRC 102203	99,71	MZ956132
	<i>Corynebacterium striatum</i> 23086514 MLD	2,28	<i>Corynebacterium striatum</i> 143 RLT	2,16	<i>Corynebacterium</i> <i>striatum</i> ATCC 6940	99,77	MZ956136
	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,02	<i>Enterococcus faecalis</i> 20247_4 CHB	2,29	<i>Enterococcus</i> <i>faecalis</i> ATCC 19433	100	MZ956134
DFI-30	<i>Enterococcus faecalis</i> 20247_4 CHB	2,43	<i>Enterococcus faecalis</i> 20247_4 CHB	2,36	<i>Enterococcus</i> <i>faecalis</i> ATCC 19433	100	MZ960142
	<i>Escherichia coli</i> MB11464_1 CHB	2,38	<i>Escherichia coli</i> MB11464_1 CHB	2,35	<i>Escherichia coli</i> NBRC 102203	99,57	MZ960143
	<i>Staphylococcus warneri</i> Mb18796_1 CHB	1,94	<i>Staphylococcus warneri</i> CCN	1,85	<i>Staphylococcus</i> <i>warneri</i> AW 25	99,93	MZ960144
DFI-31	<i>Streptococcus agalactiae</i>	2,39	<i>Streptococcus agalactiae</i>	2,41	<i>Streptococcus</i> <i>agalactiae</i> ATCC 13813	99,93	MZ960149
	<i>Escherichia coli</i> DH5alpha BRL	2,29	<i>Escherichia coli</i> DH5alpha BRL	2,29	<i>Shigella</i> <i>dysenteriae</i> ATCC 13313	99,78	MZ960146
	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,34	<i>Enterococcus faecalis</i> ATCC 7080 THL	2,26	<i>Enterococcus</i> <i>faecalis</i> ATCC 19433	100	MZ960150

Supplementary Table S2. Temperature program used for specific primers sets in order to detect investigated antibiotic-resistance genes.

Temperature program					Set
30 cycles					
94°C	94°C	60°C	72°C	72°C	1, 2
10 min	40 s	40 s	60 s	7 min	
36 cycles					
94°C	94°C	52°C	72°C	72°C	3-6
10 min	30 s	40 s	50 s	5 min	
30 cycles					
95°C	94°C	57°C	72°C	72°C	7
15 min	30 s	90 s	90 s	10 min	
30 cycles					
94°C	94°C	60°C	72°C	72°C	8
3 min	30 s	30 s	60 s	7 min	

Wszystkie dane dotyczące sekwencji potwierdzające wnioski z tego badania zostały zdeponowane w GenBank pod adresem URL:

<https://www.ncbi.nlm.nih.gov/sites/myncbi/1F1i8raBplj5d/collections/61834280/public/>



Publikacja [P7]

Silver nanoparticle targets fabricated using chemical vapor deposition method for differentiation of bacteria based on lipidomic profiles in Laser Desorption/Ionization Mass Spectrometry

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Article

Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry

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Abstract: The global threat of numerous infectious diseases creates a great need to develop new diagnostic methods to facilitate the appropriate prescription of antimicrobial therapy. More recently, the possibility of using bacterial lipidome analysis via laser desorption/ionization mass spectrometry (LDI-MS) as useful diagnostic tool for microbial identification and rapid drug susceptibility has received particular attention because lipids are present in large quantities and can be easily extracted similar to ribosomal proteins. Therefore, the main goal of the study was to evaluate the efficacy of two different LDI techniques—matrix-assisted (MALDI) and surface-assisted (SALDI) approaches—in the classification of the closely related *Escherichia coli* strains under cefotaxime addition. Bacterial lipids profiles obtained by using the MALDI technique with different matrices as well as silver nanoparticle (AgNP) targets fabricated using the chemical vapor deposition method (CVD) of different AgNP sizes were analyzed by the means of different multivariate statistical methods such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), sparse partial least squares discriminant analysis (sPLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The analysis showed that the MALDI classification of strains was hampered by interference from matrix-derived ions. In contrast, the lipid profiles generated by the SALDI technique had lower background noise and more signals associated with the sample, allowing *E. coli* to be successfully classified into cefotaxime-resistant and cefotaxime-sensitive strains, regardless of the size of the AgNPs. AgNP substrates obtained using the CVD method were used for the first time for distinguishing closely related bacterial strains based on their lipidomic profiles and demonstrate high potential as a future diagnostic tool for the detection of antibiotic susceptibility.

Keywords: bacteria; chemical vapor deposition; lipids; mass spectrometry; silver nanoparticles

1. Introduction

The development of new diagnostic methods that facilitate the initiation of targeted antimicrobial therapy through rapid and accurate identification of microorganisms is constantly driven by the global threat of numerous infectious diseases [1]. Among the methods that have significantly contributed to the transformation of the field of microbial diagnostics are mass spectrometry platforms targeting microbial products, mostly proteins [2,3]. In view of this, pathogens identification by matrix-assisted laser desorption/ionization

time-of-flight mass spectrometry (MALDI-ToF MS) analysis of high abundance proteins (ribosomal among others) is emerging as the dominant technology in many clinical laboratories [4]. Although protein-based MALDI-ToF MS platforms revolutionized the workflow of routine clinical laboratories around the world, nevertheless, application of such approach meets some constraints such as a commonly encountered failure to differentiate some closely related species (e.g., *Escherichia coli* vs. *Shigella*), laborious protein extraction procedures required for microorganisms encased in complex, thick cell walls, poor efficiency in detecting pathogens directly in the clinical specimens as well as difficulty in differentiating antimicrobial-resistant strains [4–6]. Because, after proteins, lipids are the major functional and structural component of cells and play an important role in many cellular processes such as membrane formation, energy storage, and cell signaling, they are therefore characterized by a wide variety of characteristics, such as the backbone and the headgroups, the number of fatty acids, and the chemical moieties they are modified with [7,8]. The diversity of lipids, especially glycolipids which showed species-specific characteristics within bacteria, has the potential to make them useful biomarkers for microbial identification in much the same way that the protein-based platforms are used to identify bacteria [1,5]. Moreover, recent studies have shown that microbial lipid signatures generated during MS analysis can be even more powerful than those based on the proteomics [9–11]. Therefore, the possibility of using bacterial lipidome analysis as a complementary method to existing MALDI diagnostic platforms is currently receiving particular attention from researchers because bacterial lipids are present in large quantities and can be easily extracted similar to ribosomal proteins [4]. In addition, it has been noted that the lipid profiles of drug-resistant strains differ significantly from those of susceptible strains, regardless of whether the antibiotic action directly targets membrane lipids or other cellular components [12,13].

Despite the significant progress that has been made in lipid analysis using MS, there is still a great need for their further development, especially with respect to bioinformatics resources, including the creation of robust and accurate databases containing organism-specific lipid species to support clinical applications of bacterial lipidomics [14]. Moreover, it is known that the sensitivity of the MALDI-MS analysis for distinct classes of biomolecules can vary when employing different MALDI matrices. In view of this, Perry and colleagues [15] revealed significant matrix effects on the relative signal intensities observed for different lipid subclasses that brought about the generation of unique lipid profiles. It means that during the search for optimal conditions for the generation of lipid profiles, the matrix selection is a key factor that should be considered, especially as some lipid-derived compounds are exclusively detectable if the most appropriate matrix is used [16].

A technique that may be more appropriate for use in this type of analysis is surface-assisted laser desorption/ionization (SALDI), in which target plates contain various micro- or nanoscale structures [17,18]. Silver nanostructures are among the most commonly used nanomaterials in SALDI MS. They enable efficient absorption of laser radiation and energy transfer providing desorption of both ionic and non-ionic analyte molecules. Additionally, silver nanoparticles (AgNPs) exhibit a relatively high salt tolerance and the ability to accurately calibrate the internal spectrum on silver cluster signals [19]. Silver-assisted LDI has already been found to be a powerful cationization agent for MS analysis of various lipids and olefinic species such as cholesterol and fatty acids [20].

The main goal of the study was to differentiate cefotaxime-resistant and -sensitive *E. coli* strains based on their lipidomic profiles in laser desorption/ionization mass spectrometry using the MALDI technique and silver nanoparticle targets fabricated using the chemical vapor deposition method (CVD)—SALDI approach. *E. coli* is a model representative of Enterobacterales; the group demonstrated widespread resistance against beta-lactams, including cefotaxime (cephalosporins third generation belonging to last-resort medicaments), which is, therefore, crucial for the health care system and from the epidemiological point of view. It is known that distinguishing *E. coli* from its close

relatives, including subspecies, differences in virulence level, and antibiotic resistance, can be problematic; however, as in the case of other Enterobacterales, resistance against cefotaxime can be detected using the MALDI approach based on the indication hydrolysis of the antibiotic molecules. Thus, the project aimed to check if such resistance can be detected using other LDI techniques, which analyze the bacterial lipidomic profiles. The performance of the classification was evaluated using different statistical methods—PCA, PLS-DA, sPLS-DA, and OPLS-DA.

2. Materials and Methods

2.1. Bacterial Sample Preparation

Two strains of *Escherichia coli* isolated from diabetic foot were selected for analysis. The selected strains showed different sensitivity to β -lactam antibiotics, including cefotaxime. The resistance of isolates to this group of antibiotics was detected using the MALDI technique and confirmed using multiplex PCR. The cefotaxime-sensitive strain was designated as *E. coli* DFI30 [MZ960143], and the resistant strain as DFI4 [MZ918945] (manuscript under review). Strains were grown overnight in 5% sheep blood-supplemented medium (Columbia Blood Agar; Oxoid, UK) prior to analysis. A loop of 1 μ L of bacterial biomass was suspended in 50 μ L of antibiotic solution (0.5 mg/mL cefotaxime dissolved in MBT STAR Buffer). The samples were incubated with agitation (ca. 500 rpm) for 2 h at 37 °C. Incubation time was set following the manufacturer's guideline for cefotaxime resistance detection using the MBT STAR BL assay to check whether this time is enough to distinguish isolates based on their lipidomic profiles. After incubation time, the samples were centrifuged (1 min, 13,000 rpm) and lipid extraction was performed from the biomass in the sediment. The extracts were prepared using the classical Folch method, a widely used standard method for lipid extraction in many laboratories. This method involves partitioning lipids in a biphasic mixture of chloroform and methanol. The bacterial biomass was suspended in 1.5 mL of a chloroform/methanol mixture (2:1, *v:v*) and placed in an ultrasonic bath for 10 min. An amount of 0.5 mL of 0.05 M NaCl was added to the sample and vortexed for 10 min. The samples were then centrifuged (15 min, 6000 rcf) and the chloroform layer was collected in a separate tube. Chloroform was evaporated and the obtained precipitate was dissolved in methanol and TA30 30% acetonitrile (CAN), 69.9% water, 0.1% trifluoroacetic acid (TFA). The samples prepared in this way were applied in an amount of 1 μ L to the appropriate plates.

2.2. SALDI Targets Preparation

The stainless steel plates were covered with a silver coating consisting of densely packed silver nanoparticles (AgNPs) using a chemical vapor deposition (CVD) technique. Before the deposition process, the substrate surfaces were degreased by washing them in an ultrasonic bath with distilled water containing a non-ionic surfactant for 45 min (twice). Prepared plates were immersed in the acetone (analytical grade) for 30 min, the distilled water for 10 min, and after drying in an Ar stream, their surface was activated in 0.1% trifluoroacetic acid solution for 20 min. After drying in an argon stream, the prepared substrate was placed in a CVD reactor. All CVD experiments were carried out using *hot wall* reactor (own construction) using the $[\text{Ag}_5(\text{O}_2\text{CC}_2\text{F}_5)_5(\text{H}_2\text{O})_3]$ as a precursor. The synthesis and physico-chemical properties of the mentioned above precursor were earlier described [21,22]. Metallic AgNPs were deposited under conditions collected in Table 1.

The morphology of created surfaces with silver nanoparticles was studied using a scanning electron microscope (SEM, Quanta 3D FEG, Huston, TX, USA). The structure of AgNP coatings was investigated using an energy-dispersive X-ray diffractometer (Quantax 200 XFlash 4010, Bruker AXS GmbH, Karlsruhe, Germany) with a copper monochromator and $\text{CuK}\alpha$ radiation ($\lambda = 0.15418$ nm). XRD patterns were collected in the 2θ range 10–80°, step 0.02°, and time 20 s. The Sartorius MCA2.7S-2S00-M microbalance (Sartorius Lab Instruments GmbH & Co. KG, Goettingen, Germany) was applied to determine the weight of the reference sample before and after the CVD process. The stainless steel (H17) reference

samples of sizes 1×1 cm were placed in the CVD reactor together with the investigated sample to obtain similar deposition conditions.

For the purposes of the MALDI experiments, Ag films were prepared in real time, and the storage time of samples (closed box, room temperature, limited access to light) was not longer than 2–3 days.

Table 1. Deposition parameters AgNP coatings.

Precursor	$\text{Ag}_5(\text{O}_2\text{CC}_2\text{F}_5)_5(\text{H}_2\text{O})_3$
Precursor weight (mg)	5, 10, 15
Vaporization temperature (T_V) ($^\circ\text{C}$)	230
Carrier gas	Ar
Total reactor pressure (p) (mbar)	3, 0
Substrate temperature (T_D) ($^\circ\text{C}$)	290
Substrates	stainless steel (H17)
Deposition time (min)	60
Sample heating time (min)	30 (Ar/ H_2 (3:1%))

2.3. Mass Spectrometry Analysis

MALDI measurements were performed on the Bruker 384 ground steel target. The matrices for MALDI analysis CHCA (α -Cyano-4-hydroxy-cinnamic acid, Bruker Daltonik GmbH, Bremen, Germany), DHB (2,5-dihydroxybenzoic acid, Bruker Daltonik GmbH, Bremen, Germany), and super-DHB (90:10 2,5-DHB:2-hydroxy-5-methoxy-benzoic acid, Sigma Aldrich, Steinheim, Germany) were prepared according to the manufacturer's recommended protocols (Bruker Daltonics GmbH, Bremen, Germany) as follows—(1) CHCA saturated in TA30, (2) 20 mg/mL DHB in TA30, and (3) 50 mg/mL super-DHB in TA50 (50:50 ACN:0.1% TFA in water). An amount of 0.5 μL of each sample was placed directly on the target and air-dried, then 0.5 μL of matrix was applied to the spots. We chose MALDI matrices offering sufficient sensitivities for lipids in positive ion mode MS analysis, among which DHB is recognized as the most common MALDI matrix in the lipid field [15].

SALDI targets after CVD were used with Bruker MTP Slide-Adapter II. Volumes of 0.5 μL of samples were placed directly on target plate, air-dried, and inserted into MS apparatus for measurements.

MS experiments were performed using a Bruker ultrafleXtreme time-of-flight mass spectrometer equipped with a SmartBeam II laser (355 nm and frequency 2 kHz) in positive ion reflectron mode. The measurement range was m/z 60–1500, and suppression was turned on for m/z lower than 59. The number of laser shots was 1500 (3×500 shots) for each sample spot. The first accelerating voltage was held at 25.08 kV and the second ion source voltage at 22.43 kV. Reflector voltages accounted for 26.64 and 13.54 kV. The value of detector gain for the reflector was $2.51 \times$. The value of the global attenuator offset accounted for 30%. Mass calibration was performed with FlexAnalysis 3.3 (Bruker Daltonics GmbH) using the cubic enhanced model and internal standards, silver ions and clusters from Ag^+ to Ag_4^+ for SALDI targets and matrix peaks for MALDI. The mass list for each sample was created using the 'centroid' peak detection algorithm and signal to noise (S/N) threshold equal to 3.

2.4. Analysis of MS Results

Statistical analysis of the results was performed with the use of the MetaboAnalyst 5.0 web service [23]. The interquartile range method was used for data filtering. Data were normalized by sum, cube root transformed, and default Pareto scaling was applied. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), sparse partial least squares discriminant analysis (sPLS-DA), orthogonal projections to latent structures discriminant analysis (OPLS-DA), and analysis of variance (ANOVA) statistical methods were used. In addition, a random forest classification method and

hierarchical clustering dendrograms with Euclidean distance measures and the Ward clustering algorithm were applied.

3. Results

The use of the selected conditions during the CVD process resulted in the production of the coatings consisting of the dense-packed silver particles uniformly covering the entire surface of the stainless steel plates (Figure 1A). The use of different masses of solid Ag precursor under similar deposition conditions (Table 1) allowed the control of the surface morphology of the deposited layers as well as the size of the deposited AgNPs. Layers, which consisted of dense-packed silver nanoparticles, similar in shape to a sphere, were produced in the case of low precursor concentrations in vapors (precursor weight—5–15 mg). Medium grain sizes of AgNPs changed from 50 ± 10 nm up to 240 ± 80 nm for coatings produced using 5 and 15 mg of the precursor, respectively (Table 2). The metallic silver was identified basing the reflection peaks, which were found at 38.2° , 44.3° , 64.6° , and 77.6° assigned to planes indexed as (111), (200), (220), and (311), respectively (Figure 1B) [24].

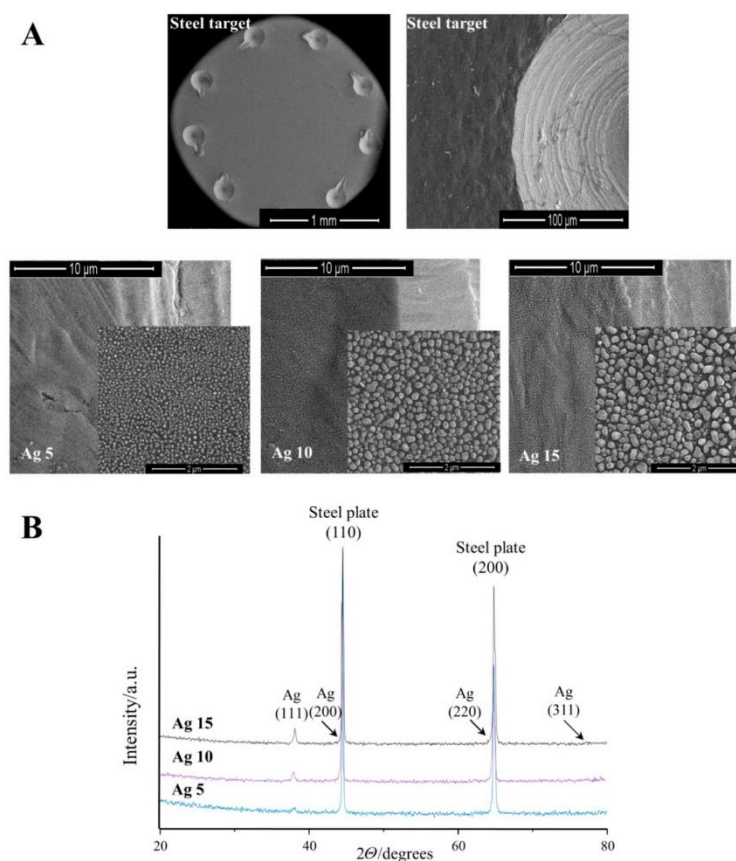


Figure 1. SEM images (A) of the bare plates as well as silver coatings obtaining using the CVD process with different amounts of the precursor (5, 10, and 15 mg). Registered XRD patterns for the studied plates (B) confirmed the deposition of the silver nanoparticles on the surface of the steel substrates. Ag 5, 10, 15—silver coating obtained using 5, 10, and 15 mg of the precursor; a.u.—arbitrary units.

Table 2. AgNP films deposited by CVD technique.

Target	Precursor Weight (mg)	Percentage Substrate Mass Increase after the CVD Process (wt.%)	AgNPs Medium Grain Size (nm)
Ag 15	15	0.06	240 ± 80
Ag 10	10	0.04	150 ± 50
Ag 5	5	0.03	50 ± 10

The precursor weight and the deposition time (1h) guarantee the deposition of uniform monolayers (over the entire surface of the substrate), composed of the spherical AgNPs. Analysis of data presented in Table 2 showed direct dependency between the weight of the used precursor and the size of deposited grains. Maintaining the repeatability of both factors mentioned above allows for precise control of the size of the deposited AgNPs and their packing density.

Figure 2 shows the results of statistical analysis of data from measurements of lipid extracts of strains DFI4 and DFI30 by the MALDI-MS technique using three matrices: α -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), and super-DHB. From analyzing charts, it can be concluded that only the sPLS-DA score plot (Figure 2C) presents completely separated groups. The other three used statistical methods, PCA (Figure 2A), PLS-DA (Figure 2B), and OPLS-DA (Figure 2D), did not allow for complete separation of analyzed strains. In Figure 2B,D, it can be seen that the samples that most affect the separation of groups are the extracts measured using the CHCA matrix. Therefore, it can be concluded that the matrix used has a decisive effect on the separation of groups in the MALDI method. This is confirmed by the dendrogram made for the same data (Figure 3B).

The large effect of the method can also be seen in the statistical analysis of all results divided into two groups, the DFI4 and DFI30 strains (Figure 4), and four groups, DFI4-SALDI, DFI4-MALDI, DFI30-SALDI, and DFI30-MALDI (Figure 5). None of the statistical methods allowed the complete separation of strains when two groups were used. The data that determined this were samples from MALDI measurements (Figure 4). When four groups were used, extracts from strains DFI4 and DFI30 measured by MALDI were clustered completely (Figure 5A), or almost completely overlapping (Figure 5B,C). Analysis of the VIP scores (variable importance in projection) showed remarkable differences between the LDI method used regarding significant m/z signals that distinguished examined isolates (Figure S1). Furthermore, the ANOVA test showed that, considering the nine most discriminating m/z values obtained for DFI4 and DFI30 bacterial lipids samples, in the case of the SALDI approach, the variation between *E. coli* isolates was much higher than in the case of the MALDI one (Figure S2). These results suggest a high degree of method influence on the spectral data over the sample data for the MALDI-MS technique. Such a phenomenon was reflected in the set of lipids that differentiate investigated *E. coli* isolates which differ considerably between MALDI and SALDI (Table 3).

Table 3. List of ions and lipids found by PLS-DA statistical analysis of bacterial lipids samples' mass spectra.

Lipid	Ion Formula	Experimental <i>m/z</i>	Calculated <i>m/z</i>	Reg. ^a	
				DFI4	DFI30
SALDI					
FA 6:0;O	[C ₆ H ₁₂ O ₃ + H] ⁺	133.074	133.0859	+	-
FA 5:1	[C ₅ H ₈ O ₂ + Na] ⁺	123.132	123.0416	+	-
FA 6:3;O3	[C ₆ H ₆ O ₅ + H] ⁺	158.963	159.0288	+	-
FA 30:1;O2	[C ₃₀ H ₅₈ O ₄ + K] ⁺	521.409	521.3967	+	-
LPG 17:1	[C ₂₃ H ₄₅ O ₉ P + K] ⁺	535.1725	535.2433	+	-
FA 7:1	[C ₇ H ₁₂ O ₂ + Na] ⁺	151.075	151.0729	+	-
FA 4:1	[C ₄ H ₆ O ₂ + K] ⁺	124.936	124.9999	+	-
MALDI					
CAR 3:1	[C ₁₀ H ₁₇ NO ₄ + H] ⁺	216.052	216.1230	+	-
FA 6:2;O4	[C ₆ H ₈ O ₆ + K] ⁺	214.9205	214.9952	+	-
TG 64:3	[C ₆₇ H ₁₂₄ O ₆ + H] ⁺	1026.025	1025.9471	-	+
CoA 4:1;O2	[C ₂₅ H ₄₀ N ₇ O ₁₉ P _{3S} + H] ⁺	868.085	868.1385	+	-
PC 16:4	[C ₂₄ H ₄₀ NO ₈ P + K] ⁺	540.132	540.2123	+	-
FA 6:2;O3	[C ₆ H ₈ O ₅ + K] ⁺	198.955	199.0003	+	-
CAR 14:1;O2	[C ₂₁ H ₃₉ NO ₆ + K] ⁺	440.173	440.2409	+	-

^a Regulation of the intensity in samples. FA—fatty acid; LPG—lyso-phosphatidylglycerol; CAR—acyl carnitine; TG—triacylglycerol; CoA—acyl CoA; PC—glycerophosphocholine.

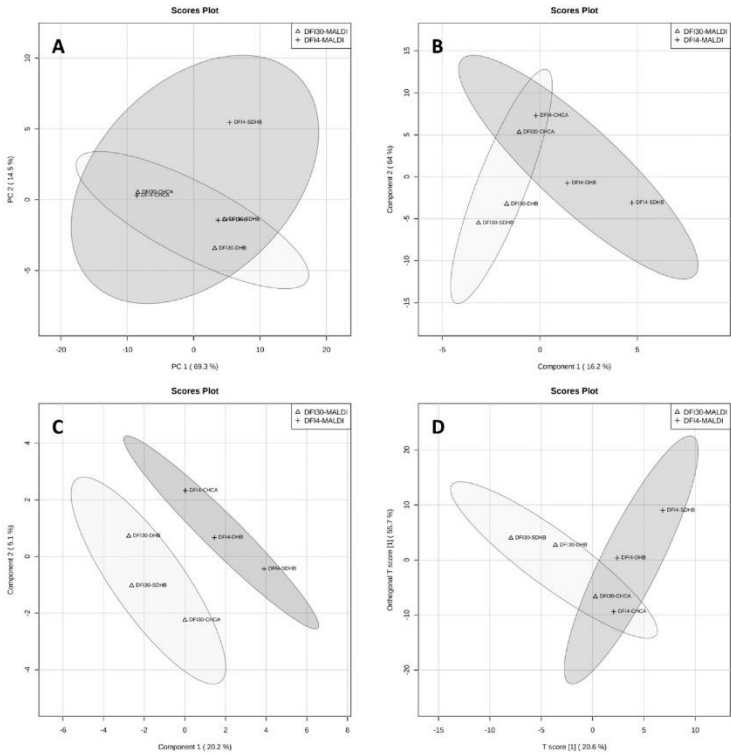


Figure 2. Graphical representation of statistical analysis of MS data from MALDI experiment: PCA component 1 vs. 2 (A), PLS-DA component 1 vs. 2 (B), sPLS-DA component 1 vs. 2 (C), and OPLS-DA (D). Dark-grey area represents data for DFI-4 strain while light-grey represents DFI-30 strain.

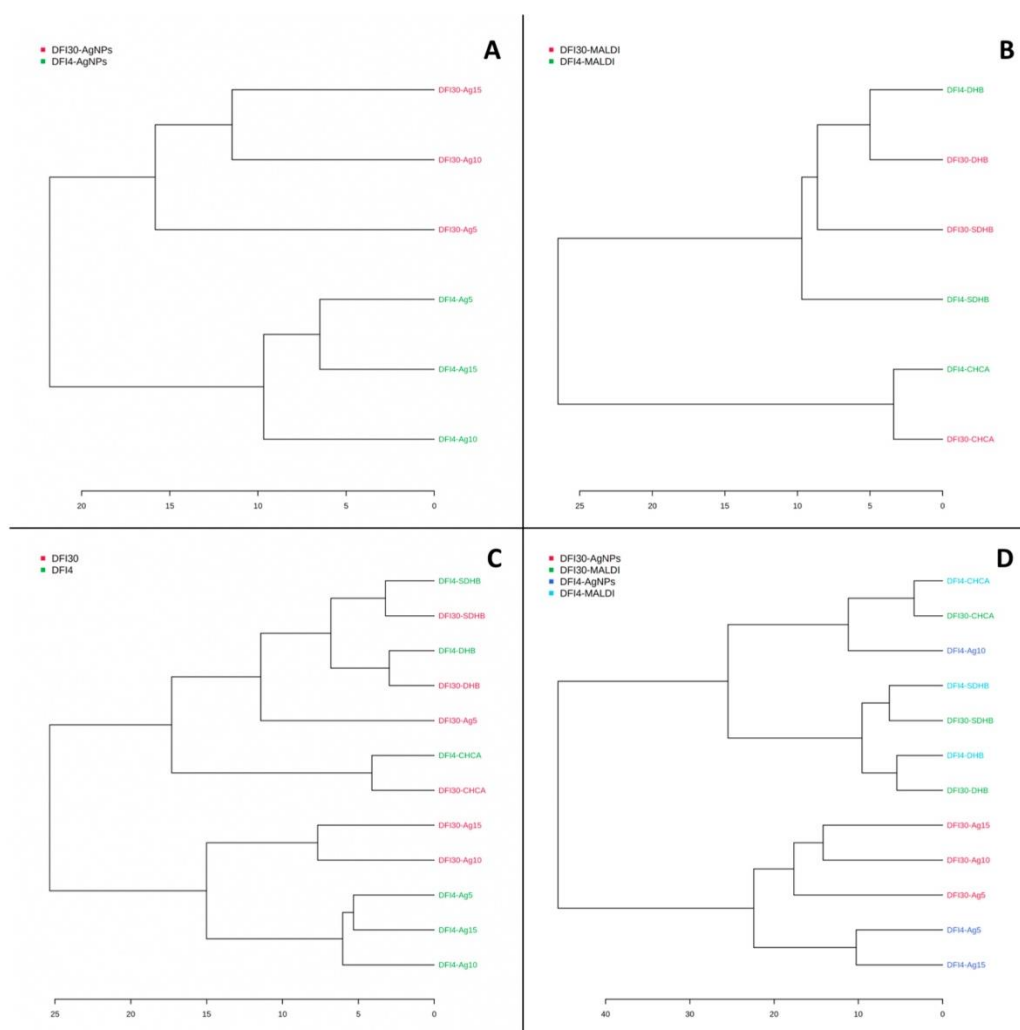


Figure 3. Dendrograms for MS data from SALDI/NALDI (A), MALDI (B) experiments and with the use of two groups (C) and four groups (D) for all data.

The SALDI method based on plates with silver nanostructures applied by the CVD technique shows a complete separation of the two tested strains by all the statistical methods used (Figure 6). In addition, the generated dendrogram first shows the grouping of samples by strain and then by the data recorded on the different plates (Figure 3A). Additionally, when all results are analyzed across two or four groups, data from SALDI-MS measures show more variation than data from MALDI (Figures 4 and 5). This implies a smaller effect of the SALDI plates used on the spectral data than is the case with the MALDI method. This can be explained by the presence of less chemical background in the form of additional signals from the matrix used (Table 4).

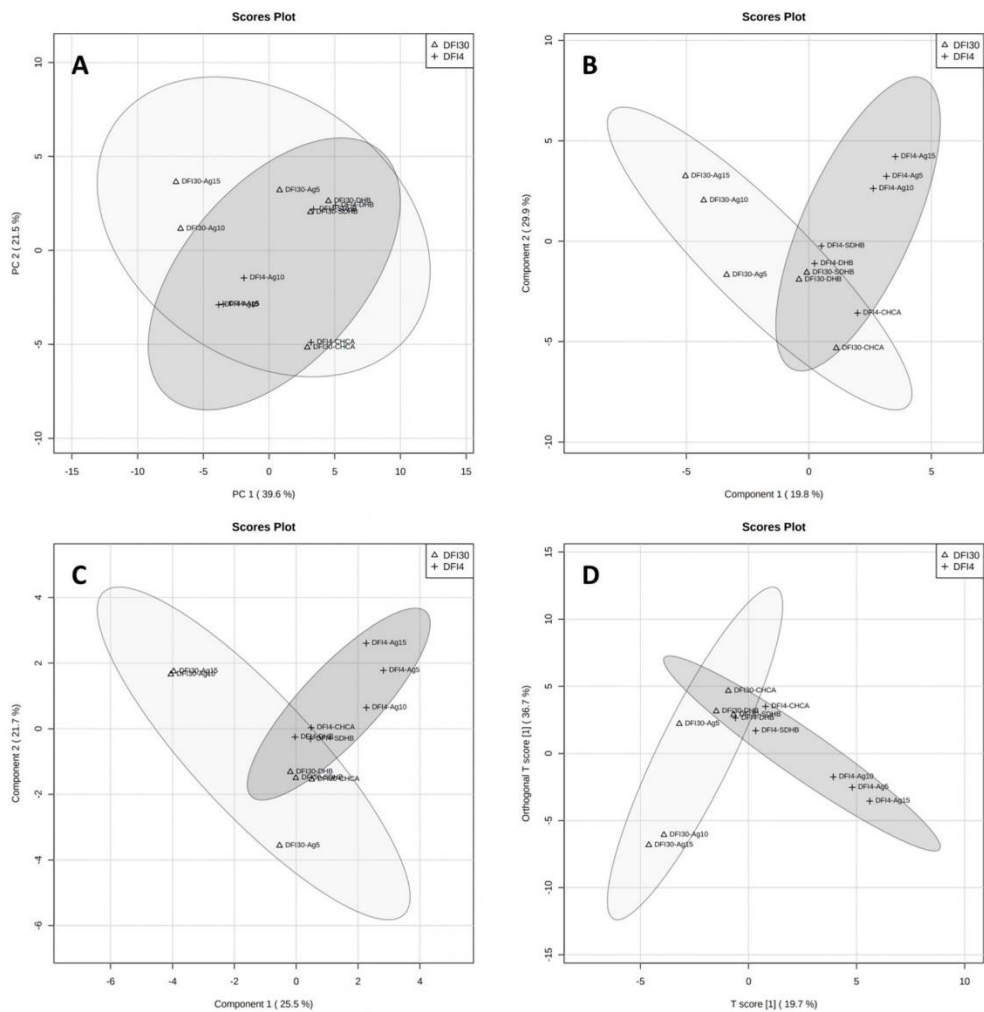


Figure 4. Graphical representation of statistical analysis of MS data from MALDI and SALDI/NALDI experiment with two groups: PCA component 1 vs. 2 (A), PLS-DA component 1 vs. 2 (B), sPLS-DA component 1 vs. 2 (C), and OPLS-DA (D). Dark-grey area represents data for DFI-4 strain while light-grey represents DFI-30 strain.

Table 4. Number of the signals in MS spectra depending on the LDI method used—MALDI vs. SALDI.

Matrix/Nanostructures	Blank MS	Signals Number (S/N $\geq$ 3)	
		<i>E. coli</i> DFI4	<i>E. coli</i> DFI30
DHB	90	262	241
Super-DHB	88	177	144
CHCA	307	447	485
Ag5	65	462	402
Ag10	47	366	315
Ag15	56	399	358

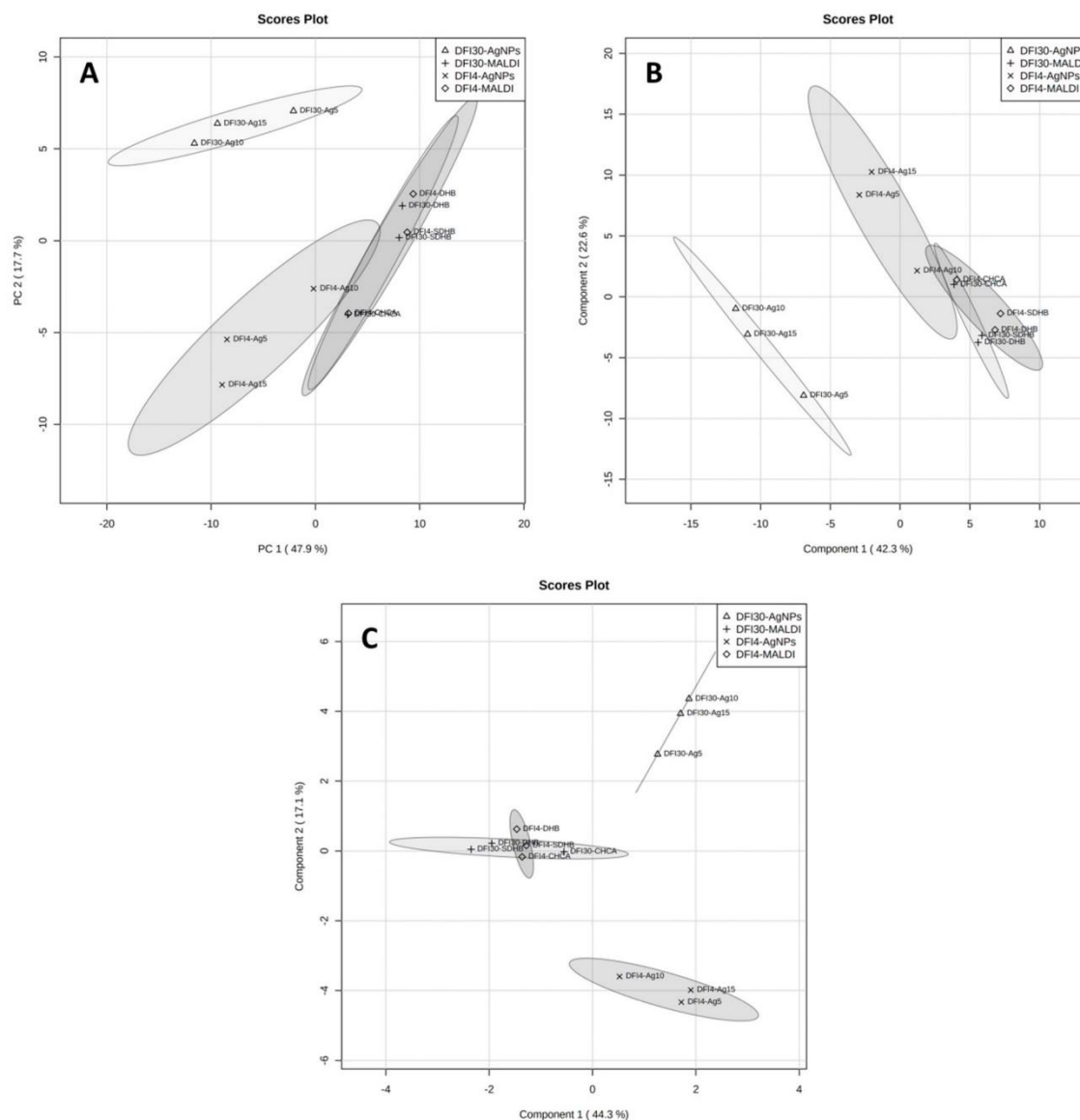


Figure 5. Graphical representation of statistical analysis of MS data from MALDI and SALDI/NALDI experiment with four groups: PCA component 1 vs. 2 (A), PLS-DA component 1 vs. 2 (B), and sPLS-DA component 1 vs. 2 (C).

In the case of SALDI wafers coated with silver nanostructures using the CVD technique, only three groups of high-intensity signals originating from Ag^+ to Ag_3^+ silver clusters are present on the spectra, while in the MALDI method there is a significant number of peaks originating from the applied matrix (Figure S3).

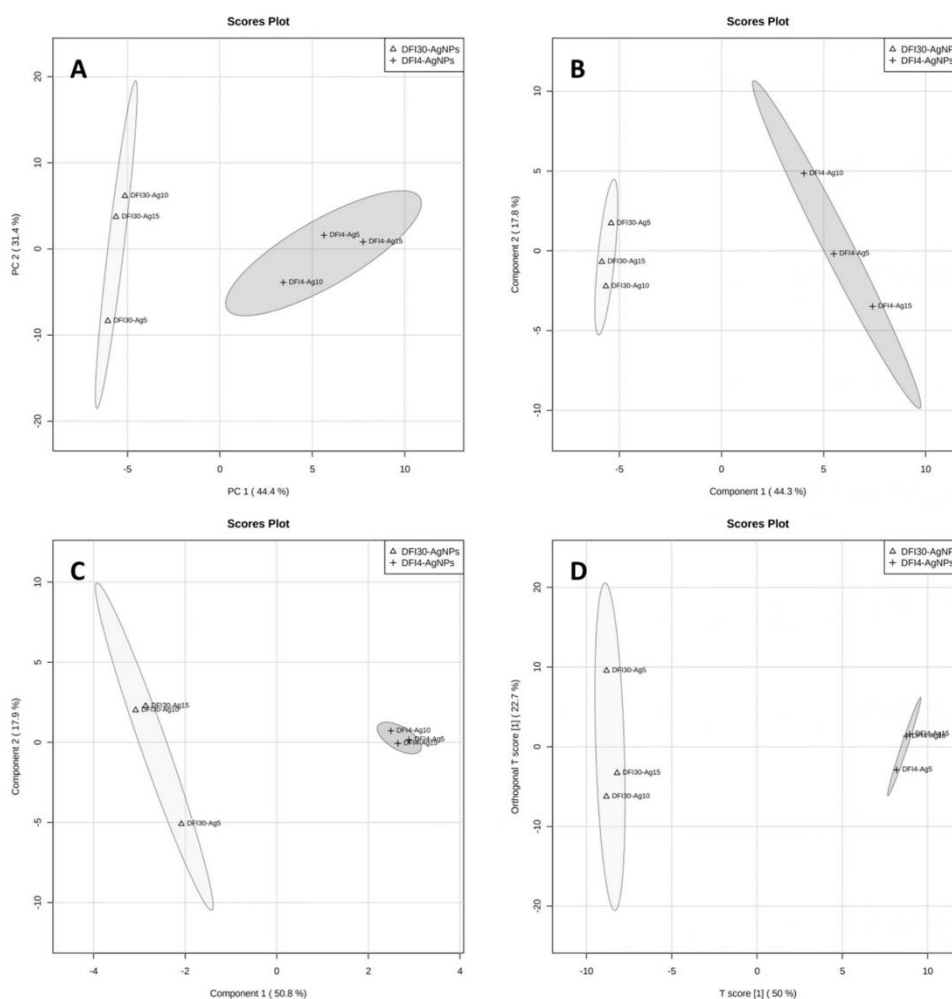


Figure 6. Graphical representation of statistical analysis of MS data from SALDI/NALDI experiment: PCA component 1 vs. 2 (A), PLS-DA component 1 vs. 2 (B), sPLS-DA component 1 vs. 2 (C), and OPLS-DA (D). Dark-grey area represents data for DFI-4 strain while light-grey represents DFI-30 strain.

4. Discussion

The clustering of *E. coli* lipid extracts observed during the study mainly depends on the matrix used (MALDI method) and may be due to interference of peaks from the matrix. It is known that standard MALDI matrices are low-molecular-weight organic acids that produce a variety of matrix-related ions, which cause interfering signals in the low-mass range as a consequence [25]. Indeed, analysis of the blank MS spectra performed for SALDI targets and MALDI matrices showed that the latter contain significantly more signals than the former. Moreover, extracts analyzed using the CHCA matrix, characterized by a significantly higher number of signals from the matrix itself (307, $S/N \geq 3$), showed a more distinctive MS pattern than the other two matrices. This effect may also explain the high similarity between the MS profiles of the tested *E. coli* strains when using the CHCA matrix. Such phenomenon was not observed in the case of SALDI targets with different AgNP sizes, where differences in the number of signals derived from blank MS spectra were comparatively minor. On the contrary, both investigated bacterial strains were clearly

divided regardless of the silver particles' size. The observed phenomenon may be due to both lower background noise and more MS signals generated from the sample itself (ca. two times more compared to the MALDI mode). Similar results were obtained for metabolomic profiling of two mold species using the SALDI target, with gold nanoparticles obtaining more than three times as many signals and matched metabolites compared to the MALDI method [26]. As Hansen and colleagues noted [27], the nanosubstrates employed in SALDI-MS experiments offer a clear background in the low m/z range, thus favoring the analysis of small molecules such as lipids (mostly < 1000 Da). In addition, the observed higher number of peaks in the SALDI variant may arise from its higher efficiency in the detection of lipids with low ionization efficiency, such as neutral lipids. Indeed, silver nanoparticles were proven to be effective substrates for analyzing a wide range of the lipids such as glycerolipids, sterol lipids, fatty acids, and sphingolipids in positive ionization mode [28]. Furthermore, it was shown that the detection of neutral lipids, which can be challenging in conventional MALDI-MS measurements due to ion suppression caused by phospholipids, can be easily performed using the SALDI method [29,30]. The mass spectrometry imaging of *Bacillus subtilis* colony biofilms by Lukowski and colleagues [31] revealed that different ionization techniques (metal-assisted vs. MALDI) led to the identification of a unique subset of molecular species, where the application of the Au-covered targets enabled the identification of more small molecules and neutral lipids compared to the use of the DHB matrix.

Chemical vapor deposition (CVD) belongs to the targets fabrication strategy that allows exact control of the nucleation and growth of metallic layers with strictly defined structure, morphology, physicochemical and biological properties, and is also characterized by high purity [22]. Indeed, all SALDI targets obtained were characterized by uniform coatings with silver particles of similar shapes and did not exhibit the sweet-spot phenomenon. Such characteristics may explain the similar classification of lipid extracts depending on the bacterial strain, despite the observed differences in the size of AgNPs between the prepared SALDI targets. It may also suggest that the morphology of the silver substrate had a more significant effect on SALDI-MS performance than the size of the AgNPs concerning lipid profiling, which is consistent with the common claim that the nature and morphology of the substrate have a significant effect on the ionization efficiency of the analytes [32]. Nevertheless, the size-dependent phenomenon of the ionization efficiency of small-molecule compounds during SALDI-MS analysis has been previously reported in the literature and found to be significant [33]. As for silver, Ding et al. [34] found that AgNPs of various sizes had different ionization efficiencies for amyloid-beta peptides. The authors noted that the small-size AgNPs (2.8 ± 1.0 nm) showed the best SALDI-MS performance, where ion yields were nearly two- and fourfold higher than those from the 12.8 ± 3.2 nm and the 44.2 ± 5.0 nm AgNPs, respectively. The observed phenomenon was considered to be related to differences in the specific surface area of AgNPs—the larger the surface area, the more peptide molecules, and laser energy could be absorbed.

Our study showed that a 2 h incubation with the addition of an antibiotic (cefotaxime) was sufficient to separate resistant and susceptible *E. coli* strains based on their lipidomic profiles. Similarly, Liang et al. [1] investigated that Gram-negative bacteria belonging to the ESKAPE pathogens (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *P. mirabilis*) could distinguish between colistin-susceptible and -resistant strains using MALDI-MS analysis based on the presence of colistin-resistance-associated ions. The same authors in another paper [4] showed that antimicrobial-resistant ESKAPE isolates are chemically distinct from their susceptible counterparts. This led to clustering together colistin-resistant *K. pneumoniae* and *A. baumannii* strains based on the mass spectra of their glycolipids during a performed hierarchical cluster analysis. It should be mentioned that both works are related to the detection of the antimicrobial resistance that is mediated via direct modification of membrane lipids—lipid A in this case—thus the observed differences in the profiles of the lipids studied were limited to mass shifts in the lipid A molecules. Nevertheless, Schenk and colleagues [35] found a correlation of lipid profiles with sensitivity to antibiotics other than

polymyxins. The authors revealed a variation in lipid content (altered distribution of the fatty acids and glycerophospholipids) in the closely related strains of *E. coli* exposed to sublethal concentrations of norfloxacin using MALDI coupled to Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR). In turn, Xie et al. [36] used multiple reaction monitoring profiling (MRM profiling) of lipids to distinguish strain-level differences in microbial resistance in *E. coli* that had been cultured with and without beta-lactam antibiotic exposure. The MRM-profiling method was able to distinguish between resistant and non-resistant *E. coli* strains treated with amoxicillin or amoxicillin with clavulanate at $1/2\times$ and $1\times$ the minimum inhibitory concentration. Our study showed that cefotaxime-resistant and -sensitive *E. coli* strains also show a different lipid pattern when cultured in the presence of this antimicrobial agent. However, using a matrix, especially CHCA, hinders the grouping of the strains. On the contrary, using the SALDI technique with silver particle coating overcame the obstacles encountered during MALDI analysis regardless of the size of the AgNPs, which was possible by obtaining homogeneous silver films using the CVD technique. To our knowledge, this is the first work related to applying the SALDI technique for distinguishing sensitive and resistant bacterial strains based on their lipidomic profiles. The SALDI method outperformed the MALDI technique in small molecule analysis for closely related strains' discrimination. It should be further investigated to find a suitable diagnostic tool for early drug resistance detection.

5. Conclusions

Lipidomic profiles of cefotaxime-resistant and cefotaxime-sensitive *E. coli* strains obtained by laser desorption/ionization mass spectrometry analysis differ as influenced by the presence of the antibiotic in the culture medium. MALDI mode clustering of lipid profiles is hampered by interference with matrix-derived ions, particularly CHCA ions. This obstacle was overcome by using the SALDI technique, in which CVD-fabricated silver nanoparticle targets allowed the strains to be clearly grouped regardless of the size of the AgNPs. SALDI AgNP targets obtained via the CVD method are characterized by low background noise and more MS signals derived from the samples, most probably related to the higher lipid ionization efficiency. AgNP substrates obtained using the CVD method were used for the first time for distinguishing closely related bacterial strains based on their lipidomic profiles and demonstrate high potential as a future diagnostic tool for detection of antibiotic susceptibility.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/antibiotics12050874/s1>, Figure S1. PLS-DA VIP scores for MS data from SALDI/NALDI (A), MALDI (B) experiments and with the use of two groups (C) and four groups (D) for all data. Figure S2. ANOVA box plots for the nine most discriminating m/z values obtained for DFI4 and DFI30 bacterial lipids samples with use SALDI and MALDI approach. Figure S3. Overview of blank MS spectra performed for SALDI targets with CVD-applied silver nanostructures (A–C) and MALDI matrices (D–F).

Author Contributions: Conceptualization, M.Z. and P.P. (Paweł Pomastowski); methodology, A.A., A.R., P.P. (Piotr Piszczek); validation, A.A.; formal analysis, E.M., A.A., J.W.-S.; writing—original draft preparation, A.A. and M.Z.; writing—review and editing, P.P. (Paweł Pomastowski) and P.P. (Piotr Piszczek); visualization, A.A.; project administration, M.Z.; funding acquisition, M.Z. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz (KB 68/2019, 29 January 2019).

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the need to access the commercial software used to generate data.

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Publikacja [P7]
Supplementary materials

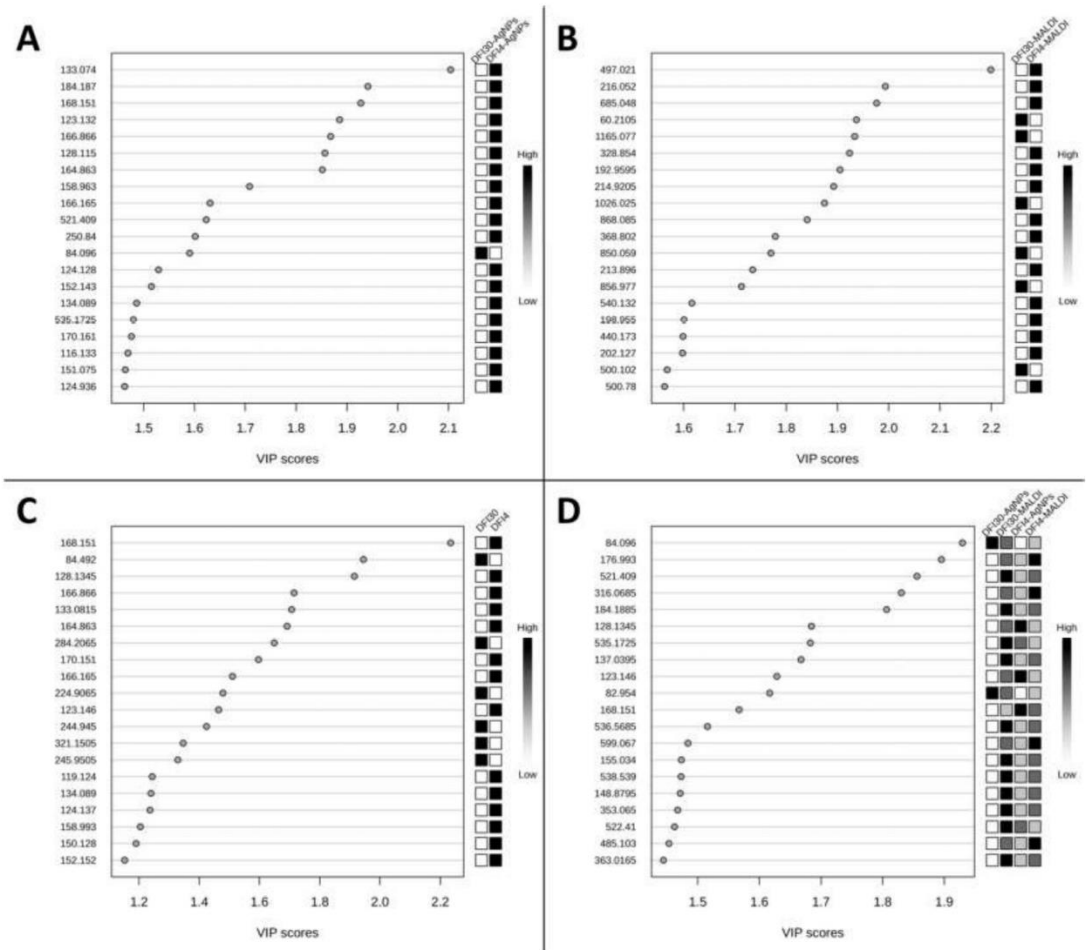


Figure S1. PLS-DA VIP scores for MS data from SALDI/NALDI (A), MALDI (B) experiments and with the use of two groups (C) and four groups (D) for all data.

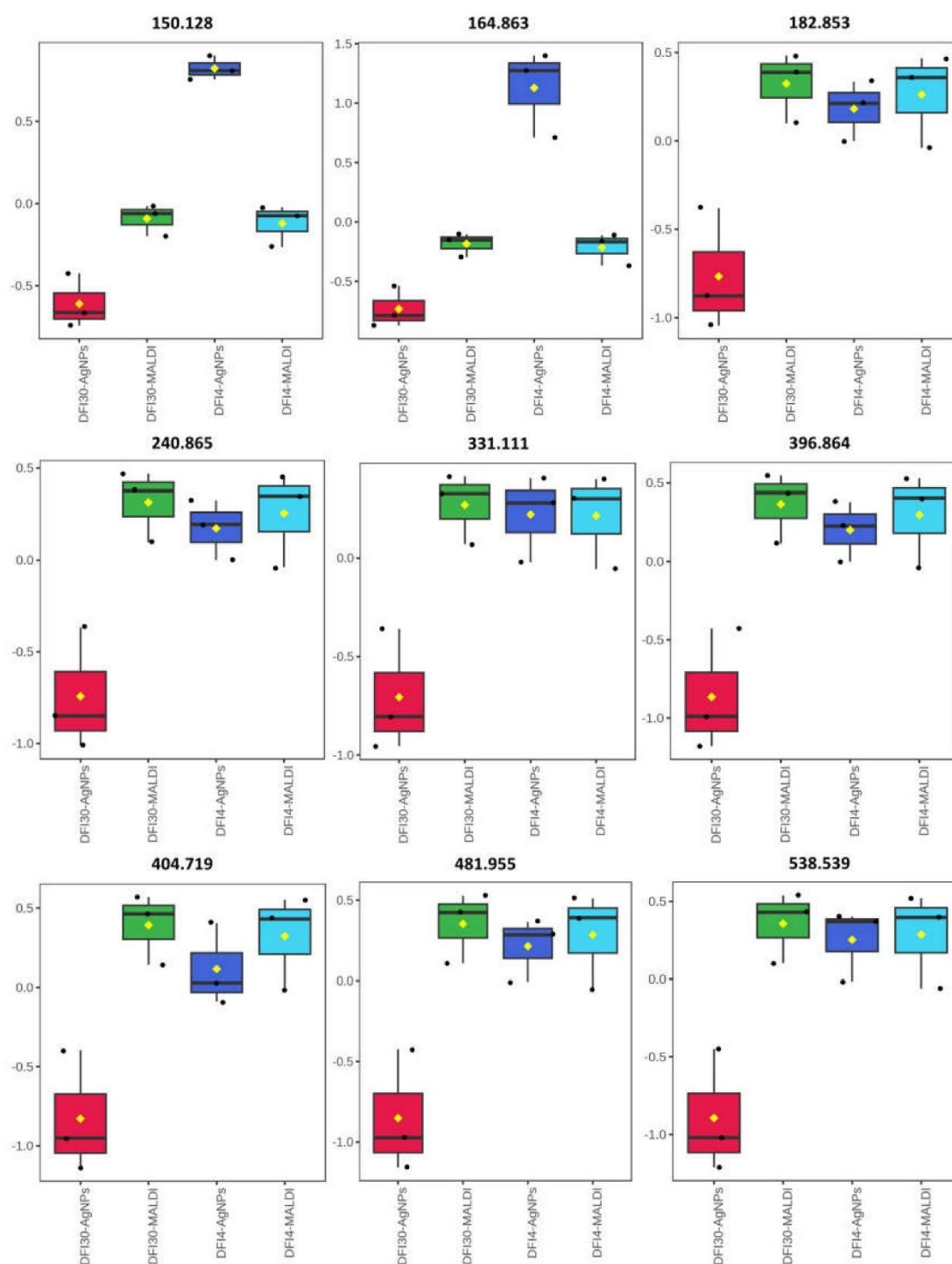


Figure S2. ANOVA box plots for the nine most discriminating m/z values obtained for DF14 and DF130 bacterial lipids samples with use SALDI and MALDI approach.

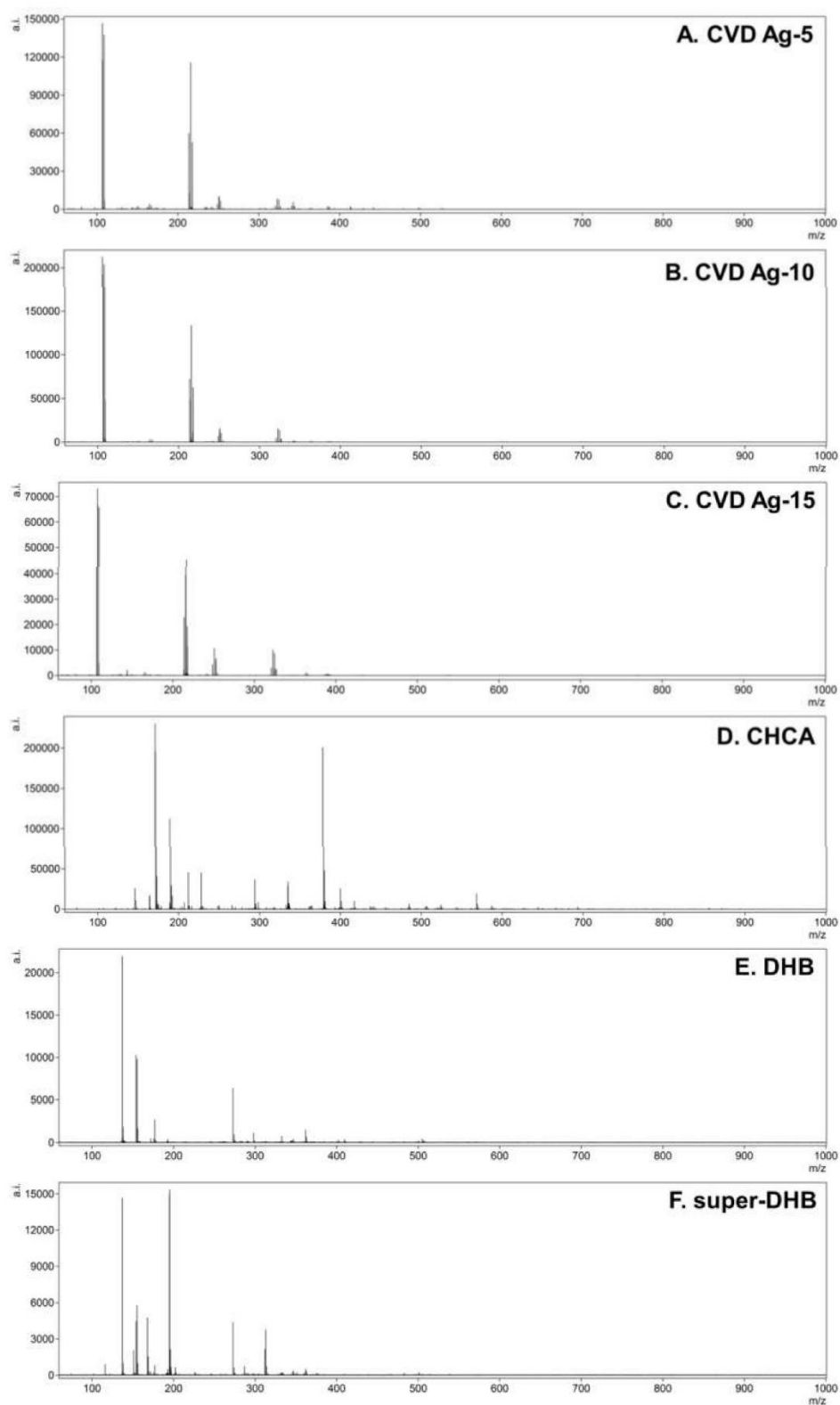


Figure S3. Overview of blank MS spectra performed for SALDI targets with CVD-applied silver nanostructures (A-C) and MALDI matrices (D-F).

Publikacja [P8]

**Advances in LDI-MS analysis: the role of CVD-synthesized silver nanoparticles
in efficient ionization of low-molecular-weight compounds**

E. Sibińska, J. Walczak-Skierska, A. Arendowski, P. Piszczek, A. Radtke, P. Pomastowski,
Advances in LDI-MS analysis: the role of CVD-synthesized silver nanoparticles in efficient
ionization of low-molecular-weight compounds **praca w recenzji**

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Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in Enhancing Detection of Low-Molecular-Weight Biomolecules

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1 **Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in**
2 **Enhancing Detection of Low-Molecular-Weight Biomolecules**

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Running title:

CVD-synthesized AgNP suitability for LDI-MS

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Abstract

In this investigation, we detail the synthesis of silver nanoparticles (AgNPs) via a precise Chemical Vapor Deposition (CVD) methodology, aimed at augmenting the analytical performance of Laser Desorption Ionization Mass Spectrometry (LDI-MS) for the detection of low-molecular-weight analytes. Employing a precursor supply rate of 0.0014 mg/s facilitated the formation of uniformly dispersed AgNPs, characterized by SEM and AFM to have an average diameter of 33.5 ± 1.5 nm and a surface roughness (Ra) of 11.8 nm, indicative of their homogeneous coverage and spherical morphology. XPS and SEM-EDX analyses confirmed the metallic silver composition of the nanoparticles, with Ag peak splitting reflecting the successful synthesis of metallic Ag. Comparative analytical evaluation with traditional MALDI matrices revealed that AgNPs significantly reduce signal suppression, thereby enhancing the sensitivity and specificity of LDI-MS for triglycerides and saccharides. Notably, the application of AgNPs demonstrated a superior linear response for triglyceride signals, with regression coefficients surpassing 0.99, markedly outperforming conventional matrices. The study further extends into quantitative analysis through NALDI, where AgNPs exhibited enhanced ionization efficiency, characterized by substantially lower limits of detection (LOD) and quantification (LOQ) for triglycerides, as opposed to saccharides. These findings underscore the potential of CVD-synthesized AgNPs to revolutionize the analytical detection and quantification of low-molecular-weight compounds in LDI-MS, presenting a novel pathway for broadening analytical applications in chemistry.

Key words: Laser Desorption Ionization Mass Spectrometry, Chemical Vapor Deposition, silver nanoparticles, triglycerides, saccharides

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40 Introduction

41 Nanoparticles (NPs) are defined as structures with at least one dimension ranging from 1 to
42 100 nanometers (nm) [1]. As the size of these particles decreases, the proportion of atoms on
43 the surface relative to those inside the particle increases, leading to significant alterations in
44 their physical and chemical properties. In comparison to their larger counterparts, NPs exhibit
45 variations in a range of characteristics, including density, solubility, spectroscopic properties,
46 melting points, surface tension, mechanical strengths, electrical and thermal conductivities,
47 magnetic responses, crystalline structures, and catalytic activities [2]. Noble metal
48 nanoparticles, particularly those of silver (Ag) and gold (Au), are of paramount importance in
49 various spectroscopic methods owing to their distinct optical properties. These nanoparticles
50 are capable of interacting with different forms of radiation, such as ultraviolet (UV), visible,
51 and infrared (IR) light, through mechanisms that include absorption, scattering, and surface-
52 enhanced processes.

53 The valence electrons of atoms located on the surface of the metal nanoparticle, known as
54 surface plasmons, can absorb electromagnetic radiation. This absorption leads to the collective
55 oscillation of these electrons, creating a coherent motion known as a *Localized Surface*
56 *Plasmon Resonance* (LSPR). The movement is influenced by the restoring forces associated
57 with the positively charged nuclei and the overall electron cloud. After the cessation of the
58 external stimulus, the oscillating electrons return to a state of equilibrium as a result of the
59 attractive Coulombic forces between electrons and nuclei. To observe this phenomenon, the
60 incident electromagnetic wave on the plasmonic nanoparticle must possess a frequency that
61 matches the vibration frequency of the localized surface plasmons [3–5]. This process is
62 manifested by an increase in light extinction (absorption, scattering), as well as the creation of
63 strong electromagnetic fields around the nanoparticle, which are responsible for the
64 effectiveness of photocatalytic properties. Absorption of radiation with energy equal to or
65 greater than the band gap energy causes the induction of electronic transitions in plasmonic
66 metals (interband and intraband transitions). These electrons constitute charge carriers referred
67 to as hot electrons, which can migrate to all available unoccupied states, including molecules
68 adsorbed on the nanoparticle surface, through an indirect charge transfer pathway. This
69 contributes to the direct photocatalysis of analytes adsorbed on the surface [6]. The frequency
70 can be adjusted by selecting the size, shape, and material type of the nanoparticles. In the case
71 of small-sized silver and gold nanoparticles, luminescence tends to shift towards the blue
72 spectrum, while larger ones exhibit a shift towards the red spectrum. These parameters allow
73 for control over resonance and adaptation to desired wavelengths for planned applications [7].

74 The unique optical properties of metallic NPs find application in various analytical techniques
75 for detecting biological molecules. In surface spectroscopy, NPs serve to amplify spectroscopic
76 signals, enhancing sensitivity and enabling the detection of substances even at low
77 concentrations. This is exemplified in techniques such as surface-enhanced Raman scattering
78 (SERS) [8], metal-enhanced fluorescence (MEF) [9], and surface-enhanced infrared absorption
79 (SEIRA) [10]. In mass spectrometry, metallic NPs act as matrices in nanoparticles-based Laser
80 Desorption/Ionization (NALDI), facilitating rapid and sensitive analysis of chemical
81 compounds [11]. This technique presents a promising alternative to Matrix-Assisted Laser
82 Desorption/Ionization (MALDI). Organic matrices employed in MALDI exhibit strong
83 absorption of laser radiation, resulting in spectra cluttered with numerous signals originating
84 from the matrix itself, its fragments, and various adducts. This abundance of signals
85 complicates the interpretation of spectra, particularly for molecules with a mass below 500 Da

[12]. There is a compelling argument for the application of noble metal nanoparticles in LDI techniques, given their (i) relatively high tolerance to salts, (ii) elimination of suppression from matrix-related ions, (iii) generation of highly reproducible signals, and (iv) potential for internal calibration [11]. Several papers have demonstrated the application of silver nanoparticles (AgNPs) in detecting various low-mass compounds in LDI MS, providing evidence of their value in the detection and quantification of pure compounds such as nucleosides and nucleic bases [13], carboxylic acids [14], lipids [15], drug metabolites [16] and the analysis of complex biological tissues with the use of MS imaging [17].

The burgeoning interest in silver nanoparticles has placed the spotlight on their synthesis, stabilization, and characterization, marking these areas as hotbeds of intensive research in recent years. A pivotal aim in the advancement of nanotechnology is the continuous improvement and standardization of methods for nanomaterial synthesis and surface modification. Such efforts are crucial for generating materials that are not only more stable but also exhibit improved uniformity in shape and particle size. Metal nanoparticles are produced via various methods, broadly categorized into two main strategies: the top-down (destructive) method and the bottom-up (constructive) method. The bottom-up approach is particularly noteworthy for its enhanced control over the formation and chemical composition of the final product, a vital aspect for crafting materials with the desired specific physicochemical properties [18]. This category also encompasses chemical vapor deposition (CVD), a technique that, according to our prior studies, shows promise in the synthesis of silver nanoparticles for the analysis of low-molecular-weight compounds in mass spectrometry [12,15]. In the CVD process, nanoparticles are formed on a substrate by the sublimation of a precursor. This precursor is transported in gaseous form to the substrate, where it undergoes chemical reactions—typically thermal decomposition for metals—leading to the deposition of a thin nanoparticle layer [19].

The aim of this study was to utilize the chemical vapor deposition (CVD) technique for synthesizing a uniform layer of silver nanoparticles (AgNPs) on a steel substrate, and to conduct a comprehensive characterization of the resultant system. Following this, we assessed the feasibility of employing this system in laser desorption/ionization mass spectrometry (LDI MS) for the analysis of low-molecular-weight compounds, including lipids and saccharides. Significantly, this research presents, for the first time, the application potential of AgNPs synthesized via this method in the quantitative analysis of triglycerides (TGs), marking a novel contribution to the field of analytical chemistry and nanotechnology.

Methodology

Synthesis of silver nanoparticles

Silver nanoparticle layers were synthesized using the chemical vapor deposition (CVD) method, and a hot-wall reactor was employed in all deposition processes. The precursor for AgNPs was a chemical compound with the molecular formula $[\text{Ag}_5(\text{O}_2\text{CC}_2\text{F}_5)_5(\text{H}_2\text{O})_3]$ (solid state). In all processes, 5 mg of the precursor was applied, the sublimation temperature (T_v) was set at 240°C, the deposition temperature (TD) was 290°C, argon was used as the carrier gas, and the deposition time (t) was 60 min.

AgNPs layers were fabricated on steel substrates (stainless steel H17). The substrate preparation process involved degreasing its surface using Viruton Extra, enabling washing and

disinfection of steel substrates in an ultrasonic bath (3×15 min). Substrates were then rinsed in distilled water and stored in anhydrous ethanol (EtOH). Before the deposition process, the substrate was dried in a stream of Ar, and the surface was activated by immersion in a 0.1% solution of trifluoroacetic acid (TFA). After drying the substrate surface in a stream of Ar, the sample was placed in the CVD reactor.

Characteristics of the obtained AgNP

In order to perform analyzes enabling the characterization of the obtained plates, nanoparticles were deposited under the same conditions on a substrate (H17 steel) with dimensions of 1x1 cm. The produced AgNPs layers were characterized using scanning electron microscopy (SEM), SEM Energy Dispersive Spectroscopy (SEM EDX), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), ultraviolet-visible diffuse reflectance spectroscopy (UV-Vis-DRS), and Raman spectroscopy.

Sample preparation

Analysis involved the use of lipid standards, including TG Internal Standard Mixture UltimateSPLASH™ ONE, 16:0 PG (phosphatidylglycerol), and 18:0 PC (phosphatidylcholine) (Avanti Polar Lipids), along with saccharides such as lactose and glucose (Sigma Aldrich). TG Internal Standard Mixture contained a mixture of 9 triglycerides dissolved in a mixture of dichloromethane: methanol (vol, 1:1). The remaining standards were in the form of powder, which was weighed and dissolved in appropriate solvents (see Tab. 1) to obtain an initial concentration of 1 mg/mL. The solutions were applied to a steel plate coated with nanoparticles in a volume of 0.5 µL. After drying, the target was inserted into the MS apparatus for measurements.

Table 1. List of compound standards used for research. Concentration refers to the initial concentration from which dilutions were subsequently made.

Compound	Formula	Molar mass	Concentration	Solvent
LIPIDS				
TG Internal Standard	14:0-13:0-14:0 TG-d5	C ₄₄ H ₇₉ D ₅ O ₆	713.66	25 (µg/mL)
	14:0-15:1-14:0 TG-d5	C ₄₆ H ₈₁ D ₅ O ₆	739.67	50 (µg/mL)
	14:0-17:1-14:0 TG-d5	C ₄₈ H ₈₃ D ₅ O ₆	767.71	75 (µg/mL)
	16:0-15:1-16:0 TG-d5	C ₅₀ H ₈₉ D ₅ O ₆	795.74	100 (µg/mL)
	16:0-17:1-16:0 TG-d5	C ₅₂ H ₉₃ D ₅ O ₆	823.77	125 (µg/mL)
	16:0-19:2-16:0 TG-d5	C ₅₄ H ₉₅ D ₅ O ₆	849.78	100 (µg/mL)
	18:1-17:1-18:1 TG-d5	C ₅₆ H ₉₇ D ₅ O ₆	875.80	75 (µg/mL)
	18:1-19:2-18:1 TG-d5	C ₅₈ H ₉₉ D ₅ O ₆	901.81	50 (µg/mL)
	18:1-21:2-18:1 TG-d5	C ₆₀ H ₁₀₃ D ₅ O ₆	929.85	25 (µg/mL)
	16:0 PG	C ₃₈ H ₇₄ O ₁₀ PNa	744.95	1 mg/mL
	18:0 PC	C ₄₄ H ₈₈ NO ₈ P	790.20	1 mg/mL
	myristic acid	C ₁₄ H ₂₈ O ₂	228.38	1 mg/mL
	stearic acid	C ₁₈ H ₃₆ O ₂	284.48	1 mg/mL
SACCHARIDES				
	glucose	C ₆ H ₁₂ O ₆	180.16	1 mg/mL
	lactose	C ₁₂ H ₂₂ O ₁₁	342.30	1 mg/mL

For selected standards, a series of dilutions were made, ensuring that the final standard solution contained 0.02 µg/mL. All prepared dilutions were plated on a steel plate coated with nanoparticles, and MALDI plate in a volume of 0.5 µL. In the case of the MALDI technique,

a ground steel plate was used and the samples were covered with two different matrices: α -cyano-4-hydroxycinnamic acid (HCCA, Bruker Daltonics, Bremen, Germany) and 2,5-dihydroxybenzoic acid (DHB, Bruker Daltonics, Bremen, Germany). The matrices were prepared by dissolving in a mixture of 50% acetonitrile, 47.5% water and 2.5% trifluoroacetic acid. The HCCA matrix concentration was 10 mg/mL and DHB was 20 mg/mL.

Sample analysis via LDI MS

LDI mass spectrometry experiments were performed in positive reflectron mode using Bruker ultrafleXtreme equipped with a SmartBeam II laser (355 nm and frequency 2 kHz). Each spot underwent a total of 10,000 laser shots. This number of laser shots was distributed among five points symmetrically positioned around the center of the spot. At each point, 2,000 laser shots were conducted, employing the default random walk approach (random points with 50 laser shots each). The measurement range encompassed m/z 100-2000, with suppression typically activated for ions with m/z values lower than 80. The data was calibrated and examined utilizing FlexAnalysis (version 3.3) employing a centroid peak detection algorithm. Mass calibration for a plate coated with silver nanoparticles relied on internal standards, utilizing signals from ^{107}Ag and ^{109}Ag ions along with their Ag_2 and Ag_3 adducts (quadratic mode). For the MALDI technique, cesium iodide mixed with a DHB matrix was employed. For quantitative analyses, four spectra were collected for each sample dilution.

Results

Characterization of AgNP

Employing a precursor supply rate of 0.0014 mg/s in the chemical vapor deposition (CVD) reactor (totalling 5 mg over 60 minutes) led to the development of dispersed silver nanoparticle (AgNP) nuclei. Scanning Electron Microscopy (SEM) studies revealed a monolayer of AgNPs consisting of particles with an average diameter of 33.5 ± 1.5 nm, uniformly distributed across the entire surface (Fig. 1A). Atomic Force Microscopy (AFM) further corroborated the monolayer's morphology, showcasing individual nanoparticles (Fig. 1B). Both techniques confirmed the spherical shape of the nanoparticles. Analysis of the roughness parameters over a surface area of $1 \mu\text{m}^2$ yielded an average roughness (R_a) of 11.8 nm and a root mean square roughness (R_q) of 14.0 nm. SEM Energy Dispersive X-ray (EDX) analysis verified the presence of AgNPs on the substrate surfaces (Fig. 1C). X-ray Photoelectron Spectroscopy (XPS) examinations of the layer's surface indicated it is composed of metallic silver grains (Fig. 2), with characteristic peaks such as C(1s), O(1s), and Ag(3d) being observed. The Ag(3d_{5/2}) and Ag(3d_{3/2}) peaks appeared at binding energies of 368.26 eV and 374.27 eV, respectively, and the 6 eV splitting of the Ag 3d doublet confirms the formation of metallic silver [20]. High-resolution analysis of the Ag 3d_{5/2} and Ag 3d_{3/2} peaks indicates the potential presence of Ag 3d oxide traces (Fig. 2C), which may be attributed to factors such as residual undegraded precursor materials or incompletely decomposed trifluoroacetic acid species (Fig. 2D). The Ultraviolet-Visible Diffuse Reflectance Spectroscopy (UV-Vis-DRS) spectrum of the silver nanoparticles deposited on steel substrates is shown in Figure 3A. The absorption spectrum displays a subtle band at 420 nm, highlighting the need for careful interpretation. The formation of nanoparticles involves concurrent nucleation and growth of crystals, a critical aspect to consider when analyzing the spectrum. Raman spectroscopy data (referenced in Fig. 3B) reveal the presence of signals around 670 and 1255 cm^{-1} . These findings suggest that the

laser radiation employed in Laser Desorption/Ionization (LDI) technology is scattered by the silver nanoparticles, a phenomenon that underpins the analysis of these signals.

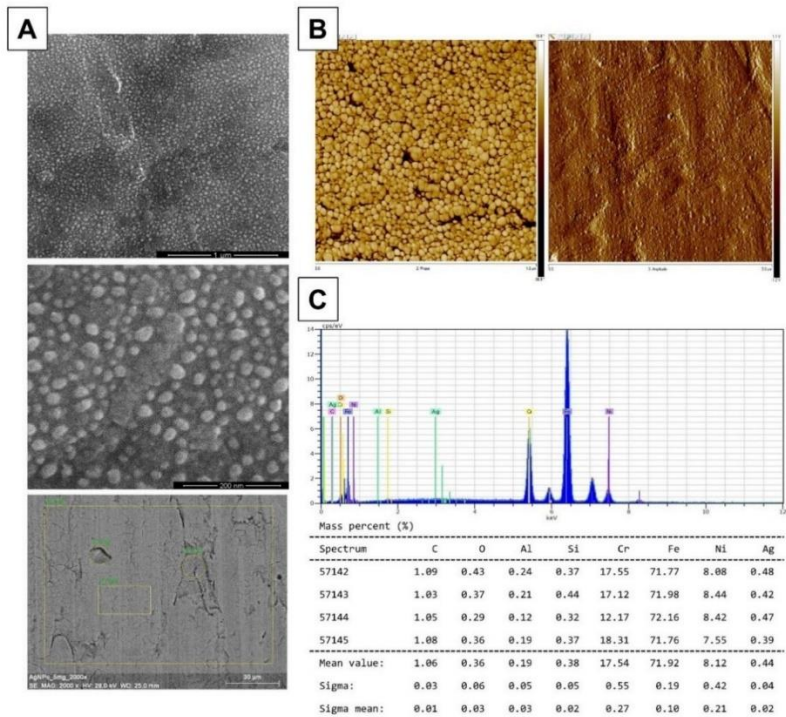


Figure 1. A - The scanning electron microscope (SEM); B - Atomic force microscopy (AFM) images of the AgNPs layer deposited on the surface of stainless steel (H17) using CVD technique. C - SEM-EDX analysis of the AgNPs layer.

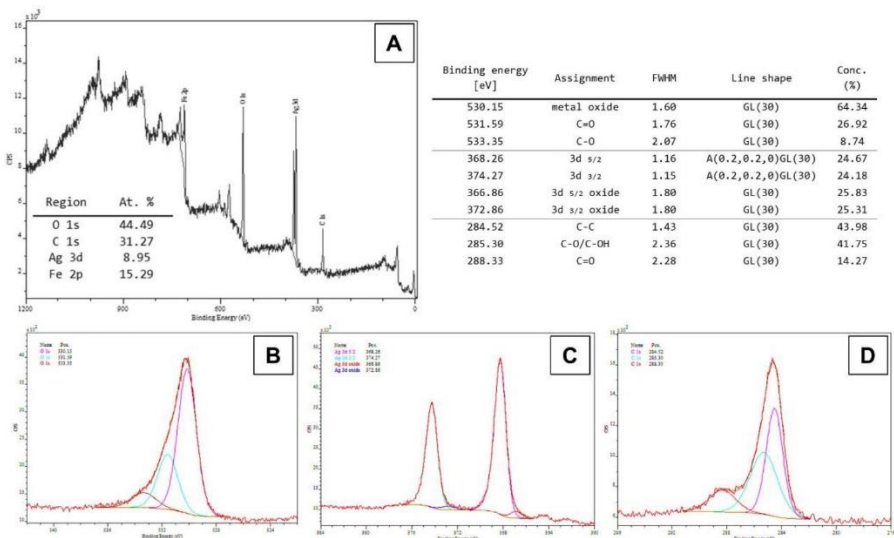


Figure 2. A - X-ray photoelectron spectroscopy (XPS) wide-scan spectrum of Ag-NPs layer obtained at -5 V. B-D - Deconvolution XPS peaks of regions: O 1s, Ag 3d, C 1s. Table presents surface chemical composition of AgNPs. Data processing was performed using the CasaXPS program, a Shirley baseline was used to cut off the background, and the signals

209 were decomposed into mixed Lorentz and Gaussian lines. In the case of metallic states, an asymmetric function was used for
210 simulation. Multiplet structures were simulated based on the models included in this publication [21].

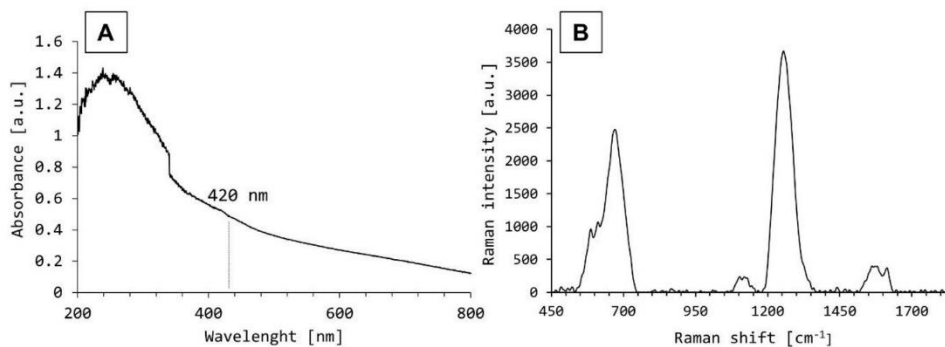


Figure 3. A - UV-Vis-DRS spectrum and B - Raman spectrum of AgNPs layer.

Comparison of signal suppression in MALDI and AgNPs based LDI

Mass spectra of the synthesized AgNPs layer and the most commonly utilized matrices in the MALDI technique were recorded across a mass-to-charge ratio (m/z) range of 100 to 2000 in positive ion mode (refer to Fig. 4). On average, the AgNPs produced 33 (± 8) signals, a figure markedly lower than that observed for standard matrices: HCCA (α -Cyano-4-hydroxycinnamic acid) generated 64 (± 8) signals, and DHB (2,5-dihydroxybenzoic acid) produced 46 (± 6) signals. Notably, for the majority of signals from AgNPs, the intensity was below 20% of the most intense signal ($m/z = 215.83$). A similar pattern was observed for DHB, whereas for HCCA, the intensity of most signals reached at least 20% of the highest signal intensity. The signals emanating from the nanoparticles were predominantly identified as clusters of silver isotopes (^{107}Ag and ^{109}Ag) and their adducts with Na, K, and NH_4 ions (detailed in Fig. 4B, with further information in Supplementary Table S1). In contrast, organic matrices demonstrated a propensity for cluster formation (including 2M, 3M, 4M, $\text{M}^+[\text{alkali ions}]$, etc., where M represents the matrix) and fragmentation ($\text{M}-\text{H}_2\text{O}$, $\text{M}-\text{COOH}$, etc.), resulting in fewer than half of all signals being identified (as listed in Table S1).

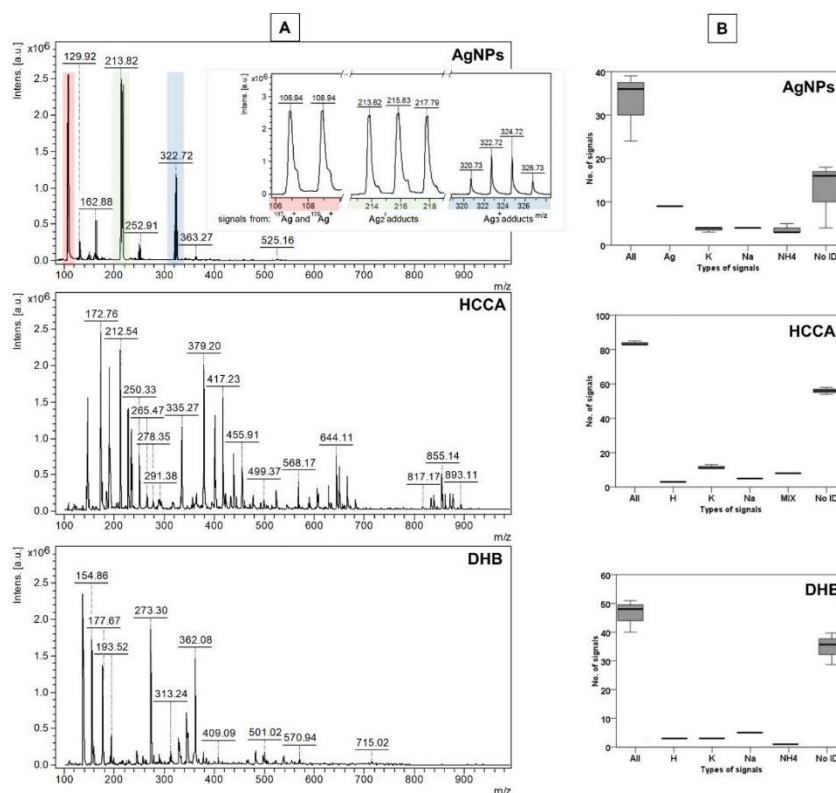


Figure 4. A – Comparison of mass spectra generated by the surface covered with AgNPs and the matrices used (HCCA, DHB). B – A box plot showing the average number ($n = 3$) of all signals recorded in individual mass spectra, the identified types of adducts with Na^+ , K^+ , NH_4^+ , H^+ or with at least two different ions (MIX), and not identified signals (No ID).

Qualitative analysis of standards via NALDI

To assess its potential for detection, the AgNPs layer, synthesized through the CVD technique, was tested in the LDI technique by recording mass spectra of standards. Low-molecular-weight compounds belonging to various classes of lipids (PC, PG, TG, stearic acid, myristic acid) and saccharides (glucose, lactose) were used as standards.

Fig. 5 displays the recorded spectra along with identified signals arising from adducts of the analyzed compounds with other ions. Notably, none of the analyzed compounds exhibited an adduct with a proton, a characteristic feature of the standard MALDI technique. Instead, all analyzed compounds appeared in the spectra as adducts with sodium ions, albeit with varying signal intensities. In comparison to internal silver signals originating from the plate surface, the most intense sodium adducts were recorded for PG, some TG, and glucose, and the lowest for PC. However, it's important to note that the initial concentration of triglycerides was significantly lower, ranging from 8 to even 40 times less than the concentrations of the other standards. Adducts with potassium ions were present in the spectra of compounds, except for PC. However, in most cases, they were barely noticeable against the background noise. Relatively high intensity of these signals was observed for PG and 4 out of 9 TGs. For TGs, signals of analytes with a single ^{13}C isotope were also observed. Only on the spectra of fatty acids and saccharides adducts with silver ions (both isotopes ^{107}Ag and ^{109}Ag) were present.

250 Their intensity varied; in the case of fatty acids, it was significantly higher for stearic acid, and
 251 in the case of sugars, for the monosaccharide - glucose.

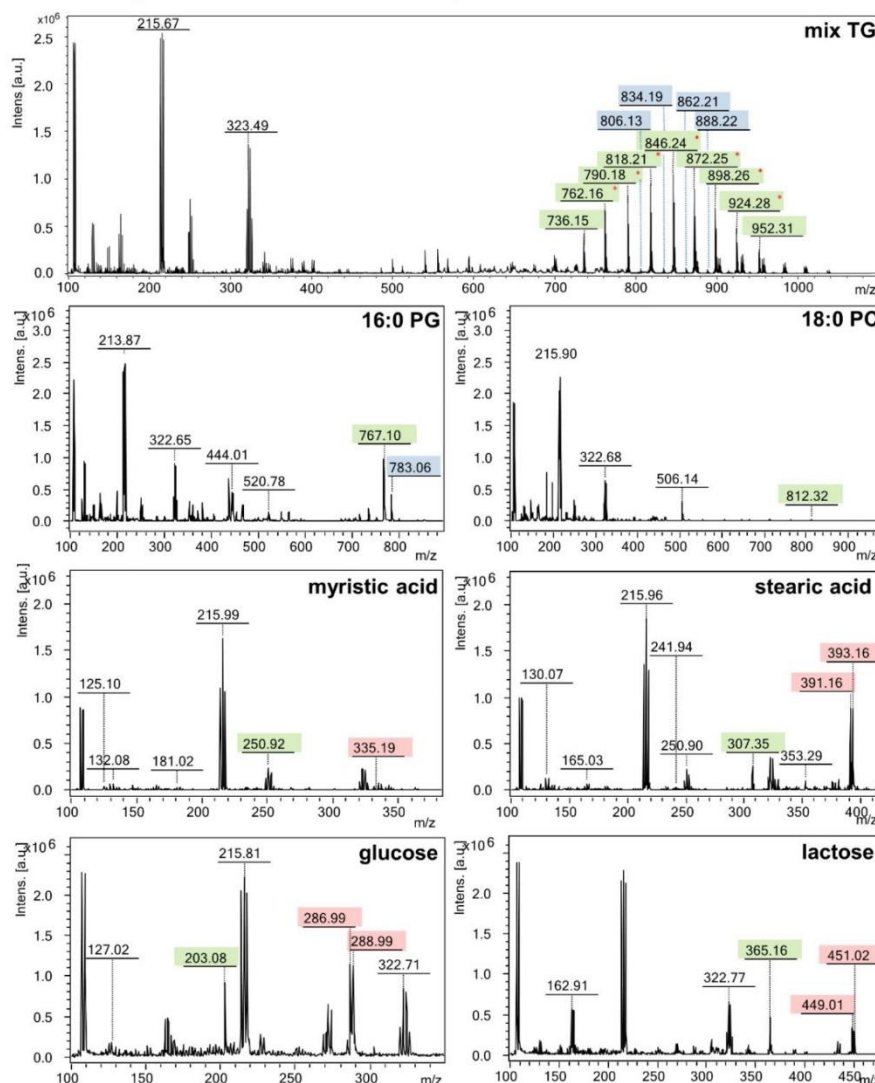


Figure 5. MS spectra recorded for the tested standards with the use of AgNPs synthesized by the CVD technique. The colors indicate the signals coming from the adducts of the analyzed compounds with sodium ions (green), potassium (blue) and silver isotopes (red); (red)* reports that this analyte was also identified with a single ^{13}C isotope.

In the presented study, fragmentation spectra were recorded for lipid molecules (triglycerides, phospholipids, fatty acids). Supplementary Table S2 depicts the identified molecular fragments of triglycerides, phospholipids, and fatty acids containing sodium, silver, and potassium adducts. The analysis encompasses various types of adducts, facilitating a comprehensive understanding of the ionization and fragmentation processes of these compounds in mass spectrometry techniques.

In the realm of triacylglycerol species, a diverse array exists, each characterized by the total length and saturation level of its acyl chains. Isobaric TAGs, while sharing the same number

of carbon atoms and double bonds, exhibit variations in the length, position, and configuration of their acyl chains. These chains occupy three distinct positions within the TG structure, denoted as the *sn*-1, *sn*-2, or *sn*-3 positions. Regiospecific analysis is primarily concerned with determining the orientation of the acyl chains at the *sn*-1(3) and *sn*-2 positions. Mass spectral fragmentation emerges as a valuable tool in elucidating the stereochemistry and regiospecific identification of TGs [22].

Figure 6 depicts the proposed fragmentation pathway of the sodium adduct of the triglyceride molecule $[M + Na]^+$ at m/z 736.15, corresponding to 14:0-13:0-14:0 TG-d5. Molecular ion underwent fragmentation, resulting in a peak at m/z = 486.88, corresponding to the loss of the 14:0 fatty acid as $[M - R_1COO]^+$, located at the *sn*-1 position. The loss of fatty acids at the *sn*-2 position is energetically less favorable compared to *sn*-1 and *sn*-3 positions, attributed to molecular steric properties [22,23]. Additionally, a peak at m/z = 508.81 was observed, corresponding to the loss of the 14:0 fatty acid located at the *sn*-3 position and the attachment of a sodium ion $[M - R_3COO + Na]^+$. Fragments identified at m/z 197.34 and 211.75 correspond to the acyl group of tridecanoic acid ion $[R_2CO]^+$ and acyl group of myristic acid ion $[R_1CO]^+$, respectively. Furthermore, a peak at m/z = 265.18 was observed, corresponding to the molecular sodium adduct of methyl ester of myristic acid $[C_{13}COOCH_3 + Na]^+$. The ion detected at m/z 392.68 can be described as the $[R_1CO + 74 + {}^{109}Ag]^+$ ion, corresponding to the acyl group of myristic acid and glycerol (mass 74 corresponds to $C_3H_6O_2$ molecule) along with the ${}^{109}Ag$ adduct [9]. Silver-containing molecular fragment adducts were also observed in the spectrum: m/z = 592.59 $[M - R_1COO + {}^{107}Ag]^+$, m/z = 618.98 $[M - R_1COOH + {}^{109}Ag + Na]^+$, and m/z = 700.25 $[M - R_1COOH + {}^{107}Ag_2]^+$, resulting from bond cleavage upon laser irradiation of the triglyceride molecule. Additionally, silver ions at m/z 109.57 and m/z 647.17 were observed in the NALDI spectrum, corresponding to ${}^{109}Ag^+$ and ${}^{109}Ag_6^+$, respectively. The presence of silver ions is induced by laser irradiation, enabling silver nanoparticles to replace commonly used protonating agents in MALDI MS [23].

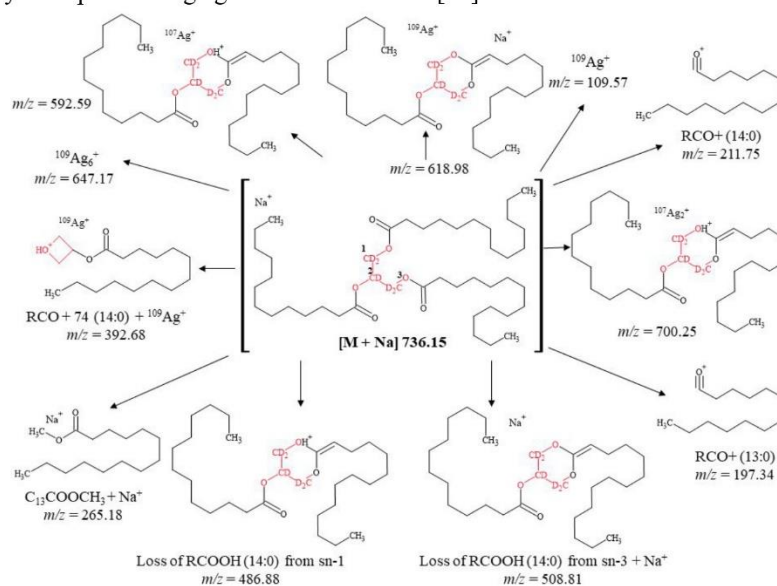


Figure 6. Fragmentation pathway of the sodium adduct of the 14:0-13:0-14:0 TG-d5.

As depicted in Fig. 7, the $[M + Na]^+$ ion of PC 18:0-18:0 was detected at m/z 812.32. Additionally, the spectrum exhibited the ion at m/z 267.15 corresponding to the moiety of

stearic acyl $[M+RCO]^+$. In MALDI mode, the most characteristic ion of lipids compounds containing choline in their structure was observed at m/z 184, corresponding to choline phosphate $[C_5H_{15}NPO_4]^+$. This ion serves as a "fingerprint" of molecules in the PC class [24,25]. Through NALDI, phosphatidylcholine fragmentation was achieved. Although the ion at m/z 184 was not observed in the spectrum, a prominent fragment at m/z 198.15 appeared, which likely constitutes a characteristic fragment for lipid compounds containing choline. The ion at m/z 198.15 correspond to methyl phosphorylcholine $[C_6H_{16}NPO_4]^+$. The another ions at m/z 146.97 and 86.14 corresponding to sodium adduct with ethenyl hydrogen phosphate $[C_2H_4PO_4 + Na]^+$ and „dehydrocholine” $[C_5H_{12}N]^+$. The small intense signal at m/z 442.86 probably corresponding to loss of „dehydrocholine” and moiety of stearic acyl and connection of sodium adduct $[C_{21}H_{40}PO_6 + Na]^+$. Also observed in the fragmentation mass spectra was a fragment equal to m/z 468.82, which probably corresponds to the silver adduct $[C_{21}H_{44}O_4 + ^{109}Ag]^+$. The ion at m/z 505.84 correspond to $[M + H - C_{18}H_{36}O_2]^+$. Furthermore, the m/z ions 606.87 and 628.80 corresponding to $[M - C_5H_{15}NPO_4]^+$, $[M - C_5H_{15}NPO_4 + Na]^+$ resulted from the neutral loss methyl phosphorylcholine and neutral loss methyl phosphorylcholine with connection sodium adduct, respectively. Peak at m/z 753.72 corresponded to $[M - C_3H_{10}NP + Na]^+$ (loss of trimethylamine).

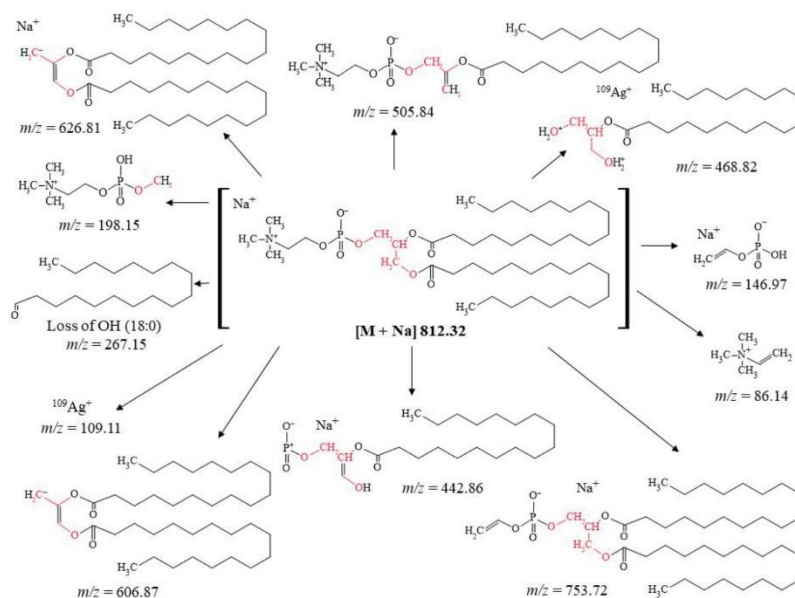


Figure 7. Fragmentation pathway of the sodium adduct with PC 18:0-18:0.

Comparison of AgNPs assisted LDI and MALDI in TG analysis

The Matrix-Assisted Laser Desorption/Ionization technique employs organic compounds as matrices to mediate energy transfer to the analyte, thus aiding in its ionization. However, this approach results in the mass spectrum being crowded with numerous matrix-derived signals, particularly in lower measurement ranges. This complexity hampers the method's effectiveness for analyzing low-molecular-weight compounds, such as lipids and sugars.

To evaluate the comparative utility of the MALDI technique against the silver nanoparticle (AgNP) layer synthesized via the Chemical Vapor Deposition (CVD) technique, a mixture of

triglycerides was analyzed, with various adducts observed in the NALDI spectra within the m/z range of 700-1000. Mass spectra for dilutions of triglyceride mix standards were recorded using both NALDI and MALDI techniques. Two matrices were employed: α -Cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB). Figure 8A presents a comparison of the mass spectra for the triglycerides mixture at its initial concentration, obtained via AgNPs-assisted and matrix-assisted LDI techniques.

The intensity of signals derived from TGs, specifically those forming adducts with sodium, in spectra obtained using silver nanoparticles, was approximately 50% greater than those recorded with DHB and about 70% higher compared to HCCA. Additionally, the spectra from the nanoparticle-based approach revealed the formation of adducts between the analytes and potassium, as well as analytes incorporating a single ^{13}C isotope bound to sodium (as detailed in Fig. 8B). Such adduct signals were not detected with conventional matrices. In every instance, about half of the signals could not be identified, predominantly appearing in the m/z range of 300 - 1000. This suggests the signals may originate from adducts of triglyceride fragments with various ions (in both MALDI and NALDI spectra) or from fragments of the matrix (in MALDI spectra). For NALDI, fewer signals were identified in this range (averaging about 8, all below m/z 350) and were considered control signals (generated directly by the plate overlaid with AgNPs). The employment of organic matrices notably reduced the number of control signals, with HCCA suppressing signals up to m/z 1200 and DHB up to approximately m/z 540. The signal-to-noise (S/N) ratios for all unidentified signals in nanoparticle-assisted techniques were generally below 30-40, mirroring those observed with the DHB matrix. However, with HCCA, some signals showed significantly higher S/N values, in a few instances exceeding 100.

Figure 8C shows a comparison of signal intensities recorded for successive dilutions of the triglyceride mixture. Utilization of nanoparticles enhanced the sensitivity, allowing for the detection of lower concentrations of TGs compared to the MALDI technique. The DHB matrix had higher sensitivity than HCCA. Based on the signals of TG adducts with sodium ions calibration curves depicting the linear relationship between the intensity of the obtained signals and the concentration of the analyte were plotted. The values of regression coefficients for individual TGs and methods are summarized in Tab. 2. For the DHB matrix, this correlation was plotted (at least 3 points obtained) for 7 out of the 9 tested TGs, with each regression coefficient consistently lower than 0.905. In the case of the HCCA matrix, the relationship was plotted for only 4 TGs, with the regression coefficient found to be lower than 0.686. The results for DHB and HCCA indicate that there is no linear dependence of signal intensity on the concentration of tested triglycerides. The occurrence of such a relationship was observed only when using the AgNP-assisted method, with a R^2 of 0.986 or higher. Fig. 9 shows a comparison of the tested dependency for the three most intense TGs signals.

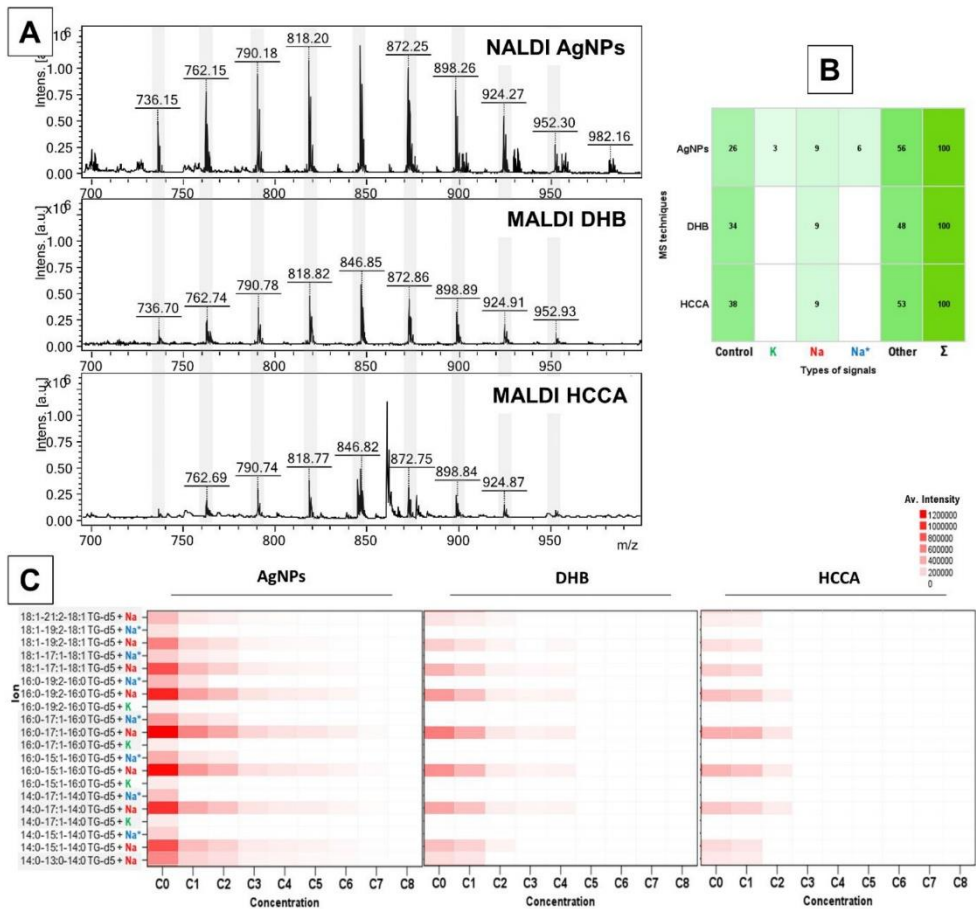


Figure 8. A - Mass spectra in the m/z range of 700 - 1000 recorded for a mixture of triglyceride standards (initial concentration - C0) using AgNPs- and matrix-assisted LDI techniques. B - Average number of signals ($n=4$) appearing in the recorded spectra of the TG mix standard, divided into those coming from nanoparticles or matrix (Control), adducts with ions and unidentified ones (others). C - Average intensity of signals ($n=4$) originating from triglyceride adducts with Na and K ions in spectra recorded using AgNPs and HCCA and DHB matrices; * marked compound containing ^{13}C .

Table 2. Regression coefficients calculated for the plotted relationship between signal intensity and triglyceride concentration.

Triglycerides	M + [adduct]	R ²		
		AgNPs	DHB	HCCA
18:1-21:2-18:1 TG-d5	929.85 + [Na]	0.986	-	-
18:1-19:2-18:1 TG-d5	901.81 + [Na]	0.990	0.902	-
18:1-17:1-18:1 TG-d5	875.80 + [Na]	0.996	0.905	-
16:0-19:2-16:0 TG-d5	849.78 + [Na]	0.997	0.899	0.590
16:0-17:1-16:0 TG-d5	823.77 + [Na]	0.993	0.889	0.432
16:0-15:1-16:0 TG-d5	795.74 + [Na]	0.997	0.882	0.596
14:0-17:1-14:0 TG-d5	767.71 + [Na]	0.996	0.866	0.686
14:0-15:1-14:0 TG-d5	739.67 + [Na]	0.992	0.718	-
14:0-13:0-14:0 TG-d5	713.66 + [Na]	0.994	-	-

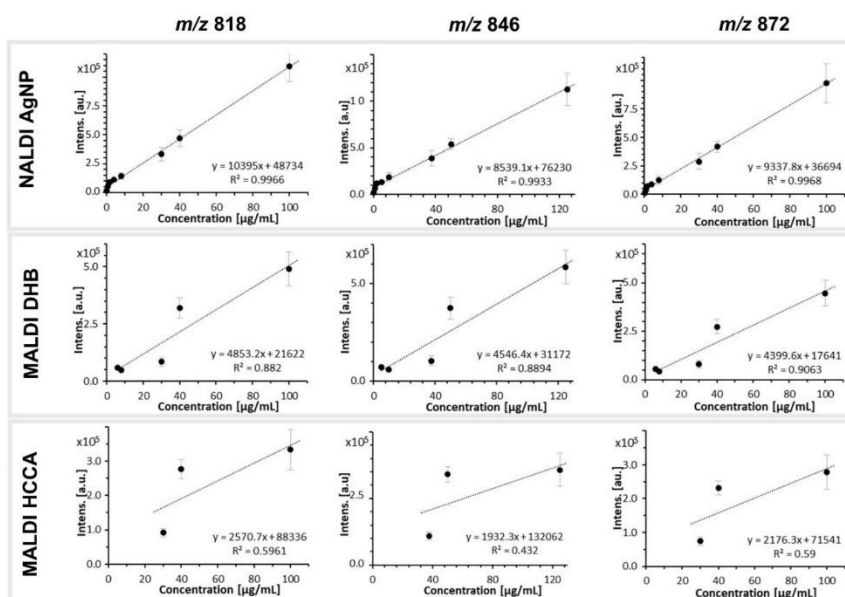


Figure 9. Example calibration curves illustrating the dependence of the signal intensity of triglycerides with m/z values of 818, 846, and 872 on their concentration, plotted based on spectra recorded using the LDI technique assisted by AgNPs and matrices (HCCA and DHB).

Quantitative analysis of standards via NALDI

The AgNPs under examination demonstrated varying capacities for ionization and, hence, the detection of the analyzed lipids and sugars. Consequently, compounds exhibiting relatively intense molecular adducts with sodium (for triglycerides) or silver ions (for saccharides) were chosen for quantitative analysis. Mass spectra for sample dilutions were acquired using LDI facilitated by the synthesized AgNPs. From these spectra, calibration curves illustrating the linear relationship between signal intensity and analyte concentration were generated, as previously described. These calibration curves were established using 6-7 data points for sugars and 8 data points for triglycerides, uniformly spanning the concentration range (see Table 3). Among the 11 tested analytes, 14 linear correlations were noted. For 12 of these 14 correlations, the regression coefficient surpassed 0.99, indicating a strong linear relationship. The use of AgNPs notably improved the ionization efficiency for triglycerides over sugars. Sodium adducts of TGs were characterized by significantly lower limits of detection (LOD) and quantification (LOQ) values (several nanograms per milliliter) compared to silver adducts of sugars (several micrograms per milliliter). The lowest recorded values for LOD and LOQ were 0.010 ± 0.003 ng/mL and 0.017 ± 0.005 ng/mL, respectively, for the 14:0-13:0-14:0 TG-d5, whereas the highest were 3.582 ± 0.454 ng/mL for LOD and 5.970 ± 0.757 ng/mL for LOQ, both for glucose.

Table 3. Details of the correlation between the signal intensity and the concentration of the tested standards obtained using the LDI technique supported by CVD-synthesized silver nanoparticles;
^aLOD – limit of detection calculated based on S/N ratio of 3; ^bLOQ – limit of quantification calculated based on S/N ratio of 5; SD – standard deviation, R² - regression coefficient

Compound	Analyte + [adduct]	Linearity range [μg/mL]	R ²	Curve equation	LOD ^a ± SD [μg/ml]	LOQ ^b ± SD [μg/ml]
14:0-13:0-14:0 TG-d5	713.66 + [Na] ⁺	0.04 – 25	0.9942	y = 19898x + 13693	0.010±0.003	0.017±0.005
14:0-15:1-14:0 TG-d5	739.67 + [Na] ⁺	0.08 – 50	0.9924	y = 14712x + 22858	0.014±0.006	0.023±0.009
14:0-17:1-14:0 TG-d5	767.71 + [Na] ⁺	0.12 – 75	0.9957	y = 11581x + 37044	0.014±0.004	0.024±0.006
16:0-15:1-16:0 TG-d5	795.74 + [Na] ⁺	0.16 – 100	0.9966	y = 10395x + 48734	0.014±0.005	0.023±0.008
16:0-17:1-16:0 TG-d5	823.77 + [Na] ⁺	0.20 – 125	0.9933	y = 8539.1x + 76230	0.012±0.003	0.021±0.005
16:0-19:2-16:0 TG-d5	849.78 + [Na] ⁺	0.16 – 100	0.9968	y = 9337.8x + 36694	0.019±0.007	0.031±0.011
18:1-17:1-18:1 TG-d5	875.80 + [Na] ⁺	0.12 – 75	0.9964	y = 10225x + 6499.6	0.026±0.009	0.043±0.015
18:1-19:2-18:1 TG-d5	901.81 + [Na] ⁺	0.08 – 50	0.9902	y = 10789x - 1680.9	0.027±0.001	0.044±0.02
18:1-21:2-18:1 TG-d5	929.85 + [Na] ⁺	0.2 – 25	0.9861	y = 11698x - 3080.4	0.064±0.020	0.107±0.33
glucose	180.16 + [Na] ⁺	4 – 500	0.9902	y = 934.12x + 31426	1.622±0.081	2.703±0.135
glucose	180.16 + [¹⁰⁷ Ag] ⁺	8 – 500	0.9908	y = 1627.5x + 30079	3.582±0.454	5.970±0.757
glucose	180.16 + [¹⁰⁹ Ag] ⁺	8 – 500	0.9847	y = 1543x + 37393	2.927±1.060	4.878±1.767
lactose	342.20 + [¹⁰⁷ Ag] ⁺	8 – 500	0.9919	y = 661.71x + 25959	1.882±0.700	3.137±1.166
lactose	342.20 + [¹⁰⁹ Ag] ⁺	8 – 500	0.9933	y = 558.38x + 22024	2.192±0.466	3.653±0.777

Discussion

For silver nanoparticles, surface plasmon resonance manifests across a broad spectral range, extending from ultraviolet to microwaves. This wide-ranging SPR capability renders AgNPs exceptionally versatile, making them a popular choice in mass spectrometry for detecting various low-molecular-weight compounds. Their sensitivity can reach femtomolar levels for certain substances. Utilizing nanoparticles in laser desorption/ionization offers a novel strategy to overcome the limitations faced in traditional MALDI techniques, which rely on organic compounds as matrices. In this study, we employed chemical vapor deposition to fabricate a uniform layer of AgNPs on a steel plate surface. We then evaluated the effectiveness of this nanoparticle layer in LDI mass spectrometry using lipid and saccharide standards and compared the performance to that of conventional MALDI techniques. Organic matrices used in MALDI exhibit significant absorption coefficients at the MS laser wavelength (Nd:YAG - 355 nm), promoting their evaporation and facilitating the transport of analyte molecules into the gas phase. In this phase, ions (such as H⁺ and Na⁺) exchange between the matrix and the analyte, enabling the analysis of the latter. However, these matrices exhibit pronounced selectivity, especially towards polar compounds, which complicates the ionization and analysis of different types of compounds, including lipids, via MALDI. As a result, the selection of an appropriate matrix for each analyte type is largely empirical. Ideally, the chosen matrix should enable the acquisition of high-resolution spectra with excellent signal-to-noise (S/N) ratios, while maintaining a moderate matrix background and minimizing analyte fragmentation [26]. The challenges outlined stem significantly from the analysis of low-molecular-weight

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4 414 compounds, such as lipids, which, due to their smaller mass, absorb radiation at shorter
5 415 wavelengths similar to the matrix, leading to increased fragmentation. Artifacts arising from
6 416 in-source decay complicate or skew the interpretation of spectra, a notable issue in lipidomics
7 417 where fragments can exhibit various isomeric forms [27]. In the MALDI technique, lipid
8 418 ionization predominantly occurs through the formation of adducts with small cations,
9 419 particularly sodium, which is ubiquitously present in samples. This interaction lowers the
10 420 threshold energy required for molecule ionization and predisposes many lipid classes to
11 421 fragmentation, especially enhancing the formation of fragment ions related to polar head
12 422 groups [27,28]. The matrix is approximately a thousand-fold excess compared to the analyte,
13 423 which means that the resulting analyte fragments may interact with matrix fragments in the ion
14 424 source, which further complicates the interpretation of the signals. The matrix is typically
15 425 present in a concentration approximately a thousand times greater than that of the analyte.
16 426 Consequently, fragments of the analyte may interact with matrix fragments within the ion
17 427 source, further obscuring signal interpretation. Additionally, the sequence in which the analyte
18 428 and matrix are applied to the target plate, along with the homogeneity of the resultant
19 429 cocrystals, significantly influences both the accuracy and repeatability of quantitative analyses
20 430 [26]. In this study, the two matrices most frequently utilized in positive ion mode—2,5-
21 431 dihydroxybenzoic acid (DHB) and α -cyano-4-hydroxycinnamic acid (HCCA)—were
22 432 employed to explore these phenomena.

23 433 Unique properties of silver nanoparticles, can be adapted to the LDI applications by choosing
24 434 the method of their synthesis, which determines their shape, size, etc. The literature indicates
25 435 that the most frequently used methods for the synthesis of silver nanoparticles for LDI are
26 436 based on chemical reduction of silver nitrate [29–31] and electrochemical growth on the
27 437 cathode surface [32,33]. Although the methods are simple and cheap, it very often leads to
28 438 uncontrolled aggregation and heterogeneous structures. Applying analytes to such a surface
29 439 may result in uneven coverage, which results in poor repeatability. To prevent this, additional
30 440 stabilizers can be used during the synthesis, which may complicate the spectra interpretation.
31 441 In turn, methods such as electron beam lithography or focus ion beam although they provide
32 442 high control over the created structure, are not suitable for routine applications due to time and
33 443 cost of process. Yagnik et al. utilized sputter coating, a form of Physical Vapor Deposition
34 444 (PVD), to achieve a uniform layer of various nanoparticles, including metals, which notably
35 445 reduced the occurrence of "sweet spots" [34]. The signal intensities they observed in the
36 446 positive ion mode for a range of analytes, including lipids, were significantly higher than those
37 447 obtained with organic matrices. Similarly, Prysiashnyi demonstrated the effective use of a
38 448 surface coated with AgNPs via sputter coating for the analysis of drugs, sugars, and lipids,
39 449 including fatty acids [35].

40 450 In the present study, we adopted a Chemical Vapor Deposition (CVD) technique, which is
41 451 recognized for its superior precision over PVD, to deposit a uniformly epitaxial layer of silver
42 452 nanoparticles on a steel plate surface. Analysis of the resultant AgNP layer confirmed the
43 453 formation of a single layer composed of evenly dispersed structures, each measuring
44 454 approximately 33.5 ± 1.5 nm in diameter. The X-ray Photoelectron Spectroscopy (XPS)
45 455 findings confirmed the metallic nature of the nanoparticles [33]. The Raman spectrum suggests
46 456 that the nanoparticles have a shape most similar to spherical [36]. The percentage of silver on
47 457 the surface was 8.95%, which indicates that the layer formed is very thin. This contributed to
48 458 difficult characterization based on, among others, UV-VIS-DRS [37], or the inability to
49 459 determine gap energy. The high content of oxygen (44%) and carbon (31%) on the surface

most likely indicates the sorption of O₂ and CO₂ from the atmosphere by nanoparticles, which could partially contribute to the oxidation of the nanoparticle surface.

Mass spectra obtained in the positive mode from the synthesized layer of AgNPs exhibited a reduced number of signals, potentially minimizing signal suppression issues commonly encountered during the analysis of low-molecular-weight compounds, in contrast to materials traditionally employed for this purpose. DHB and HCCA tend to form a large number of clusters and adducts with various ions, the general formula of which can be written as: $M_{DHB} = nM + p[M - H_2O] - xH + y[\text{alkali metal ion}]$; $M_{HCCA} = nM - xH + y[\text{alkali metal ion}]$ ($n = 0, 1, 2, 3, \dots$; $p, x, y \geq 0$) [38]. In our investigation, we noted a significant presence of silver cluster ions (Ag⁺, Ag²⁺, Ag³⁺) within the nanoparticle spectra. Additionally, adducts of sodium and, to a lesser extent, potassium were identified. This observation aligns with the findings of Guan et al. [39] who reported similar adduct formation patterns and ion preferences in their study using PVP-capped AgNPs for lipid analysis via MALDI MSI. Our data indicate a marked affinity of fatty acids' carboxyl groups for sodium ions, corroborating with predictions from theoretical frameworks like the modified Poisson–Boltzmann model, which incorporates a finite ion size of 8 Å to accommodate the hydrated Na⁺ cation diameter of 7.2–8.0 Å [40]. Density Functional Theory (DFT) and Molecular Dynamics (MD) simulations further elucidate the stability and interaction energies between fatty acids and silver ions, showcasing the dynamic and, at times, transient nature of these interactions. Specifically, secondary oxidation of AgNPs during laser desorption/ionization, as explored in the DFT study by Gallegos et al. [41], determines the stability of compounds, whereas MD simulations reveal Ag-NPs' clearance properties stemming from weak interactions with fatty acids. Moreover, the interaction energy between stearic acids and a single silver ion is quantified at -2.79 kcal/mol, which is notably lower (by an order of magnitude) than that with sodium ions, highlighting the specificity of these interactions [42]. In the context of the silver cluster-stearic acid complex, molecular orbitals (MO) analysis demonstrated a bonding interaction, which dissociated after 5 picoseconds. This interaction was characterized by an orbital overlap between the stearic acid and the silver cluster when they were approximately 2.33 Å apart. The complex dissociated as the distance increased to 10.09 Å, underscoring the critical role of spatial proximity in the stability of these molecular interactions. Based on these observations, we can conclude that sodium adducts of fatty acids are more preferable in AgNPs laser desorption/ionization than silver adducts of fatty acids, indicating a selective interaction that may influence the analytical outcomes of such processes. Furthermore, employing AgNPs for the analysis of low-molecular-weight compounds helps mitigate some of the challenges associated with matrix interference. The efficacy of the produced nanoparticles in LDI analyses was evaluated using standards from various classes of lipids (phosphatidylcholine (PC), phosphatidylglycerol (PG), triglycerides (TG), myristic acid, stearic acid) and saccharides (glucose, lactose). Detection was carried out in the positive ion mode, which is more commonly used and often offers greater sensitivity compared to the negative ion mode [43]. In all spectra signals were observed coming from metal adducts mainly with sodium, less potassium, and in the case of sugars and fatty acids, also with both silver isotopes. In the case of lipids, the best ionization was obtained for PG and TG standards. Metal nanoparticles, particularly silver nanoparticles, are highly valued for their capacity to detect neutral lipids, positioning them as among the most effective and stable matrices for lipid analysis via laser desorption/ionization mass spectrometry. AgNPs facilitate the detection of analyte adducts with silver, appearing as doublets ([M+¹⁰⁷Ag]⁺, [M+¹⁰⁹Ag]⁺), by forming weak cationic complexes with alkenes. This mechanism exhibits high

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4 506 selectivity for lipids containing olefins, such as cholesterol, triglycerides, and fatty acids [44].
5 507 Jackson et al. highlighted the use of AgNPs in positive mode lipid analysis, noting their
6 508 exceptional selectivity in ionizing triglycerides, which are typically challenging to detect
7 509 amidst more positively charged phosphatidylcholine species when organic matrices are
8 510 employed [45]. Furthermore, it was observed that triglycerides predominantly also formed
9 511 adducts with sodium when analyzed using AgNPs.
10 512 The examined in our study compounds were not detected in the proton adduct form,
11 513 potentially may attributed to their stability. In certain cases, such as TG adducts with H⁺ ions,
12 514 their stability might be insufficient to endure the flight distance in the analyzer without
13 515 noticeable fragmentation when compared to Na⁺ adducts [46]. Prysiaznyi et al. suggested that
14 516 the release of adducts with Ag⁺ ions depends on the size of nanoparticles and laser fluence [47].
15 517 They noticed that by increasing the size of silver nanoparticles or laser fluence, the release of
16 518 riboflavin-Ag⁺ connections was higher. Moreover, the formation of adducts with silver ions
17 519 depends on the amount of silver nanoparticles on the surface [48], the percentage of which was
18 520 relatively low in this study. Based on mass spectra, Sherrod et al. noticed that the preference
19 521 for binding silver to olefins depends on the number, position and configuration of double bonds
20 522 [49]. Steric factors may also explain why we observe silver ions in the case of fatty acids (single
21 523 chain) and sugars (curved ring with a large number of -OH bonds), but not in lipids composed
22 524 of two/three chains (PC, PG, TG). Moreover, the more frequently observed formation of
23 525 adducts with sodium than with silver in this study may result from their different
24 526 electrochemical potential.
25 527 The mechanism of ionization in LDI techniques using silver nanoparticles is a complex and
26 528 not fully understood process, especially since it proceeds differently in positive and negative
27 529 ionization modes. To explain the desorption process, the thermally driven mechanism
28 530 commonly used in MALDI has been broadly applied in the metallic nanoparticle assisted LDI
29 531 techniques. The laser energy disrupts the binding interaction between the sample and the
30 532 surface of the silver plate, transitioning it into the gas phase [11]. This possibility is related to
31 533 the Surface Plasmon Resonance occurring in nanoparticles, which is responsible for heating
32 534 and a strong local electromagnetic field; both effects can facilitate the ejection of atoms or even
33 535 small clusters. One of the latest theories describing the involvement of SPR postulates that in
34 536 the positive mode, sodium ions act as a secondary positive charge carrier [50]. Sodium ions are
35 537 excited to a high-energy state and react with adjacent analytes to form adducts. Positively
36 538 charged adducts are immediately transferred to the external field by electrostatic repulsion, as
37 539 a result of which they reach the mass analyzer. This theory explains why we observe mainly
38 540 sodium adducts in the spectra obtained.
39 541 Generally, AgNPs act as a photocatalyst, helping to desorb and ionize analytes. The intricate
40 542 process involves resonance-excited nanoparticles enhancing their cross-section by up to 10
41 543 times, leading to the localization of a substantial amount of light energy near the surface of
42 544 plasmonic nanostructures. This energy dissipates through processes like photon emission (light
43 545 scattering) and the induction of electronic transitions in plasmonic metals (via radiation
44 546 absorption). Following light excitation, electrons in metallic nanostructures undergo transitions
45 547 (interband and intraband), creating energetic charge carriers, known as hot carriers. These hot
46 548 electrons, possessing ample energy, can migrate to all available unoccupied states, including
47 549 molecules adsorbed on the nanoparticle surface, through an indirect charge transfer pathway.
48 550 In the excited state, the chemical bond of the molecule may elongate or even dissociate,
49 551 facilitating subsequent chemical transformations. However, if the energy deposited on the
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molecule is insufficient to overcome the reaction energy barrier, the excited adsorbate reverts back to the ground state [6]. This theory poses an interesting hypothesis that the amount of energy absorbed by nanoparticles affects the possibility of ionization or potential degradation of the molecules. This explains why fragmentation was observed for some of the tested standards (PC), while for others only ionization was observed (sacharides). Triglyceride fragmentation in the present study occurred to a small extent and the loss of one of the chains was mainly involved. While, in the case of PC 18:0-18:0 fragmentation was more noticeable, and follows the loss of an aliphatic chain or a choline-containing fragment.

Different classes of lipids have different detection limits in LDI methods, which is determined by the structure and charge of the functional groups included in them; some will be easily protonated (like PC) and will be sensitively detected as a positive ion, while others may be deprotonated and exist in the form of anions (like PA or fatty acids) [26]. This partially coincides with the results obtained in this study. Moreover, lipid analysis using the MALDI technique presents challenges, not only because of the matrix-related signals in the spectra but also due to the selectivity of matrix on the detection of individual lipids. In positive ion detection the DHB matrix is especially recommended for lipid analyses [43,51], explaining the superior results obtained for TGs with it compared to HCCA. The situation is similar in the case of the analysis of saccharides, whose ionization and detection capabilities also depend on their structure and the matrix used [52].

The application of nanoparticles allowed us to obtain linear correlations ($R^2 > 0.99$ for tested sacharides and TG standards) of signal intensity and concentration, which was not possible using the MALDI technique. The reasons for obtaining a reproducible signal can be found in the distribution of the factors responsible for ionization. The AgNP layer, synthesized through chemical vapor deposition, exhibited a homogeneous distribution of nanoparticles. Conversely, in the case of the MALDI technique, a non-homogeneous mixture of analysis and matrix is observed during the drying which results in a highly irreproducible signal. In the case of DHB, the poor spot-to-spot reproducibility is caused by the formation of nonhomogeneous needle-shaped large crystals by this matrix. The literature provides evidence that the utilization of AgNPs enables quantitative analysis of low-weight-molecules using the LDI technique, for instance, in the case of carboxylic acids [14], amino acids [53] or some medicines like trimethoprim [48].

Conclusion

The detection of low molecular weight compounds remains a significant challenge in MALDI mass spectrometry. An approach that is becoming increasingly popular is the replacement of classic matrices with metal nanoparticles, which, thanks to their different optical properties, interact with laser radiation and enable sample ionization. This approach is not only beneficial due to significantly reduced matrix background signals, but also provides greater selectivity, sensitivity and effectiveness of analytical techniques. Proposed technique for silver nanoparticle synthesis via CVD showcased the capability to detect various lipid and saccharide classes. The obtained nanostructures were characterized by high selectivity towards triglycerides, the analysis of which using MALDI is extremely difficult due to the ongoing fragmentation, which was insignificant in our studies. The obtained AgNPs, thanks to their uniform deposition on the surface of the plate, made it possible to plot linearity ranges, which is not possible using the MALDI technique. Supporting the ionization of an analyte by

nanoparticles is an extremely difficult process to characterize, however, we believe that the plasmonic and photocatalytic properties of metallic nanoparticles play a significant role in it. These findings underscore the need for further exploration into controlling the size and deposition thickness of AgNP layers via techniques such as CVD and ALD. This ongoing research promises a deeper understanding of ionization mechanisms and holds potential for broader applications of NALDI techniques in low molecular weight compound analysis.

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Data availability statement

All data generated or analyzed in this study are included in this published article.

Authorship contribution statement

E. Sibińska: Conceptualization, Methodology, Formal analysis, Investigation, Software, Validation, Writing – original draft. *J. Walczak-Skierska*: Methodology, Investigation, Software, Writing – original draft, Supervision. *A. Arendowski*: Methodology, Investigation, Software. *A. Ludwiczak*: Statistical analysis. *P. Piszczek*: Methodology, Formal analysis, Investigation, Writing – review & editing. *A. Radtke*: Methodology, Writing – review & editing. *D. Gabrys*: Project administration. *P. Pomastowski*: Conceptualization, Supervision, Resources, Writing – review & editing, Project administration.

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775 **Supplementary Table S1.** Observed and theoretical masses of identified signals on mass spectra registered for AgNPs
776 synthesized via CVD technique and standard MALDI matrices: HCCA, DHB.

AgNPs			
Cluster composition		Molecular weight	
		observed	teoretical
1.	$^{107}\text{Ag}^+$	106.94	106.91
2.	$^{109}\text{Ag}^+$	108.94	108.90
3.	$^{107}\text{Ag} + \text{NH}_4^+$	124.99	124.94
4.	$^{109}\text{Ag} + \text{NH}_4^+$	126.98	126.94
5.	$^{107}\text{Ag} + \text{Na}^+$	129.92	129.89
6.	$^{109}\text{Ag} + \text{Na}^+$	131.92	131.89
7.	$^{107}\text{Ag} + \text{K}^+$	145.87	146.00
8.	$^{109}\text{Ag} + \text{K}^+$	147.87	148.00
9.	$^{107}\text{Ag} + \text{Na}^+ + \text{H}_2\text{O}$	148.91	148.92
10.	$^{109}\text{Ag} + \text{Na}^+ + \text{H}_2\text{O}$	150.90	150.92
11.	$^{107}\text{Ag} + \text{K}^+ + \text{O}$	162.88	162.00
12.	$^{109}\text{Ag} + \text{K}^+ + \text{O}$	164.88	164.00
13.	$^{107}\text{Ag}_2^+$	213.82	213.81
14.	$[\text{}^{107}\text{Ag} + \text{}^{109}\text{Ag}]^+$	215.83	215.81
15.	$^{109}\text{Ag}_2^+$	217.79	217.81
16.	$^{107}\text{Ag}_2^+ + \text{NH}_3 + \text{H}_2\text{O}$	248.85	248.86
17.	$[\text{}^{107}\text{Ag} + \text{}^{109}\text{Ag}]^+ + \text{NH}_3 + \text{H}_2\text{O}$	250.94	250.86
18.	$^{109}\text{Ag}_2^+ + \text{NH}_3 + \text{H}_2\text{O}$	252.88	252.86
19.	$^{107}\text{Ag}_3^+$	320.73	320.72
20.	$[\text{}^{107}\text{Ag}_2 + \text{}^{109}\text{Ag}]^+$	322.72	322.71
21.	$[\text{}^{107}\text{Ag} + \text{}^{109}\text{Ag}_2]^+$	324.72	324.71
22.	$^{109}\text{Ag}_3^+$	326.73	326.71
23.	$^{107}\text{Ag} + \text{}^{109}\text{Ag}_2 + \text{K}^+$	363.77	343.71
24.	$^{109}\text{Ag}_3 + \text{K}^+$	365.78	363.81

HCCA			
Cluster composition		Molecular weight	
		observed	teoretical
1.	$\text{M} + \text{H}^+$	190.63	190.18
2.	$\text{M} + \text{NH}_4^+$	207.53	207.21
3.	$\text{M} + \text{Na}^+$	212.52	212.16
4.	$\text{M} + \text{Na}^+ + \text{H}^+$	213.45	213.17
5.	$\text{M} + \text{K}^+$	228.38	228.27
6.	$\text{M} + \text{K}^+ + 2\text{H}^+$	230.37	230.28
7.	$\text{M} + 2\text{Na}^+ - \text{H}^+$	234.39	234.14
8.	$\text{M} + 2\text{Na}^+$	235.56	235.15
9.	$\text{M} + \text{Na}^+ + \text{K}^+ - \text{H}^+$	250.32	250.25

10.	M+2K ⁺ -H ⁺	266.22	266.36
11.	2M+H ⁺	379.17	379.34
12.	2M+Na ⁺	401.13	401.33
13.	2M+K ⁺	417.22	417.43
14.	2M+Na ⁺ +K ⁺ -H ⁺	439.13	439.42
15.	2M+2K ⁺	455.90	456.53
16.	3M+H ⁺	568.16	568.13
17.	3M+K ⁺	606.10	606.09
18.	3M+Na ⁺ +K ⁺ -H ⁺	628.10	628.07
19.	3M+2K ⁺ -H ⁺	644.11	644.05
20.	3M+Na ⁺ +2K ⁺ -2H ⁺	666.09	666.03
21.	3M+3K ⁺ -2H ⁺	682.07	682.00
22.	4M+2K ⁺ -H ⁺	833.13	833.09
23.	4M+Na ⁺ +2K ⁺ -2H ⁺	855.13	855.07
24.	4M+3K ⁺ -2H ⁺	871.12	871.04
25.	4M+Na ⁺ +3K ⁺ -3H ⁺	893.12	893.03
26.	5M+Na ⁺ +2K ⁺ -2H ⁺	1044.14	1044.11
27.	5M+3K ⁺ -2H ⁺	1060.13	1060.09
28.	5M+Na ⁺ +3K ⁺ -3H ⁺	1082.14	1082.07
29.	5M+4K ⁺ -3H ⁺	1098.12	1098.04
30.	6M+3K ⁺ -2H ⁺	1249.14	1249.13
31.	6M+Na ⁺ +3K ⁺ -3H ⁺	1271.13	1271.09
32.	5M+4K ⁺ -3H ⁺	1287.11	1287.08

DHB

Cluster composition		Molecular weight	
		observed	teoretical
1.	M+H ⁺	154.84	155.15
2.	MH+H ⁺	155.81	156.14
3.	M-H2O+Na ⁺	159.78	159.09
4.	M+Na ⁺	177.65	177.11
5.	M+K ⁺	193.51	193.22
6.	2M+NH ₄ ⁺ -2H2O	290.19	290.07
7.	2M-2H2O+H ⁺	273.29	273.22
8.	2M-H2O+Na ⁺	313.22	313.22
9.	2M-H2O+K ⁺	329.12	329.32
10.	2M+Na ⁺	331.06	331.23
11.	2M+(2Na) ⁺	345.09	354.22
12.	2M+K ⁺	347.05	347.34

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778 **Supplementary Table S2.** Observed and theoretical masses of identified signals on mass spectra registered for AgNPs
779 synthesized via CVD technique and standard MALDI matrices: HCCA, DHB.

Compound	X + Na	X + Ag	Fragmentation ions
14:0-13:0-14:0 TG-d5	265.18		C ₁₅ H ₃₀ O ₂ + Na
		392.70	C ₁₇ H ₃₃ O ₃ + ¹⁰⁹ Ag
	508.81		C ₃₀ H ₅₁ D ₅ O ₄ + Na
		592.60	C ₃₀ H ₅₁ D ₅ O ₄ + ¹⁰⁷ Ag
	618.99	618.99	C ₃₀ H ₅₁ D ₅ O ₄ + H + ¹⁰⁹ Ag + Na
		700.26	C ₃₀ H ₅₁ D ₅ O ₄ + ¹⁰⁷ Ag ₂
14:0-15:1-14:0 TG-d5	736.15		C ₄₄ H ₇₉ D ₅ O ₆ + Na
	263.73		C ₁₅ H ₂₈ O ₂ + Na
		391.76	C ₁₇ H ₃₃ O ₃ + ¹⁰⁹ Ag - 2H
	534.83		C ₃₂ H ₅₃ D ₅ O ₄ + Na
		618.48	C ₃₂ H ₅₃ D ₅ O ₄ + ¹⁰⁹ Ag - H
14:0-17:1-14:0 TG-d5	762.16		C ₄₆ H ₈₁ D ₅ O ₆ + Na
		398.12	C ₁₇ H ₂₇ D ₅ O ₃ + ¹⁰⁹ Ag + H
	561.83		C ₃₄ H ₅₇ D ₅ O ₄ + Na - H
		647.14	C ₃₄ H ₅₇ D ₅ O ₄ + ¹⁰⁷ Ag - H
	790.18		C ₄₈ H ₈₅ D ₅ O ₆ + Na
16:0-15:1-16:0 TG-d5	561.85		C ₃₄ H ₅₇ D ₅ O ₄ + Na - H
		647.16	C ₃₄ H ₅₇ D ₅ O ₄ + ¹⁰⁷ Ag - H

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	818.21		$C_{30}H_{89}D_5O_6 + Na$
	347.80		$C_{20}H_{37}O_3 + Na - H$
16:0-17:1-16:0 TG-d5	591.04		$C_{36}H_{61}D_5O_4 + Na$
	846.24		$C_{52}H_{93}D_5O_6 + Na$
	317.10		$C_{19}H_{34}O_2 + Na$
16:0-19:2-16:0 TG-d5	374.92		$C_{20}H_{35}O_3 + Na$
	617.00		$C_{38}H_{63}D_5O_4 + Na$
	724.99	724.99	$C_{38}H_{63}D_5O_4 + ^{109}Ag + Na$
	872.25		$C_{54}H_{96}D_5O_6 + Na$
	347.81		$C_{20}H_{36}O_3 + Na$
18:1-17:1-18:1 TG-d5	617.00		$C_{38}H_{63}D_5O_4 + Na$
		702.62	$C_{38}H_{63}D_5O_4 + ^{109}Ag$
	898.26		$C_{56}H_{97}D_5O_6 + Na$
	317.93		$C_{19}H_{34}O_2 + Na$
18:1-19:2-18:1 TG-d5	375.85		$C_{20}H_{35}O_3 + Na + H$
	592.91	592.91	$C_{20}H_{61}O_3 + ^{109}Ag_2 + Na - H$
	643.02		$C_{40}H_{65}D_5O_4 + Na$
		727.90	$C_{40}H_{65}D_5O_4 + ^{109}Ag$
	924.28		$C_{58}H_{99}D_5O_6 + Na$
	363.88		$C_{21}H_{39}O_3 + Na + H$
18:1-21:2-18:1 TG-d5	671.07		$C_{42}H_{69}D_5O_4 + Na + H$
		754.79	$C_{42}H_{69}D_5O_4 + ^{107}Ag$
	952.31		$C_{60}H_{103}D_5O_6 + Na$
	221.10		$C_5H_{11}PO_6 + Na$
PG 16:0-16:0	507.56		$C_{22}H_{45}PO_9 + Na$
	767.10		$C_{38}H_{75}PO_{10} + Na$
	783.06		$C_{38}H_{75}PO_{10} + K$
	146.98		$C_2H_4PO_4 + Na$
	442.86		$C_{21}H_{40}PO_6 + Na$
		468.83	$C_{21}H_{44}O_4 + ^{109}Ag$
PC 18:0-18:0	628.81		$C_{39}H_{73}O_4 + Na$
	753.73		$C_{41}H_{78}PO_8 + Na$
	812.32		$C_{44}H_{88}NPO_8 + Na$
	250.92		$C_{13}H_{27}COOH + Na$
$C_{13}H_{27}COOH$		335.19	$C_{13}H_{27}COOH + ^{107}Ag$
		337.19	$C_{13}H_{27}COOH + ^{109}Ag$
	307.35		$C_{17}H_{35}COOH + Na$
$C_{17}H_{35}COOH$		391.16	$C_{18}H_{35}COOH + ^{107}Ag$
		393.16	$C_{18}H_{35}COOH + ^{109}Ag$

Podsumowanie i wnioski

Praca [P1] stanowi przegląd literatury istotny dla zrozumienia problemu badawczego jakim jest rola nowoczesnych technologii analitycznych w analizie mikroorganizmów. Badania przeprowadzone w ramach pracy [P2] umożliwiły opracowanie metody do oznaczania wrażliwości szczepów bakteryjnych na antybiotyki z zastosowaniem elektroforezy kapilarnej. Prace [P3] – [P8] koncentrowały się na wykorzystaniu technik laserowej desorpcji jonizacji do analizy mikroorganizmów, mając na celu rozwiązanie często występujących problemów analitycznych.

Przedstawione w rozprawie doktorskiej badania pozwoliły na sformułowanie następujących wniosków:

1. Zastosowanie kontrolowanej agregacji komórek bakteryjnych przy użyciu jonów Ca^{2+} stanowi innowacyjną metodę pre-koncentracji tych komórek, co pozwala na uzyskanie pojedynczego, wyraźnego sygnału na elektroforegramie. Taki proces agregacji ułatwia analizę elektroforetyczną, umożliwiając dokładniejszą charakterystykę próbek bakteryjnych. Interesującym aspektem tego podejścia jest obserwacja, iż populacje komórek żywych i martwych wykazują odmienne czasy elektromigracji, co jest bezpośrednio związane ze zmianą ładunku powierzchniowego komórek. Ładunek ten zmienia się w zależności od stanu funkcjonalnego bakterii, co pozwala na różnicowanie między komórkami żywymi a martwymi. Na podstawie analizy czasu elektromigracji opracowano równanie umożliwiające określenie procentowego udziału żywych i martwych komórek bakteryjnych w badanej próbce. Kluczowym elementem metodyki jest uprzednia inkubacja komórek z odpowiednim antybiotykiem, co pozwala na ocenę ich żywotności w kontekście odporności na działanie antybiotyków. To podejście otwiera nowe możliwości w diagnostyce mikrobiologicznej, umożliwiając szybką i precyzyjną ocenę skuteczności antybiotykoterapii. Należy podkreślić, że choć zaproponowana metoda może zostać zastosowana do analizy działania różnych antybiotyków, dla każdego badanego szczepu bakterii niezbędne jest indywidualne wyznaczenie czasów migracji komórek żywych i martwych. Jest to istotne dla zapewnienia wiarygodności i dokładności wyników, co stanowi fundamentalny warunek skutecznej aplikacji tej techniki w praktyce diagnostycznej. Proponowane rozwiązanie ma potencjał, aby znacząco przyspieszyć proces identyfikacji i oceny wrażliwości bakterii na antybiotyki, co jest kluczowe dla optymalizacji terapii antybiotykowej i ograniczenia rozprzestrzeniania się oporności na antybiotyki w populacjach bakteryjnych [P2].
2. Implementacja podejścia kulturomicznego w procesie hodowli mikroorganizmów z próbek klinicznych stanowi znaczący postęp w zakresie ich identyfikacji przy

wykorzystaniu techniki MALDI-TOF MS. Integracja metod kulturomicznych z technikami spektrometrii masowej umożliwia nie tylko szybką i dokładną identyfikację, ale także pozwala na uzyskanie bardziej reprezentatywnego obrazu populacji mikroorganizmów obecnych w specyficznych środowiskach, takich jak rany stopy cukrzycowej. Dzięki temu, możliwe jest głębsze zrozumienie dynamiki mikrobiomu rany, co ma bezpośrednie implikacje dla opracowywania skuteczniejszych strategii leczenia. Kulturomika jako metoda umożliwiająca standaryzację etapu hodowli mikroorganizmów, oferuje unikalną możliwość dostosowania procesów hodowlanych do specyficznych wymagań poszczególnych mikrobiomów. Taka personalizacja etapu hodowli znacząco przyczynia się do zwiększenia wykrywalności mikroorganizmów, co jest kluczowe w kontekście rutynowych analiz mikrobiologicznych. Poprzez optymalizację warunków hodowli, kulturomika pozwala na maksymalizację efektywności identyfikacji patogenów, co jest niezbędne dla szybkiego diagnozowania infekcji i wdrażania celowanej terapii antybiotykowej. Ponadto, zastosowanie podejścia kulturomicznego w badaniach mikrobiologicznych stanowi istotny krok w kierunku rozwoju spersonalizowanej mikrobiologii klinicznej, która uwzględnia unikalne właściwości mikrobiomu pacjenta. Takie podejście może znacząco przyczynić się do poprawy skuteczności leczenia, zmniejszając ryzyko wystąpienia oporności na antybiotyki oraz promując szybszą regenerację tkanki w procesie gojenia ran. W konsekwencji, integracja kulturomiki z techniką MALDI-TOF MS otwiera nowe perspektywy w diagnostyce i terapii infekcji bakteryjnych, podnosząc standardy opieki nad pacjentami z ranami cukrzycowymi i innymi stanami klinicznymi wymagającymi precyzyjnej identyfikacji mikroorganizmów [P3].

3. Wykorzystanie techniki MALDI-TOF do analizy lipidów ekstrahowanych z komórek bakteryjnych otwiera nowe możliwości w zakresie mikrobiologicznej diagnostyki i badania. W przeprowadzonych badaniach zastosowałam tę technikę do zarejestrowania profilów lipidowych dla 39 izolatów bakteryjnych pochodzących ze stopy cukrzycowej, co pozwoliło na szczegółową charakterystykę lipidomu tych mikroorganizmów. Obserwacje wykazały znaczącą zmienność w składzie jakościowym lipidów między bakteriami Gram-dodatnimi i Gram-ujemnymi, co podkreśla złożoność ich struktur lipidowych oraz potencjał lipidów jako biomarkerów w mikrobiologicznej diagnostyce. Uzyskane wyniki potwierdzają tezę, iż sygnały lipidowe, otrzymywane podczas analizy MALDI, mogą służyć jako specyficzne chemiczne odciski palców, umożliwiające precyzyjne różnicowanie między poszczególnymi mikroorganizmami. Taka charakterystyka lipidowa oferuje unikalne perspektywy dla identyfikacji i klasyfikacji mikroorganizmów, stanowiąc komplementarne podejście do tradycyjnych metod proteomicznych. W szczególności, możliwość dokładnej analizy lipidomu bakteryjnego może przyczynić się do lepszego zrozumienia mechanizmów adaptacyjnych bakterii do różnorodnych środowisk, w tym środowiska ran cukrzycowych, które są znane

z wysokiej bioróżnorodności mikrobiologicznej. Tym samym, zastosowanie techniki MALDI w analizie lipidów bakteryjnych reprezentuje innowacyjne podejście w bioanalityce, pozwalając na rozwijanie nowych metod diagnostycznych i terapeutycznych. Potencjał lipidów jako biomarkerów w różnicowaniu mikroorganizmów otwiera nowe ścieżki w poszukiwaniu efektywnych strategii leczenia infekcji, szczególnie w kontekście rosnącej oporności na antybiotyki. Przyszłe badania w tej dziedzinie mogą przyczynić się do opracowania nowych narzędzi diagnostycznych, które będą wspierać szybką i precyzyjną identyfikację patogenów, co jest kluczowe dla skutecznego leczenia infekcji bakteryjnych [P4].

4. W ramach przeprowadzonych badań zauważono, że jakość oprogramowania oraz obszerność i aktualność bazy danych mają istotny wpływ na skuteczność i dokładność identyfikacji mikroorganizmów w technice spektrometrii mas MALDI-TOF. Analiza porównawcza dwóch systemów spektrometrycznych, Bruker BioTyper oraz Zybion EXS2600, wykazała, że obydwa oferują wysoką efektywność w identyfikacji izolatów klinicznych, osiągając odpowiednio 95,6% i 92,4% wiarygodnych identyfikacji. Co więcej, dla 89,5% izolatów uzyskano zbieżne wyniki identyfikacji pomiędzy oboma systemami, co podkreśla ich równoważną wartość analityczną w kontekście rutynowych procedur diagnostycznych w mikrobiologii. Takie wyniki demonstrują, że zarówno Bruker BioTyper, jak i Zybion EXS2600 stanowią efektywne narzędzia, umożliwiające szybką i precyzyjną identyfikację większości izolatów klinicznych, co jest kluczowe dla szybkiego postawienia diagnozy i wdrożenia odpowiedniego leczenia. Dodatkowo, walidacja nowych spektrometrów mas, takich jak seria EXS, jest procesem wymagającym szczegółowej analizy wielu czynników, w tym odpowiedniego doboru matrycy, starannej preparatyki próbek oraz konstrukcji i ciągłej aktualizacji repozytoriów baz danych. Te aspekty mają bezpośredni wpływ na jakość oraz wiarygodność wyników identyfikacji, co z kolei przekłada się na ogólną skuteczność zastosowania technologii MALDI-TOF w diagnostyce mikrobiologicznej. Proces walidacji musi więc uwzględniać kompleksową optymalizację tych elementów, aby zapewnić maksymalną precyzję i efektywność analiz. Wyniki tych badań wskazują na konieczność ciągłej pracy nad doskonaleniem metod spektrometrii mas oraz związanych z nimi baz danych i oprogramowania, aby móc nieustannie poprawiać jakość i szybkość diagnostyki mikrobiologicznej [P5].
5. Technika MALDI dostarcza w zdecydowanej większości precyzyjne wyniki identyfikacji. Rozbieżność pomiędzy identyfikacją w oparciu o proteom i genom zaobserwowano tylko dla szczepów blisko spokrewnionych [P6].
6. Jednym z mechanizmów obronnych bakterii na działanie antybiotyków jest ich hydroliza. Technika MALDI umożliwia detekcję produktów hydrolizy, co pozwala na wykrycie oporności znacznie szybciej niż w przypadku zastosowania metod konwencjonalnych lub genomowych (molekularnych). Z jej wykorzystaniem uzyskano

bardziej precyzyjne wyniki niż w przypadku metod konwencjonalnych, które wiele wyników sklasyfikowały jako niepewne [P6].

7. Chemiczne osadzanie z fazy gazowej zapewnia równomierne pokrycie powierzchni płytki nanocząstkami metalicznego srebra. Otrzymane AgNP charakteryzują się jednorodnym kształtem i rozmiarem. Wraz ze wzrostem masy użytego prekursora wzrasta rozmiar nanocząstek. Otrzymane nanostruktury umożliwiają jonizację i desorpcję związków o niskiej masie cząsteczkowej, co pozwoliło na zarejestrowanie profili lipidowych szczepów bakteryjnych. Stosując AgNP możliwe było rozróżnienie dwóch bliskospokrewnionych szczepów bakteryjnych w oparciu o zarejestrowane profile, co nie było możliwe przy zastosowaniu standardowo stosowanych w technice MALDI matryc [P7].
8. W ramach mojej rozprawy doktorskiej po raz pierwszy przeprowadzone zostały badania, które wykazały, że nanocząstki srebra uzyskane za pomocą metody chemicznego osadzania z fazy gazowej (CVD) skutecznie wspomagają procesy jonizacji i desorpcji analitu poprzez mechanizmy fotokatalizy. Istotnym aspektem, który znacząco przyczynia się do efektywności tych procesów, jest zjawisko powierzchniowego rezonansu plazmonowego (SPR). Zjawisko to, charakterystyczne dla nanocząstek metalicznych, polega na rezonansowej oscylacji swobodnych elektronów na powierzchni nanocząstki pod wpływem padającego światła. SPR nie tylko zwiększa lokalne pole elektromagnetyczne, ale również poprawia absorpcję światła, co bezpośrednio wpływa na zwiększenie efektywności fotokatalizy. Kluczowe dla uzyskanych wyników okazało się równomierne rozmieszczenie nanocząstek na badanej powierzchni. Dzięki temu możliwe jest osiągnięcie jednorodnej jonizacji i desorpcji analitów, co jest niezbędne do przeprowadzenia precyzyjnych analiz ilościowych. Szczególnie znaczące jest to w kontekście związków o niskiej masie cząsteczkowej (LMW), takich jak lipidy i węglowodany, które często stanowią wyzwanie dla tradycyjnych metod spektrometrycznych ze względu na ich skłonność do fragmentacji lub niską efektywność jonizacji. Zastosowanie nanocząstek srebra w technice SALDI (*Surface Assisted Laser Desorption/Ionization*) otwiera nowe możliwości dla analizy ilościowej tego rodzaju związków. Technika ta, wykorzystując unikalne właściwości nanocząstek srebra, umożliwia nie tylko efektywną jonizację i desorpcję, ale także zwiększa stabilność i wiarygodność wyników analiz ilościowych. W ten sposób, badania przeprowadzone w ramach mojej rozprawy doktorskiej demonstrowują potencjał wykorzystania nanocząstek srebra otrzymanych metodą CVD w zaawansowanych metodach spektrometrycznych, oferując nowe perspektywy dla precyzyjnej analizy związków o niskiej masie cząsteczkowej w różnorodnych próbkach biologicznych i chemicznych [P8].

Wnioski wyciągnięte z przeprowadzonych badań w ramach niniejszej rozprawy doktorskiej podkreślają **istotność**, jaką rozwój metod spektrometrycznych, szczególnie technik mas spektrometrii z laserową desorpcją/ionizacją (LDI), może odegrać w przyszłości w procesie identyfikacji mikroorganizmów. Badania te rzucają światło na znaczny potencjał tych technik, wskazując na możliwość ich szerszego zastosowania, które może zrewolucjonizować tradycyjnie stosowane protokoły diagnostyczne w laboratoriach mikrobiologicznych. W kontekście globalnym, jak i w Polsce, obserwuje się trend wzrostowy w zakresie implementacji technik spektrometrycznych w środowiskach szpitalnych, co jest odpowiedzią na rosnącą potrzebę szybkiej i precyzyjnej identyfikacji patogenów. Rozwój i wdrożenie zaawansowanych protokołów wykorzystujących te technologie może znacząco przyczynić się do poszerzenia naszego zrozumienia charakterystyki szczepów bakteryjnych, co ma bezpośrednie przełożenie na efektywność diagnostyki i terapii różnorodnych stanów chorobowych oraz zakażeń. Ponadto, opracowanie i wdrożenie nowych metod opartych na spektrometrii mas LDI mogłoby umożliwić dokładniejsze badanie mechanizmów antybiotykooporności, co jest kluczowe w kontekście rosnącego problemu oporności bakterii na dostępne antybiotyki. Takie podejście nie tylko zwiększyłoby skuteczność walki z patogenami, ale również pozwoliłoby na opracowanie bardziej celowanych strategii leczenia, minimalizując ryzyko rozwoju dalszych form oporności. W rezultacie, wnioski płynące z niniejszej rozprawy doktorskiej stanowią fundament dla dalszych badań i rozwoju w dziedzinie bioanalitiky oraz diagnostyki laboratoryjnej. Przyszłe badania powinny skupić się na dalszym rozwijaniu i optymalizacji technik spektrometrycznych, tak aby maksymalnie wykorzystać ich potencjał w kontekście identyfikacji mikroorganizmów i walki z antybiotykoopornością. Innowacyjne i interdyscyplinarne podejście do diagnostyki mikrobiologicznej, oparte na zaawansowanych metodach analitycznych, może znacząco przyczynić się do poprawy jakości opieki zdrowotnej oraz skuteczności leczenia infekcji bakteryjnych, co jest niezwykle istotne w obliczu współczesnych wyzwań medycznych.

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Stáže

- 04 – 24.07.2022 Bruker Daltonics (Bremen, Niemcy)
Tematyka stażu związana była z zastosowaniem techniki MALDI w analizie mikroorganizmów i detekcji antybiotykooporności.
- 01 – 14.04.2023 Polmlek (Grudziądz, Polska)
Tematyka stażu związana była z mikrobiologicznymi procesami produkcji sera

Szkolenia

- 05/2023 "qPCR Gene Expression"
szkolenie przeprowadzone przez ThermoFisher Scientific (Polska)
- 04/2022 "MALDI imaging"
szkolenie przeprowadzone przez Bruker Daltonics (Niemcy)

Publikacje naukowe

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6. D. Błońska, **E. Maślak**, O. Drews, B. Oberheitmann, M. Kostrzewa, K. Sparbier, B. Buszewski, MALDI-TOF jako metoda wykrywania aktywności inhibitorów beta-laktamaz u bakterii Gram-ujemnych, XIII Polska konferencja chromatograficzna, Katowice (Polska) **25-28.06.2023**

7. **E. Maślak**, D. Błońska, O. Drews, B. Oberheitmann, K. Sparbier, M. Kostrzewa, M. Złoch, A MALDI-TOF-based inhibitor approach for the detection of beta-lactamase activity in Gram-negative bacteria, Deutschen Gesellschaft für Hygiene und Microbiology (DGHM), Lubeka (Niemcy) **18-20.09.2023**
8. **E. Sibińska**, M. Złoch, A. Arendowski, I. Arendowska, J. Rudnicka, J. Walczak-Skierska, K. Rafińska, P. Pomastowski, D. Białczak, Izolacja i identyfikacja szczepów *Lactococcus lactis* i *Weissella cibaria* z kiszonek oraz badanie ich właściwości jako potencjalnych kultur starterowych, Polski Kongres Serowarski, Żnin (Polska) **9-11.10.2023**

Finansowanie badań i wyjazdów zagranicznych

- * Granty Młodych dla uczestników Studiów Doktoranckich, Szkoły Doktorskiej oraz młodych pracowników Wydziału Chemii 2021
Tytuł grantu: „Synteza nanocząstek metali stosowanych w technice obrazowania NALDI”
- * Trzytygodniowy staż naukowy realizowany w ramach Projektu PROM – Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej
Termin stażu: 04.07.2022 – 24.07.2022
Ośrodek w którym zrealizowano staż: Bruker Daltonics (Brema, Niemcy)
- * Dofinansowanie na wyjazd na konferencję w ramach konkursu IDUB (Kopenhaga, Dania, maj 2023)
- * Dofinansowanie na wyjazd na konferencję w ramach konkursu IDUB (Kluż-Napoka, Rumunia, wrzesień 2023)

Współorganizacja warsztatów i konferencji

- | | |
|---------|--|
| 09/2023 | współorganizacja konferencji naukowej, UMK
65. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, Toruń (Polska) |
| 12/2023 | współorganizacja szkolenia dla studentów Weterynarii VI roku, UMK
pt. „MALDI TOF MS – podstawy techniki i identyfikacja mikroorganizmów” |
| 03/2023 | współorganizacja warsztatów dla pracowników i doktorantów UMK
pt. „Technika MALDI w rutynowej diagnostyce klinicznej – warsztaty z identyfikacji patogenów klinicznych i detekcji antybiotykooporności” |

Oświadczenia współautorów



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Niniejszym oświadczam, że jako autor przedłożonej rozprawy doktorskiej mój wkład w powstanie poniższych artykułów:

- [P1] B. Buszewski, **E. Maślak**, M. Złoch, V. Railean-Plugaru, E. Kłodzińska, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* 2021, 39:116250

polegał na współudziale w opracowaniu ogólnej koncepcji manuskryptu, przeglądzie literatury, przygotowaniu wstępnej wersji i korekcie manuskryptu.

- [P2] **E. Maślak**, W. Kupczyk, V. Railean-Plugaru, P. Pomastowski, M. Jackowski, B. Buszewski, Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches, *Electrophoresis* 2022, 43(6)

polegał na współudziale w opracowaniu ogólnej koncepcji manuskryptu, wykonaniu analiz CE i pomiarów OD, przeglądzie literatury, przygotowaniu wstępnej wersji i korekcie manuskryptu.

- [P3] M. Złoch, **E. Maślak**, W. Kupczyk, M. Jackowski, P. Pomastowski, B. Buszewski, Culturomics Approach to Identify Diabetic Foot Infection Bacteria, *International Journal of Molecular Sciences* 2021, 22(17):9574

polegał na współuczestnictwie w opracowaniu koncepcji badań, zaplanowaniu i przeprowadzeniu większości doświadczeń, opracowaniu i interpretacji uzyskanych wyników, a także na współudziale w przygotowaniu wstępnej wersji manuskryptu.

- [P4] J. Walczak-Skierska, F. Monedeiro, **E. Maślak**, M. Złoch, Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS, *Analytical Chemistry* 2023, 95(1)

polegał na współudziale w opracowaniu ogólnej koncepcji manuskryptu, współuczestnictwie w przygotowaniu i analizie próbek, korekcie manuskryptu

- [P5] **E. Sibińska**, A. Arendowski, P. Fijałkowski, P. Pomastowski, D. Gabryś, Comparison of Bruker Microflex LT and Zybion EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* 2023, 116150

polegał na opracowaniu koncepcji badań, zaplanowaniu i przeprowadzeniu większości pomiarów, opracowaniu i interpretacji uzyskanych wyników oraz współudziale w przygotowaniu wstępnej wersji manuskryptu i jego korekty.

- [P6] M. Złoch, **E. Maślak**, W. Kupczyk, P. Pomastowski, Multi-Instrumental Analysis Toward Exploring the Diabetic Foot Infection Microbiota, *Current Microbiology* 2023, 80(8):271

polegał na współuczestnictwie w opracowaniu koncepcji badań, zaplanowaniu i przeprowadzeniu większości doświadczeń, opracowaniu i interpretacji uzyskanych wyników, a także na współudziale w przygotowaniu wstępnej wersji manuskryptu.

E.S.



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- [P7] **E. Maślak**, A. Arendowski, M. Złoch, J. Walczak-Skierska, A. Radtke, P. Pomastowski, P. Piszczek, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* 2023, 12(874):1-17

polegał na współdziale w przeprowadzeniu badań i interpretacji uzyskanych wyników oraz w przygotowaniu wstępnej wersji manuskryptu.

- [P8] **E. Sibińska**, J. Walczak-Skierska, A. Arendowski, A. Ludwiczak, P. Piszczek, A. Radtke, P. Pomastowski, Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in Enhancing Detection of Low-Molecular-Weight Biomolecules, *Journal of the American Society for Mass Spectrometry*, Manuscript ID: js-2024-00071h

polegał na współuczestnictwie w opracowaniu koncepcji badań, zaplanowaniu i przeprowadzeniu wszystkich pomiarów z wykorzystaniem LDI, opracowaniu i interpretacji uzyskanych wyników oraz współdziale w przygotowaniu wstępnej wersji manuskryptu i jego korekty.

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OŚWIADCZENIE

Jako Współautor następujących publikacji:

- [P1] B. Buszewski, E. Maślak, M. Złoch, V. Railean-Plugaru, E. Kłodzińska, **P. Pomastowski**, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* 2021, 39:116250
- [P2] E. Maślak, W. Kupczyk, V. Railean-Plugaru, **P. Pomastowski**, M. Jackowski, B. Buszewski, Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches, *Electrophoresis* 2022, 43(6)
- [P3] M. Złoch, E. Maślak, W. Kupczyk, M. Jackowski, **P. Pomastowski**, B. Buszewski, Culturomics Approach to Identify Diabetic Foot Infection Bacteria, *International Journal of Molecular Sciences* 2021, 22(17):9574
- [P5] E. Sibińska, A. Arendowski, P. Fijałkowski, **P. Pomastowski**, D. Gabryś, Comparison of Bruker Microflex LT and Zybto EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* 2023, 116150
- [P6] M. Złoch, E. Maślak, W. Kupczyk, **P. Pomastowski**, Multi-Instrumental Analysis Toward Exploring the Diabetic Foot Infection Microbiota, *Current Microbiology* 2023, 80(8):271
- [P7] E. Maślak, A. Arendowski, M. Złoch, J. Walczak-Skierska, A. Radtke, **P. Pomastowski**, P. Piszczek, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* 2023, 12(874):1-17
- [P8] E. Sibińska, J. Walczak-Skierska, A. Arendowski, A. Ludwiczak, P. Piszczek, A. Radtke, D. Gabryś, **P. Pomastowski**, Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in Enhancing Detection of Low-Molecular-Weight Biomolecules, *Journal of the American Society for Mass Spectrometry*, Manuscript ID: js-2024-00071h

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na pełnieniu nadzoru merytorycznego tj. współudziale w opracowaniu koncepcji, pomocy w planowaniu eksperymentów, interpretacji i dyskusji wyników, oraz redagowaniu i korekcie manuskryptów. Wyniki prac [P5] oraz [P8] są bezpośrednim rezultatem kierowanego przeze mnie projektu pt. „Nowe podejście do obrazowania i szybkiej identyfikacji mikrobiomu w ocenie skutków radioterapii”, finansowanego przez Narodowe Centrum Nauki w ramach programu OPUS 20 (2020/39/B/NZ7/02733).



Toruń, 20.02.2024

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Jako Współautor następujących publikacji:

- [P1] B. Buszewski, E. Maślak, **M. Złoch**, V. Railean-Plugaru, E. Kłodzińska, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* 2021, 39:116250
- [P3] **M. Złoch**, E. Maślak, W. Kupczyk, M. Jackowski, P. Pomastowski, B. Buszewski, Culturomics Approach to Identify Diabetic Foot Infection Bacteria, *International Journal of Molecular Sciences* 2021, 22(17):9574
- [P4] J. Walczak-Skierska, F. Monedeiro, E. Maślak, **M. Złoch**, Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS, *Analytical Chemistry* 2023, 95(1)
- [P6] **M. Złoch**, E. Maślak, W. Kupczyk, P. Pomastowski, Multi-Instrumental Analysis Toward Exploring the Diabetic Foot Infection Microbiota, *Current Microbiology* 2023, 80(8):271
- [P7] E. Maślak, A. Arendowski, **M. Złoch**, J. Walczak-Skierska, A. Radtke, P. Pomastowski, P. Piszczek, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* 2023, 12(874):1-17

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na współudziale w opracowaniu koncepcji, w planowaniu eksperymentów, interpretacji i dyskusji wyników, oraz w przygotowaniu pierwszych wersji prac. Wyniki prac [P3], [P4] oraz [P6] są bezpośrednim rezultatem kierowanego przeze mnie projektu pt. „Wykorzystanie techniki MALDI-TOF/MS w monitorowaniu i szybkiej diagnostyce rozwoju zakażenia stopy cukrzycowej”, finansowanego przez Narodowe Centrum Nauki w ramach programu SONATINA 2 (2018/28/C/ST4/00434).





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W TORUNIU
Wydział Nauk Biologicznych
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Toruń, February 20, 2024.

Dr. Viorica Railean

Faculty of Biological and Veterinary Sciences
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STATEMENT

As a co-author of the following publications:

- [P1] B. Buszewski, E. Maślak, M. Złoch, **V. Railean-Plugaru**, E. Kłodzińska, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, TrAC Trends in Analytical Chemistry 2021, 39:116250, [doi: 10.1016/j.trac.2021.116250](https://doi.org/10.1016/j.trac.2021.116250).
- [P2] E. Maślak, W. Kupczyk, **V. Railean**, P. Pomastowski, M. Jackowski, B. Buszewski, Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches, Electrophoresis 2022, 43(6), [DOI: 10.1002/elps.202200096](https://doi.org/10.1002/elps.202200096).

part of doctoral dissertation of Mrs. Ewelina Sibińska MSc, I declare that my involvement encompassed contributing to the development of the work's conceptual framework, conducting analyses utilizing the flow cytometry technique (FC), and collaborating on the preparation of the initial manuscripts.

.....

Toruń, 15.02.2024

Dr. Fernanda Monedeiro

STATEMENT

I declare that, as Co-author of the following article,

[P4] J. Walczak-Skierska, **F. Monedeiro**, E. Maślak, M. Złoch, Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS, Analytical Chemistry 2023, 95(1)

being a part of the PhD thesis of Mrs. Ewelina Sibińska MSc., that my contribution was to performing and interpreting statistical analyses.



.....

Gliwice, 15.02.2024

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OŚWIADCZENIE

Jako Współautor następujących publikacji:

- [P5] E. Sibińska, A. Arendowski, P. Fijałkowski, P. Pomastowski, **D. Gabryś**, Comparison of Bruker Microflex LT and Zybion EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* 2023, 116150
- [P8] E. Sibińska, J. Walczak-Skierska, A. Arendowski, **D. Gabryś**, P. Piszczyk, A. Radtke, P. Pomastowski, Advances in LDI-MS analysis: the role of CVD-synthesized silver nanoparticles in efficient ionization of low-molecular-weight compounds

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na pozyskaniu materiału biologicznego od pacjentów. Wyniki obu prac są bezpośrednim rezultatem kierowanego przeze mnie projektu pt. „Nowe podejście do obrazowania i szybkiej identyfikacji mikrobiomu w ocenie skutków radioterapii”, finansowanego przez Narodowe Centrum Nauki w ramach programu OPUS 20 (2020/39/B/NZ7/02733).





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Prezes: prof. zw. dr hab. Bogusław Buszewski, dr h.c. mult. czł. rzecz. PAN & EASA,

Toruń 15 luty 2024 r.

OŚWIADCZENIE

Jako współautor następujących publikacji:

- [P1] **B. Buszewski**, E. Maślak, M. Złoch, V. Railean-Plugaru, E. Kłodzińska, P. Pomastowski, *A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques*, Trends in Analytical Chemistry (TrAC), **39** (2021) 116250,
- [P2] E. Maślak, W. Kupeczyk, V. Railean-Plugaru, P. Pomastowski, M. Jackowski, **B. Buszewski**, *Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches*, Electrophoresis, **43(6)**(2022)
- [P3] M. Złoch, E. Maślak, W. Kupeczyk, M. Jackowski, P. Pomastowski, **B. Buszewski**, *Culturomics Approach to Identify Diabetic Foot Infection Bacteria*, International Journal of Molecular Sciences, **22(17)** (2021) 9574

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej z d. Maślak oświadczam, że mój wkład polegał na współudziale w opracowaniu koncepcji badań i korekcie manuskryptów oraz pełnieniu nadzoru merytorycznego nad realizacją eksperymentów.

Jednocześnie informuję, że prace [P1] oraz [P2] są bezpośrednim rezultatem kierowanego przeze mnie projektu pt. „Nowe podejście w identyfikacji mikroorganizmów ze szczególnym uwzględnieniem wirusów”, finansowanego przez Narodowe Centrum Nauki w ramach programu OPUS_19 (2020/37/B/ST4/02136).

PREZES ZARZĄDU

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Toruń, 19.02.2024

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OŚWIADCZENIE

Jako Współautor następujących publikacji:

- [P2] E. Maślak, W. Kupczyk, V. Railean-Plugaru, P. Pomastowski, **M. Jackowski**, B. Buszewski, Viability study of clinical bacterial strains by capillary electrophoresis and flow cytometry approaches, *Electrophoresis* 2022, 43(6)
- [P3] M. Złoch, E. Maślak, W. Kupczyk, **M. Jackowski**, P. Pomastowski, B. Buszewski, Culturomics Approach to Identify Diabetic Foot Infection Bacteria, *International Journal of Molecular Sciences* 2021, 22(17):9574

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na pozyskaniu materiału biologicznego od pacjentów.


Uniwersytet Mikołaja Kopernika w Toruniu
Collegium Medicum w Bydgoszczy
Katedra i Klinika Chirurgii Ogólnej i Gastroenterologicznej i Onkologicznej
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OŚWIADCZENIE

Jako Współautor następujących publikacji:

[P5] E. Sibińska, A. Arendowski, **P. Fijałkowski**, P. Pomastowski, D. Gabryś, Comparison of Bruker Microflex LT and Zybion EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* 2023, 116150

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na współudziale w przeprowadzeniu analiz MALDI.

.....
Fijałkowski Piotr

Toruń, 19.02.2024

dr hab. Ewa Kłodzińska

Instytut Medycyny Innowacyjnej, Klinika Długowieczności

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ul. Zabłocie 25 lok. 4, 30-701 Kraków

Wyższa Szkoła Inżynierii i Zdrowia w Warszawie

Biegły sądowy z zakresu badań chemicznych i kryminalistycznych przy SO w Warszawie

Ambasador Fundacji World Healthy and Living Foundation

OŚWIADCZENIE

Jako Współautor następującej publikacji:

- [P1] B. Buszewski, E. Maślak, M. Złoch, V. Railean-Plugaru, **E. Kłodzińska**, P. Pomastowski, A new approach to identifying pathogens, with particular regard to viruses, based on capillary electrophoresis and other analytical techniques, *TrAC Trends in Analytical Chemistry* 2021, 39:116250

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na współudziale w opracowaniu koncepcji i w przygotowaniu wstępnej wersji manuskryptu.

dr hab. Ewa Kłodzińska, prof. IS-PIB

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OŚWIADCZENIE

Jako Współautor następujących publikacji:

- [P4] **J. Walczak-Skierska**, F. Monedeiro, E. Maślak, M. Złoch, Lipidomics Characterization of the Microbiome in People with Diabetic Foot Infection Using MALDI-TOF MS, *Analytical Chemistry* 2023, 95(1)
- [P7] E. Maślak, A. Arendowski, M. Złoch, **J. Walczak-Skierska**, A. Radtke, P. Pomastowski, P. Piszczek, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* 2023, 12(874):1-17
- [P8] E. Sibińska, **J. Walczak-Skierska**, A. Arendowski, A. Ludwiczak, P. Piszczek, A. Radtke, D. Gabryś, P. Pomastowski, Advances in LDI-MS Analysis: The Role of CVD-Synthesized Silver Nanoparticles in Enhancing Detection of Low-Molecular-Weight Biomolecules, *Journal of the American Society for Mass Spectrometry*, Manuscript ID: js-2024-00071h

wchodzących w skład rozprawy doktorskiej Pani mgr Eweliny Sibińskiej, oświadczam, że mój wkład polegał na współpracy w przeprowadzeniu analiz lipidomicznych i interpretacji uzyskanych danych oraz w przygotowaniu i korekcie pierwszych wersji manuskryptów.

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Jako Współautor następujących publikacji:

- [P5] E. Sibińska, **A. Arendowski**, P. Fijałkowski, P. Pomastowski, D. Gabryś, Comparison of Bruker Microflex LT and Zymote EXS2600 MALDI TOF MS systems for the identification of clinical microorganisms, *Diagnostic Microbiology and Infectious Disease* 2023, 116150
- [P7] E. Maślak, **A. Arendowski**, M. Złoch, J. Walczak-Skierska, A. Radtke, P. Pomastowski, P. Piszczek, Silver Nanoparticle Targets Fabricated Using Chemical Vapor Deposition Method for Differentiation of Bacteria Based on Lipidomic Profiles in Laser Desorption/Ionization Mass Spectrometry, *Antibiotics* 2023, 12(874):1-17
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Streszczenie

Tematyka badań niniejszej rozprawy doktorskiej koncentruje się na wykorzystaniu zaawansowanych technologii w laboratoriach mikrobiologicznych w celu identyfikacji, analizy i określenia lekowrażliwości drobnoustrojów o istotnym znaczeniu dla szerzenia się zakażeń i rozprzestrzeniania genów antybiotykooporności. Dzięki dokładnej i precyzyjnej analizie mikrobiomu możliwe jest zapewnienie bezpieczeństwa żywności, utrzymanie higieny publicznej oraz wdrażanie terapii i strategii prewencyjnych w ochronie zdrowia ludzi i zwierząt.

Głównym celem rozprawy jest rozwój efektywnych narzędzi diagnostycznych, umożliwiających wiarygodną identyfikację czynników patogennych oraz ocenę ich antybiotykooporności, szczególnie poprzez wykorzystanie techniki laserowej desorpcji/ionizacji oraz techniki elektromigracyjne. Rozprawa ma na celu nie tylko rozwój metodologiczny, ale również weryfikację skuteczności różnych podejść omicznych w kontekście analiz próbek klinicznych, co umożliwi ocenę ich przydatności w rutynowej diagnostyce mikrobiologicznej. Badania skupiają się wokół gatunków mikroorganizmów stanowiących kluczowe czynniki etiologiczne stanów chorobowych dotykających znaczną część społeczeństwa, m.in. infekcji trudno gojących się ran, takich jak zespół stopy cukrzycowej, oraz tych towarzyszących rozwojowi nowotworu prostaty.

W kontekście technik elektromigracyjnych przedstawiono potencjał wykorzystania elektroforezy kapilarnej do pre-koncentracji komórek bakteryjnych jako innowacyjnej metody oceny ich wrażliwości na różne klasy antybiotyków. W części poświęconej spektrometrii mas, szczególną uwagę poświęcono technice MALDI, dla której obserwuje się ciągły wzrostowy wykorzystania w środowisku szpitalnym. Przedstawiono możliwości i ograniczenia podejścia proteomicznego i lipidomicznego w procesie identyfikacji oraz podejścia metabolicznego, które w oparciu o detekcję hydrolizy enzymatycznej umożliwia określenie wrażliwości/oporności patogenów na antybiotyki. W pracy poruszono również tematykę związaną z przygotowaniem próbek, w szczególności z wpływem wdrożenia podejścia kulturomicznego na proces identyfikacji mikrobiomu. W celu przezwyciężenia niektórych problemów wynikających z konieczności zastosowania klasycznych matryc organicznych w technikach laserowej desorpcji/ionizacji zaproponowano podejście wykorzystujące nanocząstki srebra jako efektywne narzędzie jonizacji. Syntezę nanocząstek przeprowadzono przy użyciu metody osadzania z fazy gazowej, co umożliwiło uzyskanie warstwy charakteryzującej się wysoką jednorodnością i wykazującej potencjał w analizach związków o niskich masach cząsteczkowych. W pracy zaprezentowano możliwość ich zastosowania w rozróżnianiu szczepów bliskospokrewnionych. Integralną częścią pracy stanowią analizy porównawcze nowo opracowanych metod z tradycyjnymi podejściami, co pozwala na określenie ich zalet, ograniczeń oraz potencjalnych obszarów zastosowania.

Przedstawione w pracy badania prezentują innowacyjne i interdyscyplinarne podejście do diagnostyki mikrobiologicznej, oparte na zaawansowanych metodach analitycznych, które może znacząco przyczynić się do poprawy jakości opieki zdrowotnej oraz skuteczności leczenia infekcji bakteryjnych, co jest niezwykle istotne w obliczu współczesnych wyzwań medycznych.

Abstract

The research presented in this doctoral dissertation focuses on using advanced technologies in microbiological laboratories to identify, analyze, and determine the drug susceptibility of microorganisms that play a significant role in spreading infections and the disseminating antibiotic-resistant genes. It is possible to ensure food safety, maintain public hygiene, and implement therapies and preventive strategies in human and animal health protection through precise and accurate microbiome analysis.

The main objective is to develop effective diagnostic tools that precisely identify pathogenic factors and determine their antibiotic-resistance level, mainly through laser desorption/ionization techniques and electromigration techniques. The dissertation aims not only at methodological development but also at verifying the effectiveness of various omics approaches in clinical sample analysis, enabling assessment of their utility in routine microbiological diagnostics. The research focuses on microorganisms of clinical significance, including those involved in diabetic foot infections and those associated with the development of prostate cancer, which affect a significant portion of the population.

Regarding electromigration techniques, the potential use of capillary electrophoresis for pre-concentration of bacterial cells as an innovative method for assessing their sensitivity to various classes of antibiotics is presented. In the section dedicated to mass spectrometry, particular attention is paid to MALDI technique, which is witnessing an increasing trend of implementation in hospital settings. In the section on mass spectrometry, special attention was given to the MALDI technique, which has been steadily increasing in hospital settings. The possibilities and limitations of proteomic and lipidomic approaches in the identification process and the metabolic approach based on enzymatic hydrolysis detection enabling the determination of pathogen susceptibility/resistance to antibiotics are discussed. The dissertation also addresses sample preparation issues, particularly the impact of implementing the culturomic approach on the microbiome identification process. To overcome some of the problems associated with the use of classical organic matrices in laser desorption/ionization techniques, an approach utilizing silver nanoparticles as an effective ionization tool is proposed. The synthesis of nanoparticles was conducted using the chemical vapor deposition method, allowing the production of a layer characterized by high uniformity and showing potential in analyzing low molecular weight compounds. The possibility of their application in distinguishing closely related strains is also presented. Comparative analyses of newly developed methods with traditional approaches, allowing determination of their advantages, limitations, and potential application areas, are an integral part of the work.

The research presented in this dissertation introduces an innovative and interdisciplinary approach to microbiological diagnostics based on advanced analytical methods, which can

significantly contribute to improving healthcare quality and the effectiveness of bacterial infection treatment, particularly crucial in the face of contemporary medical challenges.