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Katarzyna Joanna Pauter-Iwicka

Badanie molekularnych mechanizmów biotransformacji antybiotyków dla potrzeb diagnostyki biomedycznej

Rozprawa doktorska

Praca wykonana pod kierunkiem
Prof. zw. dr hab., dr h.c. Bogusława Buszewskiego, czł. rzecz. PAN
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promotor pomocniczy: dr Viorica Railean

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Spis treści

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WYKAZ SKRÓTÓW

BGE	Bufor separacyjny (ang. *Background Electrolyte*)
CZE	Elektroforeza strefowa (ang. *Capillary Zone Electrophoresis*)
DAD	Detektor diodowy (ang. *Diode Array Detector*)
DNA	Kwas dezoksyrybonukleinowy (ang. *Deoxyribonucleic Acid*)
EPS	Zewnątrzkomórkowe substancje polisacharydowe (ang. *Extracellular Polysaccharide Substances*)
ESI	Jonizacja przez rozpylanie w polu elektrycznym (ang. *Electrospray Ionization*)
GC	Chromatografia gazowa (ang. *Gas Chromtography*)
HPLC	Wysokosprawna chromatografia cieczowa (ang. *High Performance Liquid Chromatography*)
LC	Chromatografia cieczowa (ang. *Liquid Chromtography*)
MALDI - TOF MS	Spektrometria mas z laserową jonizacją/desorpcją próbki wspomagana matrycą z analizatorem czasu przelotu (ang. *Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry*)
MRSA	Gronkowiec złocisty oporny na metycylinę (ang. *Methicyllin Resistant Staphylococcus aureus)*
MS	Spektrometria mas *(*ang. *Mass Spectrometry*)
MS/MS	Tandemowa spektrometria mas *(*ang. *Tandem Mass Spectrometry*)
PBP	Białka wiążące penicylinę (ang. *Penicillin Binding Proteins*)
RP-HPLC	Układ faz odwróconych (ang. *Reverse Phase*)
rRNA	Rybosomalny kwas rybonukleinowy (ang. *Ribosomal Ribonucleic Acid*)
SEM	Skaningowy mikroskop elektronowy (ang. *Scanning Electron Microscope*)
TDM	Terpia monitorowania stężenia leków (ang. *Therapeutic Drug Monitoring*)
TLC	Cienkowarstwowa chromatografia cieczowa (ang. *Thin Layer Chromatography*)
UPLC	Ultrasprawna/ultraszybka chromatografia cieczowa (ang. *Ultra Performance Liquid Chromatography*)
UV-VIS	Spektroskopia w nadfiolecie i świetle widzialnym (ang. *Ultraviolet-Visible Spectroscopy*)
VOC	Lotne związki organiczne (ang. *Volatile Organic Compounds*)

Wprowadzenie

Choroby o etiologii bakteryjnej stanowią jedno z największych wyzwań współczesnej medycyny i farmakoterapii. Obecnie coraz więcej uwagi poświęca się poszukiwaniu nowych cząsteczek biologicznych, tzw. biomarkerów, które w przyszłości mogłyby odegrać ważną rolę we wczesnej, nieinwazyjnej diagnostyce zakażeń bakteryjnych. Nowe podejścia analityczne stosowane przez współczesną naukę pozwalają na poszukiwanie markerów bakterii patogennych w płynach ustrojowych. Niezwykle istotne byłoby zidentyfikowanie jednego czułego markera, którego oznaczenie i identyfikacja w próbce ułatwiłyby potwierdzenie i interpretację zakażenia bakteryjnego.

Obecnie, w leczeniu zakażeń bakteryjnych strategia antybiotykoterapii została uznana za kamień milowy. Współcześnie, antybiotyki są jedną z najczęściej stosowanych grup leków, obok środków przeciwbólowych i przeciwgorączkowych. Ponadto powszechnie uznaje się, że nadużywanie antybiotyków może wiązać się z nawrotami pojedynczych infekcji, bezpośrednią toksycznością, a następnie niewydolnością narządową, a przede wszystkim z pojawieniem się zjawiska oporności bakterii. Dlatego, w dobie narastającej antybiotykooporności, prosta, szybka i tania identyfikacja zakażenia bakteryjnego oraz określenie odpowiedzi na antybiotyki są niewątpliwie niezbędne do optymalizacji terapii indywidualnego pacjenta oraz zmniejszenia ryzyka wystąpienia zjawiska oporności. Bardzo ważne jest również upowszechnienie świadomości, że optymalizacja antybiotykoterapii to nie tylko koncentracja na sile terapeutycznej, ale również minimalizacja ryzyka powstania oporności w trakcie terapii, zarówno patogenów zakaźnych, jak i fizjologicznej flory bakteryjnej.

Dostępne narzędzia ułatwiają lekarzom wprowadzenie pacjenta do terapii farmakologicznej. Uwzględniają one indywidualną zmienność metabolizmu pacjenta, a tym samym pozwalają na dobór farmaceutyku i jego dawki w celu uzyskania maksymalnej skuteczności leczenia. Jednak niepowodzenia obecnie stosowanych metod diagnostycznych, skłaniają naukowców i lekarzy do poszukiwania nowych leków oraz nowych sposobów postawienia właściwej diagnozy i zastosowania odpowiedniego leczenia w kierunku źródła choroby.

Z tego względu nadrzędnym celem przedstawionej rozprawy doktorskiej było opracowanie nowej procedury analitycznej mającej na celu ocenę wartości terapeutycznej i przydatności leków przeciwbakteryjnych i ich metabolitów jako wyznaczników antybiotykooporności w oparciu o analizę profili białkowych i metabolicznych.

Niniejsza praca doktorska składa się z pięciu publikacji naukowych opublikowanych w specjalistycznych czasopismach z listy filadelfijskiej Journal Citation Reports (JCR):

[P1] K. Pauter, M. Szultka-Młyńska, B. Buszewski, *Determination and identification of antibiotic drugs and bacterial strains in biological samples*, Molecules (2020) 25, (11), 1-45. IF = 4.927 PM = 140.

[P2] J. Walczak-Skierska, M. Szultka-Młyńska, **K. Pauter**, B. Buszewski, *Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD*, Journal of Pharmaceutical and Biomedical Analysis (2020) 184, 113187. IF = 3.571 PM = 100.

[P3] K. Pauter, M. Szultka-Młyńska, M. Szumski, A. Król-Górniak, P. Pomastowski, B. Buszewski, *CE-DAD-MS/MS in the simultaneous determination and identification of selected antibiotic drugs and their metabolites in human urine samples*, Electrophoresis (2021) 0, 1-13. IF = 3.595 PM = 70.

[P4] K. Pauter, V. Railean-Plugaru, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, B. Buszewski, *Identification, structure and characterization of Bacillus tequilensis biofilm with the use of electrophoresis and complementary approaches*, Journal of Clinical Medicine (2022) 11, (3), 722. IF = 4.964 PM = 140.

[P5] K. Pauter-Iwicka, V. Railean, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, D. Błońska, W. Kupczyk, B. Buszewski, *Characterization of the salivary microbiome before and after antibiotic therapy via separation technique,* Applied Microbiology and Biotechnology (2023) 107, (7-8), 2515-2531. IF = 5.560 PM = 100.

1. Cele badawcze

Nadrzędnym celem badań zrealizowanych w ramach niniejszej rozprawy doktorskiej było opracowanie nowej procedury analitycznej mającej na celu ocenę wartości terapeutycznej i przydatności leków przeciwbakteryjnych i ich metabolitów jako wyznaczników antybiotykoodporności w oparciu o analizę profili białkowych i metabolicznych [**P1**].

Podczas realizacji pracy doktorskiej, wyodrębniono następujące cele szczegółowe:

- Dobór warunków separacji wybranych antybiotyków i ich metabolitów z zastosowaniem chromatografii cieczowej [**P2**]
- Oznaczenie i identyfikacja wybranych antybiotyków i ich metabolitów w próbkach moczu ludzkiego za pomocą elektroforezy kapilarnej [**P3**]
- Ocena skuteczności wybranych antybiotyków wobec bakterii tworzących biofilm z zastosowaniem elektroforezy kapilarnej i technik pokrewnych [**P4**]
- Identyfikacja mikrobiomu śliny człowieka i monitorowanie zmian profilu molekularnego pod wpływem wybranych antybiotyków [**P5**]

Poszczególne etapy pracy doktorskiej przeprowadzono z zastosowaniem poniższych technik instrumentalnych:

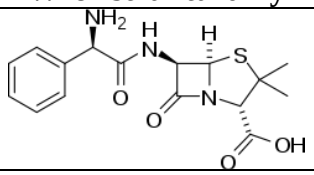
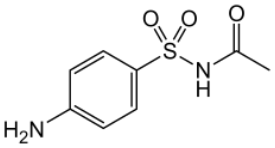
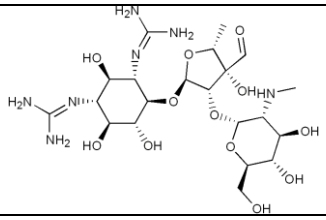
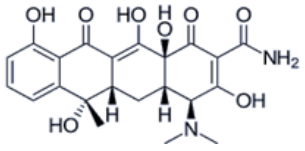
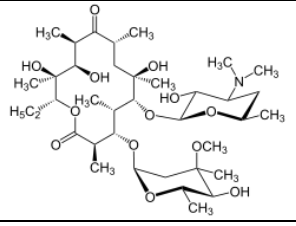
- wysokosprawna chromatografia cieczowa (HPLC) z detektorem DAD
- elektroforeza kapilarna (CE) z detektorem DAD oraz sprzężona z tandemowym spektrometrem mas (CE -MS/MS)
- spektrometria mas z jonizacją/desorpcją laserową wspomaganą matrycą oraz analizatorem czasu przelotu (MALDI-TOF MS)
- mikroskop fluorescencyjny
- skaningowy mikroskop elektronowy (SEM)
- pomiar potencjału zeta

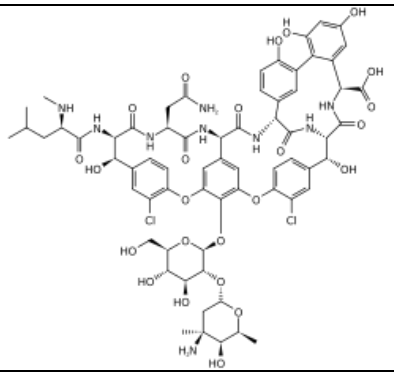
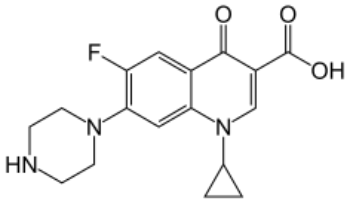
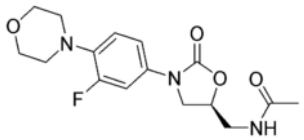
2. Problem badawczy

2.1. Leki przeciwbakteryjne

Antybiotyki obejmują zróżnicowaną grupę związków chemicznych, zarówno naturalnych, jak i syntetycznych, które wykazują działanie przeciwbakteryjne - są zdolne do zabijania lub hamowania wzrostu mikroorganizmów. Istnieje kilka sposobów podziału leków przeciwbakteryjnych, ale najpopularniejsze schematy klasyfikacji opierają się na ich strukturze molekularnej, sposobie działania i spektrum aktywności [1]. Leki należące do tej samej grupy strukturalnej będą miały podobną skuteczność i toksyczność. Niektóre popularne grupy antybiotyków to β-laktamy, makrolidy, tetracykliny, chinolony, aminoglikozydy, sulfonamidy i glikopeptydy, oksazolidynony [1-3]. Przykłady leków przeciwbakteryjnych z poszczególnych grup, a także struktury chemiczne oraz stałe dysocjacji zostały przedstawione w **tabeli 1**.

Tabela 1. Podział antybiotyków w oparciu o spektrum przeciwbakteryjne.

Grupa antybiotyków	Przykład	Wzór strukturalny	pK_{-COOH}	pK_{-NH3+}
β-lakatamy	**ampicylina** inne przykłady: oksacylina, cefotaksym		3.24	7.44
sulfonamidy	**sulfacetamid** inne przykłady: sulfadiazyna, sulfametoksazol		4.30	2.14
aminoglikozydy	**streptomycyna** inne przykłady: gentamycyna, kanamycyna		12.55	10.8
tetracykliny	**tetracyklina** inne przykłady oksytetracyklina, doksycyklina		-2.20	8.24
makrolidy	**erytromycyna** inne przykłady: azytromycyna, spektynomycyna		12.44	8.38

glikopeptydy	**wankomycyna** inne przykłady: teikoplanina, bleomycyna		2.99	9.38
chinolony	**cyprofloksacyna** inne przykłady: lewofloksacyna, moksyfloksacyna		5.76	8.68
oksazolidynony	**linezolid** inne przykłady: tedizolid		14.55	-0.66

**pK*- *stała dysocjacji*

W przeważającej części przeprowadzonych badań antybiotyki są aktywne wobec bakterii Gram-dodatnich i w mniejszym stopniu wobec bakterii Gram-ujemnych. W przypadku badań mechanizmu oddziaływania antybiotyków na bakterie występują antybiotyki bakteriobójcze (dla przykładu wankomycyna, amoksycylina, cefuroksym) i bakteriostatyczne (dla przykładu cykloseryna, linezolid, azytromycyna). Antybiotyki znajdują szerokie zastosowanie w lecznictwie ludzi i zwierząt. Celem ich stosowania jest wyeliminowanie drobnoustrojów patogennych z miejsca infekcji lub z chorego organizmu. Może to nastąpić wtedy, gdy bakterie wykazują wrażliwość na podany lek, a dawka leku osiągnie w tkance lub płynie ustrojowym pewną wartość progową, powyżej której bakterie nie są zdolne do przeżycia.

Silna aktywność przeciwdrobnoustrojowa antybiotyków skłania badaczy do analizy mechanizmów tego zjawiska. Mechanizm antybakteryjny jest niezwykle zróżnicowany i nie ma uniwersalnego wytłumaczenia działania wszystkich antybiotyków. Sukces związków przeciwbakteryjnych zawdzięcza się w dużej mierze ich selektywnemu działaniu na komórki bakteryjne i jednoczesnemu braku działania na komórki zwierzęce. Związane jest to z ich budową oraz przebiegającymi w nich szlakami metabolicznymi. Na **rysunku 1** zaprezentowano docelowe miejsca działania poszczególnych grup antybiotyków wraz z ich przykładami. Przedstawione mechanizmy działania związków przeciwbakteryjnych (**Rys. 1**), zostały szczegółowo opisane poniżej:

a) blokowanie poprzez zahamowanie syntezy ściany komórkowej bakterii. Dochodzi do zahamowania ostatniego etapu syntezy ściany komórkowej (reakcja jest katalizowana przez enzym transpeptydazę), w wyniku czego ściana staje się nieszczelna. Wewnątrzkomórkowe stężenie soli jest znacznie większe niż na zewnątrz, w związku z tym komórka pęcznieje i dochodzi do jej lizy (pęknięcia);
b) hamowanie poprzez zaburzenie metabolizmu komórki. W tym przypadku dochodzi do zahamowania metabolizmu drobnoustrojów;
c) oddziaływanie na strukturę błony cytoplazmatycznej;
d) hamowanie syntezy białek;
e) hamowanie transkrypcji oraz replikacji kwasu DNA. Antybiotyki działając enzymem – gyrazą DNA powodują zahamowanie transkrypcji i replikacji kwasu nukleinowego. W konsekwencji uniemożliwia to poprawne odczytanie kodu genetycznego.

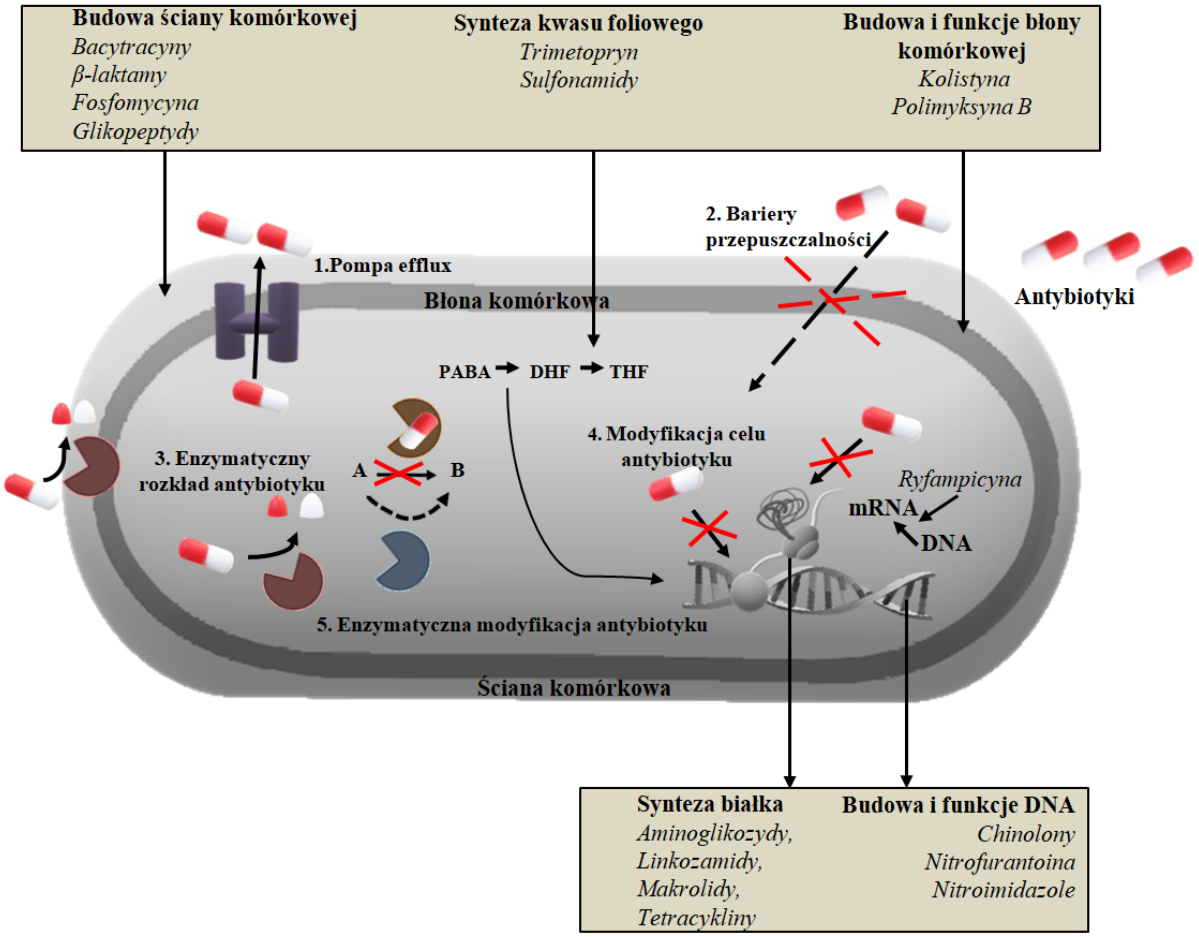
Jak można zauważyć na **rysunku 1**, docelowym miejscem działania antybiotyków może być ściana komórkowa, której syntezę na różnych etapach powstawania zaburzają m.in. β-laktamy, glikopeptydy oraz fosfomycyna.

Istota działania przeciwbakteryjnego tych leków polega na hamowaniu tworzenia mostków łączących podjednostki peptydoglikanu w integralną całość. Proces ten katalizowany jest przez enzymy bakteryjne zwane białkami wiążącymi penicyliny (*ang. Penicillin Binding Proteins, PBP*), zlokalizowane w błonie komórkowej bakterii, które wiążą antybiotyk. W wyniku trwałego związania z antybiotykiem, funkcja enzymów (PBP) zostaje zablokowana, a w konsekwencji dojrzewanie i podziały komórkowe zostają zahamowane [4]. Antybiotyki (sulfonamidy, trimetoprym) mogą również wpływać na aktywność ważnych szlaków metabolicznych w komórce. Jednym z najbardziej znanych przykładów jest hamowanie syntezy kwasu foliowego, co prowadzi do zaburzenia syntezy DNA (**Rys. 1**) [5].

Natomiast, środki przeciwbakteryjne z grupy polimyksyn wpływają na rozpad błony komórkowej i zwiększenie jej przepuszczalności dla jonów. Polimyksyny posiadają specyficzną budowę, która pozwala im wiązać się ze składnikami lipidowymi błony komórkowej, powodując utratę jej szczelności.

Daptomycyna jest również antybiotykiem zaburzającym funkcjonowanie błony komórkowej. Mechanizm działania daptomycyny polega na jej nieodwracalnym wiązaniu się z błoną komórkową bakterii Gram-dodatnich, w obecności jonów wapnia. Efektem tego działania jest tworzenie kanałów prowadzących do depolaryzacji błony komórkowej i wypływu potasu oraz innych jonów z wnętrza komórki. W wyniku tego procesu dochodzi do zniszczenia błony protoplazmatycznej i poważnego zaburzenia syntezy makrocząsteczek [6].

Stosunkowo duża grupa różnych antybiotyków hamuje syntezę białek na wielu etapach, od inicjacji translacji do właściwej elongacji łańcucha. Z syntezą białek związane są specjalne struktury komórkowe zwane rybosomami. Rybosom bakteryjny składa się z kwasów rybonukleinowych (rRNA) i białek. Rozdziela się on na dwie podjednostki- dużą (50S) i małą (30S).



Rys. 1. Miejsca działania antybiotyków w komórce bakteryjnej oraz mechanizmy oporności bakterii na antybiotyki.

Ze względu na wiele klas antybiotyków hamujących syntezę białek, molekularne mechanizmy ich działania są różne. Zazwyczaj cząsteczki leków wiążą się z różnymi cząsteczkami białka rybosomalnego lub cząsteczkami rybosomalnego RNA zarówno w podjednostce 30S, jak i 50S, powodując śmierć komórki. Do leków przeciwbakteryjnych zaburzających syntezę białek należą aminoglikozydy, makrolidy, linkozamidy oraz tetracykliny [7]. Natomiast, podstawową klasą antybiotyków zaburzających syntezę DNA są chinolony i ich pochodne, takie jak fluorochinolony (leki II i III generacji). Antybiotyki te są specyficznymi inhibitorami domen ligazowych topoizomerazy II (gyrazy) oraz domen topoizomerazy IV.

W wyniku działania domeny nukleolitycznej dochodzi do fragmentacji DNA w komórce [8]. Oprócz wpływu na syntezę DNA i okres półtrwania, istnieje grupa antybiotyków wpływających na syntezę RNA (ansamycyny), do której należy powszechnie znana ryfampicyna. Wiąże się ona specyficznie z bakteryjną polimerazą RNA w pobliżu miejsca aktywnego i uniemożliwia wydłużanie łańcucha RNA.

2.2. Antybiotykooporność

Od czasu wprowadzenia pierwszych antybiotyków do leczenia zakażeń bakteryjnych, lekooporność patogenów stała się poważnym problemem zdrowotnym. Przyczynami są różne czynniki, takie jak nieodpowiedzialne dawkowanie antybiotyków, naturalnie występujące mutacje oraz przenoszenie szczepów lekoopornych [**P1**, 9].

Oporność na antybiotyki może być określona na podstawie informacji genetycznej zakodowanej w chromosomie lub w elementach ruchomych, takich jak plazmidy, transpozony i integrony. Bakterie mogą być naturalnie oporne na określoną grupę antybiotyków lub mogą nabywać oporność w wyniku różnych procesów genetycznych, w tym mutacji, transferu genów oporności, a także poprzez bezpośredni kontakt z komórkami.

Molekularne mechanizmy oporności na środki przeciwdrobnoustrojowe u bakterii zostały zobrazowane na **rysunku 1**. Przekazywanie genów odpornościowych odbywa się na drodze horyzontalnego transferu genów. Wektorami niosącymi takie geny są najczęściej plazmidy (zwane plazmidami *R-odporności*), które w procesach koniugacyjnych mogą być przenoszone z komórki dawcy do komórki biorcy. Oporność na antybiotyki może być również determinowana przez ruchome elementy genetyczne, takie jak transpozony czy integrony, które są jednym ze źródeł powstawania szczepów bakteryjnych opornych na kilka chemioterapeutyków jednocześnie. Zmienność genomu prowadzi do zmiany metabolizmu komórki, co skutkuje pojawieniem się enzymów o szerokim spektrum działania, w tym inaktywujących antybiotyki. Kolejnym czynnikiem mechanizmu oporności na antybiotyki jest fakt, że bakterie mają do dyspozycji pompy efflux. Pompy te zlokalizowane w błonie cytoplazmatycznej (**Rys. 1**) są białkami transportującymi substancje toksyczne, w tym antybiotyki, na zewnątrz komórki bakteryjnej. Pompy efluksowe występują zarówno w komórkach bakterii Gram-dodatnich, jak i Gram-ujemnych i są one ważnym narzędziem inicjowania antybiotykooporności, w tym rozwoju oporności wieloczynnikowej [10].

Acar i Moulin [11] opisali, iż zdolność bakterii do nabywania oporności na antybiotyki zależy od zdolności poszczególnych bakterii do przystosowania się do presji selektywnej stosowanego antybiotyku. W zaproponowanej przez nich klasyfikacji, wyróżniono następujące mechanizmy oporności na leki przeciwbakteryjne:

1) efflux – usuwanie antybiotyku z komórki bakteryjnej przy pomocy pomp białkowych;
2) zmniejszenie przepuszczalności błony komórkowej bakterii;
3) modyfikacja antybiotyku w jego nieaktywnej formie z udziałem enzymów wytwarzanych przez bakterie; mogą one zmieniać antybiotyk wewnątrz lub na zewnątrz komórki bakteryjnej, eliminując jego działanie przeciwbakteryjne;
4) zmiana celu działania antybiotyku, zmniejszająca jego powinowactwo do niego;
5) mutacje bakteryjne skutkujące eliminacją bakterii opornych na antybiotyk;
6) występowanie mieszanej populacji bakterii wrażliwych i opornych na antybiotyki [11].

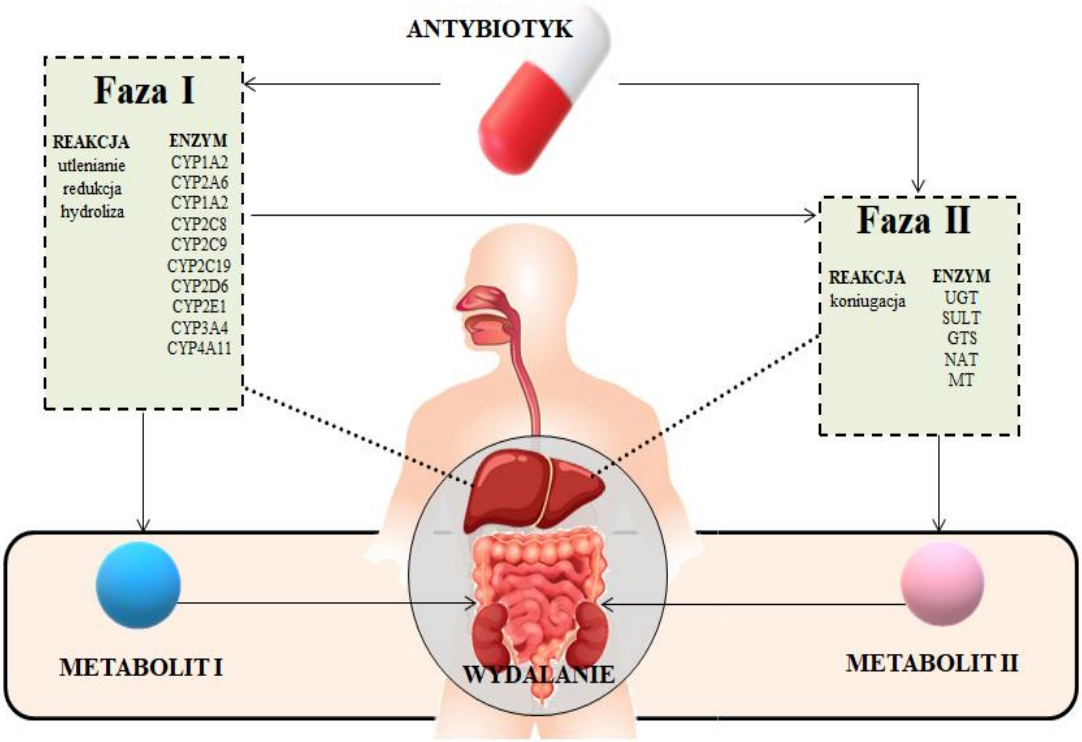
Stale rozwijające się mechanizmy oporności znacząco utrudniają właściwy dobór antybiotyków w leczeniu zakażeń bakteryjnych. Ważnym czynnikiem w selekcji opornych populacji drobnoustrojów jest zastosowanie odpowiedniego antybiotyku. Jednym z czynników zapobiegających szybkiemu rozprzestrzenianiu się lekoopornych szczepów bakteryjnych może być monitorowanie występowania antybiotykooporności i jej mechanizmów oraz rozsądne podawanie i przyjmowanie leków przeciwbakteryjnych.

2.3. Metabolizm leków przeciwbakteryjnych

Każda substancja czynna, którą dostarczamy do naszego organizmu musi przejść kilka etapów, aby uzyskać odpowiedni efekt farmakologiczny. Antybiotyki ulegają biotransformacji - modyfikacji biochemicznej, nie tylko w wątrobie, ale również w nerkach, krwi i ścianach jelita cienkiego. Metabolizm antybiotyków obejmuje różne procesy, które dzieli się na reakcje fazy I i fazy II (**Rys. 2**) [12].

Reakcje fazy I prowadzą do powstania produktów pośrednich w procesach utleniania, redukcji i deaminacji. Natomiast reakcje fazy II polegają na sprzęganiu produktów końcowych z kwasem glukuronowym, kwasem siarkowym, glutationem i glicyną oraz mogą prowadzić do metylacji lub acetylacji. Faza I zachodzi głównie przy udziale oksydoreduktaz i hydrolizy, w przeciwieństwie do fazy II, która odbywa się przy udziale transferaz katalizujących reakcje koniugacji (glukorylotransferazy) lub enzymów cytozolowych (sulfotransferaza, N-acetyltransferaza). Powstające w ten sposób metabolity różnią się właściwościami i można je podzielić na aktywne, nieaktywne, toksyczne lub takie, które pod wpływem odpowiednich czynników fizycznych ulegają przekształceniu w związek pierwotny.

W większości przypadków pod wpływem biotransformacji powstają związki nieaktywne, które w krótkim czasie są eliminowane z organizmu.

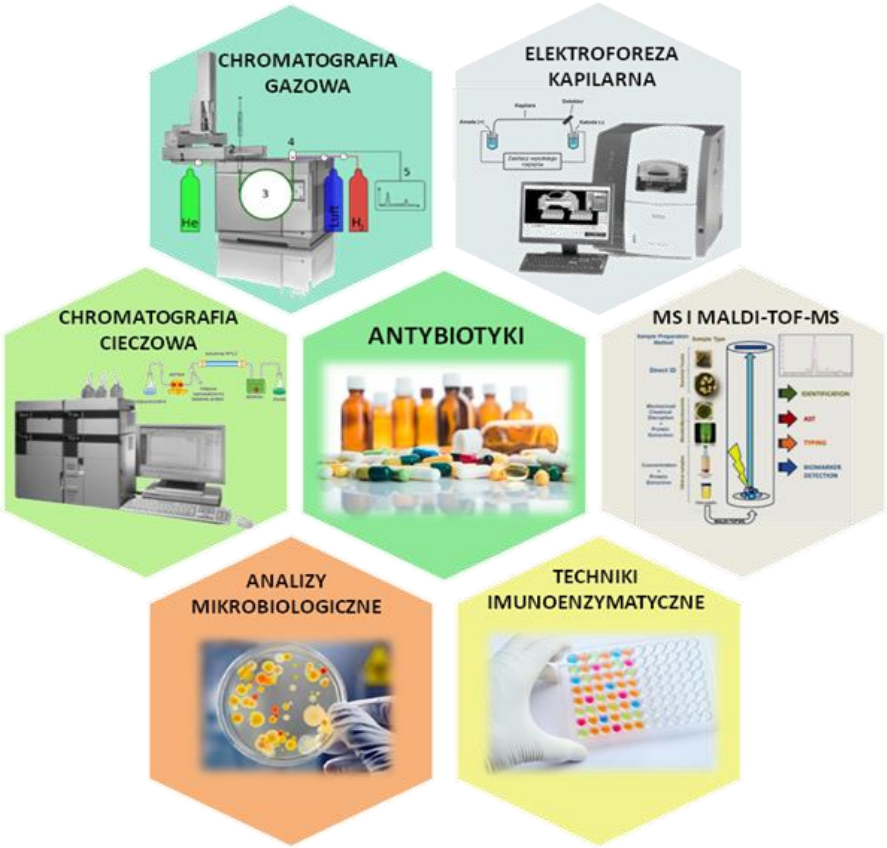


Rys. 2. Koncepcja metabolizmu antybiotyków w wątrobie.

Aktywne metabolity mogą jednak powstawać zarówno z aktywnych, jak i nieaktywnych związków macierzystych. Podczas, gdy nieaktywny lek jest przekształcany w organizmie w aktywny metabolit, dochodzi do konwersji proleku w lek. W przypadku, gdy lek macierzysty jest aktywny, to powstałe aktywne metabolity mogą mieć działanie synergistyczne lub addytywne i mogą przedłużać i/lub wzmacniać jego działanie. Metabolity te mogą charakteryzować się mniejszą toksycznością niż związek macierzysty lub inną aktywnością farmakologiczną. Cecha ta jest widoczna w rozwoju nowych leków. Procesy metaboliczne mogą również prowadzić do utworzenia substancji toksycznych, które zwiększają szkodliwość stosowanego leku, lub do powstawania metabolitów, które w odpowiednich warunkach środowiskowych ponownie wchodzą w skład związku macierzystego. W zależności od rodzaju powstałego metabolitu może wzrosnąć aktywność przeciwdrobnoustrojowa lub toksyczność związku macierzystego, co może skutkować szeregiem ograniczeń związanych z przyjmowaniem antybiotyków [13].

2.4. Metody oznaczania antybiotyków i ich metabolitów

Strategia antybiotykoterapii uważana jest za kamień milowy w leczeniu zakażeń bakteryjnych. Jednak, jednym z najważniejszych narzędzi w optymalizacji leczenia poszczególnych pacjentów jest zastosowanie indywidualnego podejścia podczas przyjmowania farmaceutyków, czyli terapii monitorowanej [14]. Celem tej terapii jest dokładna ocena stanu klinicznego oraz zapewnienie pomiaru stężenia leku i metabolitów w płynach biologicznych. Kontrola stężenia leków i ich metabolitów w czasie rzeczywistym jest jedną z najskuteczniejszych metod personalizacji terapii. Ponadto uwzględnia ona indywidualne cechy pacjenta oraz potencjalne interakcje podawanych farmaceutyków z innymi związkami endogennymi. Możliwość monitorowania wczesnych zmian chorobowych poprzez poszukiwanie potencjalnych biomarkerów wraz z oznaczaniem leków i ich metabolitów w monitorowanej terapii wymaga opracowania nowych metod i ich zastosowania w badaniach próbek biologicznych. W pracy przeglądowej [**P1**] - *Determination and identification of antibiotic drugs and bacterial strains in biological samples* opisano i szczegółowo przedyskutowano dotychczasowo stosowane techniki do oznaczania antybiotyków, które zostały zobrazowane na **rysunku 3**.



Rys. 3. Techniki stosowane do oznaczania antybiotyków.

Ponadto, w pracy [**P1**] zestawiono najczęściej wykorzystywane warunki chromatograficzne oraz elektroforetyczne w zależności od grupy oznaczanych antybiotyków oraz matrycy, z której zostały one wyizolowane.

Jednymi z pierwszych metod oznaczania antybiotyków w matrycach biologicznych były techniki immunoenzymatyczne, które charakteryzują się szeroką oznaczalnością, wysoką czułością oraz krótkim czasem analizy, ponieważ nie wymagają odrębnych technik izolacji z materiału biologicznego. Niestety, metody te nie są wolne od wad. Istotnym problemem w stosowaniu tych testów jest niespecyficzność reakcji na poszczególne antybiotyki z danej grupy. Mogą również wystąpić reakcje krzyżowe wywołane przez inną grupę związków lub wynikające z wpływu matrycy biologicznej. Ponadto elementem utrudniającym pełną ocenę zależności ilościowej w badanym materiale jest częste zjawisko jednoczesnego oznaczania ze związkiem macierzystym jego metabolitów, których obecność może zaburzać wartości bezwzględne uzyskanego wyniku. Stąd w ostatnich latach, do celów analityki biomedycznej najczęściej stosowane są techniki chromatograficzne połączone z metodami spektralnymi oraz metodami immunochemicznymi. Wśród technik separacyjnych najczęściej stosowane są chromatografia cieczowa *(ang. Liquid Chromatography, LC*), wysokosprawna chromatografia cieczowa (*ang. High Performance Liquid Chromatography, HPLC*) oraz chromatografia gazowa (*ang. Gas Chromatography, GC*) w połączeniu z różnymi systemami detekcji (spektrofotometrycznymi, fluorescencyjnymi, elektrochemicznymi, spektrometrią mas (*ang. Mass Spectrometry, MS*) [[**P1**], 14-15]. Warto podkreślić, iż połączenie chromatografii cieczowej ze spektrometrem mas gwarantuje wysoką selektywność, dużą czułość, rozdzielczość, powtarzalność, identyfikację przez masę i określenie struktury przez fragmentację, wraz z wszechstronnością zastosowania. Istotną zaletą metod chromatograficznych jest możliwość jednoczesnego oznaczania różnych leków i ich potencjalnych metabolitów w materiale biologicznym pobranym od pacjenta [16,17].

W ostatnich latach również techniki elektromigracyjne (elektroforeza strefowa, *ang. Capillary Zone Electrophoresis, CZE*) zyskały na znaczeniu jako wysoce wydajne narzędzie analityczne. Ich przewagą nad powszechnie stosowanymi technikami chromatograficznymi jest duża szybkość i wydajność separacji, wysoka rozdzielczość, małe zużycie odczynników oraz niskie koszty pojedynczych analiz [18]. Ponadto, ze względu na małe zużycie odczynników, zwłaszcza organicznych oraz małą produkcję odpadów powstających w procesie rozdzielania, technika ta jest powszechnie uznawana za wysoce przyjazną środowisku (*zielona chemia*) [19]. Fakt ten jest bardzo ważnym argumentem przemawiającym za rozwojem technik elektromigracyjnych w wielu dziedzinach analityki, zwłaszcza w biomedycynie i farmacji.

Antybiotyki są często stosowane w terapii skojarzonej w celu zapewnienia szerokiego zakresu działania, zmniejszenia toksyczności i poprawy skuteczności terapeutycznej. W związku z tym, konieczne jest opracowanie wielowymiarowych metod oznaczania więcej niż jednego antybiotyku w tej samej analizie wykonywanej w tym samym czasie. Ponadto, jeśli chodzi o praktykę kliniczną, konieczne jest opracowanie podejścia, które jest łatwe do zastosowania i obejmuje szeroki zakres grup antybiotyków. W ten sposób skraca się czas uzyskiwania wyników przez lekarzy. Dlatego też, pierwszym etapem badań przeprowadzonych w ramach pracy doktorskiej był dobór warunków rozdzielania, umożliwiający jednoczesne oznaczenie leków przeciwbakteryjnych. Stąd, w pracy pt. *Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD* [**P2**], opracowano procedurę oznaczania i identyfikacji 11 leków przeciwbakteryjnych oraz ich metabolitów z różnych klas terapeutycznych za pomocą chromatografii cieczowej z detekcją DAD.

Do analizy chromatograficznej antybiotyków zastosowano 4 różne fazy stacjonarne, różniące się właściwościami fizykochemicznymi i mechanizmem retencji. Jako fazę ruchomą zastosowano acetonitryl i 0,1% kwas mrówkowy w wodzie. Dodatek modyfikatora organicznego poprawiał kształt sygnałów (pików). W badaniach zastosowano elucję gradientową, w której zwiększano zawartość acetonitrylu. Ponadto, w pracy [**P2**] do optymalizacji procesu rozdzielenia antybiotyków i ich metabolitów w układzie faz odwróconych - RP-HPLC wykorzystano oprogramowanie ChromSword.

Dalszy etap badań pracy ukierunkowany był na opracowaniu metody identyfikacji wybranych antybiotyków i ich aktywnych farmakologicznie metabolitów w płynach ustrojowych za pomocą elektroforezy kapilarnej. W pracy [**P3**] opisano szybką, prostą i tanią procedurę analityczną do oznaczania i identyfikacji antybiotyków i ich aktywnych pod względem farmakologicznym metabolitów w próbkach ludzkiego moczu z wykorzystaniem metody CE-DAD-ESI-MS/MS. Natomiast technika CE-DAD została wykorzystana do optymalizacji ważnych parametrów wpływających na warunki analityczne dotyczące migracji i separacji związków docelowych, aby lepiej zrozumieć procesy zachodzące podczas przebiegu elektroforetycznego. W związku z tym, podczas opracowywania metody zbadano wpływ różnych warunków analitycznych: skład, stężenie i wartość pH buforu separacyjnego (*ang. Background Electrolyte, BGE*), czas i ciśnienie iniekcji, temperatura kapilary, wpływ modyfikatora organicznego). W opisanej pracy zastosowano strącanie białka jako prosty etap obróbki wstępnej w analizie antybiotyków i ich metabolitów w ludzkim moczu, co pozwoliło uzyskać zadowalającą wydajność ekstrakcji.

Ponadto zastosowany prosty etap przygotowania próbki klinicznej pozwolił na zmniejszenie objętości rozpuszczalników organicznych i próbek biologicznych. Zaproponowana metoda jest precyzyjna i powtarzalna przy stosunkowo krótkim czasie analizy, zarówno w zasadowym, jak i w kwaśnym buforze separacyjnym. Metoda ta, w porównaniu z konwencjonalną LC, jest prostsza i szybsza; wymaga również minimalnego przygotowania próbki. Ponadto, jest dobrą alternatywą dla konwencjonalnych metod LC, ponieważ spełnia wymogi *zielonej chemii*, gdyż wymaga małej ilości rozpuszczalników, odczynników chemicznych oraz niewielkiej ilości próbek. Implementacja proponowanej metody dla ludzkich próbek klinicznych daje możliwość oceny skuteczności antybiotyków, co może być korzystne dla optymalizacji indywidualnych antybiotykoterapii. Ponadto, ze względu na dobrą czułość, precyzję i powtarzalność, metoda CE-ESI-MS/MS może być z powodzeniem stosowana w analizie przesiewowej pacjentów w celu eliminacji kombinacji leków, które nie są wskazane do jednoczesnego przyjmowania.

Ze względu na to iż, zakażenia bakteryjne związane z biofilmem stanowią istotne wyzwanie w leczeniu farmakologicznym, dalsze badania oparte były na zaprojektowaniu komplementarnego podejścia w charakterystyce biofilmu przed i po antybiotykoterapii.

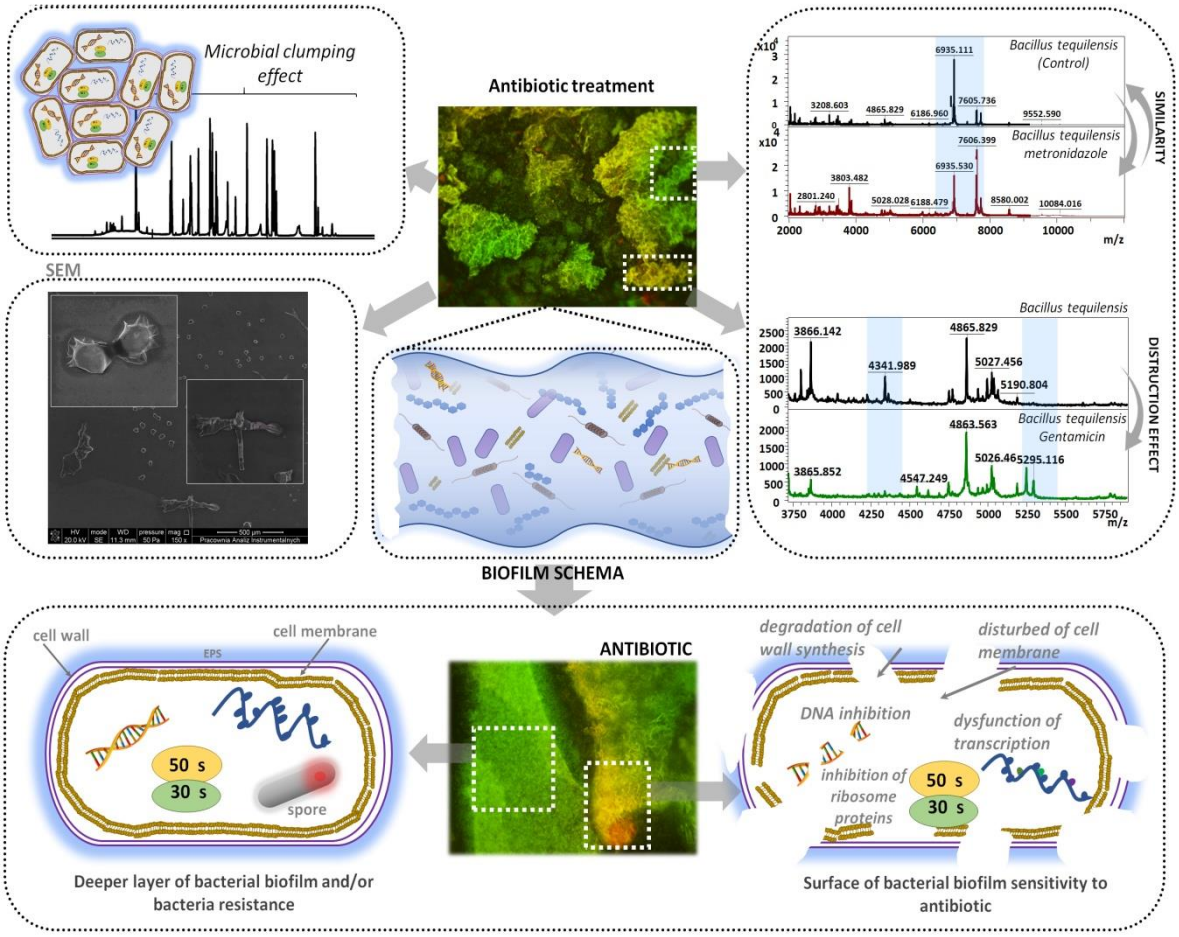
Zgodnie z wcześniejszymi doniesieniami, biofilm upośledza mechanizmy obronne człowieka i tworzy platformę dla mikroorganizmów do wykorzystania szeregu strategii zwalczania oporności na antybiotyki, w tym produkcji komórek przetrwalnikowych, wielowarstwowego systemu komórek bakterii i oporności na środki przeciwdrobnoustrojowe [20,21]. Zdolność bakterii do tworzenia biofilmu ma ogromne znaczenie zarówno ze względu na jego funkcje ochronne, jak i patogenne. Dlatego też, antybiotykoterapia miejscowa jest niewątpliwie pożądaną alternatywą dla antybiotykoterapii ogólnoustrojowej w celu zapobiegania powstawaniu biofilmu i ochrony pacjenta przed skutkami ubocznymi często długotrwałego podawania antybiotyków.

Biofilm jest nagromadzeniem komórek bakteryjnych na powierzchni w oparciu o proces aglomeracji i jest otoczony przez zewnątrzkomórkowe substancje polisacharydowe (EPS) [22,23]. Stwierdzono, że struktura EPS wykazuje specyfikę gatunkową lub szczepową zarówno pod względem składu monosacharydów, które stanowią podjednostkę EPS, jak i rodzaju wiązań chemicznych lub podstawników niesacharydowych. Niekiedy forma egzopolisacharydów może zależeć od intensywności wzrostu komórki. Wzrost składników polisacharydowych w macierzy skutkuje większą ilością wolnych grup funkcyjnych zdolnych do oddziaływania z innymi komórkami bakteryjnymi lub cząsteczkami aktywnymi, np. antybiotykami. Decyduje więc o znacznej oporności mikroorganizmów w biofilmie [24-28].

Złożoność struktury biofilmu oraz jego wysoka oporność na antybiotyki są przyczyną trudności napotykanych podczas terapii zakażeń bakteryjnych związanych z biofilmem. Poszerzenie wiedzy na temat powstawania, funkcjonowania i struktury biofilmu stanowi podstawę do poszukiwania alternatywnych metod zapobiegania i leczenia zakażeń związanych z jego występowaniem. Zgodnie z aktualnymi doniesieniami literaturowymi niezwykle istotna jest aktywność związków przeciwdrobnoustrojowych w stosunku do biofilmu [29,30]. Dlatego też, w pracy [**P4**] określono rolę ekspozycji biofilmu bakterii na antybiotyki należące do różnych grup terapeutycznych, o różnym spektrum i mechanizmie działania, a także o różnej strukturze chemicznej, takie jak amoksycylina, gentamycyna i metronidazol. Badania zostały przeprowadzone przy użyciu modelowej bakterii wytwarzającej biofilm – *Bacillus tequilensis*, wyizolowanej z miodu. Ponadto, do monitorowania zlepiania się wyizolowanych bakterii w polu elektrycznym zastosowano elektroforezę kapilarną oraz techniki komplementarne, w tym sekwencjonowanie genu 16S rRNA (do identyfikacji), pomiar potencjału zeta (do sprawdzenia stabilności dyspersji i kontroli procesu agregacji) oraz technikę MALDI-TOF MS (do określenia profilu molekularnego), a także mikroskopię fluorescencyjną (do oznaczenia żywotności komórek bakteryjnych w warunkach stresowych) i mikroskop skaningowy (do zobrazowania morfologii i struktury biofilmu).

Warto podkreślić, iż zastosowanie w pracy [**P4**] złożonego i komplementarnego podejścia do charakterystyki biofilmu zarówno przed, jak i po antybiotykoterapii, umożliwiło zaproponowanie mechanizmu działania badanych antybiotyków na zjawisko zlepiania i degradacji w komórce bakteryjnej tworzącej biofilm. Schemat opisanego mechanizmu został zobrazowany na **rysunku 4**. Dzięki zastosowaniu metody sekwencjonowania genu 16S rRNA, bakteria wyizolowana z miodu została zidentyfikowana jako model tworzenia biofilmu - *Bacillus tequilensis*. Warto zauważyć, iż, elektroforeza kapilarna okazała się użytecznym narzędziem w charakteryzowaniu biofilmu. Zastosowanie tej techniki umożliwiło obserwację zmian ruchliwości elektroforetycznej komórek bakteryjnych nieleczonych i leczonych antybiotykami (amoksycylina, gentamycyna, metronidazol). Ponadto, zauważono, że leczenie amoksycyliną i gentamycyną spowodowało zanik dużych agregatów (zjawisko zlepiana – *„clumping phenomenon''*). W tym przypadku nastąpiły silne zmiany w ładunku elektrycznym na powierzchni komórek. Bardziej uszkodzone lub wrażliwe komórki bakteryjne tworzyły bardziej rozproszony układ i mniejszą agregację w stopionej krzemionce poddanej analizie CE. Docelowe miejsca działania badanych antybiotyków zostały zobrazowane na **rysunku 4** i obejmowały: degradację syntezy ściany komórkowej; zaburzenie błony komórkowej; hamowanie syntezy DNA i białek rybosomalnych; zaburzenie transkrypcji.

Co więcej, zastosowanie komplementarnych technik pozwoliło na monitorowanie różnic w stabilności dyspersji i profilach molekularnych, a także żywotności i morfologii komórek bakteryjnych tworzących biofilm pod wpływem antybiotyków.



Rys. 4. Schemat mechanizmu reakcji biofilmu bakteryjnego - *B. tequilensis*

Dodatkowo, analiza MALDI-TOF MS wskazała na zmiany w profilach molekularnych *B. tequilensis* przed i po antybiotykoterapii, prowadząc do proponowanych mechanizmów oporności na antybiotyki. Co można zaobserwować na przedstawionym schemacie (**Rys. 4**). Natomiast zastosowanie mikroskopii fluorescencyjnej wykazało, że komórki bakteryjne w głębszych warstwach biofilmu są w stanie dostosować się do środowiska i nabyć mechanizmy oporności w wyniku działania mechanizmów molekularnych. Wreszcie, skaningowa mikroskopia elektronowa została wykorzystana do obserwacji zmian na powierzchni biofilmu pod wpływem antybiotyków zarówno przed, jak i po elektroforezie kapilarnej.

Na podstawie analiz mikroskopowych i spektrometrycznych mas zauważono, że amoksycylina i gentamycyna spowodowały degradację syntezy ściany komórkowej, zaburzenie macierzy zewnątrzkomórkowych substancji polisacharydowych otaczających biofilm (EPSs) oraz zahamowanie białek rybosomalnych i zaburzenie transkrypcji. Zjawisko to jest skorelowane również z zauważoną wyższą wartością bezwzględną potencjału zeta i mniejszą ilością zarejestrowanych sygnałów na elektroforegramach. Należy podkreślić, że wyniki uzyskane za pomocą MALDI-TOF MS, mikroskopii fluorescencyjnej i SEM były współzależne z metodą elektroforezy kapilarnej. W związku z tym, zobrazowany na **rysunku 4** mechanizm reakcji biofilmu bakteryjnego umożliwia na każdym etapie kontrolę działania antybiotyków z różnych grup terapeutycznych.

Zatem, przedstawione w pracy [**P4**] - *Identification, Structure and Characterization of Bacillus tequilensis Biofilm with the Use of Electrophoresis and Complementary Approaches* podejście może w znaczący sposób ułatwić leczenie zakażeń bakteryjnych związanych z powstaniem biofilmu.

2.5. Wpływ antybiotyków na profile molekularne mikroorganizmów

Mikrobiom jamy ustnej człowieka jest jednym z najbardziej aktywnych środowisk dla wielu gatunków bakterii, gdzie ulegają one rozległej konkurencji międzygatunkowej, tworząc wielogatunkową strukturę biofilmu. Bakterie te obecne są również w ślinie; stanowią wiele setek i tysięcy gatunków, z których część jest unikalna dla tego specyficznego siedliska [31]. W związku z tym, celem dalszych badań była identyfikacja mikrobiomu śliny człowieka, praca [**P5**] - *Characterization of the salivary microbiome before and after antibiotic therapy via separation technique*. Dodatkowo, w niniejszej pracy przeprowadzono również monitorowanie zmian zachodzących w profilach molekularnych bakterii pod wpływem przyjmowania różnych antybiotyków.

Spersonalizowane leczenie jest jednym z najważniejszych osiągnięć współczesnej medycyny [32]. Aby ta dziedzina mogła się rozwijać, konieczna jest współpraca specjalistów z zakresu biologii, genetyki, biotechnologii, bioinformatyki i farmakologii ze środowiskiem medycznym. Prowadzi to do innowacyjnego podejścia w diagnostyce, a w konsekwencji w leczeniu poprzez udoskonalenie lub dostosowanie terapii farmakologicznej do indywidualnych potrzeb pacjentów, jest to tzw. celowana terapia farmakologiczna, "*terapia szyta na miarę*" lub medycyna spersonalizowana [33]. Z drugiej strony, różnorodność i skład mikrobioty śliny wydają się wysoce istotne dla zdrowia i choroby człowieka.

Dlatego też w ostatnich latach ślina cieszy się dużym zainteresowaniem jako matryca łatwo dostępna, której skład może odzwierciedlać stan zdrowia. Zgodnie z ostatnimi doniesieniami medycznymi i aktualną wiedzą naukową, zmiana równowagi składu bakteryjnego jamy ustnej ma potencjał sygnalizowania stanów patologicznych. Dotyczy to takich chorób jak halitoza [34], próchnica [35] czy paradontoza [36], ale także chorób ogólnoustrojowych, w tym chorób układu oddechowego [37] cukrzycy [38] wraz z chorobami układu krążenia [39], czy nowotworów [40]. Uwagę zwrócono również na charakterystykę mikrobiomu śliny u osób otyłych [41]. Niemniej jednak, zdrowe bakterie ślinowe powinny być identyfikowane przede wszystkim w celu opisania zmian spowodowanych chorobą, co ostatecznie może prowadzić do opracowania narzędzi diagnostycznych w celu poprawy leczenia lub zapobiegania chorobie [42]. Dodatkowo, te kilka badań wskazuje, że biomarkery bakterii ślinowych w jamie ustnej stanowią uznane narzędzie diagnostyczne i prognostyczne dla różnych chorób. Stąd też podjęto wiele działań z wykorzystaniem metod łączonych opartych na oznaczaniu białek rybosomalnych bakterii (MALDI-TOF MS) [43] wraz z detekcją lotnych związków organicznych (VOCs) (GC-MS) [44]. Niemniej jednak, większość badań nad mikrobami jamy ustnej wykorzystuje technikę opartą na 16S rRNA [45].

W literaturze często zwraca się uwagę na drobnoustroje patogenne i ocenę ich znaczenia w etiologii i przebiegu chorób zakaźnych oraz szerzenie się lekooporności na powszechnie stosowane leki przeciwbakteryjne. Interesujące jest jednak to, czy i jakie różnice w częstości występowania kolonizacji szczepów bakteryjnych występują u osób z infekcjami bakteryjnymi poddawanych antybiotykoterapii w porównaniu z osobami bez antybiotykoterapii. W związku z tym, w pracy [**P5**] zastosowano technikę MALDI-TOF MS jako narzędzie do szybkiej diagnostyki i identyfikacji mikrobiomu śliny. Ponadto, w celu uzyskania informacji o skuteczności i dokładności badanej metody spektrometrycznej, do oznaczenia wybranych bakterii ślinowych wykorzystano również sekwencjonowanie genu 16S rRNA. Niniejsza praca dotyczyła również sprawdzenia możliwości techniki MALDI do prowadzenia badań w zakresie szybkiego monitorowania pacjentów poddanych antybiotykoterapii. Interesującym zagadnieniem było również porównanie, czy technika MALDI pozwoli na rozróżnienie każdego podawanego antybiotyku w danym okresie czasu. Ponadto, w pracy [**P5**] zbadano optymalne warunki podłoża wzrostowego do identyfikacji mikroorganizmów przy użyciu MALDI-TOF MS. Co więcej, określono i szczegółowo przeanalizowano korelację pomiędzy zmianami profilu białkowego mikrobiomu pacjentów przed i po antybiotykoterapii. Dodatkowo opisano wpływ obecności antybiotyku i patogenu na terapię pacjentów.

Warto zwrócić uwagę, iż wyniki badań wykazały istotne różnice w mikrobiomie śliny pomiędzy pacjentami nieleczonymi i leczonymi antybiotykami. Większą różnorodnością bakterii, a także pojawieniem się mikroorganizmów z rodzaju *Candida* odnotowano jedynie u pacjentów poddanych antybiotykoterapii, co wskazuje na negatywny wpływ podawania antybiotyków na mikrobom śliny pacjenta. Ponadto analiza proteomiczna wykazała wpływ antybiotykoterapii, składu i liczebności mikrobiomu śliny oraz stosowanego podłoża hodowlanego na rejestrowane profile białkowe. Ogólnie, identyfikacja mikroorganizmów za pomocą MALDI jest uważana za niezależną od kultury, ponieważ większość białek obecnych w komórkach bakteryjnych to białka rybosomalne (około 50% lub więcej), tak więc wiarygodna klasyfikacja bakterii dla większości rodzajów i gatunków jest pewna, bez względu na stosowane podłoża hodowlane. Niemniej jednak, oprócz białek rybosomalnych, badane ekstrakty bakteryjne zawierają również inne białka nierybosomalne, które są mniej lub bardziej zależne od metabolizmu. Białka takie mogą wpływać na wynik identyfikacji przede wszystkim w przypadku bakterii należących do grup blisko spokrewnionych gatunków. Jednakże wyniki naszych badań pozwoliły stwierdzić, że wpływ rodzaju podłoża na wewnątrzspecyficzne zróżnicowanie proteomu był mniejszy niż wpływ antybiotykoterapii i obecności współistniejącej mikrobioty. Wykazano, że interakcje, które zachodziły między mikroorganizmami mogą zmieniać ekspresję ich białek membranowych. Znalezione zmiany proteomiczne obejmowały białka związane z oddziaływaniami typu gospodarz-mikroorganizm, takimi jak wirulencja, adhezja i oporność. Biorąc pod uwagę wszystkie gatunki pochodzące ze śliny pacjentów leczonych i nieleczonych, wpływ na zróżnicowanie proteomiczne wzrasta w następujący sposób: rodzaj stosowanego antybiotyku > współistniejąca mikrobiota > rodzaj podłoża hodowlanego.

Wyniki przeprowadzonych badań potwierdzają, że analiza MALDI-TOF MS stanowi obiecującą metodę dla wielkoskalowego, mniej pracochłonnego, szybkiego i opłacalnego zapisu powtarzalnych profili molekularnych mikroorganizmów, w szczególności bakterii ślinowych. Warto zauważyć, że proponowane podejście umożliwia wczesne zastosowanie u pacjentów specyficznej dla danego gatunku bakterii terapii antybakteryjnej. Ponadto, uzyskane dane mogą zatem pozwolić na potwierdzenie diagnozy medycznej, a także mogą umożliwić monitorowanie leczenia chorób i opracowanie leków dla poszczególnych pacjentów. Co więcej, przedstawione badania mogą być podstawą do opracowania metod umożliwiających szybszą diagnozę i wykrywanie zmian chorobowych na poziomie komórkowym przed wystąpieniem zmian klinicznych.

Podsumowując, uzyskane wyniki sugerują, że śledzenie proteomicznej zmienności wewnątrzspecyficznej w obrębie mikroorganizmów może być obiecującym narzędziem w przyszłości do analizy skuteczności stosowanej antybiotykoterapii oraz sprawdzenia, czy mamy do czynienia z kulturami jedno- czy wielodrobnoustrojowymi z uwzględnieniem obecności innych gatunków patogennych.

2.6. Bibliografia

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3. Publikacje naukowe

3.1. *Determination and identification of antibiotic drugs and bacterial strains in biological samples*

molecules

MDPI

Review

Determination and Identification of Antibiotic Drugs and Bacterial Strains in Biological Samples

Katarzyna Pauter [1,2], Małgorzata Szultka-Młyńska [1,*] and Bogusław Buszewski [1,2]

[1] Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarin 7, 87-100 Torun, Poland; kpauter@wp.pl (K.P.); bbusz@umk.pl (B.B.)
[2] Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Wilenska 4, 87-100 Torun, Poland
* Correspondence: mszultka@umk.pl

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Abstract: Antibiotics were initially natural substances. However, nowadays, they also include synthetic drugs, which show their activity against bacteria, killing or inhibiting their growth and division. Thanks to these properties, many antibiotics have quickly found practical application in the fight against infectious diseases such as tuberculosis, syphilis, gastrointestinal infections, pneumonia, bronchitis, meningitis and septicemia. Antibiotic resistance is currently a detrimental problem; therefore, in addition to the improvement of antibiotic therapy, attention should also be paid to active metabolites in the body, which may play an important role in exacerbating the existing problem. Taking into account the clinical, cognitive and diagnostic purposes of drug monitoring, it is important to select an appropriate analytical method that meets all the requirements. The detection and identification of the microorganism responsible for the infection is also an essential factor in the implementation of appropriate antibiotic therapy. In recent years, clinical microbiology laboratories have experienced revolutionary changes in the way microorganisms are identified. The MALDI-TOF MS technique may be interesting, especially in some areas where a quick analysis is required, as is the case with clinical microbiology. This method is not targeted, which means that no prior knowledge of the infectious agent is required, since identification is based on a database match.

Keywords: antibiotics; biological samples; analytical techniques; mass spectrometry; microorganisms

1. Introduction

Microbiology is a leading science branch, which is particularly important for medicine, biotechnology, veterinary studies and agriculture. There is no doubt that microorganisms play an extremely significant role in the human life and surrounding environment. Moreover, an increasingly important role in the process of ontogenesis is attributed to the influence of various microorganisms: viruses, bacteria and fungi [1,2]. The detection of the infection in its early stages could help achieve better outcomes, and therefore, it is extremely important not only to determine changes within the body, but also to find biomarkers that characterize a given individual or population. Hence, in recent years, an increasing emphasis has been placed on the search for modern, very precise, and, above all, quick methods for the identification of microorganisms along with antibiotic drugs and their metabolites. This requires an interdisciplinary approach, in which the cooperation of specialists in medicine, chemistry, biochemistry, microbiology, molecular biology, and bioinformatics will allow the scientists to determine the immunological correlation between certain microorganisms and biomarkers (protein markers, volatile organic compounds) and the occurrence of diseases.

Over the last few years, the focus has been made on combined techniques as the main tools for solving complex analytical problems. Separation techniques (LC, GC, CE) coupled with different detection systems (FTIR, PDA, NMR and MS) permit the identification of compounds present in the raw sample. These techniques include LC-FTIR, LC-NMR, LC-MS, HPLC-PDA, HPLC-MS, GC-NMR and GC-FTIR [3]. What is especially remarkable, is that the studies carried out so far on different species and strains of microorganisms indicate a strong potential for the use of hyphenated separation techniques, especially CE-MS and GC-MS [4]. Therefore, a key step will consist in evaluating the usefulness of electromigration techniques and the technology of matrix-assisted laser desorption/ionization with time of flight (MALDI-TOF MS) for the identification and characterization of native microbial cells. In addition, the use of multi-dimensional, coupled separation techniques (LC × LC-MS/MS, GC × GC-Q-TOF/MS and LC × LC-CZE-MS/MS) will offer the possibility to prepare the metabolomic profiling of the studied biological samples in order. This techniques can be of two types—on-line or off-line procedure integration. The off-line mode is an appropriate solution for the optimization of method parameters and does not require any additional technical equipment. Although the off-line mode is very flexible, it has disadvantages in time and labor consumption, the possibility of loss or contamination of the sample and a large sample volume that may not be suitable for a very sensitive identification of proteomic samples. The alternative is to use the on-line system, which automates the sample preparation process and thus, it reduces the time of the analysis [3,5]. Moreover, the application of statistical methods will facilitate a detailed and multi-directional interpretation of the data, which can significantly contribute to progress in the detection and treatment of diseases caused by pathogens.

2. Antibiotic Drugs

Antibiotics always existed in our environment, but we did not know how to isolate and produce them. The first potentially healthy use of beer containing tetracycline was found in ancient Nubia about 350–550 years BC [6]. The modern era of antibiotics began with Alexander Fleming (1881–1955), the great antibiotic explorer. The most famous phrase of Fleming is: "Penicillin was produced by nature, I only discovered it" [7]. Today, it is estimated that there are more than 70,000 natural antibiotics [8].

Antibiotics were initially natural but nowadays they also have synthetic substances showing activity against bacteria, killing or inhibiting their growth and division. Thanks to these properties, many antibiotics have quickly found practical application in the fight against infectious diseases such as tuberculosis, syphilis, gastrointestinal infections, pneumonia, bronchitis, meningitis and septicemia [9].

From a chemical point of view, antibiotics form different groups of compounds. In general, they are low molecular weight compounds, characterized by different chemical structures, composition and physicochemical properties. A well-known group of antibiotics are β-lactams which include penicillins and cephalosporins. Other classes of antibiotics consist of macrolides, amidoglycosides, sulfonamides and tetracyclines [10]. In Figure 1, the main classification of antibiotics is presented. Moreover, antibiotics can be divided into classes with broad or narrow antibacterial spectra. Most of the studied antibiotics are active against Gram-positive bacteria and a smaller number against Gram-negative bacteria [10]. Analyzing the effect of antibiotics on bacteria, there are bactericidal (vancomycin, amoxicillin, cefuroxime) and bacteriostatic (cycloserine, linezolid, azithromycin) antibiotics. Bacteriostatic drugs cause a reversible inhibition of growth, with bacterial culture restarting after the elimination of the drug. By contrast, bactericidal drugs kill their target bacteria.

The strong antimicrobial activity of antibiotics led to the study of the mechanisms of this phenomenon. Some mechanisms of their action proved to be varied, and their place of activity may be a cell wall, whose synthesis at various stages of its formation is disturbed by penicillins, cephalosporins, cycloserine, vancomycin, and other antibiotics. The essence of the antibacterial action of these drugs is to inhibit the formation of bridges connecting the subunits of peptidoglycan into an integral whole. This process is catalyzed by bacterial enzymes called penicillin-binding proteins (PBP), located in the cell membrane of bacteria that bind the antibiotic. As a result of a permanent binding with an

antibiotic, the function of enzymes (PBP) is blocked and, as a result, the cell maturation and the cell division are inhibited [11].

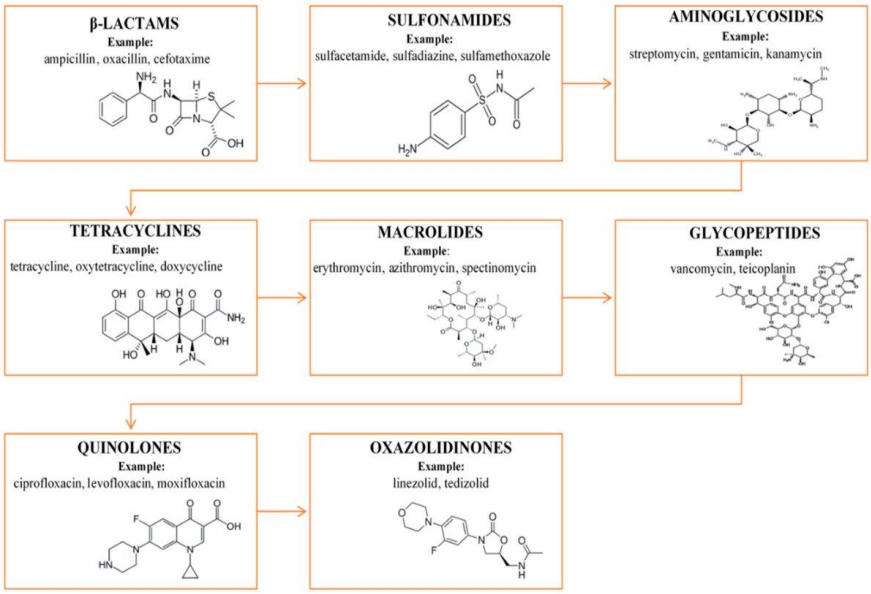

Figure 1. An overview of main antibiotics.

The other antimicrobial agents (polymyxins, polyesters) influence the breakdown of the cell membrane and the increase in its permeability to ions. Antibiotics acting on the cell membrane have a specific structure that allows them to bind with the lipid components of the cell membrane, causing the membrane to lose its tightness. Daptomycin is also an antibiotic disturbing the cell membrane functioning. The mechanism of the action of daptomycin is based on its irreversible binding with the cell membrane of Gram-positive bacteria, in the presence of calcium ions. The effect of this action is the formation of channels leading to the depolarization of the cell membrane and the outflow of potassium and other ions from the cell interior. As a result of this process, the membrane is destroyed and the macromolecular synthesis of macromolecules is seriously disturbed [12].

A relatively large group of various antibiotics inhibits protein synthesis at many stages, from the initiation of translation to the proper chain elongation. The protein synthesis is associated with special cellular structures called ribosomes. The bacterial ribosome consists of ribonucleic acids (rRNA) and proteins. It dissociates into two subunits—large (50S) and small (30S). Due to the many classes of antibiotics inhibiting protein synthesis, the molecular mechanisms of their action are different. Usually, drug molecules bind to different ribosomal protein molecules or ribosomal RNA molecules in both the 30S and 50S subunits, causing the cell death. The group of drugs disturbing protein synthesis includes aminoglycosides and macrolides [13].

The basic class of antibiotics disturbing the DNA synthesis are quinolones and their derivatives such as fluoroquinolones (II and III generation of drugs). These antibiotics are specific inhibitors of topoisomerase II ligase domains (gyrases) and IV topoisomerase domains. As a result of the nucleolytic domain's activity, the DNA in the cell is fragmented [14]. In addition to the effect on the DNA synthesis and half-life, there is a group of antibiotics that affect the RNA synthesis (ansamycins), which includes the widely known rifampicin. It binds specifically to the bacterial RNA polymerase in the vicinity of the active site and prevents the RNA chain elongation. Antibiotics (sulfonamides) may also affect the activity of important metabolic pathways in the cell. One of the best-known examples is the inhibition of folic acid synthesis, which leads to a disruption of the DNA synthesis [15].

3. Drug Metabolism

Each active substance that we deliver to our body must undergo several stages in order to obtain the appropriate pharmacological effect. Antibiotics undergo the biochemical modification (biotransformation) not only in the liver, but also in the kidneys, blood and small intestine walls. The metabolism of antibiotics involves different processes, which are divided into phase I and phase II reactions (Figure 2) [16]. Phase I reactions lead to the formation of intermediates in the processes of oxidation, reduction and deamination. Phase II reactions, on the other hand, consist in coupling the end products with the glucuronic acid, the sulphuric acid, glutathione and glycine, and may lead to methylation or acetylation. Phase I takes place mainly by oxidoreductases and hydrolysis, as opposed to phase II, which takes place by means of transferases catalyzing coupling reactions (glucuronosyltransferase) or cytosolic enzymes (sulfotransferase, N-acetyltransferase). The metabolites formed in this way differ in their properties and can be divided into active, inactive, toxic or those that are transformed into a primary compound under the influence of appropriate physical factors. Depending on the type of the metabolite produced, the antimicrobial activity and toxicity of the primary compound may increase, which may result in certain restrictions in the use of antibiotics [17].

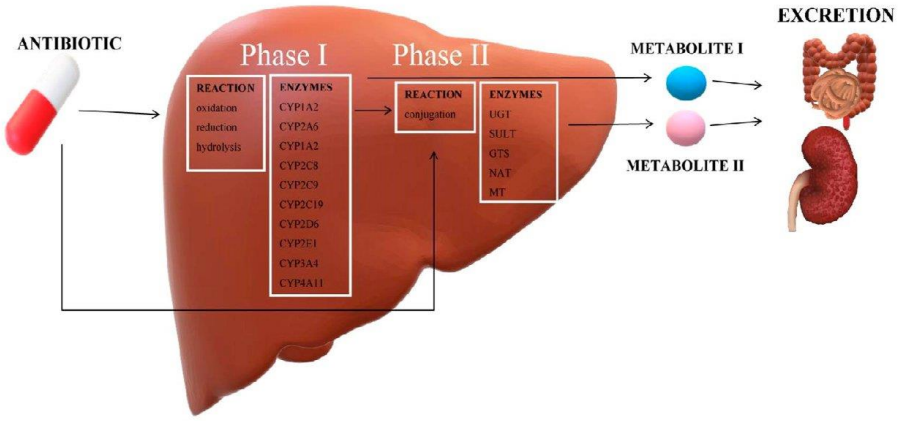

Figure 2. The concept of antibiotics' metabolism in the liver.

In most cases, under the influence of biotransformation, inactive compounds are formed, which in a short period of time are eliminated from the body. However, active metabolites may be formed from both active and inactive parent compounds. When a non-active drug is transformed into an active metabolite in the body, the conversion of prodrug to drug takes place. Where the parent is an active medicine, the resulting active metabolites may have synergistic or additive effects and may prolong and/or enhance its effects. These metabolites may have lower toxicity than the parent compound or a different pharmacological activity. This feature is visible in the development of new drugs. Metabolic processes may also result in the formation of toxic substances which increase the harmfulness of the medicine used, or in the formation of metabolites which reenter the parent compound under appropriate environmental conditions. Depending on the type of metabolite formed, the antimicrobial activity or the toxicity of the parent compound may increase, which may result in a number of restrictions related to taking the drug [17].

Penicillins are a widely used group of antibiotics. Isoxazolyl penicillins are described in more detail in all groups of penicillins. Their biotransformation results in para-hydroxy and 5-hydroxymethyl derivatives, which show a partial activity of the parent compound. However, the highest activity is observed in the oxacylin metabolite, which, isolated from urine, retains 10–20% of the activity of the primary compound. The antimicrobial activity of all metabolites originating from isoxazolyl penicillins is twice as low as that of their parent compounds. However, all substances remain active against *Staphylococcus* genus bacteria resistant to benzylpenicillins [18].

Metronidazole is a chemotherapeutic agent from the nitroimidazole group. Its biotransformation results in hydroxymethylmetronidazole. This metabolite has twice as high an activity as the parent compound in relation to bacteria of the genus *Gardnerella vaginalis* and a similar activity in relation to Gram-positive *Staphylococci* [19].

Among the antibiotics belonging to the group of lincosamides, clindamycin and its active metabolites are noteworthy. The transformation of the parent compound results in the formation of two main compounds that show an antimicrobial activity. These are clindamycin sulfoxide and *N*-demethylclindamycin. A metabolite with a higher antimicrobial activity is *N*-demethylclindamycin. This compound has twice as high an antimicrobial activity as clindamycin [20]. The antibacterial activity of selected antibiotic metabolites is presented in Table 1 [21–28].

Substances remaining in the tissues, and those which are released to the environment, can lead to the induction of bacterial resistance. Antibiotic resistance is currently a detrimental problem, therefore, in addition to the improvement of antibiotic therapy, attention should also be paid to active metabolites in the body, which may play an important role in exacerbating the existing problem.

Table 1. The antibacterial activity of selected antibiotic metabolites.

Antibiotic (Antibiotics Group)	Metabolite	Activity of the Metabolite Compared to Initial Compound	MIC (μg/mL)		Ref.
			A	M	
Metronidazole (nitroimidazole)	1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole	<*Bacteroides* spp. <*Clostridium perfringens* <*Clostridium* spp. =*Peptococcus* spp.	0.5 0.5 0.5 0.25	1.0 1.0 2.0 0.25	[21]
Metronidazole (nitroimidazole)	2-methyl-5-nitroimidazole-1-acetic acid	<*Bacteroides* spp. <*Clostridium perfringens* <*Clostridium* spp. <*Peptococcus* spp.	0.5 0.5 0.5 0.25	16.0 32.0 16.0 16.0	[21]
Clarithromycin (macrolide)	14-hydroxyclarithromycin	>*Haemophilus influenzae*	2.4	1.2	[22]
Cefotaxime (β-lactam)	desacetylcefotaxime	=*Pseudomonas aeruginosa* <*Escherichia coli* >*Proteus mirabilis* <*Shigella* spp. <*Klebsiella pneumoniae*	>128 0.25 0.5 0.125 0.25	>128 0.5 0.25 1.0 0.5	[23]
Fidaxcomicin (macrolide)	OP-1118	<*Clostridium perfringens* <*Clostridium difficile*	0.008 0.12	0.25 4.0	[24]
Tinidazole (nitroimidazole)	hydroxytinidzole	>*Gardnerella vaginalis*	32	2	[25]
Metronidazole (nitroimidazole)	hydroxymetronidazole	>*Gardnerella vaginalis*	32	4	[25]
norfloxacin (quinolone)	*N*-nitrosonorfloxacin	<*Enterococcus faecalis* <*Escherichia coli* <*Staphylococcus aureus* <*Mycobacterium gilvum* <*Pseudomonas aeruginosa*	3.01 0.05 1.6 6.2 1.6	7.5 1.9 3.8 12.5 7.5	[26]
norfloxacin (quinolone)	*N*-acetylnorfloxacin	<*Enterococcus faecalis* <*Escherichia coli* <*Staphylococcus aureus* <*Mycobacterium gilvum* <*Pseudomonas aeruginosa*	3.01 0.05 1.6 6.2 1.6	≥50 ≥50 ≥50 ≥50 ≥50	[26]
Cefetamet (β-lactam)	cefetamet pivoxil	=*Escherichia coli* =*Streptococcus pyogenes*	0.5 0.06	0.5 0.06	[27]
Ceftiofur (β-lactam)	desfuroylceftiofur	<*Salmonella* spp. <*Actinobacillus pleuropneumoniae*	1.0 0.0078	2.0 0.015	[28]

A, antibiotic; M, metabolite; <, the metabolite indicates lower antimicrobial activity than the parent compound; >, the metabolite indicates higher antimicrobial activity than the parent compound; =, the metabolite indicates comparable antimicrobial activity to the parent compound.

4. Antibiotic Resistance

Antibiotics were considered a miracle cure for many years. The pioneers of antibiotic therapy believed that these antimicrobial agents would eliminate all bacterial infections. Unfortunately, bacteria struggling to survive revealed a wide range of possibilities to gain resistance to drugs. The first signal was a large group of penicillin resistant *Staphylococcus aureus* strains, and in the early 1960s, methicillin resistant strains (MRSA) were observed [9]. The resistance of microorganisms to the introduced class of antibiotics appeared almost immediately after their first use in therapy. Such a development was predicted by Alexander Fleming. During his work on penicillin, he noted that successive generations of *Staphylococcus aureus* treated with penicillin produced cell walls that are less and less permeable to this drug. Therefore, he discovered one of the mechanisms of antibiotic resistance [29].

Antibiotic resistance can be determined from the genetic information encoded in a chromosome or in moving elements such as plasmids, transposons and integrons. Bacteria may be naturally resistant to a specific group of antibiotics or may acquire resistance through various genetic processes, including mutations, resistance gene transfer, and also through direct contact with cells. The transfer of immune genes takes place through the horizontal gene transfer. Vectors carrying such genes are usually plasmids (called plasmids *R*-resistance), which in conjugative processes can be transferred from the donor cell to the recipient cell. Antibiotic resistance can also be determined by mobile genetic elements such as transposons or integrons, which are one of the sources of bacterial strains resistant to several chemotherapists at the same time. The variability of the genome leads to a change in cell metabolism, which results in the appearance of enzymes with a wide spectrum of action, including inactivating antibiotics. Another factor of the mechanism of antibiotic resistance is the fact that bacteria have elution pumps at their disposal. These pumps located in the cytoplasmic membrane are proteins transporting toxic substances, including antibiotics, outside the bacterial cell. Efflux pumps are present in both Gram-positive and Gram-negative bacterial cells. They are an important tool for initiating antibiotic resistance, including the development of multifactorial resistance [30].

According to Acar and Moulin [31], the ability of bacteria to acquire antibiotic resistance depends on the ability of individual bacteria to adapt to the selective pressure of the antibiotic used. In the classification proposed by them, the following mechanisms of drug resistance were distinguished:

1. The active efflux, which prevents the achievement of the antibiotic target, i.e., the place where the function of the bacterial cell is damaged;
2. The reduction of the permeability of the bacterial cell membrane, which occurs when its composition and function are modified;
3. The modification of an antibiotic in its inactive form with the participation of enzymes produced by bacteria; they may change the antibiotic inside or outside the bacterial cell, removing its antibacterial effect;
4. The change of the target of the antibiotic, reducing its affinity to it;
5. Bacterial mutations resulting in the elimination of bacteria resistant by the antibiotic;
6. The occurrence of a mixed population of sensitive and resistant bacteria at antibiotic concentrations on the selection of resistant cells [31].

Molecular mechanisms of antimicrobial resistance in bacteria are provided in Figure 3. The reason for antibiotic resistance is, therefore, the evolution and exchange of genetic material through the so-called horizontal gene transfer and selection, which is, unfortunately, mainly caused by human activity. Human influence results in an improper intake of antibiotics and their use as a food additive for animals. Unfortunately, the development of antibiotic resistant strains of microorganisms is still an unresolved problem.

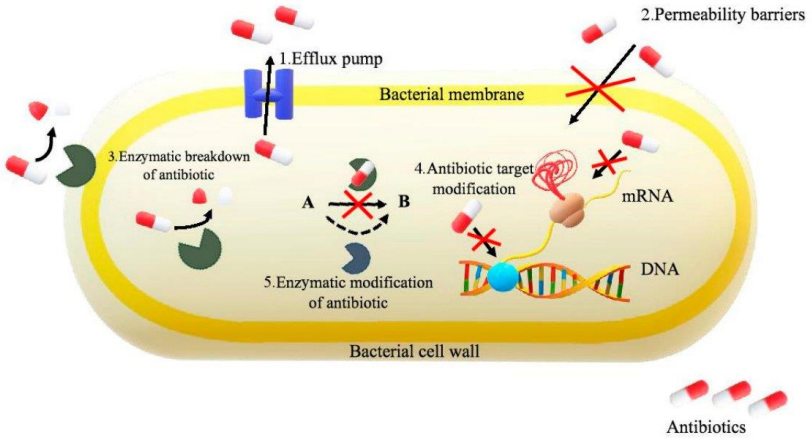

Figure 3. Molecular mechanism of antimicrobial resistance.

5. Determination and Identification of Antibiotic Drugs and Their Metabolites

5.1. Microbiological Assay

A rapid recognition of a life-threatening infection, as well as a reliable identification of the pathogen causing it and the selection of the most effective antibiotic are key factors to achieve a therapeutic success. The choice of an antimicrobial drug and its lowest concentration that prevents the visible growth of bacteria are based on the minimum inhibitory concentration (MIC). It is a value measured in vitro, which allows to determine the drug susceptibility of microorganisms. Disk diffusion, dilution methods, E-test and automated systems are commonly used MIC measurement techniques. There are two main types of dilutions: micro- and macro-dilution, with broth and agar being the most frequently used media. In the early 1870s, dilution was one of the earliest methods in the microbiological practice [32,33].

In the clinical practice, the quantitative determination of antibiotics is one of the more complex areas of the pharmaceutical analysis, especially in patients with infections difficult to treat, e.g., endocarditis. In this method, the same number of bacterial cells is added to the liquid or solid medium with the antibiotic at a certain concentration, and the growth in the presence of the antibiotic is assessed. The serial dilution method also makes it possible to determine the lowest bactericidal concentration (MBC). Currently, the main MIC determination method used in routine testing in medical microbiology laboratories around the world is the gradient diffusion method Epsilometer test (E-test®). It combines the principle of agar antibiotic diffusion with the determination of the minimum inhibitory concentration of the antibiotic by dilution in agar. Quantitative testing with the E-test is based on the diffusion of the antibiotic from the tissue paper strip, in the concentration gradient, to the medium on which the bacteria strain grows. The antibiotic gradient strip diffusion method is applicable to both the MIC determination for fast-growing aerobic bacteria such as Staphylococci, Gram-negative Enterobacteriaceae and demanding bacteria such as *Streptococcus pneumoniae* and anaerobic bacteria [34,35].

Kontopidou et al. [35] studied the antibiotic susceptibility of bacterial isolates from bronchial secretion samples. The E-test and disk diffusion were compared with the dilution technique to determine in vitro activity of five antibiotics (ciprofloxacin, piperacillin, tazobactam, meropenem and colistin). Both direct diffusion tests (E-test and disk diffusion) were susceptible to interception and could be helpful in improving the treatment of Ventilator-Associated Pneumonia (VAP) [35].

Di Bonaventura et al. [34] used E-test®, agar/broth dilution and disk diffusion methods for testing the levofloxacin susceptibility against *Staphylococcus* spp. isolated from patients with neutropenic cancer. The E-test was found to be a reliable alternative methodology to the standard test for determining the level of the levofloxacin resistance in staphylococci [34].

Gianecin et al. [36] compared the disk diffusion and agar dilution to study the antimicrobial activity of gentamicin on clinical isolates of *Neisseria gonorrhoeae*. The results indicated that the disk diffusion assay could be an acceptable method for the susceptibility of gentamicin against *Neisseria gonorrhoeae* [36].

In routine clinical management, the interpretation of the obtained drug concentration measurement requires the following conditions to be met: The knowledge of pharmacokinetics of the drug being tested, existence of a certain correlation between the drug concentration in blood and its therapeutic or toxic effects, determination of the range of therapeutic concentration of the drug being tested, as well as the development of sensitive and specific analytical methods (Figure 4) allowing to determine the drug concentration in body fluids [37–40].

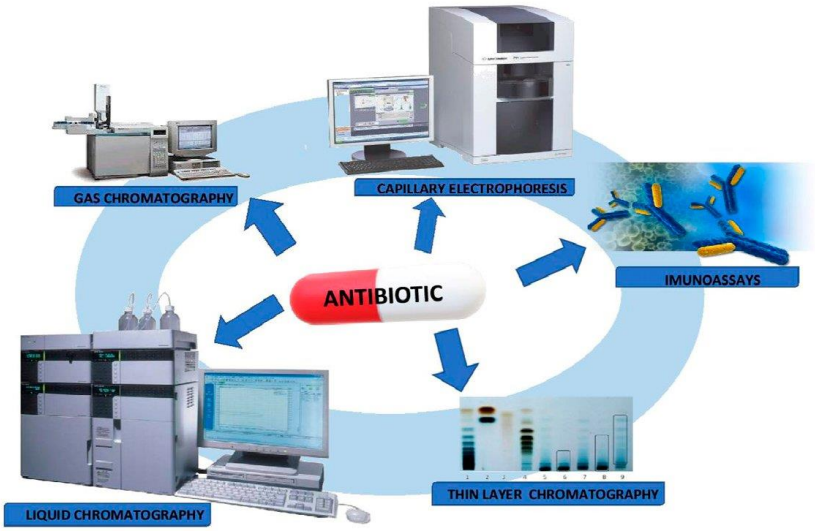

Figure 4. The analytical techniques for determination of antibiotics.

5.2. *Analytical Techniques*

High-performance liquid chromatography (HPLC) is one of the most commonly used analytical methods for the quantification and qualification analysis of antibiotics in biological samples (plasma, serum, whole blood, urine). In addition to these techniques, determinations by immunochemical tests, gas chromatography (GC), thin-layer liquid chromatography (TLC) or capillary electrophoresis (CE) are also available.

5.2.1. Immunoassays

Immunoassays are analytical methods that enable the detection of substances in clinical samples by creating a stable complex between the analyte and a specific antibody. Antigen-antibody reactions are stoichiometric; therefore, the determination of free or bound antigens leads to a direct calculation of the antibiotic level. However, the disadvantage of immunoassays is their potential lack of specificity, which may lead to cross-reactivity with metabolites, drugs or structurally related compounds [41,42].

Pastor-Navarro et al. [43] used the immunoassay to determine in human plasma the concentration of sulfasalazine. The enzyme-linked immunosorbent assay (ELISA) allowed for the detection of antibiotics at the concentration levels of 0.02 ng/mL [43].

The fluorescence polarization immunoassay (FPIA) assay of levofloxacin in urine was also described by Shanin et al. [44]. The achieved LOD value was 0.5 ng/mL [44].

Dijkstra and co-workers [45] also demonstrated the use of the tobramycin immunoassay kit for the detection of the kanamycin concentration in the serum. The results of the immunoassay method were compared with the LC-MS/MS analysis. This method is able to quantify a large range of kanamycin concentrations in a reliable and reproducible manner [45].

Merola et al. [46] presented an immunosensor technique for the determination of β-lactam antibiotic drugs in the human serum and urine. This technique showed to be very sensitive, cheap and reproducible; the LOD value was about 10^{-11} M [46].

Furthermore, the detection of antibiotic drugs from different groups based on the electrochemical Immunosensor was demonstrated in the review article by Pollap and Kochana in detail [47].

5.2.2. Chromatographic Techniques

In recent years, several rapid, sensitive and specific analytical methods for the determination of the antibiotic content in complex biological matrices have been applied in routine laboratories. These techniques are essential to provide reproducible results that can be used in clinical trials to improve the effectiveness of the antibiotic therapy. The variability of the separation mechanisms enables the identification and determination of antibiotics from different groups, including penicillins, macrolides, aminoglycosides, tetracyclines, quinolones and nitroimidazoles. The choice of the separation method is based on the properties of the analyzed antimicrobial substances, e.g., solubility in water and organic solvents or acid-base properties.

Thin Layer Chromatography (TLC)

According to the WHO European Pharmacopoeia [48], the use of liquid thin layer chromatography (TLC) is recommended for the identification of specific antibiotics (neomycin). Jain et al. [49] separated minocycline in plasma using the TLC gel coated with silica 60F254 and sprayed with a mixture of methanol-acetonitrile-isopropanol-water (5:4:0.5:0.5 (*v*/*v*)). The antibiotics were identified by the UV irradiation at 190–400 nm wavelengths. The accuracy of the method expressed as the percentage of recovery was from 95.08% to 100.6%. The method meets the acceptance criteria for validation and may be useful for the determination of minocycline in the human plasma [49]. The HPTLC method with densitometric detection for the determination of amoxicillin and ampicillin in urine samples was described by Gholipour et al. [50]. Separation was effected on titanium(IV) silicate TLC plates using a mixture of mobile phase (K_2HPO_4 (0.1 M) + KH_2PO_4 (0.1 M), 1:1 (*v*/*v*)), and the relevant compounds visualized by spraying with 1% solution of ninhydrin in ethanol. The TLC suggested technique provided a simple, accurate, and reproducible analysis of both amoxicillin and ampicillin in biological samples [50]. Unfortunately, due to the relatively low repeatability and difficult validation of results, the TLC method is not preferred for quantitative determinations.

An alternative approach consists in combining directly planar chromatography with mass spectrometry. In particular, matrix-assisted laser desorption/ionization (MALDI-TOF/MS) is a modern ionization technique that can be combined with thin layer chromatography (TLC-MALDI-TOF MS) [51].

The main positive aspects of TLC-MALDI-TOF MS were presented in the analysis of the mixture of tetracycline antibiotics. Particles of various materials (Co, TiN, TiO_2, graphite, silicon) were investigated by using suspensions of particles on eluted TLC plates. Dichloromethane, methanol and water (59:35:8, *v*/*v*/*v*) were applied ss the solvent system. Mass spectra and mass chromatograms were obtained from direct TLC plates. Before the MALDI analysis, only an unresolved spot for tetracycline and chlortetracycline were found in the TLC plate. However, the MALDI mass spectra and the graphing of individual ion chromatograms resulted in separate peaks for chlortetracycline and tetracycline.

The TLC-MALDI-TOF MS analysis of tetracyclines enabled the calculation of the R_f value of the analyte spots, which indicates good compliance with the retention factor value acquired by using the UV detection [52]

Gas Chromatography (GC)

Besides thin layer chromatography, gas chromatography (GC) is also used for the determination of antibiotics in biological fluids. However, the GC technique is very seldom used due to the need to transform drugs and their metabolites into thermostable derivatives [53,54].

Thangadurai [55] described gas chromatography with the mass spectrometric (GC/MS) detection method to determine azithromycin in biological samples (gastric cleavage samples). The sample was extracted with chloroform and cleaned up by n-hexane washing. Then, the cleaned-up extract was acetylated in the acetic anhydride-pyridine mixture (1:2). Azithromycin was analyzed by GC without derivatization. The authors used the phenylmethyl silicone bonded phase GC capillary column (0.25 µm, 30 m × 0.25 mm i.d.). The obtained detection limit was 2 $\mu g/mL^{-1}$. This method can be used to monitor the antibiotic level in biological materials for forensic and toxicological aims [55].

Chiavarino et al. [56] reported the GC method with the atomic emission detector (GC-AED) for the detection of nine suflonamides. The samples were derivatized using N-methylation. Gas chromatographic separations were achieved on 12.5 m × 0.22 mm i.d. phenylmethyl silicone column. This technique displayed linearity and may be used for the quantitative determination of suflonamides [56].

Liquid Chromatography (LC)

However, high-performance liquid chromatography (HPLC) plays an important role in the determination of antibacterial drugs in body fluids. A wide spectrum of detectors used for determination (UV, DAD, PDA, FL, MS as well as universal detectors: CAD or ELSD) and modern methods of sample preparation for the analysis enabled to obtain reproducible results, even in complex matrices [57–67]. Nonetheless, LC-MS/MS is the only technique that ensures unambiguous analysis.

Borner et al. [66] developed an HPLC assay for the determination of linezolid in human plasma and urine using a Nucleosil-100 5C18 column. The mobile phase was composed of acetonitrile/sodium acetate buffer/water 18/10/72 *v*/*v*. The elution of drugs was monitored at 250 nm. This paper addressed the compatibility of the results obtained using microbiological tests and the HPLC method in respect of serum and urine [66]. In 2009, Farshchi, Ghiasi and Bahram [68] described an HPLC protocol for the analysis of clarithromycin in the human serum after derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl). Following the liquid-liquid extraction (dichloromethane) of the antibiotic, the compounds were analyzed using HPLC with a fluorescence detector (HPLC-FL). The HPLC column used for the analysis was 150 cm long with a 4.6 mm internal diameter and a particle size of 5 µm. The authors concluded that the analysis time was reduced, the LOQ value was enhanced and the time needed for the derivatization of the clarithromycin in the human serum was also shortened [68]. Locatelli [60] and his co-researchers reported a rapid HPLC assay with the microextraction for the analysis of two fluoroquinolones (ciprofloxacin and levofloxacin) in the human sputum. Chromatographic separation was achieved by using a Gemini C18 column (250 mm × 4.6 mm i.d., 5 µm) and mobile phase was composed of a mixture of phosphate buffer (30 mM, pH 2.5, 1% triethylamine (TEA)), and acetonitrile (1% TEA) (86:14, *v*:*v*) at 1.0 mL/min flow rate. The detection of peaks was achieved by the photodiode array detector (PDA) at 295 nm for levofloxacin and at 279 nm for ciprofloxacin. The research suggested that MEPS-HPLC-PDA in off-line mode was an effective method for the quantitative determination of ciprofloxacin and levofloxacin in clinical specimens [60].

Buszewski et al. [61] described a sensitive method for the determination of five antibiotics and their metabolites in the whole blood and tissues. The analysis was carried out using the HPLC combined with tandem mass spectrometry. After the solid phase microextraction (SPME), the specimen was determined using an analytical C18 column (50 mm × 2.0 mm i.d., 4 µm) and a mobile phase consisted of water (0.1% formic acid) and acetonitrile. Detection was achieved by a triple-quadrupole mass spectrometer (HPLC-QqQ-MS) with an electrospray ionization (ESI). This is an important finding in the identification of antibiotics (amoxicillin, cefotaxime, ciprofloxacin, clindamycin and metronidazole) and their metabolites in the biological matrix using the SPME sample preparation technique. Moreover, two mass spectrometric techniques: ESI-QqQ and MALDI TOF, were demonstrated to be complementary in the determination of active compounds in clinical samples [61].

Ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) of seven antibiotics in human serum was also reported [69]. After protein precipitation, (ACN) drugs were separated by using the UPLC HSS T3 column (100 mm, 2.1 mm i.d., 1.8 µm) and a mixture of mobile phase: 5 mM ammonium acetate (pH 2.45) and acetonitrile. The authors achieved a quantification lower limit (LLOQ) of 0.1 µg/mL. In conclusion, the UPLC-MS/MS method seems to improve the limit of quantification and shorten the analysis time. The authors suggested that the proposed method is simple, fast, sensitive and suitable for clinical studies particularly in neonate patients [69].

It is also noteworthy to analyze polar drugs by hydrophilic interaction liquid chromatography (HILIC). Kathriarachchi [70] et al. performed separations of amoxicillin and metronidazole in the human serum using the HILIC technique. The chromatographic separation was obtained on the ZIC-HILIC column and the mobile phase included 0.1% (*v*/*v*) formic acid in water and 0.1% (*v*/*v*) formic acid in acetonitrile. The method was fully validated and the lowest limit of quantification was 0.0138 µg/mL for amoxicillin and 0.008 µg/mL for metronidazole. The linearity was from 0.1 µg/mL to 6.4 µg/mL for both antibiotic drugs [70].

Other examples of the determination and identification of antibiotics by liquid chromatography in different biological fluids are summarized in Table 2.

Electromigration Techniques

Electromigration techniques are also separation analytical techniques used to measure various drugs, including antibiotics, especially for polar drugs and stereoisomer analysis. Electrokinetic analyses are based on electrokinetic phenomena: electromigration of ions, charged particles and electroosmosis. These phenomena appear in solutions when charged particles are placed in an electric field, mainly with high voltage. A comparison of the separation of analytes by the capillary electrophoresis and liquid chromatography is presented in Figure 5. Depending on the separation mechanism, we can distinguish between capillary zone electrophoresis (CZE), micellar capillary electrokinetic chromatography (MEKC), capillary non-aqueous electrophoresis (NACK) and capillary isotachophoresis (CITP). The antibiotic study by capillary electrophoresis mainly includes two modes CZE as well as MEKC. A significant advantage of CE is its availability, simplicity of equipment, the use of small concentrations of organic solvents in the buffer and, above all, a short time of the analysis and high efficiency of the analytic separation.

Table 2. Identification and determination of antibiotics and their metabolites by the liquid chromatography technique.

Antibiotic/Metabolite	Matrix	Sample Preparation	Detection	Conditions	LOD/LOQ (units)	Ref.
ceftriaxone metronidazole hydroxymetronidazole	human plasma	protein precipitation (ACN)	HPLC-MS/MS *m*/*z* Q1→Q3 555.1→ 396.0 172.2→128.2 188.0→125.9	Column: Polaris 5 C18-A (150 mm × 3.0 mm i.d., 3.0 µm) Mobile phase: 10mM ammonium formate (pH 2.5)/acetonitrile (0.1% FA) gradient elution 300 µL/min, 30 °C, 5 µL	-/0.4–300 µg/mL (ceftriaxone) -/0.05–50 µg/ mL (metronidazole) -/0.02–30 µg/mL (hydroxymetronidazole)	[71]
ceftriaxone	human plasma	protein precipitation (MeOH)	LC-MS/MS *m*/*z* Q1→Q3 555.0→396.1	Column: Agilent Zorbax Eclipse Plus C18 (100 mm × 2.1 mm i.d., 3.5 µm) Mobile phase: 10mM ammonium formate/acetonitrile (2% FA) (87.5:12.5 *v*/*v*) methanol/acetonitrile (75:25 *v*/*v*) 20 mM ammonium bicarbonate gradient elution 0.4 mL/min, 40 °C, 2 µL	-/1.01–200 µg/mL	[65]
amoxicillin ampicillin cloxacillin dicloxacillin	urine	Filtration (0.45 µm)	LC-UV 210 nm	Column: Zorbax C18 (150 mm × 4.6 mm i.d., 5.0 µm) Mobile phase: 0.11M SDS/6% propanol/0.01M NaH_2PO_4 buffer (pH 3.0) mL/min, 25 °C, 20 µL	1.5–15/50 ng/mL	[58]
amoxicillin meropenem ceftazidime cefuroxime piperacillin	human plasma	protein precipitation (ACN)	UPLC-MS/MS *m*/*z* Q1→Q3 366.1→114.0 384.2→141.2 547.1→468.0 442.2→364.1 518.2→143.1	Column: Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm i.d., 1.7 µm) Mobile phase: 2 mM ammonium acetate/water (0.1% FA) 2 mM ammonium acetate/methanol (0.1% FA) gradient elution 0.4 mL/min, 50 °C, 40 µL	-/1.0–100 mg/L (amoxicillin, cefuroxime) -/0.5–80 mg/L (meropenem, ceftazidime) -/1.0–150 mg/L (piperacillin)	[72]

Table 2. *Cont.*

Antibiotic/Metabolite	Matrix	Sample Preparation	Detection	Conditions	LOD/LOQ (units)	Ref.
cefazolin cefalothin	human plasma urine peritoneal dialysate	protein precipitation (ACN) filtration (0.45 µm) direct injection	UHPLC-MS/MS *m/z* Q1→Q3 455.1→323.1 419.1→315.0	Column: Phenomenex Kinetex C8 (50 mm × 2.1 i.d., 1.7 µm) Mobile phase: 0.1% formic acid 0.1% formic acid/methanol gradient elution 50 °C, 0.2 µL	0.04–0.05/1 µg/mL (plasma) 0.46–4.6/0.1–0.2 µg/mL (urine) 0.01–0.03 /0.2 µg/mL (peritoneal dialysate)	[73]
clarithromycin	human serum	LLE (DCM) derivatization (FMOC-Cl)	HPLC-FD 265 nm (Ex) 315 nm (Em)	Column: Shimpack CLC-ODS (150 mm × 4.6 mm i.d., 5 µm) Mobile phase: 0.05 M phosphate buffer/TEA/methanol 2.0 mL/min., 58 °C, 20 µL	0.01/0.025 µg/mL	[68]
metronidazole	human plasma	LLE protein precipitation (ACN)	HPLC-UV 320 nm	Column: Eclipse XDB-phenyl (250 mm × 4.6 mm i.d., 5 µm) Mobile phase: 0.05 M sodium acetate/acetonitrile/glacial acetic acid (75:25:1 *v/v/v*) (pH 4.0) 50 µL	-/0.05–30 µg/mL	[74]
metronidazole	human feces	LLE (MeOH)	LC-MS/MS *m/z* Q1→Q3 172.2→128.0	Column: Waters Acquity UPLC BEH C18 (50 mm × 2.1 mm i.d., 1.7 µm) Mobile phase: 2 mM ammonium acetate/water (0.1% FA) 2 mM ammonium acetate/water (0.1% FA) gradient elution 0.4 mL/min, 55 °C	5/66 ng/mL	[75]
levornidazole hydroxylation metabolite N-dealkylation metabolite oxidative dechlorination metabolite	human feces	LLE protein precipitation (MeOH)	HPLC-MS/MS *m/z* Q1→Q3 220.0→128.0 236.0→171.0 202.0→128.0 299.9→128.1	Column: Atlantis T3 columns (150 mm × 2.1 mm i.d., 5.0 µm) Mobile phase: acetonitrile-methanol/water (0.5% FA) gradient elution 0.4 mL/min, 30 °C	-/0.005–2.0 µg/mL	[76]

Table 2. *Cont.*

Antibiotic/Metabolite	Matrix	Sample Preparation	Detection	Conditions	LOD/LOQ (units)	Ref.
cefepime meropenem ciprofloxacin moxifloxacin linezolid piperacillin	human serum	protein precipitation (methanol -methyl-tert-butyl ether (90:10, *v*/*v*)	HPLC-MS/MS *m*/*z* Q1→Q3 481.0→167.0 384.1→114.0 332.0→231.0 402.0→261.0 338.0→235.0 518.0→143.0	Column: Fortis C8 (100 mm × 2.1 mm i.d., 3 µm) Mobile phase: 10 mM ammonium formiate/water (0.1% FA) methanol gradient elution 0.5 mL/min, 30 °C, 15 µL	-/0.25–200 mg/L (cefepime) -/0.25–120 mg/L (meropenem, -/0.05–10 mg/L (ciprofloxacin) -/0.125–10 mg/L (moxifloxacin) -/0.125–50 mg/L (linezolid) -/0.5–400 mg/L (piperacillin)	[67]
cycloserine	human plasma	SPE (ACN)	HPLC-PDA 240 nm	Column: Allantis T3 (150 mm × 4.6 mm id, 3 µm) Mobile phase:10Mm phosphate buffer/acetonitrile (95:5 *v*/*v*) 0.4 mL/min, 30 °C, 50 µL	0.3/1.2 µg/mL	[77]
linezolid	human serum urine	dilution (acetate buffer, pH 3.5)	HPLC-UV 250 nm	Column: Nucleosil-100 5C18 (125 mm × 4 mm id, 5 µm) Mobile phase: Acetonitrile/sodium acetate buffer/water (180:100:720, *v*/*v*), (pH 3.7) 1.3 mL/min, 25 °C, 50 µL	0.07/0.14 mg/L (serum) 2.4/4.7 mg/L (urine)	[66]
fosfomycin	human plasma urine	protein precipitation (ACN) filtration (0.22 µm)	LC-MS/MS *m*/*z* Q1→Q2 137.1→78.9	Column: Merck SeQuant zic-HILIC (50 mm × 2.1 mm i.d., 5 µm) Mobile phase: 2 mM ammonium acetate/acetonitrile (15:85 *v*/*v*) 0.3 mL/min, 24 °C, 0.1µl (plasma), 0.5 µL (urine)	0.01/1.02 µg/mL (plasma) 0.01/0.1 mg/mL (urine)	[78]
amoxicillin oxacillin cloxacillin dicloxacillin	plasma whole blood urine	protein precipitation (ACN) SPE (MeOH)	HPLC-PDA 240 nm	Column: Inertsil ODS-3 (250 mm × 4.0 mm i.d., 5 µm) Mobile phase: acetonitrile (0.1% TFA) 1.0 mL/min, 1.3 mL/min, 25 °C, 20 µL	3.3–6.6/10–20 ng/mL (plasma) 6.6/20 ng/mL (whole blood, urine)	[79]

Table 2. *Cont.*

Antibiotic/Metabolite	Matrix	Sample Preparation	Detection	Conditions	LOD/LOQ (units)	Ref.
amoxicillin cefotaxime ciprofloxacin clindamycin metronidazole amoxycilloic acid 4-hydroxyphenylglycyl amoxicillin desacetyl cefotaxime 3-desacetyl cefotaxime lactone ciprofloxacin N-oxide *N*-demethylclindamycin clindamycin sulfoxide hydroxymetronidazole	whole blood surgical wound	SPME (MeOH)	HPLC-QqQ-MS *m/z* Q1→Q3 366.0→114.0 456.0→396.0 332.0→314.0 425.0→162.0 172.0→128.0 384.0→189.0 515.0→263.0 414.0→354.0 396.0→336.0 348.0→328.0 411.0→148.0 441.0→178.0 188.0→144.0	Column: Phenomex GRACE C18 (50 mm × 2.0 mm i.d., 4 µm) Mobile phase: acetonitrile/water (0.1% FA) gradient elution 0.4 mL/min, 25 °C, 5 µL	0.031/0.093 µg/mL (amoxicillin) 0.033/0.098 µg/mL (amoxycilloic acid) 0.037/0.112 µg/mL (4-hydroxyphenylglycyl amoxicillin) 0.039/0.118 µg/mL (cefotaxime) 0.041/0.123 µg/mL (3-desacetyl cefotaxime lactone) 0.044/0.131 µg/mL (desacetyl cefotaxime) 0.028/0.085 µg/mL (ciprofloxacin) 0.032/0.096 µg/mL (ciprofloxacin N-oxide) 0.033/0.098 µg/mL (clindamycin) 0.039/0.117 µg/mL (*N*-demethylclindamycin) 0.042/0.126 µg/mL (clindamycin sulfoxide) 0.043/0.129 µg/mL (metronidazole) 0.045/0.135 µg/mL (hydroxymetronidazole)	[61]
piperacillin *tazobactam*	plasma urine	ultrafiltration filtration (0.45 µm)	UHPLC-MS/MS *m/z* Q1→Q3 518.0→143.0 229.0→138.0	Column: C18 Shimadzu Shim-pack XR-ODS III (50 × 2.0 mm i.d, 1.6 µm) Mobile phase: acetonitrile (0.1% FA)/water (0.1% FA) gradient elution 1 µL	0.01/0.5 µg/mL (piperacillin) 0.01/5 µg/mL (tazobactam)	[80]

Table 2. *Cont.*

Antibiotic/Metabolite	Matrix	Sample Preparation	Detection	Conditions	LOD/LOQ (units)	Ref.
amoxicillin *ampicillin* *piperacillin* *meropenem* *cefuroxime* *ceftazidime* *cefazolin*	human plasma	protein precipitation (ACN)	UPLC-MS/MS *m/z* Q1→Q3 366.16→113.94 350.16→106.00 518.26→359.09 384.18→141.03 423.09→207.00 547.22→468.10 455.16→323.00	Column: ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm i.d. 1.6 µm) Mobile phase: acetonitrile (0.1% FA)/water (0.1% FA) gradient elution 0.4 mL/min, 50 °C, 10 µL	-/0.5–1.5 mg/L	[81]
amoxicillin *cefazolin* *cefepime* *cefotaxime* *ceftazidime* *cloxacillin* *oxacillin* *piperacillin*	human plasma	protein precipitation (ACN)	UHPLC-UV 230 nm 260 nm	Column: Hypersil Gold PFP column (100 mm × 2.1 mm i.d. 1.9 µm) Mobile phase: 10 mM phosphoric/acetonitrile gradient elution 500 µL/min, 40 °C,10 µL	-/2–100 mg/L	[82]

ACN, acetonitrile; DCM, dichloromethane; FMOC-Cl, 9-fluorenylmethyl chloroformate; LLE, liquid-liquid extraction; SDS, sodium dodecyl sulfate; SPE, solid-phase extraction; TFA, trifluoroacetic acid; SPME, solid-phase microextraction; Q1, parent ion; Q3, product ion; Ex, Excitation wavelength; Em, Emission wavelength.

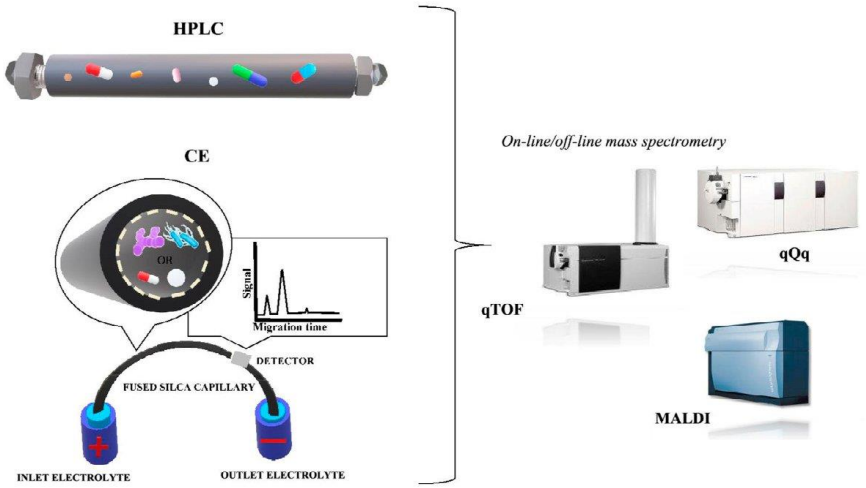

Figure 5. A comparison of the separation analysis by high performance liquid chromatography (HPLC) and capillary electrophoresis (CE).

Most of the proposed methods for the electrophoretic separation of antibiotics in different matrices are based on the use of different detection methods including spectrophotometry (UV), combined with a diode array (DAD), fluorescence (FD), electrochemical detection (ECD) or laser-induced fluorescence (LIF) [83–86]. In addition, other and more innovative detection methods have recently been used, including such methods as non-contact conductivity detection (C4D) [85,87] or potential gradient detection (PGD) [88].

Solangi et al. [89] used capillary zone electrophoresis (CZE) for the determination of two cephalosporins (cefradine and cefuroxime) in urine. The authors used 42 µm filter paper to filter these drugs and the UV detection at 214 nm. The analysis was performed at 30 kV and 25 °C using 50 mM sodium borate buffer (pH 9). The limits of the detection of two cephalosporin were from 29.0 to 30.2 µg/mL at the recovery 99–100% for cefuroxime and 1.3–1.9% for cefradine [89].

In 2015, a method of coupling CEC with mass spectrometry was also described. Hernández-Mesa et al. [90] determined five nitroimidazoles in urine samples. After SPE, drugs were analyzed by using a column packed with a mixture of Bidentate C18:Lichrospher Silica-60 (5 µm) and the background electrolyte (BGA) composed by acetonitrile, methanol and water (45:10:45 *v*/*v*/*v*). The limit of the detection of the assay was from 0.09 to 0.42 µg/mL [90]. Another report described the CE determination of ceftazidime in human plasma using a capillary column (31.5 cm × 25 µm) and 50 mM chloroacetic acid with 20% *v*/*v* methanol and 0.5% *v*/*v* coating solution of INST. The samples were deproteinized by acetonitrile. The analysis was carried out using 30 kV at 25 °C. The proper identification of ceftazidime in clinical samples constitutes an important aspect in improving the treatment of the diabetic foot [91].

Other examples of the determination and identification and determination of antibiotics and their metabolites by electrophoretic method are summarized in Table 3.

Table 3. Identification and determination of antibiotics and their metabolites by electrophoretic method.

Antibiotic/Metabolite	Matrix	Sample Peparation	Detection	Capillary Parameters	LOD/LOQ (units)	Ref.
cefazolin cefamandol cefuroxim ceftazidim ceftriaxon cefepim	serum cerebrospinal fluid sputum	lyophilization direct injection	CZE-PD 270 nm	25 mM borate buffer (pH 9.1), 50 mM SDS L_{tot} = 48 cm, L_{eff} = 40 cm, i.d. = 50 µm 20 kV, 25 °C, 2 s	0.42–0.84/ µg/mL	[92]
sulfamethoxazole N^4-acetylsulfamethoxazole trimethoprim trimethoprim 1-oxide trimethoprim 3-oxide	human serum	protein precipitation (ACN)	MEKC-DAD 260 nm 206 nm	20 mM borate buffer (pH 9.3), 25 mM SDS + 5% ACN L_{tot} = 60.2 cm, L_{eff} = 50 cm, i.d. = 75 µm 30 kV, 20 °C, 5 s	0.04–0.06/0.13–0.24 mg/L	[93]
ceftazidime cefotaxime cefuroxime ceftriaxone	wound drainage cerebrospinal fluid serum urine	filtration (0.45 µm)	CZE-UV 270 nm	25 mM borate, buffer (pH 9.2) L_{tot} = 48.5 cm, L_{eff} = 40 cm, i.d. = 50 µm 25 kV, 25 °C, 0.2 s	0.21–0.48/ µg/mL	[94]
ceftazidime	human blood	protein precipitation (ACN)	CE-DAD 200 nm 260 nm	50 mM chloroacetic acid, 20% *v/v* methanol, 0.5% *v/v* INST (pH 2.32) L_{tot} = 31.5 cm, L_{eff} = 23 cm, i.d. = 25 µm 30 kV, 25 °C, 30 s	0.42/ µg/mL	[91]
vancomycin	human serum	direct injection	MEKC-PDA 210 nm	25 mM borate buffer (pH 10.0), 100 mM SDS L_{tot} = 67 cm, L_{eff} = 50 cm, i.d. = 75 µm 25 kV, 25 °C, 4 s	1 µg/mL 1 µg/mL	[95]
daunorubicin	human plasma	SPE (MeOH)	CE-LIF 520 nm	100 mM sodium dihydrogenphosphate (pH 5.0) L_{eff} = 40 cm, i.d. = 50 µm 10 kV, 25 °C, 10 s	-/1 µg/L	[96]
cephalexi cefadroxil cefaclor ceftazidim cefsulodin cefotaxim cefamandol cefuroxim cefodizim	urine	filtration (0.2 µm)	CZE-DAD 210 nm	50 mM citrate buffer (pH 6) L_{tot} = 48.5 cm, i.d. = 50 µm 30 kV, 25 °C, 9 s	2.5–5/ µg/mL	[97]

Table 3. *Cont.*

Antibiotic/Metabolite	Matrix	Sample Peparation	Detection	Capillary Parameters	LOD/LOQ (units)	Ref.
cefadroxil cefixime cefuroxime sodium ceftriaxone sodium ceftizoxime cefaclor cefradine cefotoxime	urine	filtration (0.42 µm)	CE-UV 214 nm	50 mM sodium tetraborate buffer (pH 9.0) L_{tot} = 57 cm, L_{eff} = 50 cm, i.d. = 75 µm 30 kV, 25 °C, 4 s	0.5–5/-µg mL	[89]
moxifloxacin lomefloxacin norfloxacin ciprofloxacin ofloxacin enrofloxacin oxolinic acid flumequine	human blood	protein precipitation (MeOH)	CE-FD 240–400 nm	50 mM phosphoric acid (pH 7.55), 40% acetonitrile L_{tot} = 70 cm, L_{eff} = 55 cm, i.d. = 75 µm 50 mbar, 25 °C, 8 s	0.5–15/1.5–45 µg/L	[98]
gentamicin	smear of the wound	direct injection	CZE-DAD	TBE buffer, 0.0125% PEO (pH 8.53) L_{tot} = 33.5 cm, L_{eff} = 25 cm, i.d. = 75 µm 20 kV, 25 °C, 10 s	-/-	[99]

6. Different Analytical Techniques for the Determination and Identification of Microorganisms

The detection and identification of the microorganism responsible for the infection is also an essential factor in the implementation of appropriate antibiotic therapy. The changing epidemiology of infections, the emergence of new pathogenic bacteria and the easy spread of pathogens, including drug-resistant strains, make it necessary to improve the existing ones and to search for and develop new methods of microbial identification.

Identification is done by matching characteristics (phenotypic or genotypic) to a fixed reference organism such as the strain type. There are a number of standard methods for the detection and identification of pathogenic bacteria (Figure 6). Phenotyping methods allow the microbiologist to identify the microorganism to genus and sometimes species level based on a relatively small number of observations and tests. These methods include biotyping, serotyping, bacteriophage typing, evaluation of susceptibility profiles and protein analysis methods. Biotyping examines biochemical requirements, environmental conditions (pH, temperature, antibiotic resistance, susceptibility to bacteriocins) and physiological aspects (colony and cell morphology, cell walls and cell membrane composition such as fatty acid profiles) [100–102].

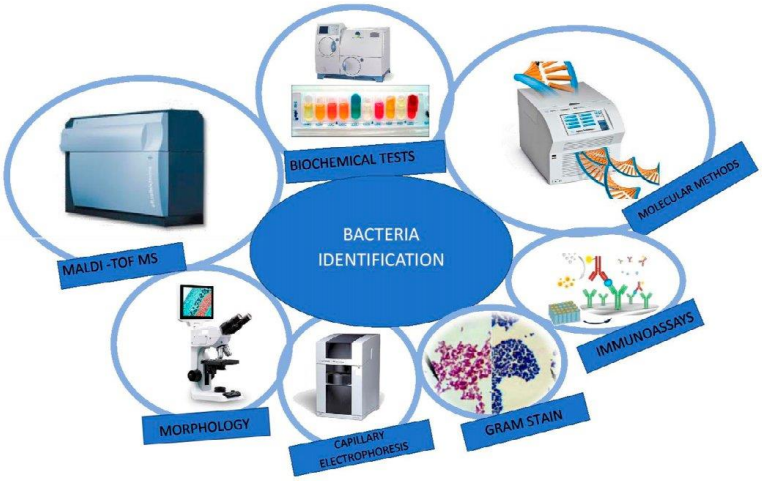

Figure 6. The popular methods to microorganism identification.

6.1. Gram Staining

A bacterial cell is an organism with a dynamic metabolism, heterogeneous in terms of structural and chemical characteristics. Microorganisms are biochemical reactors with the ability to rapidly assimilate to the environment, undergo mutation and change fast. The cell surface of bacteria consists of several components (e.g., surface proteins) forming an adherent, cohesive layer on the cell surface and affecting the physicochemical properties of the intact microbial cell. Bacteria cells can be differentiated on the basis of the properties of substances bonded to their surface.

Moreover, traditional methods of the microbial identification require the recognition of differences in morphology, growth, enzymatic activity and metabolism to identify the genera and species of bacteria. Colony morphology is usually described by a direct observation of the characteristic features of a colony—size, color, shape. The Gram staining method differentiates bacteria into two groups: violet-colored Gram-positive bacteria and pink-colored Gram-negative bacteria. Color differences result from the difference in the structure of the cell wall of both groups of bacteria (Figure 7). Gram-positive bacteria have a cell wall consisting of a thick layer of peptidoglycan, peptide bridges

and lipoteichoic acid molecules (LTA). The cell wall of Gram-negative bacteria has a more complex structure. It consists of a thin layer of peptidoglycan and an outer membrane, connected by bridges formed from the lipoprotein. The peptidoglycan and the outer membrane are separated by the so-called periplasmic space. The outer membrane with the structure of a typical protein-lipid membrane in the outer lipid layer contains lipopolysaccharide (LPS) with the composition characteristic for particular Gram-negative bacteria species. The permeation of the substance through the outer membrane is possible due to the presence of porin protein channels. In most cases, these studies are only the first stage of microbial identification, guiding the subsequent stages of microbiological investigation [103,104].

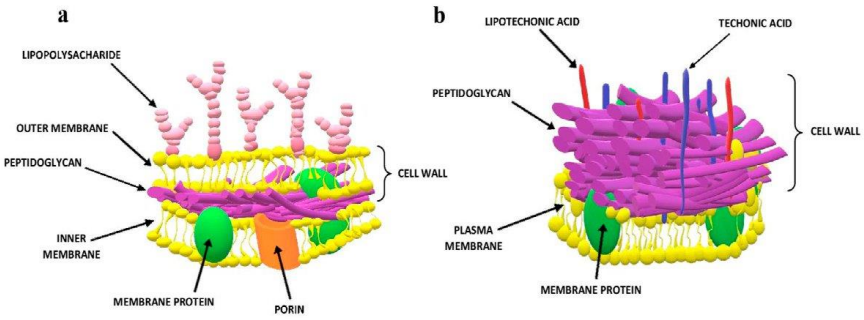

Figure 7. Differences between Gram-negative (**a**) and Gram-positive (**b**) of bacteria cell wall.

The Becerra's group [105] optimized the Gram staining procedure by comparing commonly used Gram stains and collagen counterstain. Gram staining can be a useful tool in the identification of the bacteria species such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* from clinical samples [105]. Bishop et al. [106] used the Gram staining method to determine the bacteria isolated from cerebrospinal fluid samples. They suggested that, in some cases, Gram staining provided sufficient information to start the appropriate antibiotic therapy [106].

6.2. *Biochemical Tests*

A further acceleration of the time of the detection and identification of microorganisms was possible thanks to the introduction of a new generation of automatic systems for the identification of bacteria based on biochemical properties. The identification of bacterial culture based on the principle of comparing the biochemical reaction profile with the database is most often performed using automated sets such as Analytical Profile Index API®, BD Phoenix™ Automated Microbiology System or Vitek 2 Compact [100,104]. Commercial automated systems for the identification and determination of bacterial susceptibility are usually based on the same principles as conventional tests, as they use miniaturized versions of these tests. In addition, automated systems not only determine susceptibility but also indicate the likely mechanism of antibiotic resistance, such as extended-spectrum beta-lactamases (ESBL), methicillin resistance in *Staphylococci*, glycopeptide resistance or high-degree aminoglycosides resistance in Enterococci [100,107,108].

Kierzowska and coworkers [109] described the application of the Api 20A system for the determination of various bacteria species isolated from swabs, biopsies, fluids, tissues and pus. The authors concluded that the applied biochemical test fails to give reliable results for the identification of anaerobic bacteria. Moreover, the method is time-consuming and costly [109]. Hogan et al. [110] described the use of the biochemical test, Vitek 2, to assess the susceptibility of the cultured bacterial strains to selected antibiotics. The applied method can be used as a diagnostic tool to estimate the susceptibility of antimicrobial agents to the presence of batteries, especially of the genus *Enterobacteriaceae* [110].

In comparison to traditional diagnostic methods, automated biochemical tests have more advantages. A great advantage is the possibility of a simultaneous identification and determination of the drug susceptibility of the tested microorganism (combo panels in MicroScan® WalkAway and Phoenix™ BD tests). Thanks to the use of biochemical tests, the identification time was significantly shortened. Now, instead of the few days which were needed earlier, the result is obtained within a few hours. In addition to a significant reduction in the time of the identification of the microorganism, an undoubted advantage of these systems is the ability to perform the parallel identification and/or drug susceptibility determinations for many strains of bacteria at the same time. Sensitive detection systems used in the cameras detect even the smallest, subtle changes in the growth of microorganisms, which ensures the precision of the results read out automatically. Moreover, advanced software with modern automated systems allows different ways of generating, processing, collecting and transmitting results. Unfortunately, in biochemical tests, the concentration and uniformity of suspension required for inoculation plays a very critical role in the accuracy of the identification [111–113].

The biochemical properties of proliferating microorganisms are also determined using media enriched with one or more chromogenic substrates. The inclusion of such substrates in a selective or non-selective primary medium may significantly shorten the diagnostic procedure, as cultures, isolation and identification are carried out on the same medium. Chromogenic media are widely used for screening to identify patients with antibiotic resistant bacteria such as *S. aureus* resistant to methicillin (MRSA) or vancomycin-Enterokocci (VRE). Chromogenic compounds metabolized by bacteria or fungi of certain species give colonies their characteristic color. Chromogenic substrates used in such media are usually targeted at bacterial hydrolysis—most commonly, glycosidases such as β-galactosidase or β-glucosidase. Other less frequently chosen hydrolyses are esterases or peptidases. For example, the detection of β-alanine aminopeptidase was used to detect *Pseudomonas aeruginosa*. Chromogenic media are offered by many manufacturers, including bioMérieux (chromID media), Merck (Chromocult and Fluorocult media), Bio-Rad Laboratories (Select media). Adding antibiotics to such media enables screening to detect bacteria resistant to antibiotics colonized in the respiratory tract or gastrointestinal tract of patients [107,114–116].

6.3. Immunoassays

Some *Streptococcus* species contain a unique carbohydrate molecule as part of the cell wall that can be used to distinguish them from other species. Such differences between species can be identified by the use of serological typing. Serotyping is one of the oldest immunological techniques. It is an important method of identification not only for Gram-negative bacteria such as *E. coli* and *Salmonella* spp. but also for some Gram-positive bacteria. Immunological methods use a reaction of bacterial antigens with antibodies against these antigens. Different antibody markers are used to visualize the immune response. In the fluorescence microscopy method, the marker is the fluorescence dye. In the case of latex agglutination, antibodies are coated on latex molecules. In immunoenzymatic methods, however, antibodies or antigens, depending on the variant of the method, are labelled with an enzyme. In diagnostic laboratories, fluorescence immunoanalysis and enzyme linked immunosorbent assay (ELISA) tests are commonly used, in which determinations are performed using various methods of detecting the immune response. Due to the use of antibodies for specific antigens, immunological methods allow to confirm or exclude the presence of only the desired microorganisms [117,118].

6.4. Bacteriophage Typing

Another method of phenotyping is bacteriophage typing. Bacteriophage viruses can infect host cells, causing the disintegration or incorporation of their genetic material and the expression of new proteins. These methods can be used in both single and mixed cultures where host specificity allows both detection and identification. These techniques are mainly used for research purposes and their commercial development is primarily intended for use in clinical and food microbiology [119].

6.5. Fatty Acid Profile

A more common method of bacterial identification is to characterize the types and proportions of fatty acids present in the cytoplasmic and outer membranes of bacteria. The fatty acid composition of prokaryotes can be very variable and concern the length, presence or absence of a double bond, ring or chain branching. The wealth of information contained in these compounds concerns both qualitative differences (usually at genus level) and quantitative differences (often at species level). Branched chain fatty acids are common in many Gram-positive bacteria, while Gram-negative bacteria consist mainly of simple chain fatty acids. The identification of bacteria based on fatty acid composites (profile) is widely used in clinical laboratories, public health, food and water inspection, where pathogens and other bacterial hazards must be identified routinely [120,121].

Unfortunately, these phenotypic methods are limited because microorganisms can suddenly change their phenotypic properties due to environmental changes or genetic mutations. Therefore, in order to avoid problems that may arise with phenotypic methods, identification on the basis of genotypic traits was developed. These methods include the DNA hybridization, polymerase chain reaction (PCR), rRNA 16s and 23s gene sequencing and fingerprinting (ribotyping) [100,122].

6.6. Molecular Methods

6.6.1. DNA Hybridization

The hybridization of nucleic acids involves the formation of hydrogen bonds between nucleotides of complementary single-stranded DNA or RNA molecules. Hybridization results in double-stranded molecules (hybrids) in which one thread is a DNA or RNA molecule of the tested microorganism (target) and the other is chemically, radioactively or fluorescently marked with a probe. DNA, RNA or nucleic acid molecules are used as probes. In microbiological diagnostics, the methods of solid and liquid hybridization are used. Examples of solid hybridization are Southern blot (detection of DNA acid) and Northern blot (detection of RNA acid). One of the types of hybridization is FISH (Fluorescent In Situ Hybridization), which enables the detection of a specific DNA sequence in a tested sample using a molecular probe marked fluorescently. Currently, in microbiological laboratories, the most frequently used technique is hybridization in solution. In commercial systems, labelled probes are used to detect and quickly identify the microorganism that causes the infection, while microbial detection is based on chemiluminescence or fluorescence. Examples of using the hybridization method in microbiological diagnostics are tests: AccuProbe from Gen-Prob Inc., QuickFISH and PNA FISH tests from AdvanDx [122,123].

The identification of microorganisms is increasingly carried out using hybridization with the DNA microarrays (DNA chips) technology. The marked sample (the studied microbiological material) is placed on a plate containing a DNA probe with a known nucleotide sequence. The most common probe sequences are selected from databases such as GeneBank or UniGene. Then the plate is scanned which results in a different intensity of light points, which is caused by the presence of characteristic probes for specific genes. Next, a number of fluorescence intensities are assigned to each point. The data obtained are subjected to the bioinformatic analysis. Microarrays are available in two types: oligonucleotide chip (DNA chip) and cDNA, differing in the size of the nucleic acid [124].

Jin et al. [125] used the oligonucleotide microarrays method to detect intestinal bacteria in fecal samples. The probes were projected on the base of 16S and 23 rRNA gene sequences of 15 intestinal bacteria species. The genes were amplified with two universal primers, and 22 oligonucleotide probes were used for detection. It was demonstrated that the use of the DNA microarray allows for a specific identification of bacteria species dominating in the intestinal microflora [125].

6.6.2. PCR-Based Methods

The introduction of Polymerase Chain Reaction (PCR) was one of the biggest, if not the biggest, breakthroughs in biological and chemical sciences. This method was developed by Mullis et al. [126] in the early 1980s. The technique consists in a multiple duplication of any DNA sequence using temperature-resistant polymerase and primers, i.e., short DNA chains with sequences complementary to the final synthesized DNA fragment sequences. In order to visualize the expected size and purity of the DNA molecule, the reaction product is subjected to electrophoresis in agarose gel and visualized with a DNA binding dye, e.g., ethidium bromide. For identification purposes, the 16S rRNA gene is a beneficial target for PCR amplification as it is widely distributed among bacteria and contains sufficient differences between strains and species in the DNA sequence. Microorganisms can be identified by comparing the 16S rRNA gene sequences available in databases with those of an unknown microorganism [127].

Kouidhi et al. [128] used the DNA amplification to detect typical bacteria present in the oral cavity of children with caries. *Streptococcus mutans*, *Candida albicans*, *Streptococcus salivarius* and *Streptococcus oralis* were identified in most saliva samples of children affected by tooth decay. The authors, therefore, suggested that this method may be useful in monitoring the presence of caries-specific pathogens in the oral cavity [128]. Pechorsky and coworkers [129] showed that PCR methods can be used to identify the pathogenic bacteria from blood matrices. The proposed identification method can provide useful information for the determination of blood stains in clinical laboratories [129].

Despite the development of various molecular methods, PCR remains the most widely used method, both in experimental research and in clinical laboratories. This method is often used to simultaneously detect both the PCR positive control DNA and the tested DNA in a single tube, or two different target sequences in the tested DNA. A variation of the PCR method is real-time PCR (RT-PCR), carried out in special apparatus and with appropriately prepared starters. It allows to read the result of the reaction during its course, by measuring the fluorescence of the sample, which is proportional to the amount of the product produced. Both classic and RT-PCR methods are used to determine the presence of microorganisms in bacteria, viruses and fungi. Currently, there are various PCR systems available on the market which enable the detection of microorganisms directly in the test sample (blood, serum, plasma, cerebrospinal fluid) or the presence of genes, encoding toxins or mechanisms of antibiotic resistance in the cultured bacteria [122]. Examples of such tests are GeneXpert from Cepheid, which enables the detection of e.g., MRSA in nasal swabs, positive blood culture bottle samples and wound swabs, and vancomycin-resistant enterococci (VRE) in rectal swabs [130]. The disadvantage of PCR systems is targeted testing, which means that we confirm or exclude the presence of specific microorganisms. In addition, 16S analyses of the rRNA gene sequences showed limited variability within bacterial strains such as *Bacillus cereus*. Therefore, due to high homology, this technique is not always reliable in the identification of an unknown organism [131].

PCR/ESI-MS is another microbial identification method using a combination of molecular biology techniques and mass spectrometry. Many starter pairs are used for PCR: Starters specific to entire groups of microorganisms, starters specific to species or strain, and starters aimed at antibiotic resistance genes or genes responsible for pathogenicity. After receiving PCR products, molecular masses of the DNA fragments obtained are determined using ESI-MS. The results of molecular mass determination of amplicons are species-specific code—"fingerprint", which is compared with the results stored in the database [121,127,132]. The study by Brinkman et al. [133] showed that ESI-MS PCR technology can be a useful tool in the treatment of infectious endocarditis [133].

Modern microbiological analysis is usually carried out by traditional cultures and molecular biology techniques such as PCR. However, the problem with using conventional microbiological techniques is that they are time-consuming and costly. In addition, information obtained from these tests does not provide any insight into the molecular profile of bacteria and protein expression induced by the stress factor. Therefore, an innovative analytical approach was developed, based on the electrophoretic (CZE) [134–138] and spectral analysis of microorganisms, using matrix-assisted laser desorption/ionization with time of flight (MALDI-TOF) [139–141].

6.7. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Recently, we have also used devices that allow us to identify microorganisms based on the analysis of protein profiles. Innovative mass spectrometry technology, or rather a variant of this technique, abbreviated as the MALDI-TOF, is used more and more commonly in microbiological diagnostics. In this method, the sample is subjected to a matrix that absorbs energy from the laser, resulting in the rapid heating, evaporation and ionization of the analytes; the ions are then separated based on their time taken to reach the detector, as all ions of the same charge receive the same kinetic energy highly abundant proteins, then the ribosomal proteins are analyzed [100,142–144]. Figure 8 shows a schematic diagram of the MALDI-TOF MS analysis [134,136].

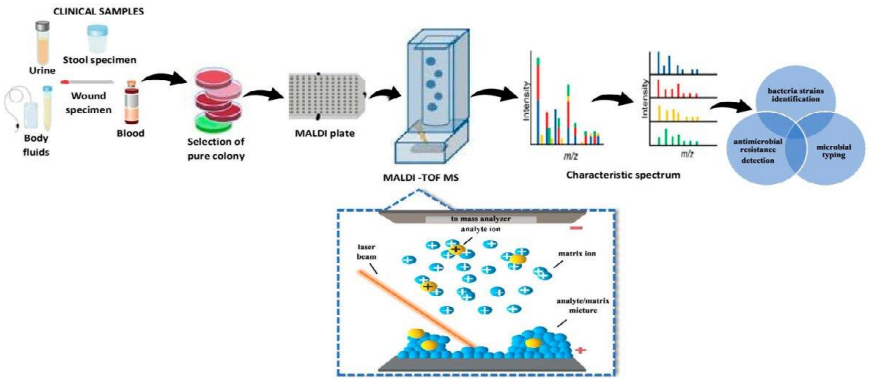

Figure 8. Schematic illustration of MALDI-TOF MS (matrix-assisted laser desorption/ionization with time of flight) analysis.

The MALDI-TOF MS technique is recommended mainly for biochemical and clinical analysis; however, the absence of an accurate database limits its technological capabilities and benefits [141–145]. The potential of the MALDI-TOF MS makes it possible to extend the applications to other fields of microbiological analysis, pharmacology, food technology or environmental analysis. Therefore, an early detection of the pathogen will facilitate an appropriate preventive action (dedicated therapy, target analysis). In the case of the medical analysis, this means selecting an appropriate treatment. The biggest drawback of the spectrometric analysis of microorganisms is the scarcity of the databases (repositories). Firstly, the limited number of producers and distributors of bacteria-identifying software results in exaggerated prices of the databases. For this reason, the spectrum of microorganisms contained therein may not coincide with the bacteria which are the subject of our research. Therefore, it is possible to combine the identification of the bacterial strain by the commercial software (Biotyper, Bruker Daltonics) with the development of cheap, fast and precise reference repositories of bacterial spectra [140,146–149]. Furthermore, coupling of molecular biology techniques with traditional culture methods in developing of the data needed to create repositories is an interdisciplinary approach to the problem of the credibility of reference strains. Another problem is related to drug resistance

mechanisms and antiseptic sensitivity. As drugs are now universally applied, the response of human immunological systems to bacterial, yeast and viral infections is getting weaker and weaker [150]. The result is the phenomenon of drug resistance in bacteria. A detailed study of the problem constitutes a modern analytical challenge.

The generated spectrum of peaks corresponding to ions of different mass to charge ratio corresponds to a unique protein profile, a specific molecular "fingerprint" of the tested microorganism. The spectrum is compared with the spectra of reference microorganisms collected in the database. The probability of correct identification is expressed by a point indicator which, depending on the value obtained, indicates a reliable identification of the micro-organism to the species reliable identification to the genus level with a probable identification to the species or the probable result of identification to the genus level. When the value of an indicator is below the accepted value, there is no reliable identification result. In microbiological laboratories, we can see systems such as Bruker's MALDI BioTyper or VITEK® MS from bioMérieux. These systems deliver results in minutes. Currently, the MALDI-TOF MS is mainly used in culture methods to confirm the identification of bacteria [146].

Mailhac et al. [151] described the use of this method to identify 45 bacterial isolates from vitreous samples. Moreover, the authors optimized the protocol for the extraction of bacterial protein. The study showed that 96% of bacterial isolates were identified by species [151]. Haiko et al. [152] demonstrated that MALDI-TOF MS can also be a diagnostic tool for determining the bacteria responsible for urinary tract infections. From 107 Gram-negative bacterial isolates tested, the MALDI-TOF MS method identified 92 (86%) of them. MALDI-TOF MS is a valuable method for a rapid diagnostic of pathogens in patients with urinary infections [152].

In addition, the MALDI-TOF MS applications in clinical microbiology go beyond the identification of microorganisms, and this technique can also be used for the rapid detection of antimicrobial resistance. It was observed that products that result from hydrolysis (e.g., β-lactams by bacterial enzymes—β-lactamases) differ in molecular weight from native antibiotic molecules. There are also studies that indicate the enormous potential of this method in a routine detection of dangerous resistance mechanisms, e.g., carbapenemase. Despite the success, there are some limitations in the use of the MALDI-TOF MS, such as the inability to determine taxonomically related bacteria, e.g., highly pathogenic *Shigella* species from commensal *Escherichia coli*, and the inability to identify *Streptococcus pneumoniae* from some commensal oral *Streptococci* species. Yet, these systems are still being improved and their sensitivity will probably increase with each next generation, which shall strengthen the position of spectrometric mass in clinical laboratories [153,154]. In Table 4, the identification of bacteria in clinical samples by using the MALDI-TOF MS technique is presented.

Table 4. Data of bacterial isolates from various clinical materials determination by MALDI-TOF MS.

Bacteria Speices	Clinical Samples	Matrix Solution	Sampling Technique	Identification System	Degree of Compliance Identification (%)	Ref.
Eschericha coli	urine	HCCA	direct application	MALDI VITEK® MS	86	[152]
Klebsiella pneumoniae					100	
Klebsiella oxytoca					67	
Citrobacter spp.					100	
Enterobacter spp.					75	
Pseudomonas aeruginosa					100	
Proteus mirabilis					100	
Eschericha coli	urine	HCCA	protein extraction	MALDI BioTyper	95	[149]
Klebsiella pneumoniae					93	
Klebsiella oxytoca					100	
Enterococcus faecium					82	
Enterococcus faecalis					90	
Pseudomonas aeruginosa					86	
Proteus mirabilis					98	
Staphylococcus epidermidis	blood	HCCA	direct application	MALDI VITEK® MS	65	[155]
Klebsiella pneumoniae					97	
Eschericha coli					93	
Staphylococcus haemolyticus					80	
Lactobacillus fermentum	saliva	HCCA	protein extraction	MALDI BioTyper	80	[156]
Lactobacillus salivarius					36	
Lactobacillus rhamnosus					75	
Lactobacillus plantarum					100	
Staphylococcus epidermidis	blood	HCCA	protein extraction	MALDI BioTyper	99	[157]
Staphylococcus hominis					100	
Staphylococcus haemolyticus					100	
Mycobacterium abscessus	sputum pus peritoneal fluid urine	HCCA	protein extraction	MALDI BioTyper	97	[158]
Mycobacterium fortuitum						
Mycobacterium avium						

Table 4. *Cont.*

Bacteria Speices	Clinical Samples	Matrix Solution	Sampling Technique	Identification System	Degree of Compliance Identification (%)	Ref.
Veillonella spp.	abdomen fluid pleural fluid bile surgical wounds pus operating material blood	HCCA	direct application	MALDI BioTyper	100	[159]
Escherichia coli *Streptoccocus aureus* *Staphylococcus epidermidis*	blood urine pus swab cerebrospinal fluid respiratory tract wound specimens	HCCA	protein extraction	MALDI BioTyper	100 100 100	[160]
Aeromonas spp.	feces	HCCA	direct application	MALDI BioTyper	100	[161]
Streptoccocus spp.	vitreous samples	HCCA	protein extraction	MALDI BioTyper	96	[149]
Escherichia coli	urine blood	HCCA	direct application protein extraction	MALDI BioTyper	94	[162]
Eggerthella lenta	blood	-	direct application	MALDI BioTyper MALDI VITEK® MS	94 100	[163]

6.8. Capillary Electrophoresis

Moreover, capillary electrophoresis has also been developed over the past decade. Although these newer methods will not replace the traditional methods of plate counting involving cultures and microscopes, their development and use will not expand further. Like other colloidal particles, the microorganism transfers the charged groups to its outer surface, and their electric double layer is created when the charged microorganism comes into contact with aqueous solution (BGE, background electrolyte). Therefore, under the influence of an electric field, bacteria show a characteristic electrophoretic mobility, which is a function of the size of the microorganism, its surface charge and the double electric layer. As we know, capillary electrophoresis quickly and effectively separates biologically important molecules such as proteins and nucleotides. These advantages can also be used for the microbial analysis, as CE methods allow a rapid and simultaneous analysis of several microorganisms in a single sample, including their identification and also quantification [164,165].

The first reports of using capillary electrophoresis for bacteria determination were published in 1987 by Hjerten et al. [166]. They described the migration of Mosaic tobacco virus and *Lactobacillus casei* bacteria in 20 mM buffer Tris-HCl (pH = 7.5); however, they did not achieve any separation. The bacteria migrated along with the electroosmotic flow and acted like units with no electric charge on the surface. In 1993, Ebersole and McCormic [167] separated four types of bacteria in TBE buffer at pH = 9.5, and by gathering particular fractions after the process of electrophoresis had been completed, they proved that the majority of bacteria (80%) were alive. The next year, Torimura [168] published his work concerning the electrophoretic behavior of nine types of bacteria, determining their electrophoretic mobility. In the 1990s, Pfetsch and Welsch [169] and Glynn [170] determined the electrophoretic mobility in different buffer solutions and proved its decrease when ionic force was growing. After 1999, Armstrong et al. [171] introduced poly(ethylene)oxide—PEO, previously used in protein separation. Adding PEO to buffer solutions caused the suppression of electroosmotic flow and significantly lowered the adhesion of bacteria cells to the internal capillary surfaces. The same research group proposed three different mechanisms of bacteria migration in the electric field using PEO, and also the creation of agglomerates by bacteria cells [172]. Zeng and Yeung [173] used a CCD camera for the visualization of cell aggregation; they observed that the cells were moving in different directions at different velocities, depending on the agglomeration size.

Buszewski et al. [135,174,175] developed a modern, extremely fast method for identifying pathogenic microorganisms based on electromigration techniques (capillary electrophoresis). In the experiments conducted so far, several bacterial strains were detected and identified, including those which are as dangerous as *Staphylococcus aureus* [174,175] and *Escherichia coli* [135].

The development of an innovative methodology to identify microorganisms, based on a rapid and selective electrophoretic and spectrometric method, can be a very good analytical solution that brings measurable results, such as a reference set and a screening method. The decrease in people's resistance to pathogens which is caused by a prolonged use of antibiotics, forces a new approach of understanding and combating drug resistance. Thus, a synthesis of a new generation of antibiotics based on metal complexes can create a desirable pharmaceutical product. The application of the MALDI technology and electromigration techniques in a microbiological analysis can become a milestone in the diagnosis and analysis of the infection. Hence, the relevant procedure should be characterized by: (i) simplicity—the equipment needed can be used in any room, and it only requires basic maintenance; (ii) speed—time of the identification of the presence of relevant bacteria in the prescribed conditions and performing the target metabolomics analysis will be no longer than 60 min—compared to the traditional methods, it is a revolutionary speed; (iii) sensitivity and reproducibility—while maintaining stable parameters of the study; thus, it is supposed to be an absolutely reproducible method.

7. Conclusions

The monitoring of therapeutic drugs (TDM) provides valuable information on the actual antibiotic concentration in body fluids. Taking into account the clinical, cognitive and diagnostic purposes of drug

monitoring, it is important to select an appropriate analytical method that meets all the requirements. One of the first methods of antibiotic determination in biological matrices were immunoenzymatic techniques, which are characterized by their wide determinability, high sensitivity and a short time of analysis, because they do not require separate techniques of isolation from the biological material. Unfortunately, these methods are not free from defects. An important problem in the use of these tests is the non-specificity of the response to the individual antibiotics of the group. Cross-reactions caused by another group of compounds or due to the influence of the biological matrix may also occur. Moreover, an element making it difficult to fully assess the quantitative dependence in the tested material is the frequent phenomenon of combined determination with the parent compound of its metabolites, the presence of which may interfere with the absolute values of the obtained result.

Therefore, chromatographic methods allowing the determination of both the active compound and its metabolites are increasingly used in everyday practice. These methods include liquid chromatography (LC), high-performance liquid chromatography (HPLC), gas chromatography (GC), which are usually coupled with a mass spectrometry detector (MS), and thin-layer liquid chromatography (TLC). The UHPLC-MS/MS method is mentioned in literature as commonly used. The combination of liquid chromatography with a mass spectrometer guarantees high selectivity, high sensitivity, resolution, repeatability, identification by mass and structure determination by fragmentation, along with the versatility of application. Capillary electrophoresis (CE) is used in new analytical methods. Both HPLC and CE are universal methods, commonly used in monitoring the concentration of antibiotics. However, an essential element of achieving reliable results of monitored drugs is their effective extraction from the biological matrix and appropriate selection of parameters of chromatographic separation and detection. In practice, the LLE method and extraction on columns filled with solid media (SPE) are the most frequently used methods. The frequency of the use of both techniques is comparable.

In recent years, clinical microbiology laboratories have experienced revolutionary changes in the way microorganisms are identified. Until now, the identification of microorganisms in clinical microbiology laboratories has been carried out mainly through the analysis of biochemical reactions and phenotypic features such as growth on different media, colony morphology and Gram staining. Combined, these routine laboratory techniques provide accurate identification of most microorganisms but are costly and time-consuming.

The development of micro and nanotechnology also allowed the use of the DNA microarrays in medical diagnostics. The advantage of this method, in comparison with the previous techniques, is their ability to study the expression of a large number of genes at the same time. Moreover, the DNA microarray requires a relatively small amount of genetic material and is highly sensitive. Unfortunately, the main barrier is the high cost of the arrays and the equipment necessary to carry out tests.

However, the MALDI-TOF MS may be an interesting alternative, especially in some areas where a rapid analysis is required, e.g., in clinical microbiology. This method is not targeted, which means that no prior knowledge of the infectious agent is required, since identification is based on a database match. The level of confidence in the match is calculated using an algorithm, thus eliminating errors of human judgment that plague traditional phenotypic analysis methods such as a morphological analysis. Although the purchase of the machine is relatively expensive, the cost per sample is very low, which translates into significant savings in laboratory operating costs. Finally, while the sample preparation stage requires a certain amount of time, the acquisition and matching of the MALDI spectrum itself is achieved in a matter of minutes. As mentioned earlier, the identification of the MALDI-TOF MS is based on the analysis of the protein spectrum of the bacterial ribosome and is therefore closely related to the analysis of the 16S rDNA gene sequence. However, due to the high similarity of these sequences in some species such as *Shigella* spp., *Escherichia coli* or *Streptococcus pneumoniae* and other members of the *Streptococcus* group (*S. mitis, S. vestibularis*), discrepancies in the identification of these species may occur. In this case, standard biochemical tests such as antigen detection or molecular methods are required. Nevertheless, the MALDI-TOF MS continues to evaluate

and improve the equipment of the microbiologist's tools. Other applications of the MALDI-TOF MS in the development and the possibility of detecting bacterial resistance aroused great interest. The detection principle is based on the hydrolysis of the β-lactam ring in the presence of bacterial enzymes. MALDI-TOF MS is also able to detect changes in the mass of antibiotics caused by the chemical modification in an antimicrobial molecule. In addition, the optimization of sample preparation protocols and the increased representation of less common microorganisms in commercial databases promise a faster and more accurate identification of microorganisms, which, as expected, will translate into better patient care.

The application of the MALDI-TOF MS, the electrophoretic approach and qPCR make a comprehensive interpretation and validation of the results possible. A promising alternative is also the use of a bacterial chip that can act as a sensor to detect bacteria from the outside environment. In addition, it can be utilized as a MALDI-TOF MS target plate for a direct detection of bacteria from clinical samples. On the other hand, along with the development of chromatographic techniques and combining them with sensitive methods of detection, the application of metabolomics has largely increased in recent years. It plays a major role in medicine and pharmacy as well as in agriculture. Thanks to it, the identification of compounds in biological samples for the purpose of clinical diagnosing of diseases is now possible. Moreover, in the pharmaceutical analysis, there exists an important trend to determine metabolic profiles after the administration of a drug in order to trace what is happening with it in the organism. Additionally, the clinical significance of pharmacokinetics stems from the need to use a personalized treatment for each patient, as in such cases, the knowledge of the drug concentration in blood and its determined physical and chemical parameters are very useful in setting a scheme of dosage.

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Abbreviations

BGE	background electrolyte
C4D	non-contact conductivity detection
CAD	charged aerosol detector
CITP	capillary isotachophoresis
Co	cobalt
CZE	capillary zone electrophoresis
DAD	diode-array detector
ECD	electrochemical detection
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector
ESBL	extended-spectrum beta-lactamases
ESI	electrospray ionization
FISH	fluorescent in situ hybridization
FL	fluorescence detector
FMOC-Cl	9-fluorenylmethyl chloroformate
FPIA	fluorescence polarization immunoassay
FTIR	Fourier-transform infrared spectroscopy
GC	gas chromatography

HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
LC	liquid chromatography
LIF	laser-induced fluorescence
LLE	liquid-liquid extraction
LTA	lipoteichoic acid molecules
MALDI	matrix-assisted laser desorption/ionization
MBC	minimum bactericidal concentration
MECK	micellar capillary electrokinetic chromatography
MEPS	microextraction by packed sorbent
MIC	minimum inhibit concentration
MRSA	methicillin-resistant Staphylococcus aureus
MS	mass spectrometry detector
NACK	capillary non-aqueous electrophoresis
NMR	nuclear magnetic resonance
PBP	penicillin binding protein
PCR	polymerase chain reaction
PDA	photodiode array detector
PGD	potential gradient detection
qQq	triple quadrupole mass spectrometer
Rf	retention factor
SPE	solid phase extraction
TBA	Tris-boran-EDTA
TDM	therapeutic drug monitoring
TEA	trietyloamina
TiN	titanium nitride
TiO_2	titanium dioxide
TLC	thin-layer liquid chromatography
TOF	time-of-flight
UHPLC	ultra-high performance liquid chromatography
UPLC	ultra-performance liquid chromatography
UV	ultraviolet
VAP	ventilator-associated pneumonia
VRE	vancomycin-resistant Enterococcus
WHO	World Health Organization

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3.2. *Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD*

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Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD

Justyna Walczak-Skierska[a,b], Małgorzata Szultka-Młyńska[a,*], Katarzyna Pauter[a], Bogusław Buszewski[a,b]

[a] Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarin 7, 87-100, Torun, Poland
[b] Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Wilenska 4, 87-100, Torun, Poland

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ABSTRACT

The separation of eleven antibiotics and ten metabolites were studied using high performance liquid chromatography. The C18-PFP octadecyl with integral PFP, C18-AR octadecyl with integral phenyl, C18-HL octadecyl and phenyl phase were used as stationary phases. Mixtures of acetontrile–0.1 % formic acid in water were investigated as mobile phases. The elution order of the target compounds was similar for all four HPLC columns applied. The best separation was obtained using the column with the pentafluorophenylpropyl chain. In addition, in order to optimize the parameters of retention elution for the column and to predict the conditions for the best separation of the active compounds studied biologically the ChromSword software was used. To obtain reliable information of the physicochemical properties and to estimate the relative biological activity of a group of the studied analytes, the QSRR approach was applied. Molecular descriptors were calculated using the HyperChem software. The study was based on multiple linear regression and the results were presented as quantitative structure–retention relationships equations. The QSRR models were determined using 16 molecular descriptors mainly related to the dipole moment (μ), the solvent accessible surface area (*SAS*), the van der Waals surface area (*VWS*), the minimum charge (δ_{min}) as well as the polar surface area (*PSA*). Moreover, structural descriptors of the target compounds were used to describe their chromatographic retention behavior under the optimized HPLC conditions.

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1. Introduction

Antibiotic drugs include a diverse group of chemical compounds, both natural and synthetic, which have an antibacterial effect - they are capable of killing or inhibiting the growth of microorganisms. There are several ways of dividing antimicrobial drugs, but the most popular classification schemes are based on their molecular structure, the mode of action and the spectrum of activity [1]. Drugs belonging to the same structural group will have similar efficacy and toxicity. Some popular groups of antibiotics include β-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulfonamides and glycopeptides [1–3].

Nowadays, antibiotics are one of the most commonly used groups of drugs, along with painkillers and antipyretics. Antibiotic therapy plays a key role in the fight against infectious diseases caused by microorganisms. The use of antibacterial drug monitoring therapy in body fluids (blood, serum, plasma, cerebrospinal fluid, saliva, urine) is a multidisciplinary activity aimed at ensuring safety and effectiveness of antibiotic pharmacotherapy, the improper use of which may be the source of many therapeutic problems [4,5]. The widespread presence of antibiotics in the environment, their use in veterinary medicine, agriculture, animal farming (as a food supplement) and the still growing human consumption of antibiotics cause bacterial resistance. Consequently, the effectiveness of these drugs in the treatment of infectious diseases is at serious risk.

The basis for monitoring the concentration of a drug in body fluids is the assumption that for most drugs, their concentration in blood correlates better with the therapeutic effect than the given dose. In practice, concentration-monitored therapy means that the antimicrobial drug can be more effective in the patient while being safer to use. In routine clinical management, the interpretation of

* Corresponding author.
E-mail address: mszultka@umk.pl (M. Szultka-Młyńska).

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the obtained drug concentration measurement requires the following conditions to be met: the knowledge of pharmacokinetics of the drug being tested, the existence of a certain correlation between the drug concentration in blood and its therapeutic or toxic effects, the determination of the range of the therapeutic concentration of the drug being tested, as well as the development of appropriately sensitive and specific analytical methods to determine the drug concentration in body fluids [6,7]. Therefore, chromatographic methods, in particular high-performance liquid chromatography (HPLC), are used to determine drug concentration [8,9]. Frequently, the separation is carried out in the reverse phase system (RP-HPLC) with the use of C18 and C8 columns and mobile phases being mixtures of water with acetonitrile or methanol. An indispensable, very important element of the analytical device is a suitable detector allowing to determine active compounds at very low levels. For HPLC, the most commonly used detectors are the UV or DAD spectrophotometer [10–13]. Recently, more and more work has also been done on the use of liquid chromatography (LC–MS/MS) [14,15] or ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) [16,17]. Furthermore, the stability assessment of drugs and metabolites in the various matrices is a fundamental factor in the validation of analytical methods. The knowledge of the most common factors influencing the stability of such analytes is fundamental in order to develop properly methods for their determination and identification.

In predicting the retention of many compounds in HPLC chromatography, the quantitative structure - retention relationships (QSRR) is used [18]. QSRR is a statistically determined relation between chromatographic parameters and values (descriptors) characterizing the structure of analyzed analytes. The QSRR model is used to describe the molecular mechanism responsible for separation in chromatographic systems. At the same time, it enables the evaluation of physicochemical properties of analytes and chromatographic stationary phases in predicting the retention of new structures. The QSRR methodology is based on the knowledge of two types of data. One of them is the chromatographic parameters obtained for a sufficiently large series of analytes. The second type of data is a set of parameters describing the molecular structure of the studied compounds. In the case of retention data, the most frequently used parameter is the n-octanol-water partition coefficient. Multiple Regression Analysis is the basic statistical method for determining the relationships between variables, which is usually used in the QSRR procedure.

The relation between the retention factor and the measure of hydrophobicity can be described using the following Eq. (1) [18]:

$$\log k_w = k_1 + k_2 \log P \quad (1)$$

where log k_w denotes the retention parameter corresponding to pure water as a hypothetical eluent and k_1, k_2 denote regression coefficients and log*P* is the logarithmic value of the n-octanol-water partition coefficient.

Physicochemical properties can be determined experimentally or theoretically based on molecular modeling methods and quantum chemical calculations.

If the results obtained during the analysis are statistically significant and physically meaningful, then the QSRR model can be used in a variety of ways. First of all, it is used to predict the retention of new analytes and to identify unknown ones. Another application of the QSRR method is to explain the molecular mechanisms of separation in a given chromatographic system. Moreover, this procedure can be used to evaluate the physicochemical properties of analytes and stationary phases (lipophilicity, dissociation constants), and to compare quantitatively the separation properties of different types of chromatographic column fillings [19–25].

In this study, the QSRR approach was used to determine the molecular mechanism of the retention of 11 antibiotics and their 9 metabolites in the four stationary phases. The use of the multiple linear regression analysis resulted more predictive and improved the QSRR equations. The molecular descriptors were calculated for each analytes by using the quantum mechanics method. The choice of active substances was made on the basis of the antibiotics and their combinations most frequently prescribed for common infections. To the best of our knowledge, the method based on high performance liquid chromatography with the diode array detector, which allows to determine 11 antibiotics and their metabolites from different therapeutic classes simultaneously, has not been developed up to now. So far, only one research paper [11] has described a similar approach, but for few of the analyzed antibiotics drugs without their metabolites and with the use of home-made HPLC column. This time we have applied more selective commercially available HPLC column for the QSRR approach regarding also metabolites of the studied antibiotic drugs belonging to different therapeutic groups. Moreover, the Chromsword software was used to optimize the process of the separation of the antibiotics and their metabolites in RP-HPLC.

2. Experimental

2.1. Chemicals and materials

In this study eleven antibiotic drugs and ten metabolites standards were used. Amoxicillin (AMOX), ampicillin (AMP), cefotaxime (CEF), ciprofloxacin (CIP), clindamycin (CLI), gentamicin (GEN), levofloxacin (LVX), linezolid (LIN), metronidazole (MET), moxifloxacin (MOXI), tetracycline (TET), and their metabolites: amoxycilloic acid (AMA), amoxicillin diketopiperazine (AMD), 3-desacetyl cefotaxime lactone (CEF-DAC-LAC), clindamycin sulfoxide (CLI-SOx), ciprofloxacin piperazinyl-N4-sulfate (CIP-PS), desmethyl levofloxacin (DEL), levofloxacin N-oxide (LVX-Ox), linezolid N-Oxide (LIN-Ox), metronidazole-OH (MET-OH), 8-hydroxy moxifloxacin (8-HYD-MOXI) were purchased from Sigma–Aldrich (Steinheim, Germany). The structure of the tested drugs and metabolites are listed in Table 1. Stock solutions of all drugs and metabolites were prepared by dissolving about 1 mg of each compounds in 1 mL composition of the mobile phase (acetonitrile/0.1 % HCOOH, 50:50, *v/v*). Acetonitrile, methanol and formic acid (all high purity) were purchased from Sigma–Aldrich (Steinheim, Germany). Ultra-pure water was obtained from a Milli-Q water system (Millipore, Bedford, MS, USA).

2.2. Instrumentation

The separation of antibiotic drugs and their metabolites was performed on a Shimadzu Prominence HPLC system (Kyoto, Japan) consisting of a quaternary solvent delivery system (LC-20AD), an autosampler (SIL-20A), a column thermostat (CTO-10AS VP), and a diode array detector (SPD-M20A) operating at 254 nm. The separation temperature was 25 °C, the flow-rate of the mobile phase was 0.5 mL/min, and the injection volume was 1 μL. The LabSolution software was used for the data analysis. The separation of all drugs and metabolites was performed on the four commercial columns: column (1) - PFP, (2) - AR, (3) - HL, (4) – Phenyl (P-300). The void volume of the column was measured by injecting thiourea, which shows no retention in the reversed phase mode (1.189 min, 1.182 min, 1.057 min, 1.177 for column (1), (2), (3) and (4) respectively).

The structure of the used columns is shown in Fig. 1. The physico-chemical properties of all the columns used in the study are summarized in Table 2.

The retention of drugs and metabolites were determined by the using ChromSword (Riga, Latva) software for the optimization the

Table 1
Chemical structures of antibiotic drugs and their metabolites.

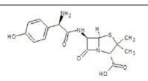
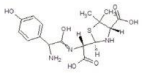
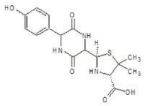
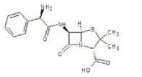
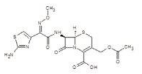
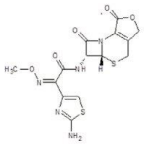
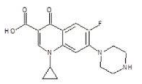
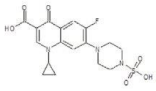
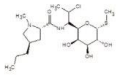
Systematic name	Shorcut	Structure	Mol. Mas [g/mol]	Log D (pH 7.4)	Log D (pH 5.5)	pKa_A	pKa_B
(2S,5R,6R)-6-([(2R)-2-amino-2-phenylacetyl]amino) −3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	AMO		365.40	−2.72	−2.04	3.23	7.43
(αR,2R,4S)-α-[[Amino(4-hydroxyphenyl)acetyl]amino]-4-carboxy-5,5-dimethyl-2-thiazolidineacetic acid	AMA		383.42	−3.12	−1.92	–	–
2-[5-(4-hydroxyphenyl)-3,6-dioxopiperazin-2-yl]-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid	AMD		365.40	−2.52	−2.14	–	–
(2S,5R,6R)-6-[(2R)-2-amino-2-phenylacetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid	AMP		349.40	−2.38	−1.59	3.24	7.44
(6R,7R,Z)-3-(acetoxymethyl)-7-(2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-aza-bicyclo[4.2.0]oct-2-ene-2-carboxylic acid	CEF		455.46	−3.35	−2.42	3.18	4.15
[5aR-[5aα,6β]]-2-amino-α-(methoxyimino)-N-(1,4,5a,6-tetrahydro-1,7-dioxo-3H,7H-azeto[2,1-b]furo[3,4-d][1,3]thiazin-6-yl)-4-thiazoleacetamide	CEF-DAC LAC		395.41	−0.10	−0.04	–	–
1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxylic acid	CIP		331.34	−2.23	−2.98	5.76	8.68
1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-sulfo-1-piperazinyl)-3-quinolinecarboxylic Acid	CIP-PS		411.40	−5.04	−4.11	–	–
methyl 7-chloro-6,7,8-trideoxy-6-{[(4R)-1-methyl-4-propyl-l-prolyl]amino}-1-thio-l-threo-α-d-galacto-octopyranoside	CLI		424.98	1.08	−0.57	12.41	7.55

Table 1 (*Continued*)

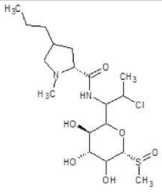
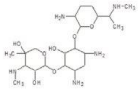
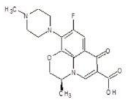
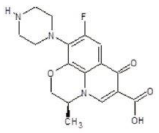
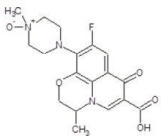
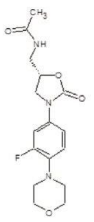
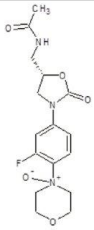
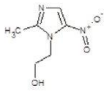
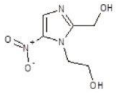
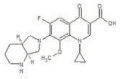
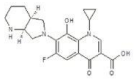
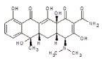
Systematic name	Shorcut	Structure	Mol. Mas [g/mol]	Log D (pH 7.4)	Log D (pH 5.5)	pKa_A	pKa_B
7-chloro-1,6,7,8-tetradeoxy-6-[[[(2S,4R)-1-methyl-4-propyl-2-pyrrolidinyl]carbonyl]amino]-1-(methylsulfinyl)-L-threo-α-D-galactooctopyranose	CLI-SOX		440.98	−0.63	−2.28	–	–
3R,4R,5R)-2-{[(1S,2S,3R,4S,6R)-4,6-diamino-3-{[(2R,3R,6S)-3-amino-6-[(1R)-1-(methylamino)ethyl]oxan-2-yl]oxy}-2 hydroxycyclohexyl]oxy}-5-methyl-4-(methylamino)oxane-3,5-diol	GEN		477.59	−8.31	−9.39	12.55	10.18
(3S)-(-)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzoxazine-6-carboxylic acid	LVX		361.36	−2.08	−1.84	5.45	6.2
(3S)-9-Fluoro-2,3-dihydro-3-methyl-7-oxo-10-(1-piperazinyl)-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic Acid; (S)-9-Fluoro-3-methyl-7-oxo-10-(piperazin-1-yl)-3,7-dihydro-2H-[1,4]oxazino[2,3,4-ij]quinoline-6-carboxylic acid	DEL		347.34	−2.75	−2.86	–	–
(3S)-9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-4-oxido-1-piperazinyl)-7-oxo-7H-Pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid	LVX-OX		377.37	−2.69	−1.21	–	–
(S)-N-({3-[3-fluoro-4-(morpholin-4-yl)phenyl]-2-oxo-1,3-oxazolidin-5-yl}methyl)acetamide	LIN		337.34	0.82	0.37	14.45	−0.66

Table 1 (*Continued*)

Systematic name	Shorcut	Structure	Mol. Mas [g/mol]	Log D (pH 7.4)	Log D (pH 5.5)	pKa_A	pKa_B
N-[[(5S)-3-[3-fluoro-4-(4-oxido-4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl]acetamide	LIN-OX		353.35	−0.54	−0.34	–	–
2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol	MET		171.15	0.05	0.05	15.44	3.09
2-(Hydroxymethyl)-5-nitro-1H-imidazole-1-ethanol	MET-OH		187.15	−0.76	−0.76	–	–
7-[(4aS,7aS)-octahydro-1H-pyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	MOXI		401.43	−1.73	−2.14	5.69	9.42
1-cyclopropyl-6-fluoro-7-((4aS,7aS)-hexahydro-1H-pyrrolo[3,4-b]pyridin-6(2 H)-yl)-8-hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid	8-HYD MOXI		387.40	−2.35	−2.02	–	–
(4S,4aS,5aS,12aS)-4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2 naphthacenecarboxamide	TET		444.43	−3.17	−2.77	−2.20	8.24

Log D – distribution coefficient at pH = 7.4 and 5.5, pKa_A – pKa strongest acidic, pKa_B – pKa strongest basic.

gradient conditions. The gradient profile was modelled on the basis retention data, such as: retention time, peak area, and peak width at 50 %, for two linear gradients from 0% to 100 % of acetonitrile in two different times (20 and 60 min). The composition of drugs and metabolites, and the optimized composition of mobile phase are presented in Table 3.

2.3. QSRR procedure

QSRR models were constructed for each column. The molecular modeling of each analytes were calculated using the HyperChem 7.5 software with the ChemPlus extension (Hyper-Cube, Waterloo, Canada). All structures before the molecular modeling were drawn using the ACD/ChemSketch Freeware (Toronto, Ontario, Canada). The geometry of the compounds was optimized by the molecular mechanics with the MM + force field method and the calculations were performed according to the Polak-Ribiere algorithm. In order to generate molecular modeling descriptors, the quantum calculations were made using the semi-empirical AM1 method. The molecular descriptors such as: the dipole moment (μ), solvent accessible (*SAS*) and van der Waals surface areas (*VWS*), hydration energy (*HE*), heat of formation (*HF*), total energy (*TE*), binding energy (*BE*), polarizability (*P*), molecular volume (*V*), molar refractivity (*R*), mass (*M*), isolated atomic energy (*IAE*), electronic energy (*EE*), core-core interaction (*CCI*), point charge (P_{ch}), minimum (δ_{min}) and maximum charge (δ_{max}), were determined. In addition, hydrogen bond acceptors (*HBA*), hydrogen bond donors (*HBD*), the polar surface area (*PSA*), and the effective partition coefficient for dissociative systems (*log D* for pH = 5.5 and 7.4) were taken from the ChemSpider database. For the metabolites the *log P* values also take from the ChemSpider database. For the drugs the *log P*, *PSA*, pKa_A and pKa_B values were taken from the DrugBank database.

To avoid overlapping information in descriptors in the regression analysis, all descriptors were correlated. In the original group of descriptors, 11 for antibiotics and 8 for metabolites respectively were strongly correlated with each other (correlation coefficient above 0.9). Then the descriptor with the highest correlation with *log k*, for each column, was used in the QSRR analysis [22]. The 16 molecular descriptors were used to determine the QSRR mod-

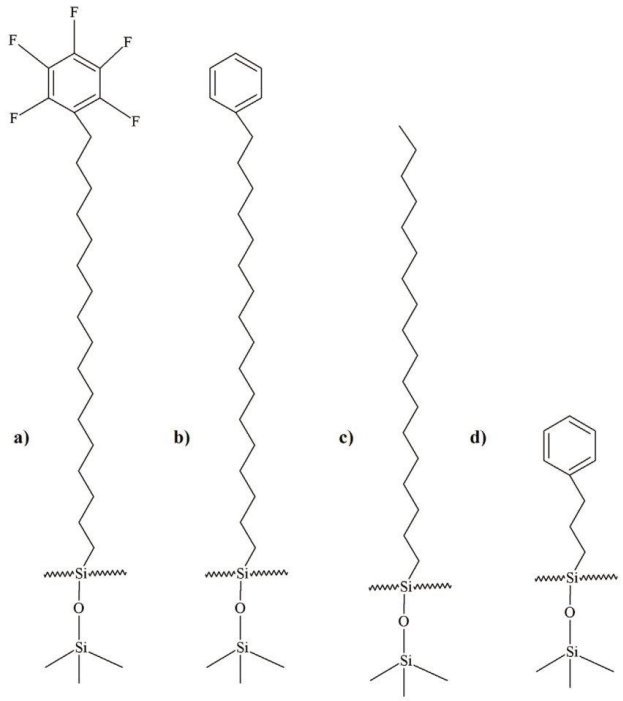


Fig. 1. Schematic structures of applied stationary phases: a) PFP, b) AR, c) HL and d) P-300.

Table 2
Physico-chemical properties of the stationary phases used in this investigation.

Stationary phase type	Abbreviation	Column dimensions [mm]	Percentage part of carbon [%]	Pore size [Å]	Surface area [m^2/g]	Active groups
ACE 5C_{18}-PFP	PFP	150 × 2.1	14.3	100	300	terminal pentafluorophenyl, octadecanol, residual silanols
ACE 5C_{18}-AR	AR		15.5	100	300	terminal phenyl, octadecanol, residual silanols
ACE 5C_{18}-HL	HL		20.0	90	400	octadecyl, residual silanols
ACE 5 Phenyl-300	P-300		9.5	300	300	phenyl, residual silanols

Table 3
Optimized gradient separation of antibiotics drugs and metabolites regarding the ChromSword software.

Stationary phase type	Analytes	Mobile phase compositions
ACE 5C_{18}-PFP	Antibiotic drugs	0–38.1 min, 0–0% ACN; 38.1–38.2 min, 0–3 % ACN; 38.2–40.5 min, 3–6 % ACN; 40.5–40.6 min, 6–20 % ACN; 40.5–45 min, 20–20% ACN
	Metabolites	0–2.2 min, 11–13 % ACN; 2.2–2.3 min, 13–14 % ACN; 2.3–30 min, 14–14% ACN
ACE 5C_{18}-AR	Antibiotic drugs	0–37 min, 0–0% ACN; 37.0–37.1 min, 0–7 % ACN; 37.1–38.3 min, 7–7% ACN; 38.3–38.4 min, 7–16 % ACN; 38.4–45 min, 16–16% ACN
	Metabolites	0–4.6 min, 1–7 % ACN; 4.6–9.6 min, 7–7% ACN; 9.6–9.7 min, 7–12 % ACN; 9.7–30 min, 12-12% ACN
ACE 5C_{18}-HL	Antibiotic drugs	0–4.3 min, 15–15 % ACN; 4.3–4.4 min, 15–18 % ACN; 4.4–5.4 min, 18–18% ACN; 5.4–5.5 min, 18–29 % ACN; 5.5–45 min, 29–29% ACN
	Metabolites	0–14.8 min, 1–4 % ACN; 14.8–17.8 min, 4–4% ACN; 17.8–17.9 min, 4–12 % ACN; 17.9–30 min, 12–12% ACN
ACE 5 Phenyl-300	Antibiotic drugs	0–31 min, 0–0% ACN; 31–45 min, 0–100 % ACN
	Metabolites	0–0.2 min, 0–0% ACN; 0.2–0.3 min, 0–35 % ACN; 0.3–30 min, 35–35% ACN

Table 4
Molecular descriptors of the tested antibiotic drugs (a) and their metabolites (b).

(a)

Antibiotic drugs	μ [D]	SAS [Å^2]	VWS [Å^2]	HE [kcal/mol]	HF [kcal/mol]	pKa_A	pKa_B	HBA	HBD	PSA [Å^2]	Log D (pH 7.4)	Log D (pH 5.5)	P_{ch} [D]	δ_{min} [e$^-$]	δ_{max} [e$^-$]	Log *P*
AMO	3.43	484.89	330.28	−17.12	−110.22	3.23	7.43	8	5	133	−2.72	−2.04	3.818	−0.3569	0.3497	−2.30
AMP	3.14	473.62	320.6	−11.18	−66.15	3.24	7.44	7	4	113	−2.38	−1.59	3.547	−0.1382	0.4242	−2.00
CEF	4.01	621.04	398.59	−13.27	−63.19	3.18	4.15	12	4	174	−3.35	−2.42	5.183	−0.1411	0.1964	−1.40
CIP	8.76	528.37	325.78	−9.93	−77.03	5.76	8.68	6	2	73	−2.23	−2.98	8.529	−0.2399	0.2787	−0.81
CLI	5.52	589.61	466.61	−10.44	−105.03	12.41	7.55	7	4	102	1.08	−0.57	4.087	−0.3483	0.2793	1.04
GEN	1.40	777.08	603.93	−12.07	109.59	12.55	10.18	12	11	200	−8.31	−9.39	1.28	−0.1668	0.2963	−3.10
LVX	8.57	547.21	347.82	−7.49	−134.46	5.45	6.2	7	1	73	−2.08	−1.84	8.242	−0.2691	0.2542	0.65
LIN	7.85	562.38	340.1	−5.27	−152.04	14.45	−0.66	7	1	71	0.82	0.37	7.703	−0.3136	0.3984	0.64
MET	3.76	336.24	189.43	−14.22	−7.99	15.44	3.09	6	1	84	0.05	0.05	3.731	−0.2190	0.1704	−0.46
MOXI	8.17	617.03	396.49	−8.84	−60.80	5.69	9.42	7	2	82	−1.73	−2.14	8.212	−0.2319	0.3229	−0.50
TET	6.59	595.09	402.9	−18.58	−255.46	−2.20	8.24	10	7	182	−3.17	−2.77	5.644	−0.2510	0.2521	−3.50

(b)

Metabolites	μ [D]	TE [kcal/mol]	VWS [Å^2]	HE [kcal/mol]	BE [kcal/mol]	HF [kcal/mol]	P [Å^3]	HBA	HBD	PSA [Å^2]	Log D (pH 7.4)	Log D (pH 5.5)	P_{ch} [D]	δ_{min} [e$^-$]	δ_{max} [e$^-$]	Log *P*
AMA	3.08	−112259.2	377.37	−22.55	−4805.44	−214.30	−117064.7	9	7	191	−3.12	−1.92	3.071	−0.3683	0.2782	2.10
AMD	0.76	−104444.1	353.41	−19.44	−4588.36	−160.99	−109032.5	8	5	153	−2.52	−2.14	1.037	−0.3677	0.3223	0.79
CEF-DAC LAC	3.97	−111052.0	379.44	2535.86	−3961.99	103.39	−115014.0	10	3	190	−0.10	−0.04	5.291	−0.8058	0.4561	−0.75
CLI-SOX	4.42	−133746.7	530.79	−131.58	−5712.60	−157.37	−139459.4	8	4	139	−0.63	−2.28	6.67	−0.9590	1.4826	0.03
CIP-PS	5.41	−125379.1	369.67	−16.63	−4821.23	−196.62	−130200.4	9	2	127	−5.04	−4.11	4.956	−0.9617	2.8269	−0.61
DEL	8.16	−106378.4	327.31	−10.32	−4578.46	−139.36	−110956.9	7	2	82	−2.75	−2.86	7.854	−0.3535	0.3299	0.15
LVX-OX	8.28	−117242.5	372.21	−17.81	−4961.80	−135.95	−122204.4	8	1	87	−2.69	−1.21	7.849	−0.3529	0.3724	−1.03
LIN-OX	8.55	−111670.2	369.43	−14.89	−4640.57	−156.51	−116310.9	8	1	85	−0.54	−0.34	7.42	−0.3849	0.3885	−2.97
MET-OH	2.98	−62244.0	192.82	−16.70	−2117.97	−46.48	−64361.5	7	2	104	−0.76	−0.76	3.781	−0.3488	0.3145	−0.81
8-HYD MOXI	7.83	−115788.0	376.32	−8.15	−5284.56	−124.39	−121072.6	7	3	93	−2.35	−2.02	8.261	−0.3509	0.3725	1.28

μ - moment dipole; *SAS* – solvent accessible surface area; *VWS* – Van der Waals surface area; *HE* – hydration energy; *HF* – heat of formation; pKa_A – *pKa* strongest acidic; pKa_B – pKa strongest basic; *HBA* – hydrogen bond acceptors; *HBD* – hydrogen bond donors; *PSA* – polar surface area; *Log D* – distribution coefficient at pH = 7.4 and 5.5, respectively; P_{ch} – point charge; δ_{min} – minimum charge; δ_{max} – maximum charge; *Log P* – logarithms of octanol/water partition coefficient ; *TE* – total energy; *BE* – binding energy; *P* – polarizability.

els. Table 4 shows the calculated molecular descriptors for each analyte.

The QSRR analysis included the use of multiple linear regression (MLR) performed using the Statistica 7.1 package (StatSoft, Tulsa, USA). Regression coefficients (± standard deviation), multiple correlation coefficients R^2, standard estimation errors *s*, and exact Fisher test values for statistical significance (*F* and *p* values) representing the significance level of equations were calculated.

3. Results and discussion

3.1. Separation of antibiotics drugs and their metabolites

The aim of the study was the chromatographic analysis of antibiotics and their metabolites by HPLC. For this purpose, 4 different stationary phases were used, which differed in their physicochemical properties and retention mechanism (Fig. 1). These stationary phases included terminal pentafluorophenyl, phenyl, and octadecyl groups (Table 2). As the mobile phase, acetonitrile and 0.1 % formic acid in water were used. The addition of the organic modifier improved the shape of the peaks. The studies used a gradient elution in which the content of acetonitrile increased. In addition, the ChromSword software was used in the study to optimize gradient elution conditions. A mixture of antibiotics and metabolites was analyzed separately because they were characterized by the same retention (the couple of antibiotic-metabolite). The composition of the mobile phase for each column (separately for antibiotics and metabolites) was adjusted individually to obtain comparable retention. Table 3 presents the composition of the mobile phase. The tested columns provides higher efficiency in RP conditions. Obtained peaks are symmetrical with a tailing factor in the range of 1.012–1.330, 0.937–1.320, 1.066–1.341, and 0.882–1.510, for PFP, AR, HL, and P-300 columns, respectively. Detailed parameters of the separations, such as selectivity (α), the theoretical plate number (*N*), and the tailing factor (F_{as}) for the tested stationary phases are presented in **S1**. The best separations of antibiotic drugs and their metabolites are presented in Fig. 2 for PFP column.

3.2. Retention mechanism

The purpose of this work was also to determine the interactions involved in the retention mechanism of the analysed compounds. Two theories that can be used to describe the retention mechanism are hydrophobic or solvophobic [18,19,21].

In the hydrophobic theory, the retention is the result of the formation of a complex substance consisting of a ligand locating on the surface of the stationary phase and molecules of the analysed substance. Whereas, according to the solvophobic theory, the retention depends on the action of the solvent and the interactions occurring in the mobile phase [18].

Moreover, the retention mechanism can also be described by specific models. The first one defines the phenomenon of dividing the analyte between the mobile and stationary phase. The second model refers to the absorption of the analysed substance on the surface of the stationary phase [18]. Both models visualized the retention of the analysed substance as a result of various interactions between the analyte, stationary and mobile phase molecules. Understanding the types of these interactions makes it easier to predict the behaviour of the analysed substances during the chromatographic process and to select appropriate conditions. Depending on the type of chemical bonded phases, the stationary phase may be characterized by one or more types of interactions. Basic intermolecular interactions include: (i) chemical interactions (hydrogen bonds, donor-acceptor interactions); (ii) physical interactions (dipole ion, dipole-dipole, induction dipole, instantaneous dipole-induced dipole); (iii) van der Waals forces and (iiii) solophobic interactions (hydrophilic, hydrophobic).

In the case of the PFP stationary phase, hydrophobic interactions, strong dipole-dipole interactions along with hydrogen and π-π bonds are responsible for the retention of analytes. The AR and Phenyl stationary phases exhibit both strong π-π and as well as dipol-dipol interactions. However, hydrophobic interactions play a dominant role in phenyl-bonded stationary phases. The C18 HL phase retention mechanism is based on hydrophobic interactions between the analysed molecule and alkyl chains of bonded stationary phase (Table 2).

3.3. QSRR analysis

The aim of the study was to determine the mechanism of the retention of antibiotics and their metabolites. The QSRR analysis was used in the research of the retention properties of various types of stationary phases such as: octadecyl bonded with integral pentafluorophenyl groups, octadecyl bonded with integral phenyl groups, phenyl and octadecyl bonded silica. On the basis of the QSRR equations obtained, the stationary phases were classified in terms of their type and the strength of the dominant interactions between the analyzed analytes. The obtained QSRR dependencies made it possible to characterize the stationary phases and to interpret the observed retention differences in the tested columns. The retention of antibiotics/metabolites constituted dependent variables, while the independent variables were descriptors. In Table 4 are listed all descriptors used in the QSRR analysis.

For the tested columns two- and three-parameter QSRR equations were determined with respect to log k. The three-parameter QSRR equation (three-dimensional quantitative structure – retention relationship) is also a useful tool to describe retention mechanisms. The three-parameter model assumes that between analytes, stationary and mobile phases there are non-covalent interactions [26]. For the tested columns, at least 3 (two parameters) and 2 (three parameters) statistically significant models based on descriptors for antibiotics were observed. In contrast, for metabolites, 1 (two parameters) and 2 (three parameters) statistically significant models were observed. In the case of the antibiotic drugs retention on the PFP column, the largest number of statistically significant QSRR equations was observed (3 two parameters and 4 three parameters). In the case of the metabolites retention on the AR and HL columns, no statistically significant two- and three-parameter QSRR equations were observed. Table 5 summarizes the two- and three-parameter QSRR equations with the highest determination coefficient for each mixture (antibiotics/metabolites) and for each column, with the significance level of 0.05. The R_{QSRR} coefficient it is in the range from 0.5624 to 0.9340 (Table 5). The QSRR equations based on descriptors derived for the HL column compared to the other columns, showed low correlation coefficients R^2 (R^2 = 0.5624 for two parameters and 0.7698 for three parameters). Low R^2 values, and thus poor predictive properties of models, can be overcome by using more descriptors in the QSRR methodology.

In the case of stationary PFP and HL phases, the largest excess of electrons on a single atom in antibiotic molecules (δ_{min}) has the greatest impact on their retention mechanism. For these phases, the δ_{min} descriptor exhibits the highest retention, because it has the highest positive value (for the PFP column for both the two- and three-parameter QSRR equation). The δ_{min} parameter describes the largest negative charge in the molecule and determines the local polarity of the analyte and the ability to participate in polar interactions [27]. This parameter probably means the participation of electrostatic interactions between antibiotics and stationary phases. Linear alkyl chains in the PFP and HL phases, they exhibit hydrophobic effects, however, ionized residual silanols can participate in electrostatic interactions. In the case of the P-300 phase,

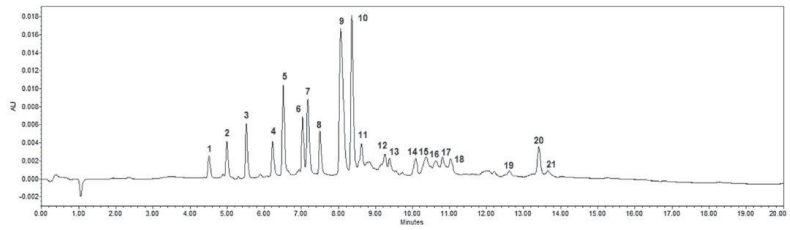


Fig. 2. HPLC-DAD chromatogram obtain with the use of ACE 5 C18-PFP column: (1) CLI-Sox, (2) GEN, (3) CLI, (4) AMA, (5) AMD, (6) MET-OH, (7) AMOX, (8) MET, (9) AMP, (10) CEF, (11) CEF-DAC-LAC, (12) DEL, (13) LVX-Ox, (14) LVX, (15) CIP-PS, (16) CIP, (17) TET, (18) 8-HYD-MOXI, (19) MOXI, (20) LIN-Ox, (21) LIN.

Table 5
Regression coefficients (k_1, k_2, k_3, k_4) with standard deviations, the square of the multiple correlation coefficient (R^2), the value of the Fisher test (F), the standard error of the estimate (s), and the level of significance of equations $p < 0.05$ of Soczewiński-Snyder relation.

Equation of QSRR	k_1	k_2	k_3	k_4	R^2_{QSRR}	F	s
PFP - metabolites							
log k = k_1 + k_2 VWS + k_3 TE	0.7476 (±0.2661)	−0.0145 (±0.0040)	0.0192 (±0.0049)		0.7106	8	0.0130
–	–	–	–	–	–	–	–
P-300 - metabolites							
log k = k_1 + k_2 P + k_3 PSA	−3.3547 (±0.1315)	0.0105 (±0.0038)	0.0033 (±0.0006)		0.8762	24	0.0007
log k = k_1 + k_2 BE + k_3 HF + k_4 PSA	−0.3517 (±0.1111)	−0.00009 (±0.00002)	0.0008 (±0.0003)	0.0034 (±0.0005)	0.9340	28	0.0006
PFP – antibiotic drugs							
log k = k_1 + k_2 μ + k_3 δ_{min}	1.1509 (±0.1686)	0.1213 (±0.0203)	2.3461 (±0.6847)		0.8239	18	0.0009
log k = k_1 + k_2 μ + k_3 SAS + k_4 δ_{min}	0.5674 (±0.2278)	0.1213 (±0.0143)	0.0009 (±0.0003)	2.1127 (±0.4899)	0.9231	28	0.0003
AR - antibiotic drugs							
log k = k_1 + k_2 μ + k_3 SAS	0.0529 (±0.3291)	0.0953 (±0.0224)	0.0013 (±0.0005)		0.7426	11	0.0043
log k = k_1 + k_2 μ + k_3 HE + k_4 PSA	0.6312 (±0.3242)	0.1147 (±0.0274)	0.0442 (±0.01776)	0.0048 (±0.0017)	0.8091	10	0.0065
HL - antibiotic drugs							
log k = k_1 + k_2 P_{ch} + k_3 δ_{min}	0.7911 (±0.2449)	0.0709 (±0.0284)	2.3229 (±0.9064)	–	0.5624	5	0.0367
log k = k_1 + k_2 HE + k_3 HBD + k_4 Log P	1.4946 (±0.2175)	0.0796 (±0.0199)	−0.0786 (±0.0272)	−0.3255 (±0.0700)	0.7698	7	0.0123
P-300 - antibiotic drugs							
log k = k_1 + k_2 VWS + k_3 δ_{min}	0.7281 (±0.1747)	−0.00002 (±0.000005)	−1.8300 (±0.6722)	–	0.6419	7	0.0164
log k = k_1 + k_2 pKa_B + k_3 δ_{min} + k_4 Log D (7.4)	0.5705 (±0.1784)	−0.0379 (±0.0152)	−2.7306 (±0.6163)	0.1066 (±0.0222)	0.7982	9	0.0079

the δ_{min} descriptor also influences the retention of the chromatographic system, but shows a high negative value (both for a two- and three-parameter equation) (Table 5). Probably the negative value of the minimum charge describes stronger intermolecular interactions between antibiotics and mobile phase molecules than antibiotics and stationary phase ligands. Electrostatic interactions (described by the δ_{min} descriptor) also play a significant role in the retention mechanism of antibiotics on stationary phases of AR and P-300.

The next descriptor that describes the retention mechanism is the dipole moment (μ). The dipole moment (μ) is a measure of the polarity of the antibiotic molecule and describes the dipole-dipole interactions between the analyte (antibiotic) and the induced mobile phase and the stationary phase [27]. This parameter was included in the QSRR equations for the PFP and AR columns (for both the two- and three- parameter QSRR equations). The participation of μ in retention for the PFP column was quite low, while for the AR column its value was significantly high. Polar functional groups in pentafluorophenyl and phenyl rings are responsible for dipole interactions, dipole-dipole and hydrogen bonds, between antibiotics and stationary ligands. At the same time antibiotic retention increases with the increasing dipole moment. In the case of the PFP column, the dipole moment occurs together with δ_{min}, which indicates the strength and orientation behavior of antibiotics in the electrostatic field (Table 5).

The point charge (P_{ch}) descriptor has little effect on the retention of antibiotics compared to the minimum charge (δ_{min}) descriptor with which it occurs in the QSRR equation (for the octadecyl phase) (Table 5). This parameter probably indicates the presence of electrostatic interactions and the presence of additional interactions, which are responsible for the retention mechanism of antibiotics on alkyl chains and the lowered amount of residual silanol groups.

Solvent accessible surface areas (*SAS*) determines the surface available in contact with the solvent, which is characterized by dispersion forces (London type) of the analyte with stationary phase particles. In the physical sense, the *SAS* parameter increases with the increasing retention. *SAS* describes the ability of analyte molecules to participate in non-specific intermolecular interactions. This parameter was determined for QSRR equations for columns with aryl ligands (Table 5). Despite the small contribution to the retention mechanism, the interactions are stronger between antibiotics and ligands of PFP and AR phase than between antibiotics and mobile phase molecules (acetonitrile). It can be concluded that with the increase in the dispersion force, the retention of the chromatographic system grows. The dispersive forces between analytes and ligands of the stationary phase are stronger than between analytes and small molecules of the mobile phase.

Another descriptor appearing in the QSRR equations is van der Waals surface areas (*VWS*). *VWS* is a physicochemical parameter, which determines the transport properties of the molecule, binding and solubility, and refers to the hydrophobic interaction between the analyte and the stationary phase. For the PFP phase (metabolite retention) and for the P-300 phase (antibiotic retention), the *VWS* descriptor is included in the two-parameter equation. Its contribution is negative in the retention of the examined systems, which indicates that non-specific intermolecular interactions are stronger between antibiotics/metabolites and mobile phase molecules than between mobile phase.

The total energy (*TE*) descriptor was determined for the PFP column. The total energy (*TE*) determines the sum of the energy of electrons and the energy of the intranuclear repulsion of molecules.

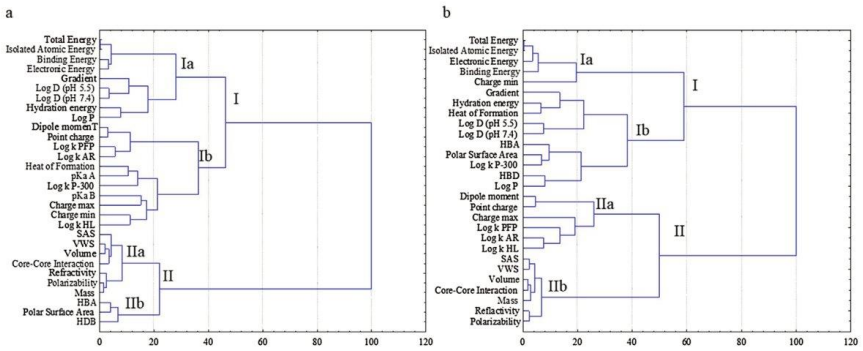


Fig. 3. Results of cluster analysis for: (a) antibiotic drugs and (b) metabolites.

Hydration energy (*HE*) was observed for columns with octadecyl chains. Hydration energy is associated with the formation of relatively strong coordination bonds between water molecules and ions. It is the energy released while antibiotics undergo hydration in the mobile phase solution. *HE* reflects hydrophobic interactions between ligands of the stationary phases and antibiotics.

The heat of formation (*HF*) is a physicochemical descriptor that reflects the differences in the mass of analytes. This descriptor describes the geometry of the molecular structure [28]. The descriptor was observed only for the P-300 column, and shows a positive effect on the retention of the metabolite mixture.

A *log D* presence was observed for the P-300 column. The effective partition coefficient for dissociative systems (*log D*) descriptor determines the polarity of the analytes tested, and its low value indicates high hydrophilicity. In addition, it can be observed that the *log D* descriptor is present along with the δ_{min} descriptor, which determines the electrostatic interactions. The occurrence of these two descriptors together in one QSRR equation may suggest that there are hydrophilic and ionic interactions in the chromatographic system studied. Moreover, *log D* also depends on the pH of the solution and thus on the dissociation constant *pKa*, which is also a descriptor occurring in the three-parameter QSRR model (Table 5). *pKa* determines the acid's ability to dissociate hydrogen ions. The *pKa* descriptor along with the δ_{min} descriptor shows a negative contribution to retention, which indicates stronger intermolecular interactions between antibiotics and mobile phase molecules than antibiotics and stationary phase ligands. The *pKa* value decreases when the negative charges in the molecule rises [29].

In the three-parameter QSRR model for the HL column, *log P* and *HBD* descriptors show a negative value. *Log P* is a physicochemical quantity that determines the hydrophobicity (lipophilicity) of an analyte. The *log P* descriptor determines the measure of the lipophilic nature of the molecule. With the increase in the *log P* value, the hydrophobic interaction between dissolved analytes and the stationary phase increases. The number of hydrogen bond donors (*HBD*) also affects the retention of antibiotics (to a very small extent). *HBD* and *HBA* are responsible for the formation of hydrogen bonds, which affect the mechanism of retention. However, the negative value of the *HBD* descriptor means that the hydrogen bonds do not significantly affect the retention of antibiotics, because the analyte molecules interact more strongly with the mobile phase molecules. Also, the high negative value of the *log P* descriptor is the same. The *HE* descriptor has the greatest influence on retention for the HL column, suggesting that hydrophobic interactions are dominant.

The binding energy (*BE*) was described for a three-parameter equation (Table 5). A negative contribution to the retention mechanism (as above) also indicates stronger interactions between the analyte and the mobile phase molecules. *BE* is the energy needed to separate the system into its components and distance them from each other like this, to stop interacting with each other.

The polar surface area (*PSA*) descriptor was described by the QSRR equation (two- and three-parameter) for the P-300 and AR columns. The *PSA* parameter shows a positive contribution to the retention mechanism of the analyzed analytes. The *PSA* descriptor determines surface diffusion, absorption, contact surface, provides information on th particle size and the polar surface area of molecules. In addition, *PSA* affects the formation of hydrogen bonds.

The *P* descriptor is responsible for the ability to create instant dipoles. Polarizability (*P*) is also defined as the dipole moment that is responsible for the dipole-dipole and π-π interaction between the analyte molecules and the stationary ligands. Interactions occur between aryl groups in metabolite molecules and ligands of P-300 stationary phase.

The presented QSRR equations in Table 5 show that the mechanism of the retention of antibiotics and their metabolites on stationary phases containing aryl groups, alkyl chains, free silanol residual is a complex phenomenon. Hydrophobic and electrostatic interactions in the alkyl phase influence the separation process. The retention mechanism of antibiotics/metabolites on columns containing aryl groups is based on hydrophobic/hydrophilic interactions, dipole-dipole interactions, π-π interactions.

3.4. Comparative analysis of the tested compounds

Cluster analysis (CA) was used to determine the similarities and differences between molecular descriptors and the chromatographic systems under study. Fig. 3 shows CA analysis for antibiotics (Fig. 3a) and metabolites (Fig. 3b). In both diagrams, two main classes (I, II) and their subclasses (Ia, Ib, IIa, IIb) can be distinguished. In the first cluster, in the subclass Ib there are chromatographic parameters, such as *pKa*, maximum and minimum charge, octanol/water partition coefficients, dipole moment (Fig. 3a). The sub-group IIb contains the number of hydrogen donors, acceptors and the polar surface area. Descriptors related to bulkiness and responsible for interactions between the analyte and the stationary phase are found in subclass IIa for antibiotics (Fig. 3a). However, electrical and mass descriptors are in subclass Ia (Fig. 3a). In the case of metabolites, the chromatographic parameters are found in cluster II, in subclass IIa (Fig. 3b). Descriptors

describing the polarity of the analyte are found in cluster I, in subclass Ib (Fig. 3b). Molecular bulkiness-related descriptors create subcategory Ia and II b (Fig. 3b). The CA analysis allows the grouping of molecular descriptors and shows the linking of individual descriptors with each other in a simple way.

4. Conclusion

The use of the QSRR analysis allowed to examine the antibiotics and their metabolites retention mechanism in commercial stationary phases. The obtained two- and three-parameter QSRR equations allowed to describe the retention of the analyzed substances in a gradient elution. The descriptors contained in the best statistically significant QSRR equations indicate the presence of dominant electrostatic, hydrophilic interactions, dipole interactions, dipole-dipole, and π-π interactions. Weak hydrophobic interactions were observed for the P-300 column, in which there are stronger interactions between analyte molecules and mobile phase particles. The order of the elution of the analyzed compounds on four stationary phases was similar. Some similarities were also observed for the columns. Stationary phases containing phenyl groups (AR and PFP) showed similarity associated with the occurrence of dipole moment descriptors in the QSRR equations. This means that the formation of dipole-dipole, and π-π interactions, between analytes and stationary phase ligands is a characteristic feature of this type of filling.

It can be concluded that the obtained mathematical models can be used to predict the retention mechanism of antibiotics and their metabolites. Moreover, the use of QSRR models also allows great potential for application in the design of new active antibacterial drugs with a specific pharmacological activity, without the need for experimentation *in vitro* in cell culture as well as *in vivo* on animals.

CRediT authorship contribution statement

Justyna Walczak-Skierska: Writing - review & editing. **Małgorzata Szultka-Młyńska:** Conceptualization, Methodology, Software, Writing - original draft. **Katarzyna Pauter:** Visualization, Investigation. **Bogusław Buszewski:** Writing - review & editing.

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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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113187.

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3.3. *CE-DAD-MS/MS in the simultaneous determination and identification of selected antibiotic drugs and their metabolites in human urine samples*

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Katarzyna Pauter[1,2]
Małgorzata Szultka-Młyńska[1]
Michał Szumski[2]
Anna Król-Górniak[1,2]
Paweł Pomastowski[2]
Bogusław Buszewski[1,2]

[1]Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Torun, Poland
[2]Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Torun, Poland

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Research Article

CE-DAD-MS/MS in the simultaneous determination and identification of selected antibiotic drugs and their metabolites in human urine samples

In this study, a new analytical method was developed and validated for the simultaneous analysis of antibiotic drugs (amoxicillin, cefotaxime, ciprofloxacin, clindamycin, linezolid, metronidazole) and their metabolites (amoxycilloic acid, amoxicillin diketopiperazine, 3-desacetyl cefotaxime lactone, clindamycin sulfoxide, ciprofloxacin piperazinyl-*N*4-sulfate, linezolid *N*-oxide, metronidazole-OH) in human urine. Capillary electrophoresis (CE) along with the tandem mass spectrometry (MS/MS) was used to determine and identify all analytes. Appropriate conditions for MS/MS measurements along with the use of the central composite design were optimized. The effects of different analytical conditions (the composition, the concentration, and the pH value of the background electrolyte, the time and pressure of the injection, the capillary temperature and influence of the organic modifier) on the migration and separation of antibiotic drugs and metabolites were examined using the CE-DAD. The analytical procedure was linear for concentrations ranging from 20 to 1000 ng/mL, with determination coefficients higher than 0.99 for all the analytes. The validated analytical procedure was then applied to the measurement of antibiotic drugs and their metabolites in human urine samples.

Keywords:
Antibiotic drugs / Capillary electrophoresis / Mass spectrometry / Metabolites / Urine samples
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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

One of the most important tools in the optimization of the treatment of individual patients is the application of an individual approach during the pharmacological treatment, that is, the monitored therapy [1]. The aim of this therapy is to accurately assess the clinical condition and provide the measurement of the drug and metabolite concentration in biological fluids. Controlling the concentration of drugs and their metabolites in the real-time treatment is one of the most effective methods of personalizing the therapy. Moreover, it takes into account the individual characteristics of the patient and the potential interaction of administered pharmaceuticals with other endogenous compounds. The possibility of monitoring early disease changes by searching for potential biomarkers along with the determination of drugs and their metabolites in the monitored therapy requires the development of new methods and their application in the study of biological samples. For the purposes of biomedical analytics, the most commonly used are chromatographic techniques coupled with spectroscopic or spectrometric methods and immunochemical methods. Among the separation techniques, it is LC and GC that are most commonly used in combination with various detection systems (spectrophotometric, fluorescence, electrochemical, and MS) [1–3]. An important advantage of chromatographic methods is the possibility to determine simultaneously various drugs and their potential metabolites in the biological material taken from a patient [4,5]. On the other hand, in the case of immunochemical

Correspondence: Professor Bogusław Buszewski, Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarin 7, 87–100 Torun, Poland.
E-mail: bbusz@umk.pl

Abbreviations: **AMA**, amoxycilloic acid; **AMD**, amoxicillin diketopiperazine; **AMOX**, amoxicillin; **CCD**, central composite design; **CEF**, cefotaxime; **CEF-DAC-LAC**, 3-desacetyl cefotaxime lactone; **CIP**, ciprofloxacin; **CIP-PS**, ciprofloxacin piperazinyl-*N*4-sulfate; **CLI**, clindamycin; **CLI-SOx**, clindamycin sulfoxide; **LIN**, linezolid; **LIN-Ox**, linezolid *N*-oxide; **LLOQ**, lower LOQ; **ME**, matrix effect; **MET**, metronidazole; **MET-OH**, metronidazole-OH; **MRM**, multiple reactions monitoring; **RE**, relative error

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methods, drug metabolites may deteriorate the analytical specificity during the performance of assay, which may require antibodies for their determination. Hence, in recent years the trend to apply LC, especially coupled with MS (LC-MS or LC-MS/MS), has been present in the scientific reports [6,7]. Most of the procedures using these techniques, described so far in the literature, allow us to determine single antibiotics or those belonging to the same group, and rarely, antibacterial agents and their metabolites [8–10]. Only a few reports contain information regarding chromatographic conditions for the separation of antibiotics from various therapeutic groups and their metabolites [11–13].

In recent years, electromigration techniques have also gained significance as a highly efficient analytical tool. Their advantage over commonly used chromatographic techniques is their high-separation speed and efficiency, high resolution, low reagent consumption, and low costs of single analyses [14]. In addition, due to the low consumption of reagents, especially organic, and the low production of waste generated during the separation process, this technique is widely recognized as highly environmental friendly ("green chemistry") [15]. This fact is a very important argument for the development of electromigration techniques in many analytical areas, especially in biomedicine and pharmacy.

The level of antibacterial substances has been hitherto determined primarily in plasma and urine. Therefore, the determination of selected antibiotics in various samples by CE versus LC is summarized in Table 1 [16–23]. What is essential is that the separation buffers prescribed for use in CE/MS are volatile acid-base types including formic acid, ammonium formate, acetic acid as well as ammonium acetate [24]. Therefore, in our work, volatile salts, such as ammonium acetate and ammonium formate, were used for buffer solutions, which is compatible with MS.

This study was focused on the simultaneous determination and identification of selected antibiotics and their pharmacology active metabolites in human urine samples. Hence, the main aim of the present study was to develop a rapid, simple, and cheap validated analytical procedure with the use of CE-DAD-ESI-MS/MS approach. However, the CE-DAD alone was used with regards to optimize the important parameters influencing the analytical conditions in case of the migration and separation selectivity of target compounds to understand better the processes taking place during an electrophoretic run. During the method development, the effects of different analytical conditions (the composition, the concentration, and the pH value of the BGE, the time, and pressure of the injection, capillary temperature, and influence of the organic modifier) were examined. While there are several methods for one or several of the analytes described here, to our knowledge no method offers the quantification and qualification of the six antibiotics presented here. The combination of a simple sample preparation procedure with a highly selective CE-MS unit was effective for analyzed drugs from different therapeutic groups. To our knowledge, this report is the first such a comprehensive study conducted for relevant antibiotic drugs and their metabolites by CE-MS from biological samples.

2 Materials and methods

2.1 Reagents and materials

All reagents were of analytical grade and solvents were of chromatographic purity. Thirteen target compounds, namely amoxicillin (AMOX) (HPLC grade, ≥98% purity), cefotaxime (CEF) (HPLC grade, ≥98% purity), ciprofloxacin (CIP) (HPLC grade, ≥98% purity), clindamycin (CLI) (HPLC grade, ≥98% purity), linezolid (LIN) (HPLC grade, ≥98% purity), metronidazole (MET) (HPLC grade, ≥98% purity), and their metabolites: amoxycilloic acid (AMA) (HPLC grade, ≥98% purity), amoxicillin diketopiperazine (AMD) (HPLC grade, ≥98% purity), 3-desacetyl cefotaxime lactone (CEF-DAC-LAC) (HPLC grade, ≥98% purity), CLI sulfoxide (CLI-SOx) (HPLC grade, ≥98% purity), CIP piperazinyl-*N*4-sulfate (CIP-PS) (HPLC grade, ≥98% purity), linezolid *N*-oxide (LIN-Ox) (HPLC grade, ≥98% purity), metronidazole-OH (MET-OH) (HPLC grade, ≥98% purity) were supplied by Sigma-Aldrich (Steinheim, Germany). Stock standard solutions were prepared by dissolving the appropriate amount of each drug or metabolite in ultrapure water to a final concentration of 1 mg/mL. Acetonitrile, ammonium acetate, ammonium formate, ammonium hydroxide solution, and formic acid were purchased from Sigma–Aldrich. The ultrapure water was produced in our laboratory using Milli-Q RG apparatus (Millipore Intertech, Bedford, MA, USA). The evaporation of samples was performed on a ScanVac speed vacuum concentrator (Kansas City, USA). Drug-free urine samples were kindly provided by Collegium Medicum, Nicolaus Copernicus University in Toruń (Poland) upon the permission of the Bioethical Commission (no. 322/2017).

2.2 Instrumentation

2.2.1 Capillary electrophoresis

Electrophoretic separations were performed using an Agilent G1600AX CE system (Agilent Technologies, Waldbronn, Germany) equipped with a DAD. ChemStation software was used for the equipment control, data collection, and processing.

The calibration curve was prepared by the quantified standard solutions of drugs, while the components were identified on the basis of their migration times and UV spectra. Prior to each analytical run, the capillary was rinsed with the 0.1 M solution of sodium hydroxide followed by ultrapure water (1 min each), then flushed with a separation electrolyte for 3 min. Fresh portions of the separation buffer were used for each run which was performed automatically before each analysis using a built-in electrolyte replenishment system. Moreover, prior to the analyses, the capillary was flushed with 1 M NaOH for 10 min followed by the ultrapure water (10 min) and BGE (30 min).

The influence of several electrophoretic separation parameters, such as the composition of the BGE (formic acid, ammonium acetate, ammonium formate), its concentration

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Table 1. Determination and identification of selected antibiotics in pharmaceutical formulation and biological samples by CE and LC with different detection systems

Antibiotics	Matrix	Analytical parameters		LOD/LOQ		Refs.
		Capillary electrophoresis	Liquid chromatography	CE	LC	
Amoxicillin ampicillin	Pharmaceutical formulation	CE-UV-VIS Fused-silica capillary (60 cm, 75 μm) phosphate-borate buffer (pH = 8.66, 1.44% SDS) 25°C, 18 kV, 214 nm	HPLC-UV-VIS HPLC column C18 (300 mm, 3.9 mm i.d., 10μm) sodium phosphate (100 mM, pH = 4.0)/methanol (amoxicillin) tetrabutylammonium hydroxide (pH = 5.0, 5 mM)/acetonitrile (ampicillin) 230 nm	0.4/0.8 μg/mL	-/-	[16]
Cefepime	urine	MEKC-UV-VIS Fused-silica capillary (60 cm, 50 μm) phosphate-borate buffer (pH = 9.1, 75 mM SDS) 25°C, 15 kV, 6 s at 68.95 mbar, 254 nm	LC-MS LC column C18 (125 mm, 4.0 mm i.d., 5.0 μm) Ammonium formate (5 mM, pH = 3.0)/acetonitrile 25°C, 10 μL, *m/z* 481.1 0.5 mL/min, gradient elution	5/2 μg/mL	0.5/0.2 μg/mL	[17]
Florfenicol	Swine plasma	CE-UV-VIS Fused-silica capillary (57 cm, 75 μm) phosphate-borate buffer (pH = 8.66, 1.44% SDS) 25°C, 25 kV, 7 s at 34.5 mbar, 200 nm	HPLC-UV-VIS HPLC column C18 (150 mm, 4.0 mm i.d., 5μm) acetonitrile/water (pH = 2.7) 25°C, 20 μL, 1.5 mL/min, 224 nm isocratic elution	0.015/0.05 μg/mL	0.03/0.1 μg/mL	[18]
Cefepime	Human plasma Human serum	MEKC-UV-VIS Fused-silica capillary (60 cm, 50 μm) phosphate-borate buffer (pH = 9.1, 75 mM SDS) 25°C, 15 kV, 6 s at 68.95 mbar, 254 nm	LC-MS HPLC column C18 (125 mm, 4.0 mm i.d., 5 μm ammonium formate (5 mM, pH = 3.0)/acetonitrile 25°C, 10 μL, *m/z* 481.1 0.5 mL/min, gradient elution	1/0.5 μg/mL	0.25/0.1 μg/mL	[19]
Ciprofloxacin	Pharmaceutical formulation	CZE-DAD Fused-silica capillary (48.5 cm, 50 μm) TRIS/hydrochloride/sodium tetraborate buffer (pH = 8.87, 15 mM) 25°C, 25 kV,5 s at 30 mbar 285 nm	HPLC- UV-VIS HPLC column C18 (150 mm, 4.6 mm i.d., 3.5 μm) phosphoric acid (25 mM, pH = 3.0)/acetonitrile 25°C, 20 μL, 278 nm 1.0 mL/min, isocratic elution	2.72/9.06 mg/L	0.11/0.35 mg/mL	[20], [21]
Gentamicin	Pharmaceutical formulation	CE-UV-VIS Fused-silica capillary (40.2 cm,75 μm) 0.35 mM cetyltrimethylammonium bromide, 90 mM sodium pyrophosphate (pH 7.4, 3% methanol) 30°C, −6.0kV, 10 s at 55 mbar, 195 nm	HPLC-DAD HPLC column C18 (150 mm, 2.1 mm i.d., 5 μm) ammonium acetate (20 mM, pH = 9.0)/methanol 30°C, 20 μL, 280 nm, 0.3 mL/min	2.09/0.67 mg/mL	0.37/1.2 μg/mL	[22],[23]

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(20–40 mM), as well as the pH (8–10), injection pressure (20–30 mbar), time (3–20 s), capillary temperature (23–35°C), and the influence of the organic modifier (methanol or acetonitrile, 5–15%), were investigated. Electrophoretic buffer solutions were prepared by dissolving the appropriate amount of ammonium formate/ammonium acetate in the ultrapure water and the pH was adjusted with the ammonium hydroxide solution. After the sonication, the buffer solutions were filtered. BGE solutions were prepared fresh daily. The pH of the buffer solutions was determined by the CX-551 pH meter (Elmetron, Zabrze, Poland). The water solution of mesityl oxide (from Sigma–Aldrich) was used as an EOF marker.

The optimized electrophoretic conditions also included the use of an extended light path (bubble cell, $l = 150$ μm) fused-silica capillary with an internal diameter of 50 μm and a total length of $L_{tot} = 64.5$ cm (effective length $L_{eff} = 56$ cm) supplied by Agilent Technologies. The detection of the analytes was performed at the following wavelengths: amoxicillin and CLI ($\lambda = 220$ nm), LIN/CEF ($\lambda = 254$ nm), CIP ($\lambda = 280$ nm), and MET ($\lambda = 320$ nm).

2.2.2 Mass spectrometry

To identify and determine the target compounds, a system consisting of a CE apparatus along with a triple quadrupole MS was used. The coupling of CE to MS was carried out through an orthogonal ESI using a G1607 Agilent CE-MS sprayer kit. The sheath liquid was 50/50 v/v methanol/water with 1% of formic acid delivered at 10 mL/min by a pump equipped with a 1:100 splitter. ESI-MS was conducted in the positive ion mode, and the capillary voltage was set at –4.0 kV. Dry nitrogen was heated to 150°C and delivered at a flow rate of 10 L/min. The pressure of nebulizing nitrogen was set at 10 psi. The lenses and block voltages were fixed using the smart option of the tune page considering compound stability as 80%. The CE and MS instruments were connected and synchronized by an external pulse signal programmed from the CE system. The mobile phase was composed of ACN and 0.1% HCOOH (30:70, v/v). Adding 0.1% formic acid to the acetonitrile/water mobile phase could improve peak shapes and increase the MS detection sensitivity. The system equipped with the ESI source operating in a positive ion mode (ESI+) was controlled by the Mass Hunter workstation data acquisition software and the Qualitative Analysis software (version B.01.00).

The instrument was operated in the extracted ion chromatogram and the product ion modes, respectively. Moreover, selected antibiotic drugs and their metabolites were detected in the multiple reactions monitoring (MRM) mode. Ionization conditions were optimized by directly injecting different solutions and bypassing the column (FIA). The mass transitions for the ions monitoring performed in the MRM mode are presented in Supporting information Table S1. Each target compound was monitored at two different transitions: the first was used to quantify (quantifier ions, *Q1*) and the second to confirm (qualifier ion, *Q3*).

Full-scan mass spectra within the mass range of m/z 100–500, and product ion spectra for mass range m/z 50–450 were recorded. The QqQ MS parameters were optimized using Central Composite Design (CCD) (Design of Experiments). The source temperature was maintained at 290–350°C, fragmentor voltage at 70–150 V, and the ionization voltage was set at 3500–4500 V. The nebulizer gas was set at 35–45 psi.

2.3 Sample preparation

Human urine samples were thawed at room temperature. The samples were diluted with deionized water in different dilution factors, filtered through a nylon filter (0.45 μm, 13 mm) and directly injected into the CE ESI-MS/MS system. A total of 300 μL of the human urine samples were pipetted to an Eppendorf tube and 600 μL ACN was added. The tubes were vortexed for 60 s. Following the centrifugation of the tube at 15 000 *g* for 15 min, 600 μL of deionized water was added to 300 μL of the obtained supernatant. The obtained mixture was vortexed for 30 s, the supernatant was filtered through a 0.22 μm microporous membrane to the clean Eppendorf vial. This study was approved by the Bioethical Committee permission (Poland, no. 322/2017).

2.4 Electrophoretic mobility calculation

The electrophoretic mobility of each analyte was determined on the basis of the monitored migration times using the Eq. (1) [25].

$$\mu = \frac{L_{eff} L_{tot}}{V} \left(\frac{1}{t_m} - \frac{1}{t_{EOF}} \right), \quad (1)$$

where μ is the electrophoretic mobility of the active compounds, L_{tot} is the total length of the capillary, L_{eff} is the effective length of the capillary, V is the applied potential, t_m is the measured migration time of the analyte, t_{EOF} is the migration time of an uncharged solute (mesityl oxide). The migration time of each analyte was reported individually.

2.5 Method validation

To confirm the suitability of the proposed method in the determination of antibiotics and their metabolites, linearity, LOD, and LOQ were calculated. The stock solutions were prepared at a concentration of 1 mg/mL to prepare calibration standards and quality controls. The stock solutions were prepared by weighing an appropriate amount of each compound and dissolving it in drug-free human urine. The 1 mg/mL working solution was prepared as a composite of all 14 compounds (drugs and metabolites). Further working calibration solutions were prepared by diluting the compound in drug-free urine at concentrations of 20–1000 ng/mL with the use

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of CE-ESI-MS/MS. The solutions were stored in the dark in brown tubes for up to 3 months at −80°C as 50 μL aliquots.

Linear regression equations were determined at six concentration levels and three consecutive repeats for each concentration. A regression analysis of the obtained data was performed and then coefficients of determination (R^2) were calculated for each compounds. The best weighting factor was determined based on the lowest value of the Σ|%RE| (relative error) and acceptable R^2 (≥0.99). The regression parameters slope and *Y*-intercept were tested using the independent samples Student's *t*-test for 95% confidence.

The lower LOD was defined as the concentration at which the *S/N* occurred more than three times in response (the peak area). Similarly, the lower LOQ (LLOQ) was defined as the concentration at which the *S/N* was not less than five times in response (the peak area) [26]. Both parameters were calculated using the calibration curve containing the smallest concentration of the analyte.

The intra- and interday accuracy and precision were assessed on the basis of quality control at LLOQ and QC samples; at low (LQC), medium (MQC), and high (HQC) concentration level they were prepared by adding working solutions of the specified concentration to the extracts of blank samples. Analyses were performed in six replications. Accuracy was determined as the RE using the Eq. (2). Precision was expressed as the %CV, which corresponds to the RSD.

$$\mathrm{RE}\,[\%] = \left(\frac{\text{measure value} - \text{theoretical value}}{\text{theoretical value}}\right) \cdot 100\%. \quad (2)$$

The selectivity of the developed method was evaluated on the basis of chromatograms obtained for extracts of samples containing analytes and those without analytes. The matrix effect (ME) was calculated according to the Eq. (3), where A_{extract} means the analyte area in the sample after extraction and A_{standard} means the analyte area in the standard solution. Sample solutions and standard solutions were prepared at three concentration levels and proceeded as above.

$$\mathrm{ME}\,[\%] = \left(1 - \frac{\mathrm{A_{extract}}}{\mathrm{A_{standard}}}\right) \cdot 100\%. \quad (3)$$

QC sample stability analyzes were also performed for each of the analytes at 2–8°C for the short (7 days) and long term (28 days). Additionally, the 24 h stabilities of all analytes were measured in standard solutions at the autosampler temperature of 10°C.

3 Results and discussion

3.1 Optimization of CE parameters

CE separation is governed by several factors, such as separation buffer selection, the buffer pH and ionic strength, the voltage applied, the capillary size and temperature, or various buffer additives, which may influence not only analytes, migration times, but also the EOF and electrical-field strength. A spectrophotometric detector (DAD) was used for the analysis of target compounds only during the optimization of the CE separation parameters.

3.1.1 The influence of the BGE composition on the electrophoretic separation

Initially, to select a BGE the common knowledge of the pK values of the studied compounds and the volatile mobile phases typical of MS were employed [27–30]. The choice of BGEs was made on the basis of physicochemical properties of the studied antibiotics, particularly, their pK values that were captured from the DrugBank database (Supporting information Table S1). Therefore, formic acid, ammonium formate, and ammonium acetate were studied as the BGEs.

Background electrolyte composition for AMOX, LIN, and MET

The pK values of the amoxicillin include 3.23 (due to –COOH substituent) and 7.43 (corresponding to the NH_2 group). Therefore, AMOX ionizable in an alkaline environment can be also detected in an acidic medium (the amphoteric behavior). LIN has two pK values of 14.45 and –0.66 as its nitrogen atom can be protonated so it can migrate as a cation. Remarkably, the effect of the pH on the resolution of MET and its metabolites was observed. When the pH was above 3.0, they were partially separated. To sum up, these compounds were completely separated at pH below 3.0. For this reason, formic acid (22 mM, pH 2.57) was chosen as the optimum separation BGE for AMOX, LIN, MET and their metabolites. The acidic conditions enabled us to gain a positive charge of the mentioned drugs and their analysis as cations with strongly suppressed electroosmosis.

BGE composition for CIP, CEF, and CLI

Other antibiotics included CIP, CLI, and CEF. They were detected only in the basic separation buffer such as ammonium acetate and ammonium formate. Nevertheless, the reproducibility of both peak areas and the migration times, using ammonium formate was not satisfactory. The peaks in subsequent runs constituted different migration times and intensity. Those results could be caused by the adsorption of the buffer constituents or the studied analytes which could influence the surface properties of the capillary and the EOF velocity. Hence, ammonium acetate was chosen for the simultaneous separation of CIP, CLI, and CEF.

3.1.2 The influence of the BGE concentration on the migration times of CIP, CEF, and CLI

In addition, the effect of the ammonium acetate concentration between 20 and 40 mM was also studied. The increase

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in the buffer concentration slowed down the EOF, giving even a better resolution but a longer migration time. Thus, the satisfactory outcomes in terms of analysis time and peak shape were observed at the concentration of 40 mM.

3.1.3 The influence of the BGE pH on the migration time of CIP, CEF, and CLI

The pH values were adjusted by adding either acetic acid or the ammonium hydroxide solution to the buffer. Hence, the next step was to study the pH of the ammonium acetate solution between 8 and 10, and finally, the pH 9.0 was selected.

The separation can be explained by taking into consideration the structural characteristics, the molecular mass, and the pK values of the studied antibiotic drugs and their metabolites. The pK values of CIP were as follows $pK_{-COOH} = 5.76$ and $pK_{-NH3+} = 8.68$, connected with the N4 deprotonation of the piperazine ring. CIP is an amphoteric compound that can be negatively or positively charged depending on the pH of the solution Therefore, an acidic or alkaline running buffer can be used for its separation. Most of the published CE methodologies are supported by the basic BGE [20,30,31], whereas the acidic separation electrolytes were investigated to a lesser extent [27,29]. Furthermore, it was noticed that the peaks of LI were detected above pH 8.0, but the satisfactory symmetric shape was achieved at the pH 9.0. The analytes (CIP, CEF, CLI) were not detectable at pH 10. By taking into account both the migration time and the resolution, we chose 40 mM ammonium acetate adjusted to pH 9.0 for the CIP and CLI separation.

3.1.4 The influence of injection time and pressure on the migration times of studied antibiotics

Thereafter, the effect of the injection time from 3 to 20 s and pressure (20–30 mbar) was examined. The modification of the injection parameters (pressure and time) did not have a significant influence on migration times. These parameters had a slight impact on the peak symmetry and shape. The optimum injection by the application of 25 mbar for 10 s was found.

3.1.5 The influence of the capillary temperature on the migration times of studied antibiotics

Additionally, the influence of the capillary temperature on the analysis was determined in the range of 23–35°C. An increase in the system temperature led to a decrease in the buffer viscosity, which increased the electrophoretic mobility and reduced the migration times. Accordingly, the optimal temperature to obtain producibility migration time with the best resolution was found at 23.0 ± 0.5°C.

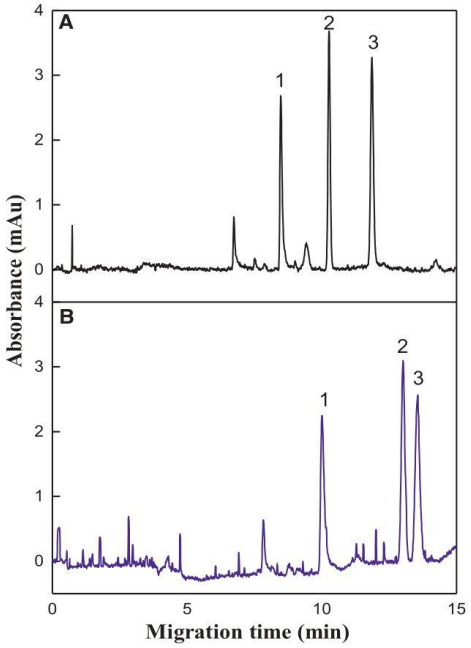

Figure 1. Influence of organic modifier: acetonitrile (**A**) and methanol (**B**) on migration time for CLI (1), CIP (2), and CEF (3). Separation conditions: ammonium acetate (40 mM, pH = 9.0), applied voltage: 30 kV, temperature: 23°C, injection pressure 25 mbar, 10 s, UV detection at 220 nm, capillary: 64.5 cm (56 cm effective length) × 50 μm, analytes concentration: 0.05 mg/mL.

3.1.6 The influence of the organic modifier on the migration times of studied antibiotics

To increase the selectivity of the separation, organic solvents (methanol, acetonitrile) were used as buffer additives. Hence, their influence on the separation was examined in the concentration range of 5–15%. The aim of their application was to examine the electrophoretic mobility of the analytes. The increased concentration of organic solvents in the buffer solutions reduced EOF and consequently increased the observed migration time. Methanol proved to be more efficient than acetonitrile. For the sake of comparison, let us point out that methanol led to a higher increase in the migration time than acetonitrile but negatively influenced the shape of the baseline; a considerable amount of noises in the baseline was observed (Fig. 1). It is noteworthy that the curve shape was closely connected with the type of the organic modifier applied. This effect can be explained by the fact that acetonitrile displayed a slight current decrease when its concentration increased, while the addition of methanol caused a sharp decrease in the current.

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3.2 Optimization of CE-ESI-QqQ-MS parameters

The CE-ESI-MS/MS system was used for the first time in the analysis of examined antibiotic drugs and their metabolites. The optimal conditions of MS parameters for the determination of target compounds were estimated using CCD as the experimental design. In this investigation, the drying gas temperature and fragmentor voltage were chosen as independent variables. The peak area at the extracted ion chromatogram was applied as a dependent variable, since the ESI-QqQ-MS sensitivity determination was the main goal of this step of the investigation.

The product ion mode was used to determine a fragment ion indispensable for the MRM mode. The optimization of the collision energy for the studied compounds was performed in the range from 10 to 40 eV. The fragmentation was performed only for parent ions, which were the most intensive at the full scan spectra (Supporting information Table S1). Parent ions were not fragmented when low values of collision energy were applied. The greatest number of specific fragmentation signals was observed in the range of 20–35 eV. A subsequent increase in energy resulted in the reduction of various product ions and a decrease in their intensity. Consequently, the optimized collision energies were selected for the studied compounds (Supporting information Table S1).

To study the drying gas temperature and the fragmentor voltage, a circumscribed CCD with three levels was used (− 1, 0, 1). These three controlled variables were studied in a multivariate study conducted along with nine experiments.

For AMOX and its metabolites, the best fit of conditions is dependent on X_2. The higher the drying gas value, the better the model fit and the larger the surface area (PP_{MRM}). The value of X_1, the voltage on the fragmentor, does not have any significance here. The results for CIP and its metabolite are similar. Conversely, the opposite is true for MET and its metabolite. The fit depends on the voltage on the fragmentor. Initially, the greater the value of X_1, the better the fit, but after reaching the optimal value for the best fit, a further increase in X_1 causes the deterioration of the result. However, the value of the drying gas is irrelevant here. The conclusion is that with the same parameters of the voltage on the fragmentor (X_1) and the drying gas temperature (X_2) for the relevant antibiotic drug, its metabolites can be determined and identified at the same time.

3.3 Method validation

3.3.1 Selectivity

The analysis of urine samples collected from different patients did not show interference of the endogenous components. The selectivity of the newly developed CE-ESI-MS/MS method was examined by comparing the data of extracted blank human urine from six different batches and samples spiked with known amounts of each studied drugs and metabolites at the LLOQ (Table 2). Results show that no interfering peaks were detected for the transitions analyzed by MRM.

3.3.2 Linearity, LOD, LLOQ, and LOQ

After comparing the two weighting models ($1/x$ and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the studied compounds was found to produce the best fit for the chromatographic response versus concentrations for all analytes in human urine. The calibration curves showed good linearity over the established concentration range of 40–1000 ng/mL for AMOX, AMA, AMD, CEF, CIP, CIP-PS, CLI, LIN-Ox, MET, MET-OH; 80–800 ng/mL for CEF-DAC-LAC, CLI-Sox, and 20–1000 ng/mL for LIN. The coefficient (R^2) of determination for all analytes was over 0.99, showing a good linearity among the concentration range (Table 2). LOD was defined as the lowest concentration of the analyte in the sample that can be detected above the baseline noise. It is expressed as a concentration at a specified *S/N*, typically, three times the noise level. LOQ was defined as the lowest concentration of the analyte in a sample that can be reproducibly quantitated above the baseline noise with *S/N* of more than 10.

3.3.3 Precision and accuracy

Precision and accuracy were expressed as the RSD and the RE at three different concentration levels of drugs, respectively. The intraday precision and accuracy were determined by analyzing six replicates of human urine samples containing both antibiotic drugs and their metabolites at different concentrations. The interday precision and accuracy were determined on five separate days at the same concentrations (RSD $\leq$ 15% and RE within $\pm$15%). The minimal differences between the calculated concentrations and the expected values indicated that intra- and interday accuracy of the tested analytical method was very good. Similar migration times and peak areas were obtained. The mean values and SD were calculated; RSD (%) values mostly lower than 4% indicate the satisfactory precision of the method. As usual, the precision for the migration times was better than for the peak areas. Accuracy and precision were determined based on the analysis of QC samples. This indicates that the current method met the criteria of accuracy and precision.

3.3.4 Stability

QC sample stability analyzes were also performed for each of the analytes at 2–8°C for the short (7 days) and long term (28 days). Additionally, the 24 h stabilities of all analytes were measured in standard solutions at a CE-ESI-MS/MS autosampler temperature of 10°C (Supporting information

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Table 2. Basic validation method for the studied antibiotic drugs and their metabolites

Analyte	Linear range (ng/mL)	R^2	LLOQ (ng/mL)	Concentration (ng/mL)	Intraday		Interday		Regression equation/95% CI[c]: slope/intercept	Recovery (%)	Matrix effect (%)
					RS[a] (%)	RE[b] (%)	RSD[a] (%)	RE[b] (%)			
AMOX	40–1000	0.9982	40	100	5.82	−2.15	6.87	−6.91	$y = 6.0349x - 1.1488$	93.8	8.22
				500	4.71	−1.47	5.21	−4.72	5.7332−6.3366/	94.1	5.31
				800	4.01	−1.14	5.03	−2.84	1.0914−1.2062	96.2	1.80
AMA	40–1000	0.9981	40	100	4.75	−4.28	7.21	−5.93	$y = 4.9170x - 1.8536$	97.7	−7.46
				500	3.21	−3.91	6.32	−4.72	4.6712−5.1629/	98.5	−6.04
				800	2.47	−2.78	4.12	−1.35	1.7609−1.9463	90.2	−4.21
AMD	40–1000	0.9964	40	100	3.36	−6.40	6.32	−8.47	$y = 3.1694x - 2.9320$	94.1	7.28
				500	2.87	−4.32	4.21	−5.32	3.0109−3.3279/	94.8	6.32
				800	1.20	−3.78	2.12	−3.14	2.7854−3.0786	95.0	2.15
CEF	40–1000	0.9966	40	100	5.42	2.87	7.98	5.87	$y = 7.2201x - 0.9176$	98.7	5.69
				500	3.17	1.42	6.13	4.32	6.8591−7.5811/	90.3	4.71
				800	1.98	1.25	4.78	2.98	0.8717−0.9635	92.5	3.22
CEF-DAC-LAC	80–800	0.9948	80	100	6.47	−6.32	8.36	−8.39	$y = 6.1654x - 3.6848$	98.0	−5.78
				400	5.85	−4.13	7.54	−7.90	5.8571−6.4737/	92.5	−4.21
				650	3.21	−1.09	6.89	−6.23	3.5006−3.8690	92.9	−4.20
CIP	40–1000	0.9977	40	100	4.44	−6.30	7.93	−9.17	$y = 3.9445x + 0.4741$	92.9	−8.45
				500	2.79	−5.41	5.71	−5.24	3.7473−4.1417/	93.3	−7.63
				800	0.88	−4.17	2.07	−3.96	0.4504−0.4978	93.6	−6.21
CIP-PS	40–1000	0.9970	40	100	5.32	−5.93	7.36	−8.32	$y = 2.1564x + 0.9428$	95.5	5.27
				500	4.87	−4.71	6.91	−7.63	2.0486−2.2642/	95.8	4.89
				800	1.09	−2.98	2.35	−6.01	0.8957−0.9899	97.2	2.75
CLIN	40–1000	0.9941	40	100	6.32	−5.63	8.71	−8.32	$y = 8.6936x - 3.8104$	92.6	−6.91
				500	4.08	−4.87	7.56	−5.93	8.2589−9.1283/	93.5	−5.03
				800	2.78	−1.23	6.32	−4.71	3.6199−4.0009	95.0	−4.72
CLI-Sox	80–800	0.9916	80	100	6.78	−6.91	8.69	−8.45	$y = 6.8543x - 2.4050$	98.0	−6.90
				400	4.11	−5.72	7.21	−7.69	6.5116−7.1970/	98.3	−5.78
				650	0.98	−2.45	3.59	−2.98	2.2848−2.5253	93.2	−4.16
LIN	20–1000	0.9961	20	100	5.72	5.78	9.12	8.63	$y = 6.9543x - 5.0439$	98.5	7.28
				500	4.78	4.27	8.36	7.20	6.6066−7.3020/	98.9	5.87
				800	1.03	4.22	4.75	6.32	4.7917−5.2961	90.3	4.32
LIN-Ox	40–1000	0.9981	40	100	6.97	−7.54	7.36	−8.91	$y = 4.0865x - 1.9641$	98.6	−5.93
				500	5.30	−7.21	6.18	−7.57	3.8822−4.2908/	90.3	−4.27
				800	3.21	−6.32	5.63	−7.40	1.8659−2.0623	91.5	−3.80
MET	40–1000	0.9941	40	100	3.78	−4.78	5.47	−6.74	$y = 7.3839x - 2.2561$	94.3	−7.93
				500	3.25	−3.64	4.82	−5.55	7.0147−7.7531/	89.5	−6.20
				800	2.97	−2.13	3.91	−1.89	2.1433−2.3689	95.7	−4.17
MET-OH	40– 1000	0.9969	40	100	8.52	6.28	8.78	7.53	$y = 5.9534x - 1.9530$	92.8	−7.89
				500	6.34	5.47	7.52	6.30	5.6557−6.2511/	93.8	−6.23
				800	2.98	3.48	6.41	4.91	1.8554−2.0507	93.5	−5.74

[a] RSD, relative standard deviation.
[b] RE, relative error.
[c] 95% confidence interval for the slope and intercept.

Table S2). The obtained results indicate that the analytes were stable in human urine samples in all studied conditions.

3.3.5 Matrix effect and extraction recovery

The ME was calculated by comparing the peak area ratios of selected analytes dissolved in the blank urine samples with that of analytes spiked in the mobile phase at corresponding concentrations. For most analytes, the ME was negligible for the assay (ME = –8.45 to 8.22%). These data confirm that the validation parameters of the developed method are acceptable (Table 2). The developed CE-ESI-MS/MS method showed good and reproducible recoveries for the analytes.

The obtained validation results showed that the assay met the desired acceptable criteria set by regulatory guidelines [26].

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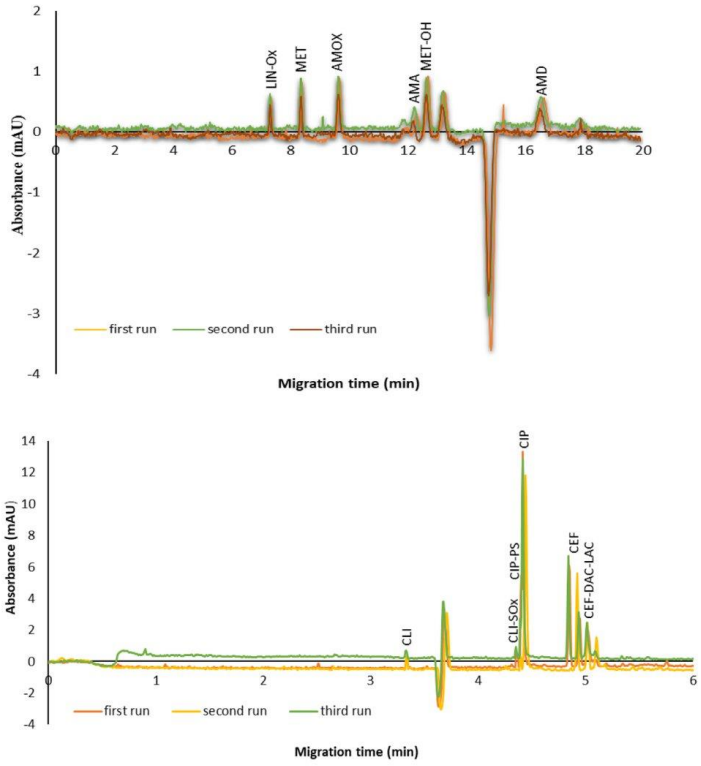

Figure 2. Reproducibility of the optimized electrophoretic method with migration time, (t_m [min] ±RSD [%]) in (A) formic acid (22 mM, pH 2.57), LIN-Ox (7.31 ± 0.18), MET (8.36 ± 0.16), AMOX (9.64 ± 0.18), AMA (12.26 ± 0.38), MET-OH (12.64 ± 0.21), LIN (12.65 ± 0.21), AMD (16.54 ± 0.28) and (B) ammonium acetate (40 mM, pH 9.0), CLI (3.33 ± 0.84), CLI-Sox (4.39 ± 0.53), CP-PS (4.43 ± 0.45), CIP (4.45 ± 0.15), CEF (4.92 ± 0.14), CEF-DAC-LAC (5.02 ± 0.19) as a separation electrolyte. Electrophoretic conditions: V = 30 kV, T = 23°C, injection pressure 250 mbar·s, UV detection at 220 nm, capillary: 64.5 cm (56 cm effective length) × 50 μm, analytes concentration: 0.05 mg/mL.

3.4 Separation of studied antibiotic drugs and their metabolites with the use of CE-DAD-MS/MS

In this study, for the sake of the electrophoretic separation, the antimicrobial agents were selected according to the most commonly prescribed antibiotics and their combinations for frequent surgical infections as well as the diabetic foot.

Taking into account the parameters of the simultaneous electrophoretic separation and sensitivity, we chose formic acid (22 mM, pH 2.57) for AMOX, LIN, and MET along with their metabolites and ammonium acetate (40 mM, pH 9.0) for CLI, CIP, CEF and their metabolites, applying a voltage of 30 kV at a temperature of 23°C and the injection pressure/time 25 mbar/10 s, and the detection at 220 nm. The order of the separation was: LIN-Ox, MET, AMOX, AMA, MET-OH, LIN, AMD and CLI, CLI-SOx, CIP-PS, CIP, CEF, and CEF-DAC-LAC in formic acid and ammonium acetate, respectively.

To control the reproducibility of the migration times and peak areas, three injections of the analytes mixture were made for three samples (Fig. 2).

3.5 Application to real samples

To demonstrate the applicability of the electrophoretic procedure developed, some samples were analyzed including a sample of human urine. The electropherograms for the real and blank samples are shown in Fig. 3. Each sample was prepared in duplicate and injected in triplicate. To elucidate the structures of the relevant metabolites, we studied the fragmentation of the [M+H]+ ions of the target compound and its metabolites using ESI-MS/MS experiments. The presence of a drug and its potential metabolites in samples from the patients were additionally confirmed by the MS/MS spectra. A notable observation from this figure is that even though this is a real sample, the electropherogram is clear (Fig. 3), which demonstrates that the clean-up step with the addition of ACN was efficient. The recovery was in the range 93.4–98.9% (±1.6–2.7).

In the samples taken from patients treated with MET, the presence of one metabolite of the parent drug: hydroxy MET (m/z = 188) was observed. For AMOX, CEF, CIP, CLI, and LIN, six metabolites were identified at m/z = 366 and 384

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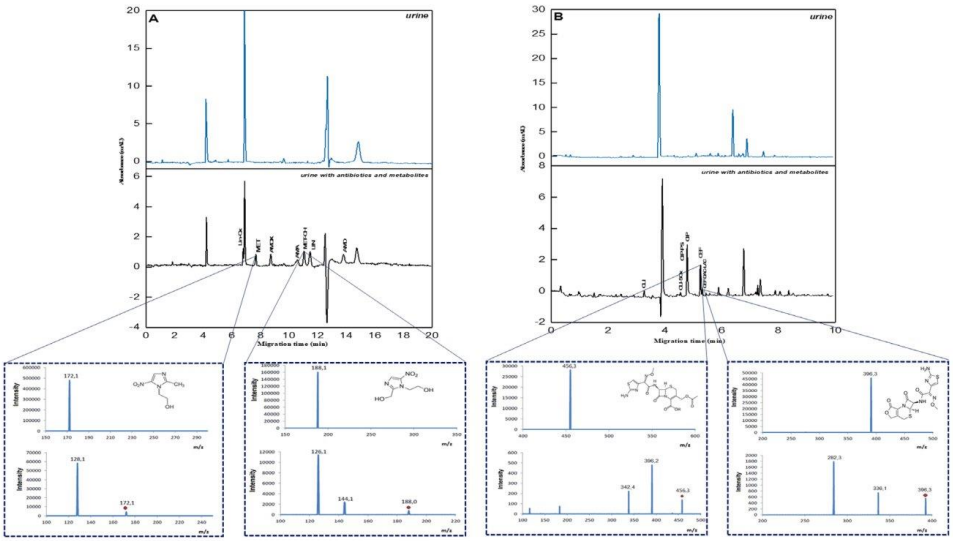

Figure 3. Electropherograms of blank urine and urine with antibiotics and metabolites using (A) formic acid (22 mM, pH = 2.57) or (B) ammonium acetate (40 mM, pH = 9.0). Electrophoretic conditions: $V = 30$ kV, $T = 23$°C, injection 250 mbar·s, UV detection at 220 nm, capillary: 64.5 cm (56 cm effective length) × 50 μm, analytes concentration: 0.05 mg/mL) and representative MS spectra as well as fragmentation MS/MS spectra for MET and MET-OH (A) and for CEF and CEF-DAC-LAC (B) of the extracted urine sample obtained from a patient after the administration of antibiotics.

(for AMOX), at $m/z = 396$ (for CEF), at $m/z = 412$ (for CIP), at $m/z = 441$ (for CLI), and at $m/z = 354$ (for LIN). Representative MS spectra and fragmentation MS/MS spectra for MET and MET-OH as well as for CEF and CEF-DAC-LAC in human urine samples are shown in Fig. 3. Acquired MS/MS spectra proved that the precursor ion for MET at $m/z = 172$ was mainly fragmented to $m/z = 128$ ($-C_2H_4O$) and for MET-OH at $m/z = 188$ to $m/z = 144$ ($-C_2H_2NO$), and $m/z = 126$ ($-C_2H_4O$). For CEF and CEF-DAC-LAC, the precursor ion was at $m/z = 456$ and 396, respectively. CEF fragmentation (MS/MS) generated mainly two product ions at m/z 342 and 396 (major product) and CEF-DAC-LAC fragmented to m/z = 282 and 336.

The identities of the analyte peaks in urine extracts were confirmed by the comparison of their migration times, UV spectra, and MS of relevant antibiotic drugs standards. Moreover, their presence in real samples was confirmed by MS/MS spectra, while collision induced the dissociation of the peak of the most intense ion.

CE is a very useful technique for the screening analysis, it also plays an important role in biomedical science [32]. Nevertheless, the main drawback is its high sensitivity of CE condition to the pH and the ionic strength change, reflected in repeatability [33]. Nowadays, the most crucial thing is the optimization of electrophoretic parameters base to receive good reproducibility. It should be pointed out that the method developed by us is rapid, simple, cheap and, most importantly, repeatable (Fig. 2). Moreover, for the analysis of antibiotic drugs and their metabolites in clinical samples, the application of the protein precipitation procedure at the preparation stage provided satisfying extraction efficiency. Moreover, good sensitivity and precision along with the high accuracy of the assay of target compounds might make the CE-ESI-MS/MS method a useful tool in clinical laboratories for the therapeutic drug monitoring of antibiotics and in forensic laboratories owing to their determination and higher levels in human fluids. Among methods available in the literature [12–20], the proposed method provided better accuracy and precision. Additionally, it is suitable to determine analytes in complex matrices, and it requires only small sample volumes. The reduction of the sample volume was also possible, because the MS detection was applied. The applied sample preparation technique is used simply to perform, limiting the volumes of organic solvents and biological samples as well.

4 Concluding remarks

Antibiotic drugs are often applied in the combined therapy to provide a wide range of activities, reduce toxicity, and improve therapeutic efficacy. Therefore, it is necessary to develop multidimensional methods for the determination of

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more than one antibiotic in the same analysis performed at the same time. The performed method can be applied in the screening analysis of patients to eliminate combinations of drugs that are not recommended for concurrent use.

Moreover, as far as clinical practice is concerned, it is necessary to develop an approach that is easy to apply and involves a wide range of antibiotics groups. Thus, the time for clinicians to obtain results is reduced. To the best of our knowledge, this is the first analysis where antibiotics and their metabolites from different therapeutic groups are determined simultaneously in urine samples with the use of the CE-ESI-MS/MS system.

In this study, the use of protein precipitation as a simple pretreatment step in the analysis of antibiotics and their metabolites in human urine resulted in the satisfactory extraction efficiency. Moreover, the simple clinical sample preparation step applied permitted to reduce the volumes of organic solvents and biological samples.

The method described is precise and reproducible with a relatively short analysis time, either in the basic or in the acidic BGE. This method, compared to conventional LC, is simpler and faster; it also requires minimal sample preparation. Moreover, this approach is a good alternative to conventional LC methods because it fulfills green chemistry demands as it requires low amounts of solvents, reagents, and minute samples. For good sensitivity, precision and repeatability, the CE-ESI-MS/MS method can only be successfully applied to determine target compounds in human biological samples as a screening tool in a clinical laboratory to drug monitoring and in forensic laboratories. The proposed method has better accuracy and precision when compared to the work available in the literature.

The obtained results can be particularly useful in the antibiotic therapy. The implementation of the proposed method for human clinical samples gives an opportunity to assess the effectiveness of antibiotics, which can be beneficial for the optimization of individual antibiotic therapies. To the best of our knowledge, no detailed information regarding the structure of their potential metabolites via such an approach has been hitherto published.

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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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3.4. *Identification, structure and characterization of Bacillus tequilensis biofilm with the use of electrophoresis and complementary approaches*

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Identification, Structure and Characterization of *Bacillus tequilensis* Biofilm with the Use of Electrophoresis and Complementary Approaches

Katarzyna Pauter [1,2], Viorica Railean-Plugaru [2], Michał Złoch [1,2], Paweł Pomastowski [2], Małgorzata Szultka-Młyńska [1] and Bogusław Buszewski [1,2,*]

[1] Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarina 7, 87-100 Torun, Poland; kpauter@wp.pl (K.P.); michalzloch87@gmail.com (M.Z.); mszultka@umk.pl (M.S.-M.)

[2] Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Wilenska 4, 87-100 Torun, Poland; rviorela@yahoo.com (V.R.-P.); pomastowski.pawel@gmail.com (P.P.)

* Correspondence: bbusz@umk.pl; Tel.: +48-56-611-4308

Abstract: Biofilm is a complex structure formed as a result of the accumulation of bacterial cell clusters on a surface, surrounded by extracellular polysaccharide substances (EPSs). Biofilm-related bacterial infections are a significant challenge for clinical treatment. Therefore, the main goal of our study was to design a complementary approach in biofilm characterization before and after the antibiotic treatment. The 16S rRNA gene sequencing allowed for the identification of *Bacillus tequilensis*, as a microbial model of the biofilm formation. Capillary electrophoresis demonstrates the capability to characterize and show the differences of the electrophoretic mobility between biofilms untreated and treated with antibiotics: amoxicillin, gentamicin and metronidazole. Electrophoretic results show the clumping phenomenon (amoxicillin and gentamicin) as a result of a significant change on the surface electric charge of the cells. The stability of the dispersion study, the molecular profile analysis, the viability of bacterial cells and the scanning morphology imaging were also investigated. The microscopic and spectrometry study pointed out the degradation/remodeling of the EPSs matrix, the inhibition of the cell wall synthesis and blocking the ribosomal protein synthesis by amoxicillin and gentamicin. However, untreated and treated bacterial cells show a high stability for the biofilm formation system. Moreover, on the basis of the type of the antibiotic treatment, the mechanism of used antibiotics in cell clumping and degradation were proposed.

Keywords: antibiotics; biofilm; *Bacillus tequilensis*; capillary electrophoresis; SEM

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1. Introduction

The bacterial infections associated with biofilm constitute a serious problem in the clinical area [1,2]. As previously described, biofilm impairs human defense mechanisms and creates a platform for microorganisms to use a range of strategies to control antibiotics resistance, including spore cell production, the multilayer system of bacteria cells and antimicrobial resistance [3,4].

Abiofilm is an accumulation of bacterial cells on the surface based on the agglomeration process and is surrounded by extracellular polysaccharide substances (EPSs) [5,6]. It was found that the structure of EPS shows species or strain specificity both in terms of the composition of monosaccharides, which constitute the subunit of EPS and the type of chemical bonds or non-saccharide substituents. Sometimes the form of exopolysaccharides may depend on the intensity of the cell growth. The increase in polysaccharide components in the matrix results in more free functional groups able to interact with other bacterial cells or active molecules such as antibiotics. Therefore, it determines a significant resistance of the microorganisms in biofilm [7–11].

Relucenti et al. [12] recommend the use of different microscopic techniques to understand the ultrastructure of the biofilm, its three-dimensional organization and the behavior of bacterial cells and their response after the antibiotic treatment. The utilization of various microscopic techniques (optic microscopy (OM); confocal laser scanning microscopy (CLSM); atomic force microscopy (AFM); scanning electron microscope (SEM)) permits imaging of the biofilm's surface [9]. Furthermore, by combined SEM imaging with a three-dimensional image analysis system, the extracellular matrix can be quantitatively analyzed [12]. Undoubtedly, one promising analytical technique employed in the investigation of microorganisms is capillary electrophoresis (CE). This tool is also used for the separation of the microorganism's analysis. Dziubakiewicz et al. [13] analyzed both Gram-positive (*Bacillus cereus*, *Bacillus subtilis*, *Sarcinalutea*, *Staphylococcus aureus* and *Micrococcus luteus*) and Gram-negative (*Escherichia coli*) bacteria by capillary electrophoresis. The authors pointed out that the modification of the surface charge of bacteria with Ca^{2+} allows their screening analysis with the pseudo-isotachophoretic mode of CE. The use of calcium ions allowed the number of signals on the electropherograms to be reduced, indicating a hampered ability to aggregate. Furthermore, the utilization of the pseudo-isotachophoresis mode of CE allowed the aggregates to be clustered, so the results improved. Moreover, the zeta potential was used as complementary studies [13]. In addition, Ca^{2+} was also used for the study of the yeast aggregation measurement [14]. However, in another work, Ruzicka et al. [15] demonstrated the application of capillary isoelectric focusing (CIEF) to detect also biofilm formation in *Staphylococcus epidermidis*. In this work, they report that the surface characteristics of *S. epidermidis*, based on the CIEF method, can be used to distinguish between positive and negative biofilm-forming strains [15]. The isoelectric points (pI) were between pI 2.6 and 2.3 for the strain that was able to create and not create the biofilm, respectively [15]. These discrepancies could be the result of the presence or absence of EPS on the bacterial surfaces. This crucial component of the biofilm layer enables the bacterial adhesion to solid substrates and promotes intercellular adhesion, contributing to biofilm creation. In addition, adhesion and biofilm layer formation were observed; the surface protein EPSs are involved in the antibiotic and immune system protection of bacterial cells [15]. Annet E. J. van Merode et al. [1] investigated the influence of zeta potential culture heterogeneity on retention and the biofilm creation by clinical isolates—*Enterococcus faecalis* strains. *E. faecalis* strains generally display subpopulations with various surface charges, which are expressed as zeta potential bimodal distributions. The heterogeneous strains tended to be trapped in more numbers on the polystyrene than the homogeneous strains. Likewise, the biofilm creation was much more complex for heterogeneous strains than for homogeneous strains [1]. Moreover, the matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) technique was also applied to the biofilm characterization. Li et al. [16] investigated imaging of a *B. subtilis* biofilm created on agar by this technique. Thus, it was possible to visualize the distribution of metabolites of the biofilm-forming cells [16].

Therefore, the main purpose of this study was to check the usefulness of the capillary electrophoresis (CE) technique for the fast screening of the effect of different antibiotics when added on the biofilm formation using model biofilm-producing bacteria—*Bacillus tequilensis*. A variety of complementary techniques were applied here: (a) 16S rRNA gene sequencing for identification; (b) capillary electrophoresis in order to monitor the bacteria clumping of isolated bacteria in the electric field under the antibiotic treatment; (c) zeta potential measurements to check the stability of the dispersion and control the aggregation process; (d) MALDI-TOF MS on molecular profile changes; (e) fluorescence microscopy to determine the viability of bacterial cells under stress conditions; and (f) scanning electron microscopy for biofilm morphology and structure imaging. Moreover, we elucidated the role of the bacteria biofilm exposure to antibiotics belonging to different therapeutic groups, with different spectrums and mechanisms of action, as along with various chemical structures, such as amoxicillin, gentamicin and metronidazole.

2. Materials and Methods

2.1. Sample Preparation

Bacillus tequilensis were chosen as a model for the biofilm formation and characterization. The isolation of the used strain was performed according to the previous protocol described by Pomastowski et al. [17]. Honey, as an accessible source, was used in the present study to isolate the *Bacillus tequilenis* strain; the isolate is a Gram-positive, non-pathogenic bacterium, which is commonly found in honey and is well known to produce biofilms. The *Bacillus* sp. are model bacterial strains for biofilm formation due to two aspects. Firstly, *Bacillus* species can produce heat-resistant endospores that play an important role in bacterial persistence and biofilm formation. Second, members of the genus *Bacillus* are known to be able to produce extracellular polymeric substances (EPS), which play a key role in the resistance of the biofilm to antibiotics by creating a mechanical barrier that restricts drug diffusion into bacterial cells. In addition, these bacteria also possess swarm motility, which can facilitate microbial survival in the environment and colonization of surfaces, leading to biofilm formation [18].

In this study, three different antibiotic drugs—amoxicillin (≥98% purity), gentamicin (≥98% purity) and metronidazole (≥98% purity)—were used. All of them were purchased from Sigma-Aldrich Chemicals (Madrid, Spain). The used concentrations in the present study were establish based on the minimal inhibitory concentration value according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for the *Bacillus* spp. [19].

A bacterial colony was grown on Tryptic Soy Agar (Sigma-Aldrich, Steinheim, Germany) over 24 h at 37 °C. Then, the fresh cells were incubated for 2 h in Mueller Hinton Broth solution (MHB, Sigma-Aldrich, Madrid, Spain) with chosen antibiotics (amoxicillin, MIC (2 μg/mL); gentamicin, MIC (0.5 μg/mL); and metronidazole, MIC (8 μg/mL). The optical density of each suspension was adjusted according to the McFarland Standard (OD600 ≈ 3). These three antibiotics with different chemical structures (aminoglycoside, nitroimidazole, β-lactam), distinct mechanisms of action (different molecular targets—inhibition of bacterial protein synthesis, disruption of cell wall biosynthesis, interaction with the DNA) as well as various MIC values (high action—gentamicin, medium—amoxicillin and low—metronidazole) were chosen in order to change the biofilm surface structure in different ways. Three antibiotics that demonstrated various modes of action were selected, knowing that they may be characterized also by their different ability to perform EPS penetration—a key factor in determining the effectiveness of an antibiotic in biofilm eradication. Untreated cells served as a control. After the incubation step, the bacterial culture was harvested by centrifugation (13,000 rpm for 15 min, 20 °C ± 0.5 °C) and washed two times with water (5000 rpm for 5 min, 20 °C ± 0.5 °C). Washed cells were resuspended in outlet background electrolyte TB—this is a buffer solution containing a mixture of Tris base, boric acid and EDTA (Tris-borate; pH 8.0) for subsequent analysis.

2.2. Molecular Identification of Bacterial Biofilm Formation

The total genomic DNA was extracted using the method provided in the DNeasy UltraClean Microbial Kit (QIAGEN, Wroclaw, Poland) and was used as template for 16S rDNA region amplification using the PCR method and the universal bacterial primers 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3). Obtained PCR products were sequenced using the Sanger dideoxy method (Genomed, Warsaw, Poland) and consensus sequences were compared with known reference 16S rRNA sequences found in the National Center for Biotechnology Information's Reference RNA sequences (refseq rna) database via the Basic Local Alignment Search Tool algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 3 November 2021)).

2.3. Capillary Zone Electrophoresis (CZE) Analysis

A CZE-UV system (Beckman Coulter, Brea, CA, USA) was used for experiments, which were performed using a fused silica capillary (length = 70 cm, inner diameter = 75 μm

and outer diameter = 375 µm, Beckman Coulter) with an effective length of 50 cm. New capillaries were rinsed prior with Milli-Q water, 1 M NaOH, 0.1 M NaOH, Milli-Q water and a running buffer, each for 2 min. Before each day, the capillary was treated with 1 M NaOH followed by distilled water for 30 min. To ensure repeatability, between each analysis, the capillary was rinsed with the background solution for 30 min. At the separations, an inlet buffer—TBH (Tris-borate, hydrochloric acid; pH 7.3)—and an outlet buffer—TB (pH 8.0)—were used. The buffer solutions were prepared by dissolving the appropriate amount of H_3BO_3 and Trizma® base in deionized water and the pH was adjusted with 0.1 M HCl (Sigma Aldrich, St. Louis, MI, USA) in case of TBH, and 0.1 M NaOH (Sigma Aldrich) in case of TB. The boric acid came from Chempur (PiekaryŚląskie, Poland). The Trometamol (Trizma® base) was purchased from (Sigma-Aldrich, USA). Each sample was injected into the capillary at 8 psi for 10 s. UV detection wavelength was set at λ = 214 nm and the capillary temperature was maintained at 23 °C. The separation procedure was adapted based on the research of Dziubakiewicz et al. [13]. Electropherograms included migration time and the peak area; they were recorded and processed using the 32 Karat v.8.0 software (Beckman Coulter, Brea, CA, USA). Fractions for the SEM analysis were collected directly into the outlet vial during the CE analysis. Thiourea was used as a determinant of electroosmotic flow (EOF), (Sigma-Aldrich, Bangalore, India).

2.4. Zeta Potential Assay

For the zeta potential of bacterial cell measurements, Zetasizer Nano ZS was used (Malvern Instruments, Malvern, UK). The bacterial suspensions in TB buffer were prepared according to the method published by Buszewski et al. [20]. Additionally, the samples were vortexed for 15 min at 22 °C, and subsequently sonicated (5 min, 25 °C). The values of the zeta potential were summarized as the average value based on the three measurements.

2.5. MALDI-TOF MS Analysis

MALDI-TOF was used to analyze the molecular profile of *B. tequilensis*. Protein extracts were obtained from bacterial cells using a quick method described by Pomastowski and co-workers [17]. To the bacterial pellets, 900 µL of ethanol was added, then centrifuged at 14,400 rpm for 5 min at 20 °C. The supernatant was removed and the pellet was dried using vacuum concentrator for 8–10 min.

The material was then dissolved in 10–12 µL of 70% formic acid (Merck, 98–100%, Darmstadt, Germany), which was followed by the addition of the same volume of acetonitrile (Fluka Analytical Sigma-Aldrich, Munich, Germany). The resulting mixture was centrifuged again at 13,000 rpm for 2 min. Finally, 1 µL of each control bacteria and bacterial suspension with antibiotics were transferred to one sample spot on a MALDI target plate and allowed to dry at room temperature before being overlaid with 1 µL of HCCA matrix solution (10 mg/mL in 47.5% water, 50% ACN, 2.5% TFA), the calibration on BTS being in the quadratic mode. The visual evaluation of the mass spectra was carried out initially with the Flex Analysis 2.4 software (Bruker Daltonik GmbH, Bremen, Germany). Then, the raw spectra were processed using the MALDI BioTyper 1.1 software (Bruker Daltonik GmbH). The obtained raw data have been additionally processed by STATISTICAL Release 7 software in order to identify the signal changes on the protein profile of untreated and treated bacteria cells. The data were visualized by the Scatterplots w/histograms model.

2.6. Fluorescence Microscopy Assay

The effect of a bacterial viability under the antibiotics treatment was investigated through the fluorescence microscopy. The bacterial cells were stained regarding the method reported previously by our group using the ethidium bromide and acridine orange as a dyes in order to differentiate the live and dead cells to be able to establish the level of the modified biofilm structure [21]. For each sample, fluorescence images were taken with a Zeiss Axio Observer.D1 (Zeiss, Oberkochen, Germany) by the Axio Vision 4.8. software (Zeiss, Oberkochen, Germany).

2.7. Scanning Electron Microscopy Assay

Scanning electron microscopy (SEM) was performed to add a visual aspect of the effects of used antibiotics on the biofilm morphology and structure. The samples were prepared in simple way by applying a small drop of bacteria cells suspension on the dedicate microscope slide. Then, the specimens were completely dried and directly examined by the scanning electron microscopy (SEM) (SEM/FIB—Quanta 3D FEG, FEI, Gräfelfing, Germany). Moreover, this technique was also applied to observe the changes in biofilm structure after the CZE-UV technique. In addition, the changes in biofilm surface were also pictured for the collected fractions using the capillary electrophoresis technique. The fractions were collected based on all recorded signals on the electropherogram.

3. Results

3.1. Molecular Identification of Bacteria Biofilm Formation

The identification of the bacterial isolates was carried out by the polymerase chain reaction (PCR) and sequencing of a conserved 16S rRNA gene fragment. For the PCR reaction, two commonly used primers, 27F and 1492R, were used. Regarding the PCR method, isolated bacteria was identified at 99.78%, as a Gram-positive *B. tequilensis* strain. The evolutionary timeline of the study bacteria was presented by constructing a phylogenetic tree (Figure 1).

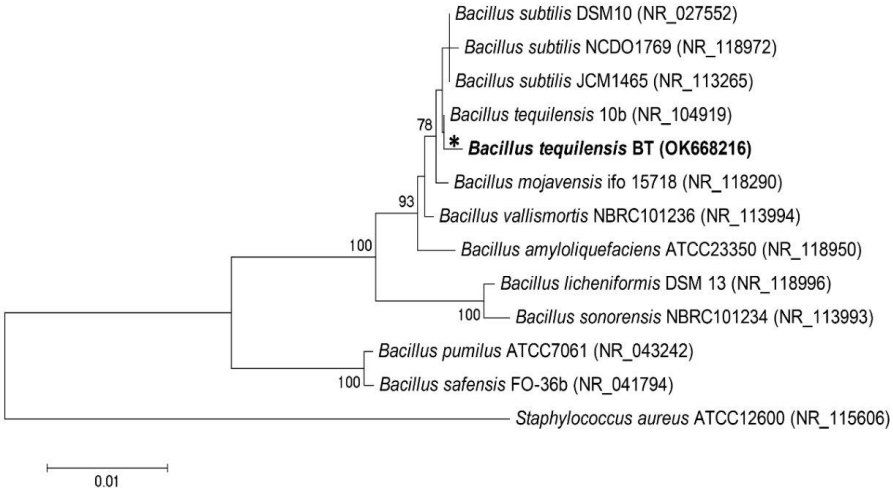

Figure 1. The evolutionary timeline of the study bacteria as a phylogenetic tree. *—indicate the isolated strain and used in present study.

We determined the evolutionary history based on the Neighbor-Joining method [22]. For the percentage of replication trees in which related taxa clustered along in the bootstrap test (500 replicates), it is presented next to the branch [23]. The tree is plotted to scale, with the branch lengths being of the same units as the evolutionary distances used to draw the phylogenetic tree inference. Therefore, the evolutionary distances were calculated according to the p-distance method [24] and given in units of the number of baseline differences for each position. The data evaluation was performed in the program MEGA7. It was observed that *B. tequilensis* next to *Bacillus subtilis* created the biofilm after 12 h of incubation, through the creation of endospores [18]. *Bacillus* sp. are model bacterial strain to the biofilm formation [25].

3.2. CZE Assay

After the molecular identification, the migration of untreated and treated bacteria cells with the chosen antibiotics was performed via the CZE-UV technique. Bacterial cells yielded signals with migration times longer than thiourea (2.58 min), confirming the negative bacterial charge. On the basis of the electropherograms (Figure 2), it can be observed that the intensity of the signals depends on the used antibiotic. In case of amoxicillin and gentamicin, the intensity of the signals was significantly lower compared to control. The large number of signals in the case of control may indicate a natural tendency to form aggregates. The sum of the surface area signal for the control was 100% (2,170,436), 14.75% (320,311) for amoxicillin, 19.67% (426,867) for gentamicin and 125.89% (2,732,344) for metronidazole. This may indicate that the remaining bacterial cells create the resistance effect. Moreover, the electropherogram of the bacterial cells treated with metronidazole was observed to be closed to the control sample. What is more, after the addition of the antibiotics, the disappearance of the last signals was registered at 12.10 ± 0.03, 12.23 ± 0.03 and 12.42 ± 0.05 min with 87.40, 23.25 and 6.45 mAU, respectively, compared to the control. This aspect indicates the presence of the clumping phenomenon (disappearance of large aggregates) (Figure 2). Based on Figure 2, it can be seen that the signal at 4.22 min in the control had the highest intensity and area (231,793).

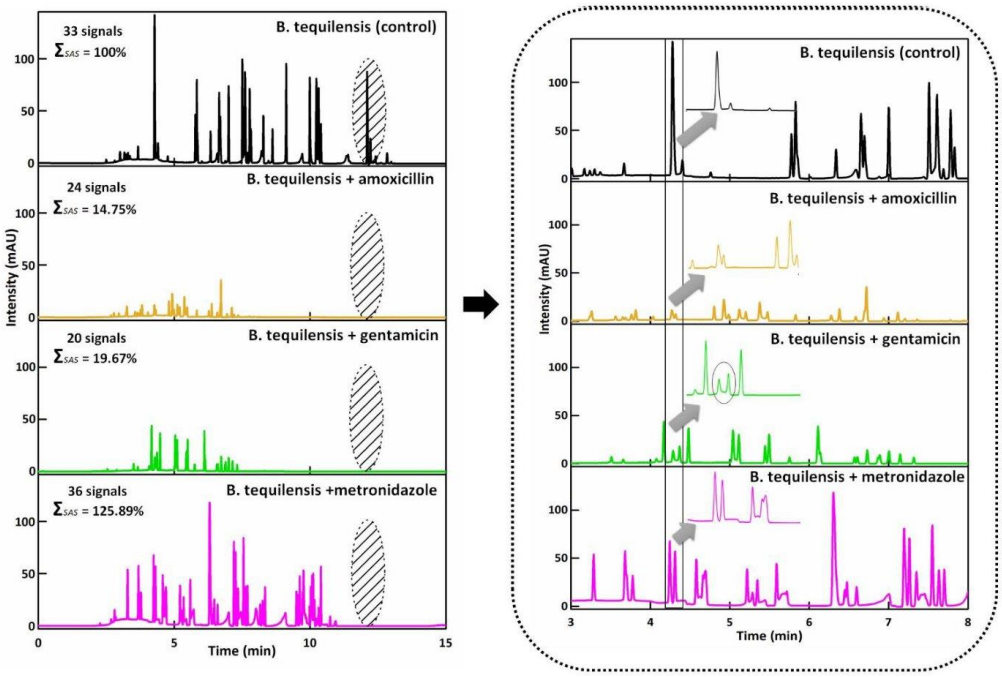

Figure 2. Electropherograms of the *B. tequilensis* treated antibiotics. Electrophoretic conditions: inlet buffer TB (pH 7.3), outlet buffer TBH (pH 8.0), U—20 kV, T—25 °C, injection—8 psi/10 s, λ = 214 nm, capillary—L_{tot} = 70 cm; L_{eff} = 50 cm; and i.d. 75 µm. ΣSAS—sum of surface area signal.

However, when the control was treated with antibiotics, the signals had a significantly lower area (amoxicillin 6152, gentamicin 15,171) and intensity. In the case of metronidazole, it can be seen that the signal was divided into two signals with the surface area signal equal to 88,124 and 63,942. In addition, in case of metronidazole, the surface area signal was noticed to be less compared to the control.

It was reported by many authors that this technique can be a promising screening tool to control the changes in bacterial cell membranes after antibiotic treatment and it may predict the mechanism of their action. Kłodzińska et al. [26] demonstrated the interactions of antibiotics with bacteria isolated from patients with postoperative wound infections by capillary electrophoresis. It was noticed that, in case of the sample after the antibiotic administration, the spectrum generated by the *Escherichia coli* cells becomes diffuse and broad. This indicates the interaction of the antibiotic (gentamicin) with the bacterial cells. Moreover, this study elucidates the action mechanism of antibiotics on *E. coli* cells, suggesting changes in cell membrane permeability and the slow lysis of cells [26].

Indeed, one of the conditions for the formation of a monolayer of bacterial cells on a given surface is the overcoming of electrostatic repulsive forces by hydrophobic interactions of an attractive nature. According to the literature data [26,27], bacterial cells are usually endowed with a negative surface electric charge, the value of which depends on, among others from the strain, chemical structure and surface of the cell. The reason for charge formation in the bacterial cell wall was the dissociation of the surface group of the molecular component of cell walls (peptidoglycan and proteins).

The surface electric charge of cells influences the rate of their movement in an electric field and also the value of their electrokinetic potential, which characterizes the electric double layer around the bacterial cell. In our study, the applied capillary electrophoresis allowed us to understand the changes occurring in the cell membrane of biofilm-forming bacteria under exposure to antibiotics.

3.3. Zeta Potential Analysis

CE was reported to constitute one of the techniques involved in the biofilm characterization. However, it is necessary to underline that each technique has its limitations. In fact, only the combination of CE with another approach can give complimentary characteristics [13,27–29]. Therefore, in the present study, the zeta potential analysis was performed in order to elucidate the impact of various antibiotics with different action spectra on the biofilm strain. For the sake of comparison, the untreated cells were also investigated as a negative control (absolute value). For untreated cells the ZP value was found to be $\xi = -43.65 \pm 1.06$ mV while for treated cells $\xi = -51.50 \pm 1.44$ mV for amoxicillin (BT-AMOX), $\xi = -48.35 \pm 0.92$ mV for gentamicin (BT-GEN) and $\xi = -55.50 \pm 1.56$ mV for metronidazole (BT-MET). As is well known, the optimal value determining the stability of the dispersion is around ± 30 mV [27]. Therefore, since the established zeta potential value ($\xi = -43.65$ mV) for the untreated bacterial cells is more than -30 mV, such a system is considered stable [27]. Furthermore, it was noticed that the ZP value of bacteria cells treated with applied antibiotics caused an increase in the dispersive stability of the system, which may be caused by the increased impact of solvation resulting in the degradation of bacterial cells into smaller micellar systems.

It was demonstrated that the exposure to antibiotics can cause changes in bacterial surface characteristics. Thornsberry et al. reported [30] that β-lactams inhibit the stabilization of new cell-wall components. It was recognized that antibiotics can cause changes in zeta potential value before any signs of cell death appear [30]. Furthermore, Kłodzińska et al. [26,27] suggest that the zeta potential measurements are useful in order to understand the separation electrophoretic behavior and provide more information about the charge that is present on the surface of the bacteria cells [26,27].

The presence of an electrical charge on the surface of microorganisms consequently has a direct impact on aggregation and adhesion to solid surfaces, and can also serve as a parameter to distinguish between different bacterial strains. The electrical properties of bacterial cells can therefore be characterized by measuring the zeta potential, as one of the electrokinetic parameters related to dispersion stability and solvation phenomena, which is the electrical potential generated between the solution and the solid layer (capillary surface, stationary phase) [1,27].

We noticed that the value of zeta potential increases; therefore, the electrostatic interactions between bacterial cells and antibiotics will be stronger, the stability of the systems will increase and the size distribution will be more homogeneous. All the zeta potential values found in this study were negative and relatively high, indicating good physicochemical stability of colloidal suspensions.

3.4. MALDI-TOF MS Analysis

The MALDI-TOF MS technique was performed to follow the molecular level changes of the untreated bacteria cells and cells affected by the antibiotic treatment. The UNIPROT database was used to associate the selected signals with the corresponding protein names and their function. The mass spectra (Figure 3A) presents the common signals recorded at 2882 and 6934 m/z in case of all investigated samples.

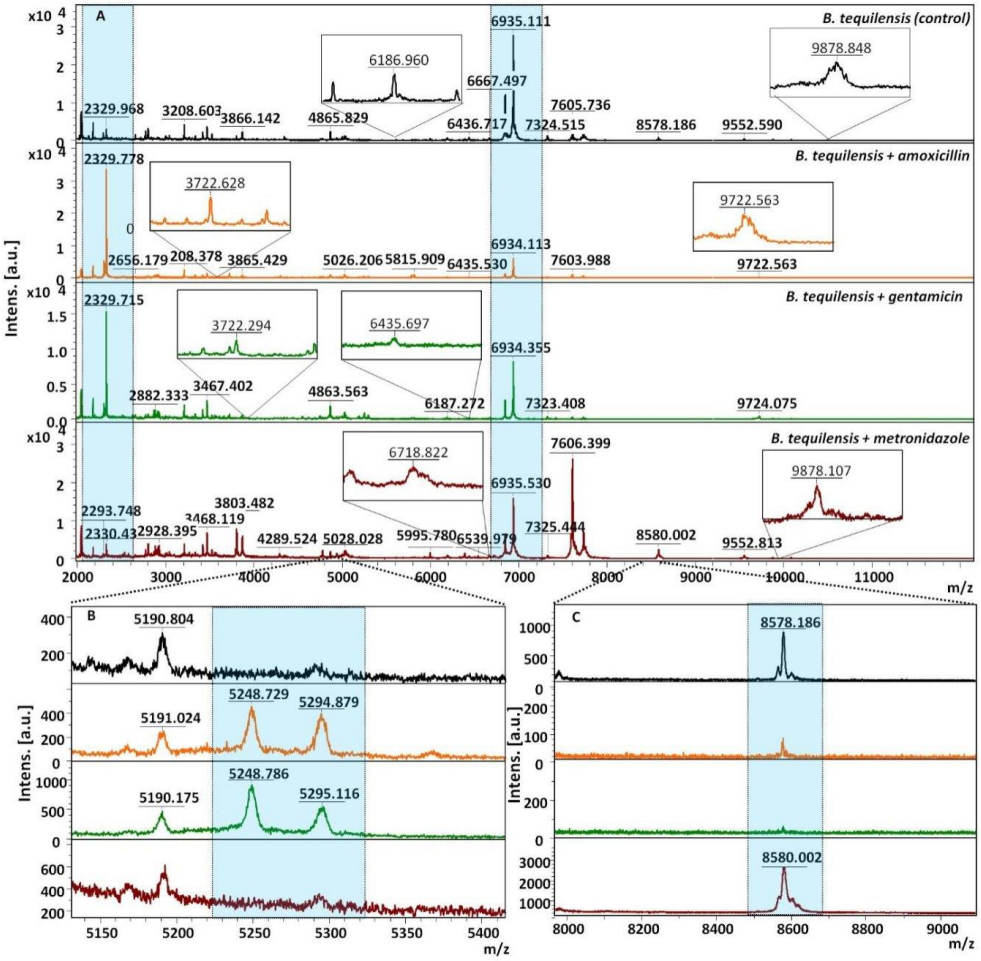

Figure 3. MALDI-TOF MS mass spectra of bacteria—*Bacillus tequilenis* after influence of antibiotic drugs. (**A**) main MS spectra, (**B**,**C**) represent the zooms MS spectra.

The protein profile and intensity of these signals are dependent on the applied antibiotic. One can also observe three signals (m/z at 5190, 6187, 6435) occurring in case of treated and untreated bacteria cells that belong to sporulation protein [18–20]. Remarkably, for BT-AMOX and BT-GEN, the characteristic protein profile was noticed (Figure 3B). The typical changes appeared at 3711, 5248 and 5294 and 9722 m/z. According to the UNIPROT database, the protein responsible for the formulation of cellular spores was identified (3711 and 5248 m/z). It constitutes a structural component of the ribosome (5248 m/z), transcription (5294 m/z) and RNA binding (9722 m/z) [31,32]. The MALDI spectra recorded in the case of the cells treated with metronidazole is the most similar to the control, based on the weak action and mechanism of action involving stimulation of production of DNA-destroying compounds of microorganisms inside cells (Figure 3C). The signals at 6720, 9552 and 9878 m/z are responsible for the constitution of the transcriptional, regulation and proper spore morphogenesis (one of the formation mechanisms of biofilm), respectively, of the ribosome [32,33]. Additionally, the disappearance of some signals (50 ribosomal protein (6720 m/z), in the spore morphogenesis (9878 m/z) was noticed [32,33]. In the case of the biofilm treatment with amoxicillin and gentamicin, the strong effect of these antibiotics and their mechanisms of action were indicated. Amoxicillin is responsible for inhibiting cell wall synthesis while gentamicin blocks the ribosomal protein synthesis.

In the case of metronidazole, the molecular profile was the most similar to the control, indicating a limited entry of antibiotic molecules into the cells and, therefore, missing access to their molecular target, which is the microbial DNA [31–33].

Figure 4 presents a comparison between the protein profiles of *B. tequilensis* cells untreated and treated with antibiotics (Figure 4A–D), representing a comparison of signals appearing noticed on MALDI-TOF mass spectra for control *Bacillus tequilensis* cells (x axis) and under antibiotics treatment (y axis) which were proteins (marked with different numbers #1–#10) identified using UNIPROT data first of all, with base proteins being listed in the figure captions with their m/z values. The recorded signals were clustered together mainly in three clusters. In the case of cells treated with metronidazole, the clustering way was close to the control samples (Figure 4A,B).

This indicates that metronidazole displays weak profile changes. In turn, in the case of the samples treated with amoxicillin and gentamicin, the clustering takes place differently; the profile clustering was very similar in those cases (Figure 4C,D), which suggested a similar effect on the proteomic profile of the *B. tequilensis*. Moreover, the cluster groups are different compared to the control. First of all, the downregulation of the proteins associated with the regulation of the protein translation and transcription along with the proper structure of the ribosomes in amoxicillin and gentamicin treated cells were noted: 6720 m/z—50S ribosomal protein L32 (structural constituent of ribosome; Uniprot, [32]), 9552 m/z—Protein Veg (regulation of transcription, DNA-templated; Uniprot, [33]). Furthermore, MS profiles of the treated cells lacked signals derived from the protein YwcE required for proper spore germination: disturbance of the spore production is mainly marked by a lack of the signal 9878 m/z in the *B. tequilensis* cells treated with gentamicin and amoxicillin.

SEM images from amoxicillin and gentamicin variants supported these findings, since disrupted cells or cells with distorted cell wall morphology were not accompanied by the presence of spore-like structures, which should appear after cell envelope destruction when the sporulation process is not disturbed. Interestingly, the addition of the metronidazole failed in the expression of the mentioned cellular components, although its mode of action is that it, when diffused into the organism, inhibits protein synthesis by interacting with the DNA. Such observations suggested different biofilm penetration abilities by amoxicillin and gentamicin compared to metronidazole; namely, the latter failed to reach the molecular target inside a cell in sufficient amounts to reveal biological activity against *B. tequilensis*.

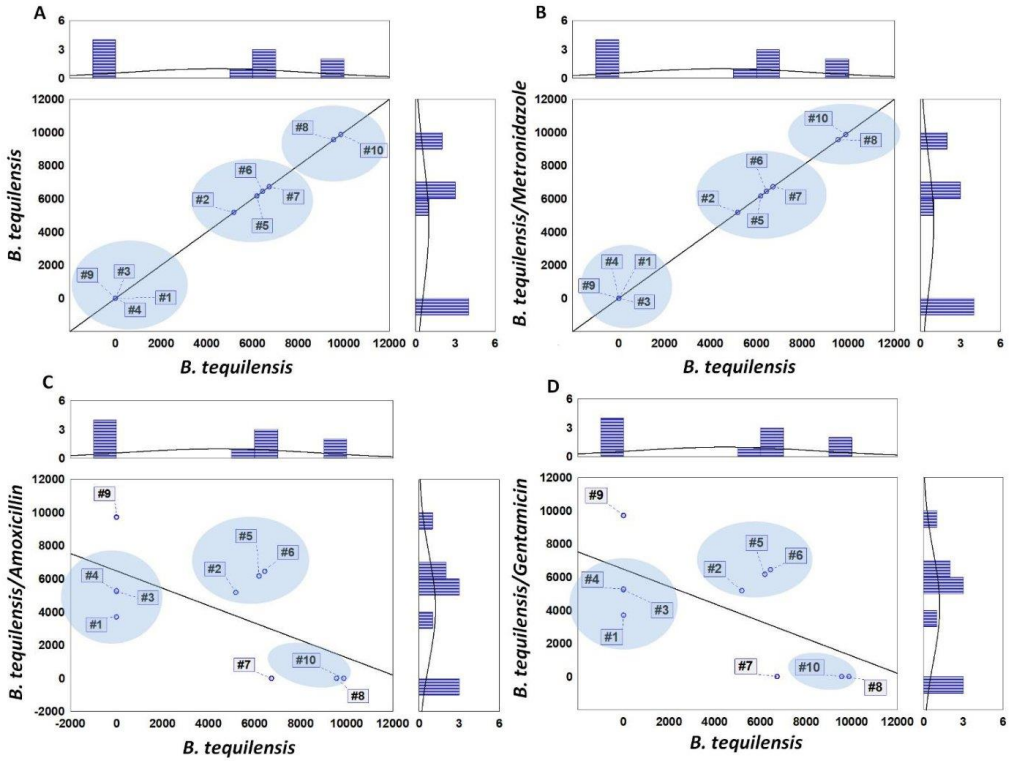

Figure 4. Comparison of signals noticed on MALDI-TOF mass spectra for *Bacillus tequilensis* (control) and under antibiotics treatment: (**A**) control, (**B**) *B. tequilensis*/metronidazole, (**C**) *B. tequilensis*/amoxicillin, (**D**) *B. tequilensis*/gentamicin. #1—3711 m/z, #2—5190 m/z, #3—5248, #4—5294 m/z, #5—6184 m/z, #6—6435 m/z, #7–6720 m/z, #8—9552 m/z, #9—9722 m/z, #10—9878 m/z.

Pereira et al. [34] evaluated the MALDI-TOF mass spectrometry to analyze the molecular profile of *Pseudomonas aeruginosa* biofilms grown on glass and plastic surfaces at different stages of biofilm development. Results from molecular studies showed that profiling based on MALDI is not able to distinguish between different stages of biofilm development, but it may be observed when biofilm cells are released in the dispersion stage, which occurred first on the polypropylene surface. Furthermore, the present study indicates that MALDI profiling may become a promising technique for a clinical diagnosis and prediction of the biofilm formation development [34]. Additionally, Si et al. studied [35] the molecular heterogeneity in *B. subtilis* colony biofilms by using the MALDI-TOF MS. In this work, they combined the MALDI and fluorescence method, which permitted the detection of distinct cell populations in the biofilm [35]. In our study, we demonstrated that matrix-assisted laser desorption time-of-flight mass spectrometry with the UNIPROT database can be used as a complimentary technique to CE to study differences in the molecular profile of *B. tequilensis*, after antibiotic treatment. Similarities of molecular profiles of control and bacteria treated with metronidazole were observed; the same was registered in electropherograms of the bacteria strain with antibiotics.

3.5. Fluorescence Microscopy

Fluorescence microscopy was performed in order to supplement the hitherto obtained observations concerning the investigation of the viability of bacterial cells under antibiotic treatment. Figure 5 depicts the fluorescence images of the untreated biofilm formed by *B. tequilensis* bacteria and treated with antibiotics. Amoxicillin and gentamicin showed reddish-yellow colored areas, indicating the presence of damage in the bacterial cells.

In addition, a significant destruction of the surface of the biofilm formation was observed, especially with gentamicin. Moreover, after the metronidazole treatment, a slight surface changes of the biofilm can be noticed. This aspect indicate the low effectiveness of the respective antibiotics. The impact of antibiotic on *B. tequilensis* (control) formation was as follows GEN > AMOX > MET.

Amoxicillin and gentamycin are the strong bactericidal antibiotics. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST), their minimum inhibitory concentration (MIC) is 2 μg/mL for amoxicillin and 0.5 μg/mL for gentamicin [19]. Therefore, both antibiotics induced bacteria biofilm damages and even bacterial cells damages of *B. tequilensis*. Notably, metronidazole had a measurable effect on the biofilm viability. However, this could be associated with its weak bactericidal activity (MIC = 8 μg/mL) [19].

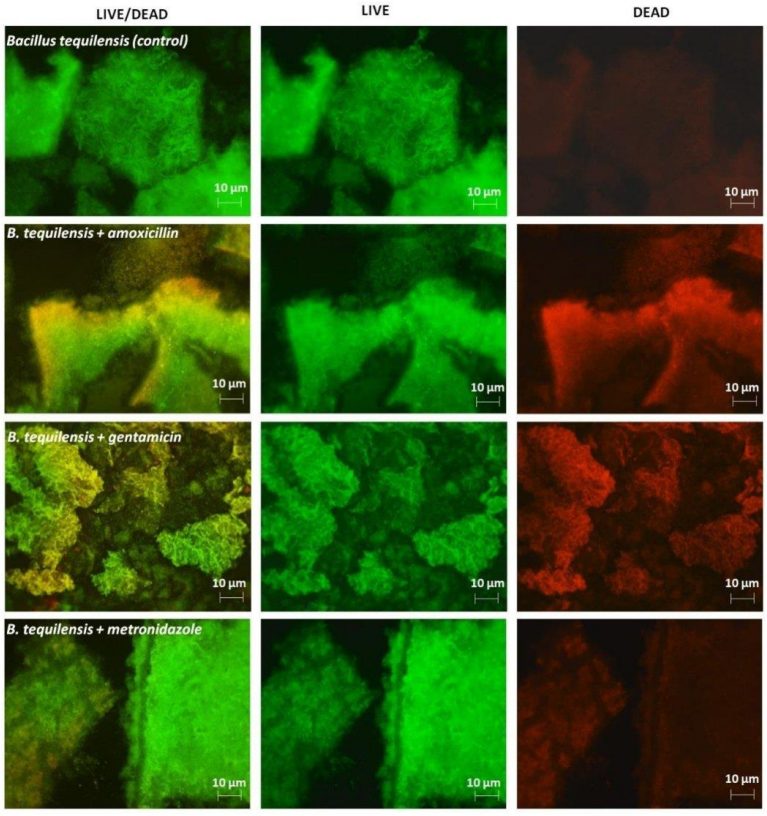

Figure 5. Fluorescence images of the biofilm formed by *B. tequilensis* bacteria treated with antibiotics, at magnifications of 100×.

On the other hand, earlier research suggested that antibiotics move through biofilms quite rapidly. However, Tseng et al. [36] analyzed the penetration of two clinically relevant antibiotics, tobramycin (aminoglycoside) and ciprofloxacin (fluoroquinolone), into *Pseudomonas aeruginosa* biofilms by using confocal fluorescence microscopy. They demonstrated that the positively charged antibiotic tobramycin is sequestered at the biofilm periphery, whereas the neutral antibiotic ciprofloxacin readily penetrates. Furthermore, they evidenced that tobramycin applied at the biofilm periphery both stimulated a localized stress response and caused the death of bacteria cells those regions, but not in the deeper layers of the biofilm [36]. In our study, a similar observation was noticed in reference to *B. tequilensis* cells treated with gentamicin. Except for the efficient mechanism action on gentamicin, the surface bacterial strain also was sensitive to the respective antibiotic, in contrast to the deeper layer as was noticed on the SEM results before and after electrophoresis (Figures 6 and 7).

Oubekka et al. [37] studied the influence of vancomycin for the biofilm created by *S. aureus* human isolates by the advanced fluorescence microscopy method (fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and fluorescence lifetime imaging (FLIM)). They demonstrated that at therapeutic concentrations of vancomycin, the biofilm matrix was not an obstacle to the diffusion reaction of the antibiotic, which can reach all cells through the biostructure [37].

To conclude, we can suggest that the bacterial cells in the deeper layers of the biofilm have the ability to adapt to the environment and acquire resistance mechanisms as a consequence of molecular mechanisms. It can be assumed that the results are correlated with the findings received by using the MALDI-TOF MS technique and CE.

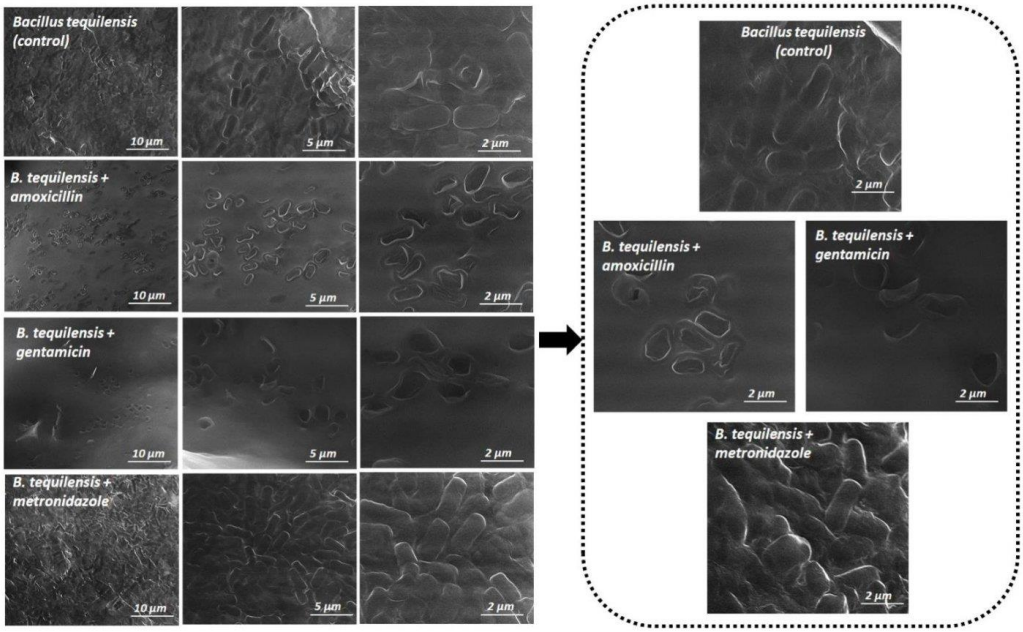

Figure 6. SEM images of the biofilm formed by *B. tequilensis* bacteria treated with antibiotics, before capillary electrophoresis at magnifications of 10.000×; 25.000× and 50.000× respectively.

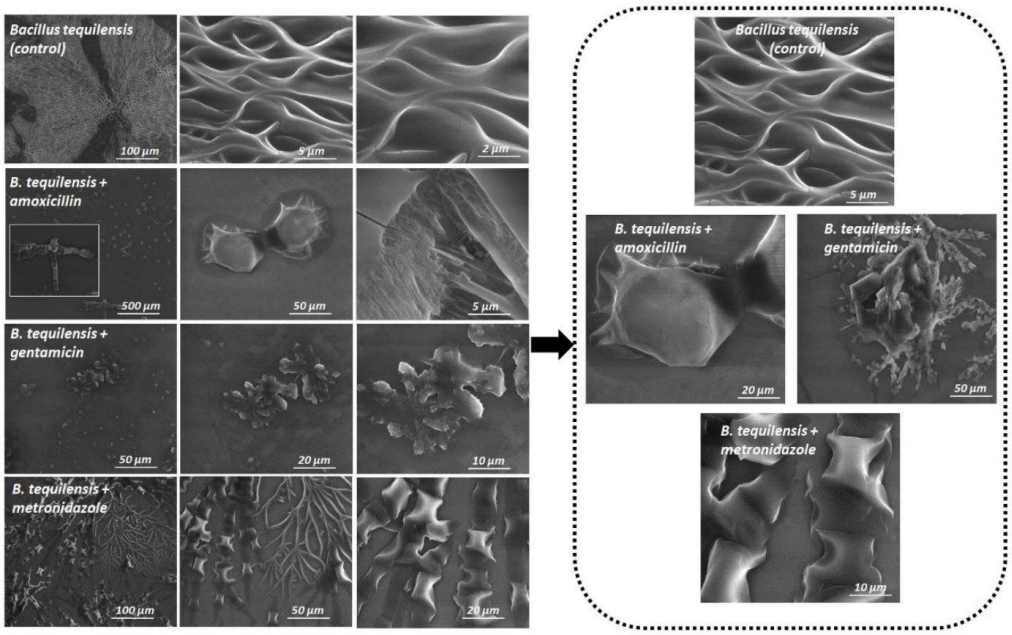

Figure 7. SEM images of the biofilm formed by *B. tequilensis* bacteria treated with antibiotics, after capillary electrophoresis, at magnifications of 1.000×; 2.500×; 10.000×; 25.000× and 50.000× respectively.

3.6. Scanning Electron Microscopy

In order to check the changes on the surface and morphology of the biofilm under the influence of tested antibiotics, scanning electron microscopy (SEM) imaging was applied. Moreover, such technique was used as a complimentary approach to prove the capillary electrophoresis results and to monitor how different antibiotics with different action mechanisms can influence the migration of the investigated bacterial cell in an electric field.

Figures 6 and 7 present the SEM images of the biofilm formation of *B. tequilensis* bacteria treated with antibiotics, before (Figure 6) and after the capillary electrophoresis analysis (Figure 7). On the basis of the SEM results, before and after electrophoresis, two aspects were observed: the changes on the surface area of the biofilm and the inhibition of the bacteria growing. As can be seen in Figures 6 and 7, the strong effect was observed for GEN and AMOX, compared to the control. Nonetheless, the effect of metronidazole was slightly similar to the untreated cells. Gentamicin significantly destroyed the bacterial cells. On the other hand, after the metronidazole treatment, the change was mainly in the biofilm surface.

Bacterial cells treated with metronidazole were less affected on the surface of the biofilm compared to the amoxicillin and gentamicin treatment. The shape of biofilm-forming cells was also found to be significantly modified depending on the antibiotic used.

After the metronidazole treatment, cells adhered to the surface were more elongated and had a narrow size compared to the amoxicillin and gentamicin exposed, whereas the bacterial cells of BT-AMOX and BT-GEN had a similar oval shape.

Remarkably, the different correlations in the change of the biofilm area and morphology in the case of treated cells were observed after the capillary electrophoresis (CZE-UV) analysis (Figure 7).

Notably, following the CZE-UV, clustering was noticed in both the treated and untreated cells of the biofilm. They created complex structures with different shapes. However, in the case of metronidazole, besides the overlapping cells with a similar pillow shape, the presence of branching, which is also present in the control, was also noticed. It was observed that bacterial cells treated with gentamicin formed more clusters than those treated with amoxicillin.

The SEM images of biofilms treated with amoxicillin and gentamicin displayed substantial changes to the biofilm structure—fewer cells and larger areas without cells. Thus, both of these antibiotics kill bacteria and clear large sections of the biofilm, thereby penetrating inside the structure to exert their bactericidal effects. The images obtained seem to reflect the proteomic changes noted during the MALDI analysis—mostly related to the disturbance of proper spore production. Indeed, a similar observation was noted in the work by James et al. 2018 [38] for the *Clostridium difficile* biofilm treated with surotomycin and fidaxomicin, where both vegetative cells and spores were killed by the aforementioned antibiotics, which were reflected in different biofilm images than the control one, thus negating the protective role of the biofilm structure in that case. Moreover, the obtained images reflected mechanisms of action under the influence of both antibiotics—amoxicillin disturbs proper cell wall formation, which prevents the proper process of cell division (whole cells but deformed morphology of the cell wall), while gentamicin deregulates the bacterial protein synthesis by irreversibly binding to 30S ribosomes and inducing a significant increase in the misreading of messenger RNA (disintegrated cells). It is also significant that one important structural feature of biofilms is that the bacteria are embedded in a self-reproducing EPS matrix. These proteins include polymers that impede the transport of antibiotics into the biofilm. Hence, the biofilm matrix can provide a protective barrier by neutralizing antibiotics [8]. However, depending on the antibiotic treatment, their penetration through the matrix is different. Andert et al. [39] studied the penetration of ciprofloxacin and ampicillin for the biofilm created by *Klebsiella pneumoniae*. They noticed that ampicillin was unable to penetrate and was captured by high concentrations of β-lactamases secreted by bacteria living in the biofilm [39]. Since amoxicillin demonstrated a visible destroying effect on the investigated *B. tequilensis* biofilm, the accumulation of antibiotic-degrading enzymes such as β-lactamases in the biofilm matrix as a defense mechanism should be excluded. Contrary to this, the penetration of the biofilm through the metronidazole seems to be limited—biofilm morphology was similar to the control. Metronidazole inhibits the bacterial protein synthesis by interacting with the DNA, thus, such an observation may suggest the presence of the extracellular DNA (eDNA) in the biofilm matrix produced by investigated *B. tequilensis* strains that interact with metronidazole and prevent it entering cells. eDNA is a significant and common component ingredient of the bacterial biofilm matrix that can increase the biofilm's resistance to certain antimicrobial agents [40].

Through this technique in our study, changes in the surface layers of the biofilm could be seen depending on the antibiotic used both before and after capillary electrophoresis (Figures 6 and 7). The changes in the surface of the bacteria cells correlate with the results in the deeper layers of the biofilm.

On the basis of the findings achieved by capillary electrophoresis, the MALDI-TOF MS technique and microscopic studies, we proposed the mechanism of action of the studied antibiotics (amoxicillin, gentamicin, metronidazole) on the biofilm formation (Figure 8).

The proposed scheme is concerned with the mechanism of studied antibiotics on the phenomenon of clumping and degradation in the bacterial cell of *B. tequilensis*. According to MALDI profiles, the molecular changes induced by the used antibiotic and identified by the UNIPROT database are related to aggregates formation, visible by CE. The creation of aggregates results in changes in the charge measured by the ZP approach. More destroyed or sensitive bacterial cells creates a more dispersive system and less aggregation in fused silica under the CE analysis. The main mechanism included: the degradation of cell wall synthesis; the disturbance of the cell membrane; the inhibition of the DNA and ribosomal proteins; and the dysfunction of transcription. Regarding observed data that can decipher

potential molecular mechanisms involved in biofilm development disturbance, both SEM images, CE analysis as well as results of the proteins' profile changes indicated significant differences between investigated antibiotics in terms of cell morphology, presence or lack of specific proteins related to spore production, protein transcription and regulation, reflected by different electrophoregram images.

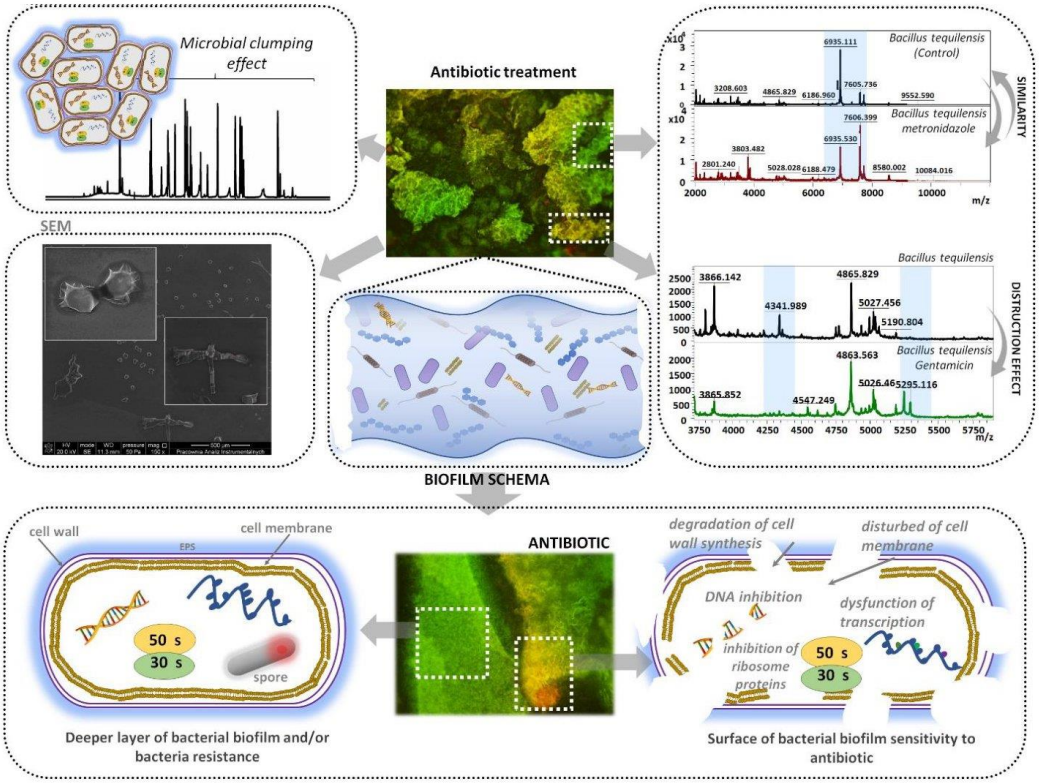

Figure 8. Scheme of mechanism of bacterial biofilm response—*B. tequilensis*.

4. Conclusions

This study is the first complex and complementary approach in the characterization of biofilm both before and after antibiotic therapy. Owing to the 16S rRNA gene sequencing method, the bacteria isolated from honey was identified as a model of biofilm formation—*Bacillus tequilensis*.

Remarkably, capillary electrophoresis was found to be a useful tool in biofilm characterization. The application of this technique enabled us to observe changes in the electrophoretic mobility of bacterial cells untreated and treated with antibiotics (amoxicillin, gentamicin, metronidazole). It was noted that the treatment with amoxicillin and gentamicin caused the disappearance of large aggregates (clumping phenomenon). In this case, there were strong changes in the electrical charge on the cell surface. Furthermore, the use of complementary techniques allowed us to monitor differences in dispersion stability and molecular profiles, along with the viability and morphology of bacterial cells creating the biofilm when exposed to antibiotics.

Once the zeta potential increases, therefore, the electrostatic interactions between bacterial cells and antibiotics will be stronger, the stability of the systems will increase and the size distribution will be more homogeneous.

The increasing of the zeta potential value indicates that strong electrostatic interactions between bacterial cells and applied antibiotics were observed. The treated and untreated bacterial suspensions showed negative zeta potential values, which is related to a good stability of colloidal systems.

The MALDI-TOF MS analysis indicated changes in the molecular profiles of *B. tequilensis* before and after the antibiotic therapy, leading to proposed mechanisms of antibiotic resistance. However, the use of fluorescence microscopy showed that bacterial cells in the deeper layers of the biofilm are able to adapt to the environment and acquire resistance mechanisms as a result of molecular mechanisms. Finally, scanning electron microscopy was used to observe changes in the biofilm surface under the influence of antibiotics both before and after capillary electrophoresis. According to microscopic and mass spectrometric analyses, it was noticed that amoxicillin and gentamicin caused the degradation of the cell wall synthesis, the disturbance of the matrix of extracellular polysaccharide substances surrounding the biofilm (EPSs) and the inhibition of ribosomal proteins and the dysfunction of transcription. This phenomenon is correlated also with the noticed higher absolute value of ZP and less registered signals on CE. It should be pointed out that the results obtained by MALDI-TOF MS, fluorescence microscopy and SEM were corelated with the capillary electrophoresis method. Therefore, in this study, we proposed a mechanism of examined antibiotics (amoxicillin, gentamicin, metronidazole) on biofilm formation, associated with the phenomenon of clumping and degradation, which may facilitate the treatment of bacterial infections related to biofilm.

Author Contributions: Conceptualization, B.B., P.P., V.R.-P. and K.P.; methodology, V.R.-P., K.P. and M.Z.; formal analysis, K.P., M.Z. and V.R.-P.; investigation, K.P. and V.R.-P.; writing—original draft preparation, K.P.; writing—review and editing, V.R.-P., B.B., M.Z., P.P. and M.S.-M.; visualization, K.P. and V.R.-P.; supervision, B.B.; project administration, M.S.-M. All authors have read and agreed to the published version of the manuscript.

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3.5. *Characterization of salivary microbiome before and after antibiotic therapy via separation technique*

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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Characterization of the salivary microbiome before and after antibiotic therapy via separation technique

Katarzyna Pauter-Iwicka[1,2] · Viorica Railean[3,2] · Michał Złoch[1,2] · Paweł Pomastowski[2] · Małgorzata Szultka-Młyńska[1] · Dominika Błońska[1,2] · Wojciech Kupczyk[4] · Bogusław Buszewski[1,2]

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Abstract
In the present research, the MALDI-TOF MS technique was applied as a tool to rapidly identify the salivary microbiome. In this fact, it has been monitored the changes occurred in molecular profiles under different antibiotic therapy. Significant changes in the composition of the salivary microbiota were noticed not only in relation to the non antibiotic (non-AT) and antibiotic treatment (AT) groups, but also to the used media, the antibiotic therapy and co-existed microbiota. Each antibiotic generates specific changes in molecular profiles. The highest number of bacterial species was isolated in the universal culture medium (72%) followed by the selective medium (48% and 38%). In the case of non-AT patients, the prevalence of *Streptococcus salivarius* (25%), *Streptococcus vestibularis* (19%), *Streptococcus oralis* (13%), and *Staphylococcus aureus* (6%) was identified while in the case of AT, *Streptococcus salivarius* (11%), *Streptococcus parasanguinis* (11%), *Staphylococcus epidermidis* (12%), *Enterococcus faecalis* (9%), *Staphylococcus hominis* (8%), and *Candida albicans* (6%) were identified. Notable to specified that the *Candida albicans* was noticed only in AT samples, indicating a negative impact on the antibiotic therapy.
The accuracy of the MALDI-TOF MS technique was performed by the 16S rRNA gene sequencing analysis—as a reference method. Conclusively, such an approach highlighted in the present study can help in developing the methods enabling a faster diagnosis of disease changes at the cellular level before clinical changes occur. Once the MALDI tool allows for the distinguishing of the microbiota of non-AT and AT, it may enable to monitor the diseases treatment and develop a treatment regimen for individual patients in relation to each antibiotic.

Key points
- *The salivary microbiota of antibiotic-treated patients was more bacteria variety*
- *MALDI-TOF MS is a promising tool for recording of reproducible molecular profiles*
- *Our data can allow to monitor the treatment of bacterial diseases for patients*

Keywords Antibiotics · Therapy · Salivary bacteria · Microbiota · Mass spectrometry · Proteomics

✉ Bogusław Buszewski
bbusz@umk.pl

[1] Department of Environmental Chemistry and Bioanalytics, Faculty of Chemistry, Nicolaus Copernicus University, Gagarina 7, 87-100 Torun, Poland

[2] Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Wilenska 4, 87-100 Torun, Poland

[3] Department of Infectious, Invasive Diseases and Veterinary Administration, Institute of Veterinary Medicine, Nicolaus Copernicus University in Torun, Gagarina 7, 87-100 Toruń, Poland

[4] Department of General, Gastroenterological&Oncological Surgery Collegium Medicum, Nicolaus Copernicus University, Torun, Poland

Introduction

Since the time when the first antibiotics were introduced to treat bacterial infections, drug resistance to pathogens has become a serious health problem. The reasons include various factors such as an irresponsible dosage of antibiotics, naturally occurring mutations, and the transmission of drug-resistant strains. Microorganisms can have both positive and negative impacts. Many of them can make food go bad and cause serious diseases. For this reason, it is extremely important to search for quick and reliable methods to identify the basic infectious agents such as bacteria, which is particularly important in the medical diagnostics (Jackowski et al. 2008; Pauter et al. 2020).

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Personalized treatments are one of the most important achievements of modern medicine (Garzón et al. 2020). For this field to develop it is necessary for specialists in the field of biology, genetics, biotechnology, bioinformatics, and pharmacology to cooperate with the medical community. This leads to an innovative approach in the diagnostics and, in consequence, in the medical treatment by improving or adapting the pharmacological therapy to the individual needs of patients, the so-called targeted pharmacological therapy, "tailor-made therapy" or personalized medicine (Borg-Bartolo et al. 2020).

On the other hand, the diversity and composition of saliva microbiota seem highly important for the human health and disease. Hence, in the recent years, saliva has attracted widespread interest as a means of simple and rapid testing because the composition of it might reflect the health status. The quick identification of the pathogen causing the infection will enable the implementation of an appropriate therapy (Jackowski et al. 2008; Pauter et al. 2020). Currently, the MALDI-TOF MS is used with great success (Złoch et al. 2020b). The worthwhile point is that this technique is often chosen in the identification of microorganisms for routine clinical testing (Hou et al. 2019; Duncan and DeMarco 2019; Van Belkum et al. 2017).

The human oral microbiome is one of the most active environments for many species of bacteria, where they undergo an extensive interspecies competition to form a multispecies biofilm structure. These bacteria are also present in saliva; they constitute many hundreds and thousands of species, some of which are unique to this specific habitat (Gao et al. 2018). *Streptococcus salivarius* is considered to be the first human oral colonizer at birth and can therefore play a role in setting up immune homeostasis and controlling the inflammatory reactions of the host. *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus anginosus* prefer to colonize on oral soft tissues and saliva, while *Streptococcus sanguinis* tends to colonize on teeth (Abranches et al. 2018). There are also opportunistic species among *Streptococcus* bacteria like *Streptococcus mutants*. Its contribution to caries development is well established (El-sherbiny 2014;Koo and Bowen 2014). Moreover, various *Lactobacillus* species, especially *L. fermentum*, *L. rhamnosus*, *L. salivarius*, *L. casei*, *L. acidophilus*, and *L. plantarum* are frequent mouth inhabitants and studies show that they antagonize harmful microorganisms and reinforce the dental health (Koll-Klais et al. 2005; Badet and Thebaud 2008; Wasfi et al. 2018).

The other group of microbes in the oral cavity includes *Candida* species, especially during a long-term antibiotic therapy (Muzyka and Glick 1995). In many individuals, *C. albicans* is a minor component of their oral flora, which does not generate any clinical symptoms (Cannon and Chaffin 1999). In contrast, when the balance of the microbiota in the oral cavity is disturbed, candida seeks to colonize the oral tissue by creating a biofilm with *Streptococcus*, which plays a pathogenic role (Tsui et al. 2016; Koo et al. 2018).

According to the recent medical reports and current scientific knowledge, a change in the balance of the oral bacterial composition has the potential to signal pathological conditions. This includes diseases such as halitosis (Haraszthy et al. 2007), caries (Guo and Shi 2013), and periodontosis (Ko et al. 2020), but also systemic diseases including breathing diseases (Gomes-Filho et al. 2010), diabetics (Sabharwal et al. 2019) along with cardiovascular diseases (Fernandes et al. 2014), and cancer (Mager et al. 2005). The characteristics of the salivary microbiome in obese subjects also received attention (Al-Rawi and Al-Marzooq 2017). Nevertheless, healthy salivary bacterium should be identified primarily to describe the changes caused by the disease, which may eventually lead to the development of diagnostic tools to improve the treatment or prevent the disease (Espuela-Ortiz et al. 2019). Additionally, these several studies indicate that salivary bacteria biomarkers in the oral cavity constitute a recognized diagnostic and prognostic tool for a variety of diseases. Hence, many activities were undertaken using hyphenated methods based on the bacterial ribosomal proteins determination (MALDI-TOF MS) (Stîngu et al. 2008; Sun et al. 2016) along with volatile organic compounds (VOCs) detection (gas chromatography-mass spectrometry, GC-MS) (Milanowski et al. 2019). Nevertheless, most of the research on oral microbes utilize the 16SrRNA-based technique (Hrynkiewicz et al. 2008).

The literature often focuses on pathogenic microorganisms and the assessment of their significance in the etiology and course of infectious diseases along with the spread of drug resistance to commonly used antibacterial drugs. However, what is interesting is the fact whether and what differences in the prevalence of the bacterial strain colonization occur in people with bacterial infections undergoing the antibiotic therapy compared to non-antibiotic therapy. Hence, in this study, the salivary microbiota after antibiotic treatment was described. The MALDI-TOF MS technique as a tool to provide a rapid diagnosis and identification of microbiota was used; different media were investigated in order to achieve a complimentary microbiota identification. At the same time, we utilized the 16S rRNA gene sequencing to determine the selected salivary bacteria in order to obtain information on the effectiveness and accuracy of the investigated spectrometric method. Additionally, the present research focused on checking the ability of the MALDI technique for the investigation of fast monitoring of the patients under antibiotic therapy. The samples from 14 patients not treated with antibiotics were intended to determine possible changes in the local population related to both local epidemiological factors and hospitalization

factors. This study was performed to determine the possible impact of the hospital environment on changes in the patient's microbiome after 10 days of hospitalization with or without antibiotic treatment. Therefore, in the population of patients subjected to antibiotic therapy, the focus was on changing the above-mentioned profile, which allowed to separate the changes resulting from the hospitalization itself from those caused by antibiotic therapy. As we have presented in the profile of patients undergoing antibiotic therapy, we have noticed an increase in the diversity of strains; the emergence of bacteria typically associated with the surgical ward. The interesting point also was to compare if the MALDI technique would differentiate each administrated antibiotic in the relevant time period. Moreover, the optimal conditions of the growth medium for the identification of microorganism by using the MALDI-TOF MS were examined. A correlation between protein profile changes of the non-AT and AT microbiota was performed and studied in details. Additionally, the impact of antibiotic and pathogen's presence on the patients' therapy was described.

Materials and methods

Saliva samples preparation protocol

In this study, 38 samples were investigated; the saliva samples were provided from fourteen non-AT and twenty-four AT patients, who were hospitalized in very serious condition. The present research involved demographic data from 38 consecutive patients admitted to the Department of General, Gastroenterological and Oncological Surgery of the Nicolaus Copernicus University in Toruń. Patient data is strictly identified and marked with both name and surname, 11-digit ID number, description of the medical history number, date of sampling and type of antibiotic, its dose, and disease being the reason for its recommendation. Patients with diseases in the oral cavity were not eligible for the study. In some of the qualified patients, there were no indications for antibiotic therapy. This group was used to determine the local status of the salivary microbiome dependent on both population and hospitalization effects as mentioned. Initially, 42 patients were qualified for the study in the proportion of 14 non-antibiotic vs 28 antibiotic/2: 1 ratio; however, due to difficulties in obtaining the appropriate sample volume, 2 patients treated with an antibiotic were disqualified from the experiment—which was also a limitation of the study. Among the patients receiving antibiotic therapy, the participants undergoing the diabetic foot, surgical wounds, sinusitis, and phlegmon have been immediately subjected to the antibiotic therapy: azithromycin, amoxicillin, ciprofloxacin, clindamycin, cefotaxime and levofloxacin, metronidazole, and piperacillin. Non-antibiotic therapy patients were selected as a control. The average age of the subjects was 58.9 years old. Of these participants, 68% were men and the rest 32% were women. The present research did not classified the patients to the specific patient data (age, sex) once we were restricted in the sample number collection. Generally, all the patients were instructed to avoid eating, drinking, and brushing their teeth for 2 h before the saliva collection.

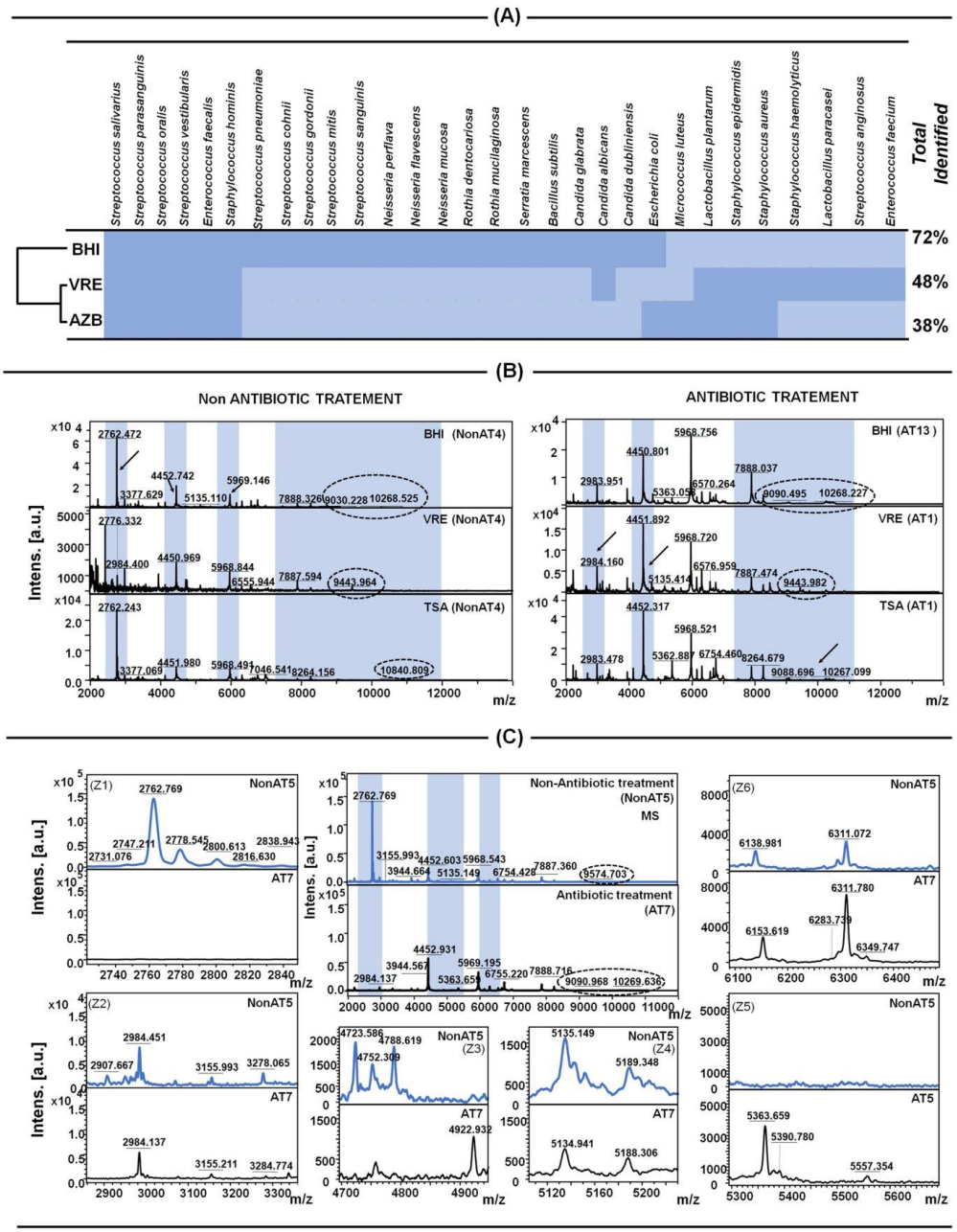
For the sample cultivation, an universal growth medium—*Brain Heart Infusion* (BHI) (Sigma-Aldrich, Germany) and two selective mediums such as the *Vancomycine Resistant Enterococci Agar Base* (VRE) (Sigma-Aldrich, Germany), and the *Azide Blood Agar BASE* (AZB) (Sigma-Aldrich, Germany) were chosen. The saliva was diluted with sterile peptone water (Sigma-Aldrich, Germany) in a 1:9 (v/v) ratio. The cultivation was performed by the serial dilution method based on the procedure of Abouassi and co-workers (Abouassi et al. 2014) with a slight modification. The peptone water was used instead of 0.9% NaCl to support the growth of the fastidious microorganisms. Subsequently, 100 µL of each suspension was streaked on the Petri dishes containing the chosen culture medium. Thereafter, they were incubated for 24 h (BHI, AZB) or 48 h (VRE) at a constant temperature of 37 °C in aerobic conditions. Moreover, the number of colony-forming units (CFU/mL) was determined by the colony counter (IUL S.A., Barcelona, Spain) and compared in non-AT and AT patients.

Identification of salivary bacterial microbiome

MALDI-TOF MS measurements

All fresh colonies isolated in the different medium (as described in "Saliva samples preparation protocol" section) were then used for the identification. Owing to problems with the identification by the MALDI-TOF MS on AZB (considered as a selective medium), the respective medium was changed to the Tryptic Soy Agar (TSA, Sigma-Aldrich, Germany). TSA is considered a universal medium and applied as a routine diagnostic medium. The colonies isolated on the AZB medium were transferred to the TSA medium, incubated for 24 h at 37 °C in aerobic conditions, then identified using the MALDI tool.

The standard extraction protocol was adopted from our previous study, Pauter et al. with some changes (Pauter et al. 2022). The modification included suspending the pellet in 150 µl of distilled water and adding 450 µl of ethanol. Afterwards, the pellet was centrifuged for 5 min at 20 °C, 14, 400 rpm, then the supernatant was removed. Subsequently, the vacuum concentration was used to dry the pellet (8–10 min). The 70% formic acid (Merck, 98–100%, Germany), acetonitrile (Fluka Analytical Sigma Aldrich, Germany), was added into the dried pellet (1,1), and then centrifuged


(A)
Streptococcus salivarius
Streptococcus parasanguinis
Streptococcus oralis
Streptococcus vestibularis
Enterococcus faecalis
Staphylococcus hominis
Streptococcus pneumoniae
Streptococcus cohnii
Streptococcus gordonii
Streptococcus mitis
Streptococcus sanguinis
Neisseria perflava
Neisseria flavescens
Neisseria mucosa
Rothia dentocariosa
Rothia mucilaginosa
Serratia marcescens
Bacillus subtilis
Candida glabrata
Candida albicans
Candida dubliniensis
Escherichia coli
Micrococcus luteus
Lactobacillus plantarum
Staphylococcus epidermidis
Staphylococcus aureus
Staphylococcus haemolyticus
Lactobacillus paracasei
Streptococcus anginosus
Enterococcus faecium
Total Identified
BHI
VRE
AZB
72%
48%
38%
(B)
Non ANTIBIOTIC TRATEMENT
ANTIBIOTIC TRATEMENT
BHI (NonAT4)
VRE (NonAT4)
TSA (NonAT4)
BHI (AT13)
VRE (AT1)
TSA (AT1)
Intens. [a.u.]
m/z
(C)
(Z1)
NonAT5
AT7
Non-Antibiotic treatment (NonAT5)
MS
Antibiotic treatment (AT7)
(Z6)
AT5
(Z2)
(Z3)
(Z4)
(Z5)

◂**Fig. 1** The abundance of isolated microorganisms in correlation with the used growth media (**A**) and the comparison between both non-AT as well as AT of the *S. salivarius* protein profiles in dependence of used culture media (**B**) and comparison between non-AT and AT patients on *S. vestibularis*, example – Z1–Z6 representing common and characteristic MS signals of each (**C**)

(2 min, 20 °C, 13, 000 rpm). Next, 1µl of the material was dropped into the MALDI target, left to dry and covered with 1 µl matrix alpha-cyano-4-hydroxycinnamic acid (HCCA) (Sigma-Aldrich, Switzerland). The external calibration of the instrument was performed using the Bacterial Test Standard, (BTS, Bruker, Bremen, Germany). Each spot was analyzed in duplicate in order to minimize the effect of changes in the sample preparation. The MALDI-TOF mass spectra measurements were carried out by an Ultraflextreme instrument (Bruker Daltonik, Bremen, Germany) operated in the positive ion mode using the BrukerBiotyper 1.1 software (Bruker Daltonik GmbH). The Flex Analysis 2.4 software (Bruker Daltonik GmbH, Bremen, Germany) was used to visualize the MS spectra. Moreover, the data were analyzed automatically by the MBT Compass software (Bruker Daltonik GmbH, Bremen, Germany) and the mass spectra were compared with the spectra of known microbial isolates from commercial libraries provided by Bruker Daltonik. Based on this data, the phyloproteomic tree (dendrogram) was prepared. The spectra match was evaluated by a proprietary algorithm and generated a logarithmic value (score) ranging from 0.0 to 3.0.

Statistical analysis

The heat maps, the hierarchical clustering analysis, and radar chat were generated using the STATISTICAL Release version 7.0 software and Microsoft Excel 2010. All raw data were taken into consideration and correlated on the basis of the used medium and antibiotic, while the identified microbiota was performed.

16S rRNA gene sequencing

To correlate the data obtained from the identification by the MALDI-TOF MS, the 16 rRNA sequencing method was performed. There was an attempt to select one species (*Neisseria perflava*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus epidermidis*, *Streptococcus salivarius*, *Streptococcus pneumoniae*, *Staphylococcus cohnii*, and *Lactobacillus plantarum*) each of the identified microbial genus. Moreover, *Bacillus subtilis* was also chosen, based on the unreliable low score (1.6) generated by the MALDI-TOF MS.

The procedure of the DNA isolation was carried out according to the protocol supplied in the DNeasyUltraClean Microbial Kit (QIAGEN, Wrocław, Poland). The polymerase chain reaction amplification was performed using universal primers as forward: 27F(5-AGAGTTTGATCMTGGCTC AG-3) and reverse primers: 1492R(5-GGTTACCTTGTT ACGACTT-3). After that, the PCR amplification products were purified and the sequencing of the amplified fragments was performed by using the Sanger dideoxy method by Genomed (Warsaw, Poland). Then, from the received sequences, the contigs were submitted using the BioEdit Sequence Alignment Editor ver. 7.2.5 software (12.11.2013) (Hall 1999). Finally, the Basic Local Alignment Search Tool (BLAST) database, available in the National Center for Biotechnology Information (NCBI) was used to compare the consensus sequences with the known 16S rRNA gene sequences deposited in the GenBank. The accession numbers of the studied DNA sequences were determined.

Ethical considerations

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethical Commission of Collegium Medicum in Bydgoszcz of Nicolaus Copernicus University in Torun, Poland, according to the agreement number 477/2021 – 14.09.2021. A written informed consent was obtained from all the participants.

Results

In the present research, the emphasis is put on the microbiota differences in patients with non- and under-antibiotic therapy. Moreover, to date, no work has been focused on the investigation of protein profile changes of isolate species identified in the patient's microbiota not undergoing and undergoing the antibiotic therapy.

The colony-forming unit results indicate that in the saliva samples collected from the AT group bacteria cells were found between 10^5 and 10^7 CFU/mL. However, the number of CFU in the non-AT patients was noticed more than 10^7 CFU/mL (64% of patients). Only in the case of non-AT2, non-AT6, non-AT7, non-AT8, and non-AT10, (36%) the bacterial count was around 10^6 CFU/mL. The slight differences in the abundance can be associated with various lifestyles and distinct genotypes of the hosts (Ling et al. 2013). Figure 1A presents, the heat map combined with the dendrogram representing the total of isolated bacteria (%) in both group of patients (AT and non-AT) and differences between medium to isolate possible microbiota. The heat map was created to show the complimentary of the culture medium applied. The most percentage of isolated bacteria were observed in the universal growing media (BHI), which reported 72%, followed by the VRE medium (48%), and AZB medium (38%). Figure 1A shows

that *Streptococcus salivarius*, *Streptococcus parasanguinis*, *Streptococcus oralis* and *Streptococcus vestibularis*, *Enterococcus faecalis*, *Staphylococcus hominis* were identified in all the cultivation medium.

Information regarding isolates identified in all the samples by the MALDI-TOF MS was presented in details (name of strain, score, and antibiotic used) in Table 1 (non-AT) and Table 2 (AT). Additionally, the used culture medium was also included. According to Tables 1 and 2, it can be observed that the log (score) value (level of identification) was in most cases above 2.0.

The only one - *B. subtilis* (AT12) was found to be below 1.7 (log (score) = 1.6). Moreover, it is necessary to underline that *S. aureus* and *S. pneumoniae* were noticed only in non-AT group, whereas *E. faecalis, E. faecium*, *S. epidermidis*, *B. subtilis*, *S.cohnii* and yeasts *C. albicans*, *C. glabrata*, and *C. dubliniensis* were found only in AT group. Based on the shown date, it can be noticed that the identification for *S. salivarius* cultivated on various medium was similar (score

Table 1 Identification results of all isolates identified in non-AT patients using MALDI-TOF MS

Patient name	Antibotic	Best much in MALDI Biotyper database	Score value
Non-antibiotic treatment (non AT)			
Non-AT1	-	*Streptococcus salivarius 0807M25049501 IBS (BHI)*	2.06
		*Streptococcus pneumoniae DSM 11868 DSM (BHI)**	2.11
		Streptococcus parasanguinis DSM 6778T DSM_2 (VRE)	1.85
Non-AT2	-	*Streptococcus vestibularis DSM 5636T DSM (BHI/VRE/TSA)*	2.11
		Streptococcus sanguinis CCUG 29269 CCUG_corr (BHI)	2.35
Non-AT3	-	*Streptococcus salivarius 0807M25049501 IBS (BHI)*	2.34
		Streptococcus mitis V17_201158 MUZ (BHI)	2.05
		Streptococcus parasanguinis 14137939_2 MVD (TSA)	1.91
Non-AT4	-	*Streptococcus vestibularis DSM 5636T DSM (BHI)*	1.93
		Streptococcus salivarius 0807M25049501 IBS (BHI/VRE)	2.07
		Streptococcus salivarius DSM 20560T DSM (TSA)	2.07
Non-AT5	-	*Rothia mucilaginosa DSM 20446 DSM (BHI)*	1.80
		Streptococcus vestibularis DSM 5636T DSM (TSA)	2.05
Non-AT6	-	*Streptococcus vestibularis DSM 5636T DSM (BHI)*	1.94
		Streptococcus parasanguinis CCUG 55521 CCUG (BHI)	2.31
		Streptococcus oralis DSM 20379 DSM (VRE/TSA)	2.24
Non-AT7	-	*Streptococcus salivarius DSM 20560T DSM (BHI)*	2.30
		Neisseria perflava DSM 18009T DSM (BHI)	2.45
		Streptococcus anginosus DSM 20563T DSM (VRE)	2.20
		*Escherichia coli ATCC 25922 CHB (TSA) **	2.29
Non-AT8	-	*Streptococcus salivarius 0807M25049501 IBS (BHI/VRE/TSA)*	2.17
Non-AT9	-	*Streptococcus oralis DSM 20395 DSM (BHI/VRE)*	2.20
		Streptococcus oralis DSM 20627T DSM (TSA)	2.26
Non-AT10	-	*Streptococcus salivarius 0807M25049501 IBS (BHI)*	2.11
		*Staphylococcus aureus ssp aureus DSM 4910 DSM (TSA) **	2.51
Non-AT11	-	*Streptococcus salivarius DSM 20560T DSM (BHI)*	2.27
		Neisseria perflava 1621 PGM (BHI)	2.35
		*Staphylococcus aureus ssp aureus DSM 4910 DSM (VRE) **	2.28
		Lactobacillus plantarum DSM 1055 DSM (VRE/TSA)	2.25
Non-AT12	-	*Neisseria flavescens C1 2 PGM (BHI)*	2.18
		Neisseria perflava DSM 18009T DSM (BHI)	2.49
		Streptococcus oralis DSM 20379 DSM (TSA)	2.48
Non-AT13	-	*Streptococcus vestibularis DSM 5636T DSM (BHI)*	2.13
		Streptococcus gordonii DSM 6777T DSM (BHI)	2.01
		Staphylococcus aureus ATCC 33591 THL (VRE/TSA)	2.12
Non-AT14	-	*Streptococcus vestibularis DSM 5636T DSM (BHI/TSA)*	2.08
		Rothia dentocariosa DSM 43762T DSM (BHI)	2.04
		Streptococcus salivarius DSM 20560T DSM (VRE)	1.80

Table 2 Identification results of all isolates identified in AT patients using MALDI-TOF MS

Patient name	Antibiotic	Best much in MALDI Biotyper database	Score
Antibotic treatment (AT)			
AT1	Azithromycin	*Streptococcus oralis DSM 20395 DSM (BHI)*	2.26
		Streptococcus salivarius 0807M25049501 IBS (VRE/TSA)	2.10
AT2	Amoxicillin	*Streptococcus parasanguinis CS 50_4 BRB (BHI)*	2.19
AT3	Piperacillin	*Staphylococcus haemolyticus Mb18803_2 (VRE)*	2.15
		*Enterococcus faecium DSM 13589 DSM (VRE) **	2.29
AT4	Ciprofloxacin + metronidazole	*Streptococcus salivarius 0807M25049501 IBS (BHI)*	2.24
		Staphylococcus hominis ssp novobiosepticus DSM 15614T DSM (AZB)	2.42
AT5	Clindamycin	*Escherichia coli DH5alpha BRL (BHI) **	2.26
		Streptococcus oralis DSM 20627T DSM (BHI)	2.08
		*Enterococcus faecalis 20247_4 CHB (VRE/TSA) **	2.46
AT6	Clindamycin	*Candida albicans ATCC 10231 THL (BHI) **	2.16
		Streptococcus sanguinis DSM 20567T DSM (BHI)	2.13
		*Staphylococcus epidermidis 10547 CHB (VRE) **	2.30
		Lactobacillus plantarum DSM 1055 DSM (TSA)	2.30
AT7	Ciprofloxacin	*Streptococcus salivarius DSM 20560T DSM (BHI)*	2.36
		Streptococcus parasanguinis 14137939_2 (BHI)	2.18
		*Enterococcus faecium DSM 17050 DSM (VRE) **	2.40
		Streptococcus vestibularis DSM 5636T DSM (TSA)	2.19
AT8	Clindamycin	*Candida glabrata DSM 11950 DSM (BHI) **	2.29
		Rothia dentocariosa B16575_bh8 IBS (BHI)	2.16
		*Staphylococcus epidermidis DSM 1798 (VRE)**	2.22
		Lactobacillus plantarum DSM 1055 DSM (VRE)	2.33
		Streptococcus salivarius 0807M25049501 (TSA)	2.06
AT9	Ciprofloxacin	*Streptococcus oralis DSM 20379 DSM (BHI/TSA)*	2.14
		Lactobacillus paracasei ssp paracasei DSM 2649 (VRE)	2.11
AT10	Piperacillin	*Streptococcus parasanguinis CS 50_4 BRB (BHI)*	2.05
		*Candida albicans DSM 6569 DSM (VRE) **	2.15
		Staphylococcus epidermidis 10547 CHB (TSA)	2.08
AT11	Metronidazole	*Rothia mucilaginosa DSM 20445 DSM (BHI)*	2.23
AT12	Piperacillin	*Streptococcus parasanguinis CS 50_4 BRB (BHI)*	2.00
		*Bacillus subtilis ssp subtilis DSM 10T DSM (BHI) **	1.60
		Streptococcus salivarius 0807M25049501 IBS (VRE)	2.21
		Staphylococcus epidermidis 10547 CHB (TSA)	2.23
AT13	Clindamycin + levofloxacin	*Streptococcus salivarius 0807M25049501 IBS (BHI)*	2.20
		Streptococcus parasanguinis 14137939_2 MVD (TSA)	2.29
AT14	Clindamycin	*Enterococcus faecalis DSM 20409 DSM (BHI) **	2.54
AT15	Clindamycin	*Candida albicans ATCC 10231 THL (BHI) **	2.04
		Serratia marcescens DSM 12481 DSM (BHI)	2.38
		*Enterococcus faecalis DSM 20409 DSM (VRE) **	2.35
		Enterococcus faecalis DSM 2570 DSM (TSA)	2.34
AT16	Clindamycin	*Candida dubliniensis 99 PSB (BHI) **	2.01
		*Enterococcus faecium DSM 13589 DSM (VRE) **	2.29
		Staphylococcus epidermidis DSM 1798 DSM (TSA)	2.06
AT17	Clindamycin	*Neisseria flavescens C1 2 PGM (BHI)*	2.27
		Micrococcus luteus IMET 11249 HKJ (TSA)	2.10
AT18	Clindamycin	*Neisseria perflava DSM 18009T DSM (BHI)*	2.18
AT19	Cefotaxime	-	

Table 2 (continued)

Patient name	Antibiotic	Best much in MALDI Biotyper database	Score
AT20	Cefotaxime	*Rothia mucilaginosa BK2995_09 ERL (BHI)*	2.37
		Streptococcus parasanguinis CS 50_4 BRB (BHI)	2.44
		Streptococcus parasanguinis 14137939_2 MVD (VRE)	2.26
		*Candida albicans DSM 6569 DSM (VRE) **	1.95
		Staphylococcus epidermidis DSM 1798 DSM (TSA)	2.13
AT21	Amoxicillin	*Staphylococcus cohnii ssp urealyticus DSM 6718T(BHI) **	2.13
AT22	Clindamycin	*Neisseria mucosa 1591 PGM (BHI)*	2.12
		Staphylococcus hominis ssp novobiosepticus DSM 15614T DSM (BHI/TSA)	2.43
		Staphylococcus epidermidis ATCC 14990T THL (VRE)	2.16
AT23	Clindamycin	*Rothia dentocariosa RV_BA1_032010_D LBK (BHI)*	2.35
		Staphylococcus hominis 18 ESL (VRE)	2.17
		Staphylococcus hominis ssp novobiosepticus DSM 15614T DSM (TSA)	2.15
AT24	Clindamycin	*Neisseria flavescens C1 2 PGM (BHI)*	2.24
		Staphylococcus epidermidis ATCC 12228 THL (VRE)	1.83
		*Enterococcus faecalis 20247_4 CHB (TSA) **	1.80

value for non-AT4 was 2.07), (Table 1). However, the protein profile of the identified bacteria differed (Fig. 1B). The comparison of the protein profile of *Streptococcus salivarius* based on the culture medium was presented in Fig. 1B. In the case of the non-AT patients, the mass spectra show that the signals at $m/z = 4451$ and $m/z = 5968$ are similar in BHI, VRE, and TSA. Moreover, it can be observed that the differences in intensities of generated signals depend on the culture medium used. However, the signal 2762 *m/z* was recorded only in the universal media (BHI, TSA). In contrast, this signal disappeared in the case of the VRE medium, and the new signal was registered at 2984 *m/z*. Moreover, the signal $m/z = 7888$ was observed in the protein profile of *S. salivarius* identified on the BHI and VRE growing media in the non- and treated group. It is notable that some signals are present only in one mass spectra of non-AT4 in the case of each medium: 9030 and 10268 *m/z* in BHI; 9443 *m/z* in VRE and 10840 *m/z* in TSA. Based on the mass spectra of *S. salivarius* in the patient group treated with antibiotics (AT13 and AT1), the common signals ($m/z = 2984$; $m/z = 4451$; $m/z = 5968$) can be noticed. Furthermore, the $m/z = 10068$ was noticed in the universal media (BHI and TSA). However, the differences in the protein profile of the studied bacteria strain were also recorded. The signals at 9090 *m/z* were observed in BHI and at 9444 *m/z* in the VRE growing medium.

It is notable that the relative intensities and the noise level of the signals of the registered protein profiles depended on the growing employed media. Hence, the use of the selective culture media that contains various components, including antibiotics, salts, and pH indicators can be the only limitation associated with the use of the mass spectrometric techniques. Some components, such as salts, are well-known inhibitors for the mass spectrometry, and various media can induce changes in the bacterial protein expression (Metwally et al. 2015). Therefore, it is supposed that the disappearance of some signals in the mass spectra recorded after the use of the VRE medium of *S. salivarius* can be correlated with the salt mixture present in this selective culture medium. Karamonová and co-workers (Karamonová et al. 2013) established the optimal cultivation media for the identification of *Cronobacter sakazakii* bio groups using the MALDI-TOF MS. They studied the universal growth media, such as TSA, BHA, Blood Agar Base (Blood Agar Base with sheep blood (5%), BA), and selective cultivation medium *Enterobacter sakazakii* Isolation Agar (ESIA). It was observed that the intensity of the recorded mass spectra was lower in the case of the ESIA medium than in the universal media (TSA, BHA, BA). It was suggested that the unsatisfied intensity of the protein profile of *Cronobacter sakazakii* CB03 can be caused by the deficient composition of the selective medium rather than the universal media and the presence of specific (selective) substances (sodium desoxycholate, sodium chloride, and crystal violet) as inhibitors of growing microbial competitors. Finally, the TSA medium was chosen to further analysis by the MALDI-TOF MS (Karamonová et al. 2013).

Additionally, the mass spectra of *Streptococcus vestibularis* between the non-antibiotic treatment (non-AT5) and the antibiotic treatment (AT7) patients were also compared (Fig. 1C). The common signals at 3944, 4452, and 5968; 6755, 7888 *m/z* (Fig. 1C) and at 2984 *m/z* (Fig. 1C-Z2), 5135; 5188 *m/z* (Fig. 1C-Z4), and 6311*m/z* (Fig. 1C-Z6) were found in both studied groups. Moreover, the signals at 2731, 2743, 2762, 2778, 2800, 2816 m/z (Fig. 1C-Z1), at 2907 *m/z* (Fig. 1C-Z2), at 4723; 4788 *m/z* (Fig. 1C-Z3),

and at 6139 *m/z* (Fig. 1C-Z6) were characteristics only for the non-AT patients. However, in the protein profile of *S. vestibularis* (AT7), the non-common signals *m/z* 4923 (Fig. 1C-Z3), *m/z* 5363, 5391, and 5557 (Fig. 1C-Z5) were also observed. According to the UNIPROT database, the common signals registered at 4452 *m/z* indicated the 50S ribosomal protein L36 (structural constituent of ribosome) (Maeder and Draper 2005) while those registered at 5968 *m/z* were found to be responsible for the defense response to bacterium (Bacteriocin-type signal sequence) (Wescombe et al. 2009). Remarkably, the disappearance of the signal at *m/z* 4723 in the AT group, responsible for the DNA binding and transpose activity can be associated with the mechanism of antibiotics, in this case, ciprofloxacin (it inhibits the DNA replication) (LeBel 1988).

In the next step, the recorded mass spectra for all the isolates were matched to the reference spectra (MSPs), and the phyloproteomic tree was created. Figure 2 represents the hierarchical clustering of the identified isolates and correlation to the reference species (from the MALDI database). Based on the MSP dendrogram (Fig. 2), 11 main clusters indicating genus (*Micrococcus, Enterococcus, Serratia, Escherichia, Rothia, Candida, Staphylococcus, Bacillus, Streptococcus, Lactobacillus,* and *Neisseria*) were noticed. The relationship between the identified microorganisms and the reference strains was found. The showing longest distance level correlation between the bacteria strain was observed in the cluster belonging to the *Staphylococcus* genus. Moreover, the cluster describing the *Enterococcus* genus indicates the shortest distance among the identified species. According to the dendrogram, a close relationship between the *Serratia* and *Escherichia* genus can be noticed. Moreover, the *Bacillus* genus was also included in the phyloproteomic tree (Fig. 2), and (marked by #), despite the low identification level using the MALDI-TOF MS technique (1.7 >). Furthermore, a close relationship between *Streptococcus salivarius* and *Streptococcus vestibularis* was noticed. According to the research previously published by our group (Złoch et al. 2020b), the problem of distinguishing those species could be overcome by using the MALDI-TOF MS to create protein and lipid profiles.

Considering the manufacturer's guidelines, the low score value of 1.6 (Table 2), only for *Bacillus subtilis* was obtained while the standard method for the bacteria identification (16S rRNA gene sequencing) was performed. Furthermore, to confirm the accuracy of the MALDI results for all the identified 11 genus of bacteria, the one species from each cluster was selected (*Neisseria perflava*, *Lactobacillus plantarum*, *Streptococcus salivarius*, *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Enterococcus faecalis*). In addition, the *Streptococcus pneumoniae* as a serious pathogen and *Staphylococcus cohnii* showing the high level of the antibiotic resistance were also chosen. However, the genus with the low abundance of percentage in the identification including *Candida*, *Rothia*, *Escherichia*, *Serratia*, and *Micrococcus* was considered in the PCR analysis.

Then, the results received by the MALDI-TOF MS (score > 2.00) were correlated with the 16S rRNA gene sequencing method (excluding the *Bacillus subtilis*). However, the low identification level was verified in the PCR analysis. On the basis of the data from the PCR assay, the value of identification was over 99.5% for all the studied bacteria species. Moreover, the following accession numbers were given to the bacteria: *B. subtilis* (MZ336018); *N. perflava* (MZ191898); *Lactiplantibacillus plantarum* (the previous form *Lactobacillus plantarum*) (A taxonomic note on the genus *Lactobacillus*) (MZ411566); *S. salivarius* (MZ191906); *S. aureus* (MZ191908); *S. epidermidis* (MZ411533); *E. faecalis* (MZ191905); *S. pseudopneumoniae* (MZ191882), and *S. cohnii* (MZ191897).

Based on the PCR method, in one case, only the identification compared to the MALDI- TOF MS was slightly different. The protein profile of *S. pneumoniae* was identified as *S. pseudopneumoniae* using the 16S rRNA gene sequencing method. Lucia Gonzales–Siles et al. studied the genomic markers for the differentiation and identification of both *Streptococcus* species. The presence of these unique markers was confirmed by the PCR with reference strains and clinical isolates (Gonzales-Siles et al. 2020).

Summarily, 29 already identified species were represented as predominant species in both non-AT and AT salivary sample groups (Fig. 3). The diversity in the salivary bacteria in the AT group vs the non-AT was observed. Figure 3 (up) illustrates the heat map representing the abundance of all identified species of microorganisms (%).

Moreover, based on the created heat map, the % distribution predominance of all identified microorganism species was performed and shown as a form of the radar chart (Fig. 3 down).

Regarding the investigated radar chat, the differences between the salivary microbiota of non-AT and AT patients were found. It can be observed that the *Streptococcus salivarius* (25%) and *Streptococcus vestibularis* (19%) dominated in the non-antibiotic treatment patients. Another predominant bacteria species in patients with the normal salivary microbiome were *Streptococcus oralis* and *Staphylococcus aureus.*

The blue area of the web chart (Fig. 3 down) shows that in the non-AT group, the 16 species of bacteria (from *S. salivarius* to *R. mucilaginosa*) were identified. Compositionally, the most abundant microorganism present in the AT patients were *Streptococcus salivarius*(11%) *Streptococcus parasanguinis* (11%), *Staphylococcus epidermidis* (12%), *Enterococcus faecalis* (9%), *Staphylococcus hominis* (8%), *and Candida albicans* (6%).

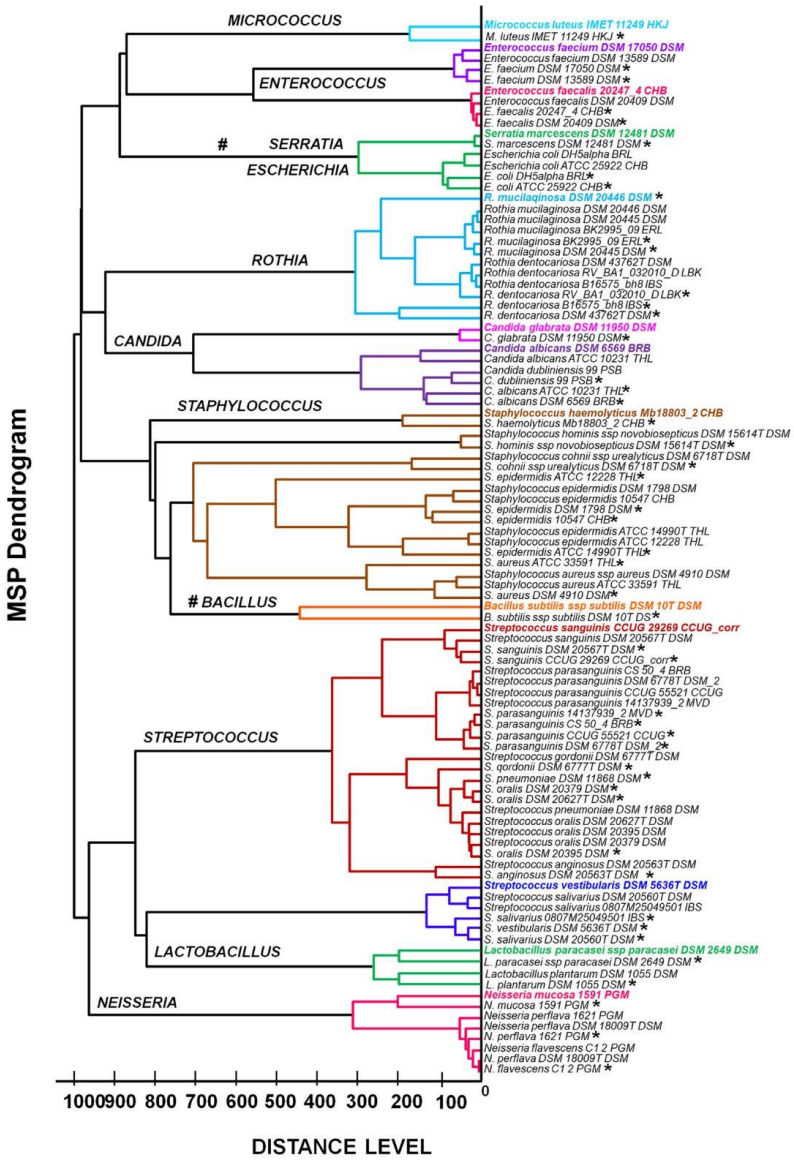


Fig. 2 The phyloproteomic tree of all identified isolates based on the obtained MSP identification via MALDI Biotyper platform

The orange color indicates that the saliva of patients under the antibiotic therapy was more bacterially rich than the non-AT group. It was also observed that pathogenic microorganisms dominated in the group. It can be assumed that the type of the antibiotic treatment influenced the salivary bacteria composition in the AT patients. In comparison with patients with normal (physiological) salivary microbiota, more diversity of microorganism and more

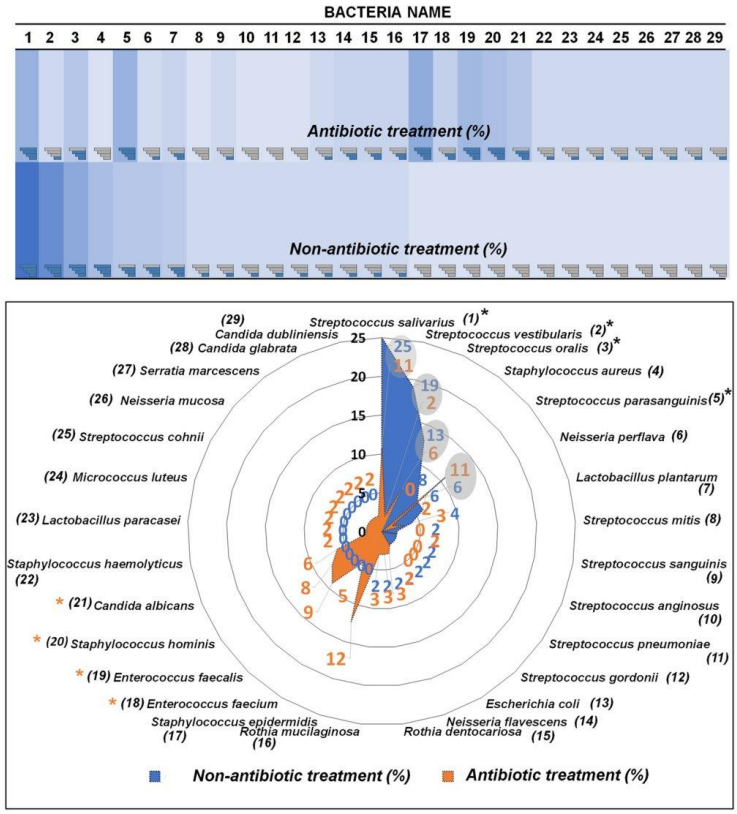


Fig. 3 The heat map (up) representing the abundance of identified isolates and radar chat (down) showing the % distribution predominance of all identified microorganism species in non-AT and AT group

abundance of pathogenic bacteria can be noticed in the AT group, which can be associated with stress conditions under the antibiotic treatment.

The correlation between the identified pathogen and the antibiotic was shown in the cluster analysis (Figs. 4 and 5). For further discussion, the most predominant species identified in both non-AT and AT samples were taken into consideration.

The analysis of the intraspecific proteomic variation within distinct microbial species derived from the saliva of patients treated and untreated with antibiotics revealed the impact of the treatment undertaken, associated microbiota, and applied culture conditions on the generated protein profiles (Figs. 4 and 5). Regarding species that occurred in both patients group, obtained results indicated the influence of the antibiotic used and the type of the culture medium on the variation in the proteomic composition of the bacteria (Fig. 4). As the example of the species *S. vestibular*is is shown (Fig. 4A), such differences cover a wide range of *m/z* with various frequencies. In that particular case, the most different protein pattern was noted for the strains isolated from the patient non-AT6 and AT7 and can be attributed to the synergic effect of the culture medium composition (TSA vs. BHI) and antibiotic treatment (ciprofloxacin). A similar effect was observed for *S. oralis* (Fig. 4B), where isolates collected from the AT patients and cultured on the BHI comprised a distinct group. Moreover, *S. oralis* isolated from the non-AT also revealed a clear grouping according to the culture medium—VRE vs. TSA. Although all *S.oralis* from the AT patients were isolated using BHI, their proteome differentiation was much higher than that of isolates detected among the non-AT, which can be associated with a different antibiotic treatment—ciprofloxacin (AT9), azithromycin (AT1), and clindamycin (AT5). Regarding the other two strains found in both patients group—*S. salivarius* and *S. parasanguinis*—the comparative analysis revealed the highest proteomic variations; however, grouping according to drug-treated and untreated patients was not as evident as in the former cases. Nonetheless, *S.*

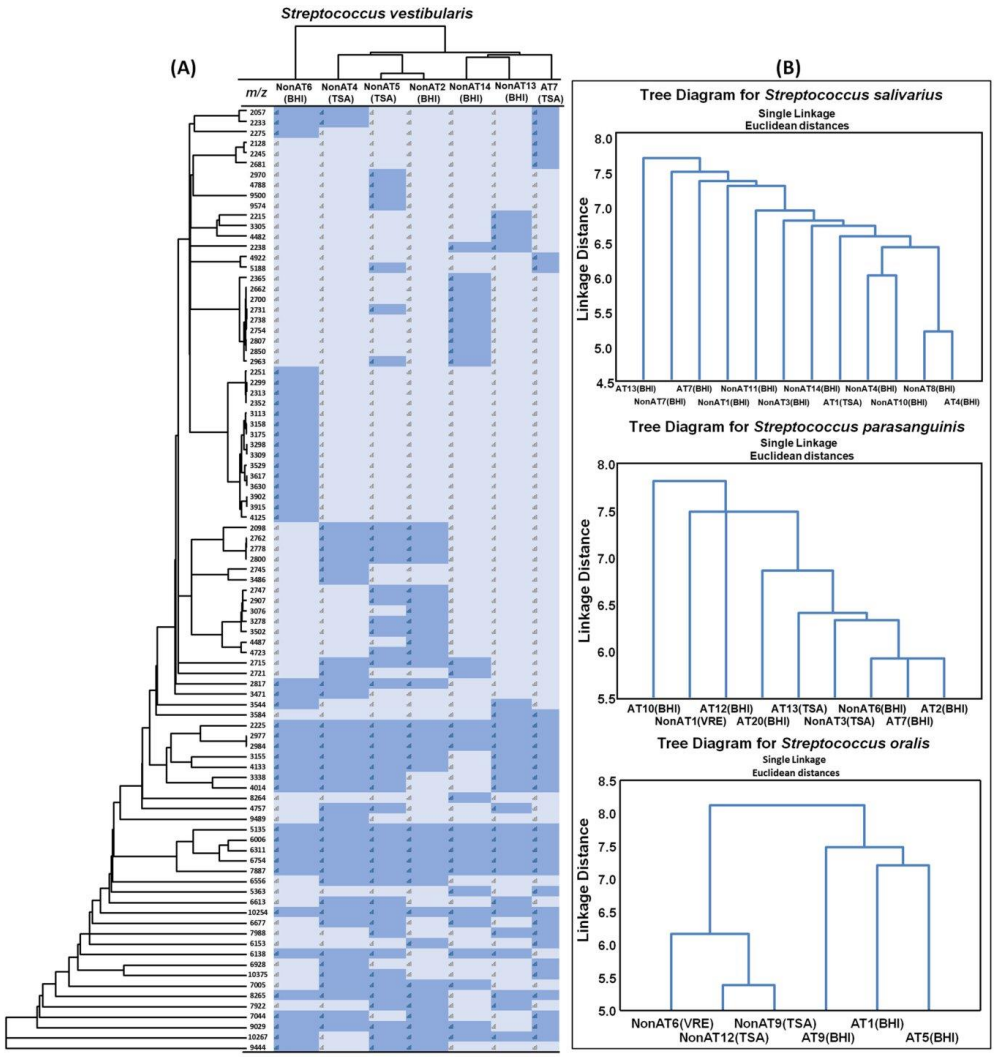


Fig. 4 Heat map (**A**) and formed clusters (vertical left) illustrating the differences between registered common and characteristic signals in both groups; hierarchical clustering distinguishing non-AT and AT for *S. vestibularis* (**A**, up), for *S. salivaris*, *S. parasanguinis*, and *S. oralis* (**B**)

parasanguinis strains isolated from the AT patients were placed into different clusters according to the type of antibiotic used, where strains from piperacillin-treated patients (AT10 and 12) were much more similar to each other than those from ciprofloxacin, amoxicillin, and clindamycin with levofloxacin (Fig. 4B). Additionally, great diversity noted for *S. parasanguinis* can be related to various microbiota found in the saliva samples. Indeed, strains isolated from samples accompanied by the presence of such microorganisms as *Candida albicans* (AT10 and 20) or *Bacillus subtilis* (AT12) demonstrated a more unique proteins pattern than those comprised of streptococci only (AT2 or non-AT6). A similar phenomenon was noted for *S. salivarius*, where the strains showing extremely different protein profiles came

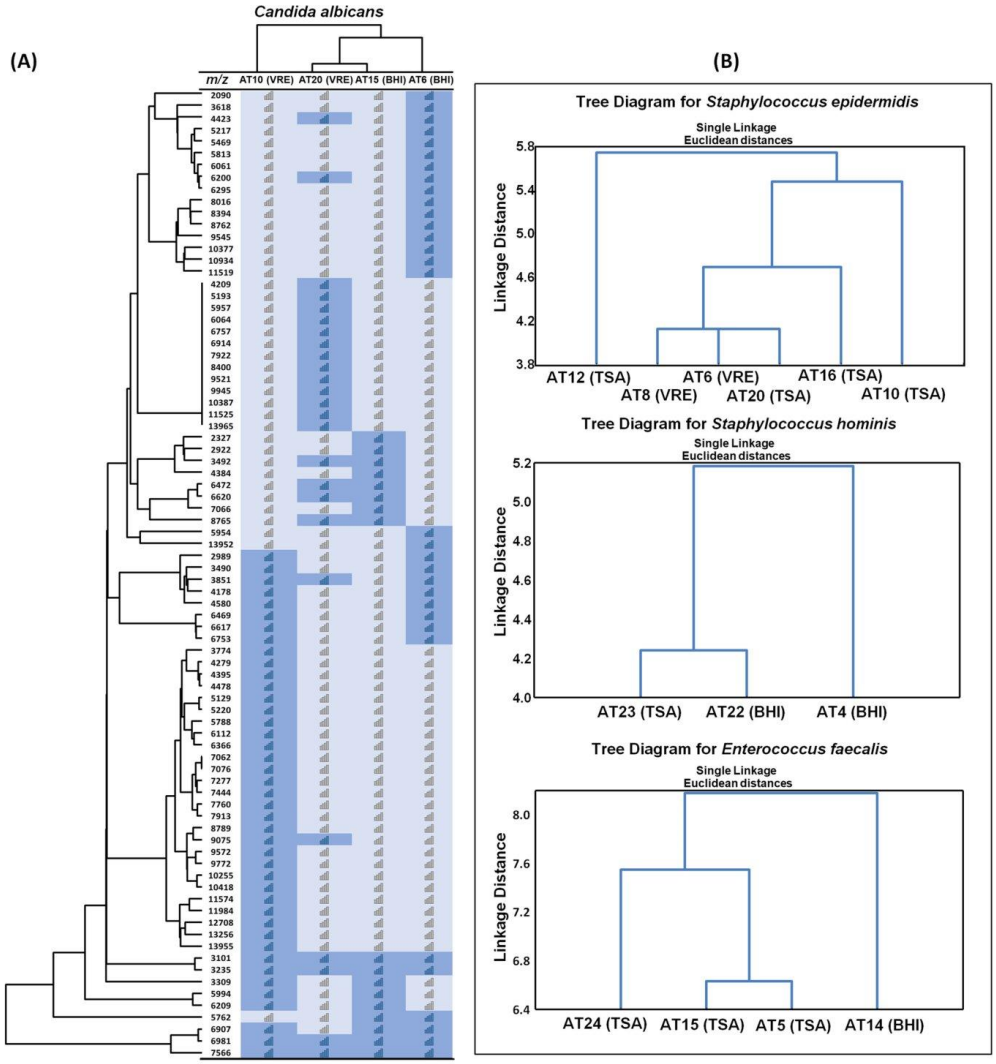


Fig. 5 Heat map (**A**) and formed clusters (vertical left) illustrating the differences between registered common and characteristic signals only in AT; hierarchical clustering distinguishing different AT for *C. albicans* (**A**, up), for *S. epidermidis*, *S. hominis*, and *E. faecalis* (**B**)

from samples significantly different in the species composition—non-AT7 (additionally *N. perflava*, *S. anginosus*, *E. coli*) vs. non-AT8 (*S. salivarius* only)—or the antibiotic used—AT13 (clindamycin with levofloxacin) vs AT4 (clindamycin with metronidazole). All in all, comparing species derived from the saliva of treated and untreated patients, the impact on the proteomic diversification increases as follows: the type of the antibiotic used>coexistent microbiota>culture medium type.

Similarly, a comparison of the proteomic diversity among microbial species that occurred only in the AT patients was performed (Fig. 5). The effect of the culture medium used was noted for *C. albicans* (Fig. 5A) along with *E. faecalis* strains (Fig. 5B). Interestingly, in the case of *E. faecalis*, the

strain isolated as a single microorganism from patient AT14 demonstrated a far more different proteins pattern compared to the rest of the strains which were isolated from samples occupied by pathogenic microorganisms like *C. albicans*, *S. marcescens*, *N. flavescens* or *E. coli*. In turn, the proteomic intraspecific diversification of the staphylococci (*S. hominis* and *S. epidermidis*) most likely resulted from the type of antibiotic treatment undertaken—ciprofloxacin with levofloxacin vs. clindamycin in the case of former and piperacillin vs. clindamycin or cefotaxime in the latter one. Moreover, among *S. epidermidis* strains the most distinct proteins profile was demonstrated by the isolate AT12—the only one that derived from the sample in which the presence of *Candida* spp. was not observed.

Discussion

Intraspecific differences in microbial protein profiles depending on culture media compositions were recognized as significant since 50% of the peaks of all bacteria are non-ribosomal proteins, which are more or less metabolic status dependent (Mellmann et al. 2008). It was proved in the work of Złoch et al. (Złoch et al. 2020a) where changing the culture conditions significantly influenced the differentiation of *S. aureus* strains based on their protein patterns. As the authors pointed out, it may result from the induction of some new metabolic pathways in bacteria leading to the appearance of more discriminant signals.

According to previous reports (Monedeiro et al. 2021; Szultka-Młyńska et al. 2021) the culture media could be useful for the separation of each bacterial strains from the sample obtained from the hospital. In this context, the present research have also used different media to select and identify the individual strain. Moreover, it has been investigated how the composition of the used media can influence the isolation and identification of each bacteria in order to establish an optimal media in this way regarding the biomedical approach. In general, the identification of microorganisms by MALDI is considered culture independent, since most of the proteins present in bacterial cells are ribosomal proteins (about 50% or more), so that reliable classification of bacteria for most genera and species is certain regardless of the culture media used. Nevertheless, in addition to ribosomal proteins, the bacterial extracts studied also contain other non-ribosomal proteins that are more or less metabolism dependent. Such proteins can affect the identification result primarily in the case of bacteria belonging to groups of closely related species (e.g., *Bacillus subtilis* or cereus group, *S. salivarius* group or *S. mitis/oralis*) leading to misidentification. Knowing that in the case of closely related species, the genomic and proteomic differences are very small, even slight variation in the culturing conditions may matter.

Nevertheless, the results of our studies showed that the impact of the culture medium type on the intra-specific variation of the proteomes was lower than the effect of the antibiotic treatment and the presence of the co-exist microbiota. It was shown that the interaction that occurred between microbes can alter the expression of their membrane proteins. Such a phenomenon was noted in the work of Kumar and Ting, where amounts of seven classes of proteins on the *S. aureus* surface were elevated upon coculturing with *P. aeruginosa* (Kumar and Ting 2015). Found proteomic changes included proteins related to host-microbe interactions such as virulence, adhesion, and resistance, which explains the increased fatality of infections with the simultaneous presence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* compared to the colonization of the individual bacterial species (Fazli et al. 2009). A similar phenomenon was observed in our studies where for instance *E. faecalis* and *S. salivarius* that co-existed with other microbial species demonstrated distinct protein patterns from the ones isolated as a monoculture. Since most of the accompanying species represented pathogenic microorganisms, the detected intra-specific proteome variation of the mentioned bacterial species may be partially explained by the horizontal transfer of the genes related to virulence factors. Bacterial genomes frequently contain a significant amount of foreign DNA, which is DNA that originated from another organism and was inserted into the genome of a bacterium (Ochman et al. 2000). The DNA mobilized into the host bacterium is referred to as mobile genetic elements (MGEs), which have a huge impact on the shape of the bacterial genomes and promote intra-specific variability (Heuer and Smalla 2007). *Enterococcus faecalis* harbors a pathogenicity island containing several virulence factors and is known for its fast adaptation to the clinical environment by the acquisition of antibiotic resistance and pathogenicity traits generating it the third leading cause of hospital-associated infections (Laverde Gomez et al. 2011). Although the horizontal gene transfers occur more frequently between closely related bacteria, they also occur among distantly related species. Nevertheless, horizontally acquired (or lost) genes can also contribute to ecological adaptation, and they are likely to be major drivers of niche differentiation among bacteria (Smillie et al. 2011). Moreover, different habitats can be expected to support different levels of intra-species diversity and to be subject to distinct selection pressures (Ellegaard and Engel 2016). Considering clinical specimens, antibiotics demonstrate highly selective pressure on the bacteria populations. Besides, causing the shifts in the species composition, it was proved that antibiotics (at certain concentrations

which depend on their class) are also responsible for the high phenotypic variation even at a single bacterial population level (Lee et al. 2018). Indeed, the presence of the antibiotic demonstrated the highest impact on the proteomic intra-specific diversity of the investigated salivary microorganisms, including *Candida* spp. Moreover, such an impact depends on the type of the antibiotic used and tested microorganisms.

Our results indicated significant differences in the saliva microbiota between non-antibiotic treatment and antibiotic treatment patients. We noticed the dominated and characteristic microorganisms in the non-AT (*Streptococcus salivarius* (25%) and *Streptococcus vestibularis* (19%), *Streptococcus oralis* (13%) and *Streptococcus parasanguinis* (6%)) and in the AT (*Streptococcus salivarius* (11%), *Streptococcus parasanguinis* (11%), *Staphylococcus epidermidis* (12%), *Enterococcus faecalis* (9%), *Staphylococcus hominis* (8%), and *Candida albicans* (6%)) groups. The salivary microbiota of antibiotic-treated patients was characterized by a more bacteria variety; the appearance of the *Candida albicans* species was noticed only in the AT patient indicating a negative impact of the antibiotic administration on the patient microbiota. Moreover, the proteomic analysis showed the influence of the antibiotic therapy, composition, and abundance of saliva microbiota and used growth medium on the recorded protein profiles. Remarkably, the MALDI-TOF MS analysis represents a promising method for a large-scale, less labor intensive, rapid, and cost-effective record of reproducible molecular profiles of microorganisms, particularly the salivary bacteria. It is notable that the proposed approach enables the early administration of the specie-specific antimicrobial therapy in the patients. Therefore, our data can allow a medical diagnosis to be confirmed and they may also enable us to monitor the treatment of diseases and develop drugs for individual patients. Moreover, the presented study can be the base to develop methods enabling a faster diagnosis and the detection of disease changes at the cellular level before clinical changes occur. To summarize, the obtained results suggest that tracking proteomic intra-specific variation within microorganisms may be a promising tool for the future use to examine the effectiveness of the applied antibiotic curation and to check whether one deals with mono- or polymicrobial cultures including the presence of the other pathogenic species.

Author contribution BB, VRP, PP, and KPI: conceptualization; KPI, VR, and MZ, MSzM: methodology; VRP and KPI: formal analysis and investigation; VRP, KPI, and MZ: writing—original draft preparation; BB, VRP, PP, KPI, MZ, and MSzM: writing—review and editing; KPI: funding acquisition; DB and WK: resources; BB: supervision. All authors read and approved the manuscript.

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Data availability Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The authors confirm that all experimental protocols were approved by the Regional Bioethics Committee of Nicolaus Copernicus University in Torun, Poland (decision no. 477/2021).

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

Conflict of interest The authors declare no competing interests.

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4. Podsumowanie

Badania przeprowadzone w ramach prac [**P2**] oraz [**P3**] umożliwiły opracowanie metod do jednoczesnego oznaczania środków przeciwbakteryjnych oraz ich metabolitów z różnych grup terapeutycznych z zastosowaniem odpowiednio chromatografii cieczowej oraz elektroforezy kapilarnej. Dalszym etapem badań była ocena skuteczności działania antybiotyków wobec bakterii tworzących biofilm, a także z monitorowaniem zmian zachodzących w profilach molekularnych mikrobiomu śliny człowieka pod wpływem różnych antybiotykoterapii osiągnięto w pracach [**P4**] oraz [**P5**].

Przedstawione w rozprawie doktorskiej badania pozwoliły na sformułowanie następujących wniosków:

1. Wyniki uzyskane w pracach [**P1**]-[**P5**] mogą być szczególnie przydatne w antybiotykoterapii. Wdrożenie proponowanych metod do oznaczania ludzkich próbek klinicznych, daje możliwość oceny skuteczności antybiotyków, co może być korzystne dla optymalizacji indywidualnych terapii.
2. Opracowana metoda CE-ESI-MS/MS, ze względu na wysoką czułość, precyzję i powtarzalność, może być z powodzeniem zastosowana w analizie przesiewowej u pacjentów w celu wyeliminowania kombinacji leków, które nie są zalecane do jednoczesnego stosowania. Doświadczenia te mogą także być wykorzystane w laboratoriach kryminalistycznych.
3. Elektroforeza kapilarna jest przydatnym narzędziem w charakterystyce biofilmu. Zastosowanie tej techniki umożliwia obserwację zmian ruchliwości elektroforetycznej i stabilność układu komórek bakteryjnych traktowanych i nietraktowanych antybiotykami z różnych klas (amoksycylina, gentamycyna, metronidazol).
4. Komórki bakteryjne traktowane antybiotykiem wykazały wzrost oddziaływania elektrostatycznego pomiędzy komórkami bakteryjnymi a antybiotykami oraz większą stabilność układów i większą homogenność.
5. Komórki bakteryjne w głębszych warstwach biofilmu są w stanie dostosować się do środowiska i nabyć mechanizmy oporności w wyniku działania mechanizmów molekularnych.
6. Antybiotyki, w tym β-laktamowy (amoksycylina) i aminoglikozydowy (gentamycyna) działając na biofilm doprowadziły do degradacji syntezy ściany komórkowej, a także do zaburzenia macierzy zewnątrzkomórkowej substancji polisacharydowych otaczających biofilm (EPSs) oraz zahamowania białek rybosomalnych i zaburzenia transkrypcji.

Zjawisko to jest skorelowane również z wyższą wartością bezwzględną potencjału zeta i mniejszą ilością zarejestrowanych sygnałów z zastosowaniem elektroforezy kapilarnej.

7. Zaproponowany mechanizm działania badanych antybiotyków (amoksycylina, gentamycyna, metronidazol) na tworzenie biofilmu, związany jest ze zjawiskiem zlepiania (forma agregacji) i degradacji, co w znacznym stopniu może ułatwić leczenie zakażeń bakteryjnych związanych z biofilmem.
8. Występują istotne różnice między mikrobiomem śliny pacjentów leczonych oraz nieleczonych antybiotykami. Mikrobiom pacjentów podanych antybiotykoterapii charakteryzuje się większą różnorodnością bakterii.
9. Stosowanie antybiotykoterapii ma duży wpływ na profil białkowy identyfikowanych mikroorganizmów, a w konsekwencji na mikrobiom pacjenta. Dodatkowo użyte podłoże do identyfikacji będzie odgrywać kluczową rolę w identyfikacji mikrobiomu.

Poza tym, zaproponowane w pracach [**P1**-**P5**] podejścia umożliwiają, potwierdzenie diagnozy lekarskiej, a także monitorowanie leczenia chorób bakteryjnych poprzez zastosowanie u pacjentów specyficznej dla danego gatunku bakterii terapii przeciwdrobnoustrojowej. Ponadto zaprezentowane badania mogą być bazą do opracowania metod umożliwiających szybszą diagnostykę i wykrywanie zmian chorobowych na poziomie komórkowym przed wystąpieniem zmian o podłożu klinicznym.

Reasumując można stwierdzić, że wszystkie przedstawione w rozprawie doktorskiej badania obejmują zagadnienia związane z mikrobiologią, chemią, medycyną i farmacją, co stanowi o ich interdyscyplinarnym podejściu. Dlatego wiedza uzyskana podczas realizacji badań może być wykorzystana w rutynowych badaniach klinicznych do kontrolowania zjawiska antybiotykooporności.

5. Streszczenie

W dobie rosnącej oporności na antybiotyki diagnostyka, która może być prostą, szybką i tanią identyfikacją infekcji bakteryjnej lub określająca odpowiedź na antybiotyki, jest niewątpliwie niezbędna do optymalizacji indywidualnej terapii pacjenta i zmniejszenia ryzyka wystąpienia oporności na antybiotyki. Z tego względu, przedstawiona praca doktorska dotyczy opracowania nowej procedury analitycznej mającej na celu ocenę wartości terapeutycznej i przydatności leków przeciwbakteryjnych i ich metabolitów jako wyznaczników antybiotykoodporności w oparciu o analizę profili białkowych i metabolicznych. Pierwszym etapem badań przeprowadzonych w ramach rozprawy doktorskiej było opracowanie prostych, stosunkowo szybkich, a także tanich metod oznaczania antybiotyków oraz ich metabolitów z różnych grup. W tym celu, najpierw uwagę skupiono na doborze warunków separacji wybranych środków przeciwbakteryjnych oraz ich metabolitów z zastosowaniem wysokosprawnej chromatografii cieczowej z detektorem diodowym (HPLC-DAD). W dalszej kolejności, opracowano i zwalidowano nową metodę analityczną do jednoczesnej analizy antybiotyków i ich metabolitów w ludzkim moczu. Do oznaczenia i identyfikacji wszystkich analitów zastosowano elektroforezę kapilarną (CE) wraz z tandemową spektrometrią mas (MS/MS). Natomiast, za pomocą CE-DAD zbadano wpływ różnych warunków analitycznych (skład, stężenie i wartość pH buforu separacyjnego, czas i ciśnienie iniekcji, temperatura kapilary oraz wpływ modyfikatora organicznego) na migrację i rozdzielenie antybiotyków i metabolitów.

Dalsze badania ukierunkowane były na ocenie skuteczności antybiotyków (amoksycylina, gentamycyna, metronidazol) wobec modelowej bakterii (*B. tequilensis*) tworzących biofilm z zastosowaniem elektroforezy kapilarnej i technik pokrewnych. Elektroforeza kapilarna wykazała zdolność do scharakteryzowania i wykazania różnic w ruchliwości elektroforetycznej pomiędzy biofilmami nieleczonymi i leczonymi antybiotykami. Zbadano również stabilność dyspersji, dokonano analizy profili molekularnych, a także przeanalizowano żywotność komórek bakteryjnych oraz ich morfologię. Badania mikroskopowe i spektrometryczne wskazały na degradację matrycy zewnątrzkomórkowych substancji polisacharydowych (ang. *Extracellular Polysaccharide Substances, EPSs*), hamowanie syntezy ściany komórkowej oraz blokowanie syntezy białek rybosomalnych przez amoksycylinę i gentamycynę. Zaobserwowano, iż komórki bakteryjne nieleczone i leczone charakteryzowały się wysoką stabilność dla systemu tworzenia biofilmu. Ponadto na podstawie rodzaju zastosowanego antybiotyku zaproponowano mechanizm działania użytych antybiotyków w zlepianiu i degradacji komórek.

Kolejnym etap badań dotyczył zastosowania techniki MALDI-TOF MS jako narzędzia do szybkiej identyfikacji mikrobiomu śliny i obserwacji zmian w profilach molekularnych w zależności od podanego antybiotyku. Istotne zmiany w składzie mikrobiomu śliny zauważono w zależności od stosowanych podłoży hodowlanych, antybiotykoterapii i współistniejącej mikrobioty.

Przedstawione w pracy metody mogą znaleźć zastosowanie w analizie przesiewowej pacjentów w celu wyeliminowania kombinacji leków, które nie są zalecane do jednoczesnego stosowania. Ponadto, zaprezentowane podejścia mogą pomóc w opracowaniu metod umożliwiających szybszą diagnostykę zmian chorobowych na poziomie komórkowym przed wystąpieniem zmian klinicznych.

6. Abstrakt

In the era of increasing antibiotic resistance, diagnostics that can be a simple, quick and inexpensive identification of bacterial infection or determining antibiotic response are absolutely necessary to optimize individual patient therapy and reduce the risk of antibiotic resistance. Therefore, the presented doctoral dissertation involves assessment of therapeutic value and usefulness of antibiotics and their metabolites as resistance markers based on the analysis of protein and metabolic profiles.

The first stage of the research performed within the scope of the dissertation was the development of simple, relatively fast, as well as low-cost methods for the determination of antibiotics and their metabolites from different groups. To this purpose, the focus of attention was initially on the choice of separation conditions for selected antibacterial agents and their metabolites using high-performance liquid chromatography with diode array detector (HPLC-DAD). Further, a new analytical method was developed and validated for the simultaneous analysis of antibiotics and their metabolites in human urine. Capillary electrophoresis (CE) combined with mass spectrometry (MS/MS) was used to determine and identify all analytes. However, the effect of different analytical conditions (composition, concentration and pH value of separation buffer, injection time and pressure, capillary temperature and the effect of organic modifier) on the migration and separation of antibiotics and metabolites was investigated using CE-DAD.

Subsequent studies were focused on estimating the efficacy of antibiotics with different action mechanism (amoxicillin, gentamicin, metronidazole) against a model of bacteria (*B. tequilensis*) forming a biofilm using capillary electrophoresis and related techniques. Capillary electrophoresis demonstrated the ability to characterize and show differences in electrophoretic mobility between untreated and antibiotic-treated biofilms. The stability of the dispersion study, the molecular profile analysis, the viability of bacterial cells and the scanning morphology imaging were also investigated. Microscopic and spectrometric studies indicated degradation of the extracellular polysaccharide substances (EPSs) matrix, inhibition of cell wall synthesis, and blocking of ribosomal protein synthesis by amoxicillin and gentamicin. It was observed that untreated and treated bacterial cells had high stability for the biofilm formation system. In addition, on the basis of the type of the antibiotic treatment, the mechanism of used antibiotics in cell clumping and degradation were proposed.

The next stage of the study involved the application of the MALDI-TOF MS technique for identification of the saliva microbiome and observation of changes in molecular profiles in dependence of the administered antibiotic. Significant changes in the composition of the saliva microbiome were noted depending on the used culture media, antibiotic therapy and coexisting microbiota.

The methods presented in this paper can be applied in patient screening analysis to eliminate drug combinations that are not recommended for co-administration. In addition, the such approaches can help in developing the methods enabling a faster diagnosis of disease changes at the cellular level before clinical changes occur.

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9. **K. Pauter**, D. Błońska, V. Railean-Plugaru, M. Szultka-Młyńska, P. Pomastowski, B. Buszewski, 26TH INTERNATIONAL SYMPOSIUM ON SEPARATION SCIENCES, 28.06-01.07.2022, Ljubljana, Słowenia, *Electrochemical simulation of selected antibiotic drugs phase I metabolism with the use of MALDI-TOF MS,* (poster)

Granty

1. Grant OPUS 11 finansowany przez Narodowe Centrum Nauki, nr 2016/21/B/ST4/02130, pt. *Nowe podejście w identyfikacji patogenów za pomocą sprzężonych technik separacyjnych (CE-LIF-MALDI-TOF-MS, LCxLC-MS/MS, GCxGC-Q-TOF) dla potrzeb spersonalizowanej diagnostyki medyczne,* kierownik: dr hab. Małgorzata Szultka-Młyńska, prof. UMK, wykonawca: mgr Katarzyna Pauter (02.01.2019 – 22.04.2020)
2. Grant Młodych Naukowców, nr 2092/2019, pt. *Oznaczanie i identyfikacja antybiotyków oraz mikroorganizmów za pomocą łączonych technik analitycznych dla celów biomedycznych,* kierownik: mgr Katarzyna Pauter
3. Grant Młodych Naukowców, nr 492/2020, pt. Zastosowanie MALDI-TOF MS w oznaczaniu i identyfikacji szczepów bakteryjnych, kierownik: mgr Katarzyna Pauter
4. Grant Preludium 19 finansowany przez Narodowe Centrum Nauki, nr 2020/37/N/ST4/02358, pt. *Ocena wartości terapeutycznej oraz przydatności nowej generacji antybiotyków i ich metabolitów jako markerów oporności za pomocą łączonych technik analitycznych,* kierownik: mgr Katarzyna Pauter (01.02.2021-31.01.2023)
5. Grant Młodych Naukowców, nr PDB/granty wydziałowe, pt. Opracowanie metody izolacji antybiotyków z matryc biologicznych (mocz, ślina, osocze) za pomocą mikroekstrakcji na upakowanym sorbencie (MEPS), kierownik: mgr Katarzyna Pauter

Staże

1. Zagraniczny staż badawczy w ramach projektu PROM - Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej na UMK (Instytut Karolinska, Wydział Biochemii Medycznej i Biofizyki w Sztokholmie (Szwecja), w terminie 17.08.-06.09. 2021)

Nagrody i osiągnięcia

1. zespołowa nagroda Rektora Uniwersytetu Mikołaja Kopernika w Toruniu - II stopnia, za osiągnięcia naukowo-badawcze uzyskane w 2019 roku
2. uzyskanie dofinansowania na 21- dniowy staż zagraniczny w ramach projektu PROM - Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej na UMK
3. zespołowa Nagroda Rektora Uniwersytetu Mikołaj Kopernik w Toruniu - II stopnia, za osiągnięcia naukowo-badawcze uzyskane w 2020 roku
4. zespołowe wyróżnienie Rektora Uniwersytetu Mikołaj Kopernik w Toruniu, za osiągnięcia naukowo-badawcze uzyskane w 2020 roku

Udział w innych projektach

1. współudział w prowadzeniu warsztatów w ramach XIX Toruńskiego Festiwalu Nauki i Sztuki, 24-28.04.2019r.
2. współudział w organizacji konferencji Metabolomics Circle 2018, 26-28.10.2018, Przysiek k. Torunia, Polska

8. Oświadczenia współautorów

UNIWERSYTET MIKOŁAJA KOPERNIKA W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Mgr Katarzyna Pauter-Iwicka
Interdyscyplinarne Centrum Nowoczesnych Technologii
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Gagarina 7, 87-100

OŚWIADCZENIE

Niniejszym oświadczam, że jako współautor następujących publikacji:

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2. J. Walczak-Skierska, M. Szultka-Młyńska, **K. Pauter**, B. Buszewski, *Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD*, Journal of Pharmaceutical and Biomedical Analysis (2020), 184, 113187, doi: htpps://doi.org/1016/j.jpba.2020.113187.
3. **K. Pauter**, M. Szultka-Młyńska, M. Szumski, A. Król-Górniak, P. Pomastowski, B. Buszweski, *CE-DAD-MS/MS in the simultaneous determination and identification of selected antibiotic drugs and their metabolites in human urine samples*, Electrophoresis (2021), 0, 1-13, doi: htpps://doi.org/10.1002/elps.202100190.
4. **K. Pauter**, V. Railean-Plugaru, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, B. Buszewski, *Identification, structure and characterization of Bacillus tequilensis biofilm with the use of electrophoresis and complementary approaches*, Journal of Clinical Medicine (2022), 11, 722, doi: htpps://doi.org/ 10.3390/jcm11030722.
5. **K. Pauter-Iwicka**, V. Railean-Plugaru, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, D. Błońska, W. Kupczyk, B. Buszewski, *Characterization of the salivary microbiome before and after antibiotic therapy via separation technique* (2023), 107, 2515-2537.

Wchodzącej w skład mojej rozprawy doktorskiej, oświadczam, że mój wkład polegał na współuczestnictwie w opracowaniu koncepcji badań, zaplanowaniu i wykonaniu doświadczeń, opracowaniu i interpretacji uzyskanych wyników, a także przygotowaniu wstępnych wersji manuskryptów.

Katarzyna Pauter-Iwicka

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Prof. zw. dr hab. dr h.c. czł. rzecz. PAN Bogusław Buszewski
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Gagarina 7, 87-100

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2. J. Walczak-Skierska, M. Szultka-Młyńska, K. Pauter, **B. Buszewski**, *Study of chromatographic behavior of antibiotic drugs and their metabolites based on quantitative structure-retention relationships with the use of HPLC-DAD*, Journal of Pharmaceutical and Biomedical Analysis (2020), 184, 113187, doi: htpps://doi.org/ 1016/j.jpba.2020.113187.
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Wchodzącej w skład rozprawy doktorskiej mgr Katarzyny Pauter-Iwickiej, oświadczam, że mój wkład polegał na współudziale przy opracowaniu koncepcji manuskryptów oraz interpretacji wyników, a także korekcie manuskryptów i pełnieniu nadzoru merytorycznego.

.....

prof. zw. dr hab. Bogusław Buszewski, dr h.c. mult

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Nauk Biologicznych
i Weterynaryjnych

Toruń, April 8, 2023.

Dr. Viorica Railean

Faculty of Biological and Veterinary Sciences
Institute of Veterinary Medicine
Department of Infectious, Invasive Diseases and Veterinary Administration,
ul. Lwowska 1, 87-100 Toruń
Interdisciplinary Center of Modern Technologies
Nicolaus Copernicus University in Toruń
ul. Wileńska 4, 87-100 Toruń

STATEMENT

As a co-author of the following publications:

1. K. Pauter- Iwicka, **V. Railean**, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, B. Buszewski, *Identification, structure and characterization of Bacillus tequilensis biofilm with the use of electrophoresis and complementary approaches*, Journal of Clinical Medicine (2022), 11, 722, doi: htpps://doi.org/ 10.3390/jcm11030722.

2. K. Pauter-Iwicka, **V. Railean**, M. Złoch, P. Pomastowski, M. Szultka-Młyńska, D. Błońska, W. Kupczyk, B. Buszewski, *Characterization of the salivary microbiome before and after antibiotic therapy via separation technique* (2023), 107, 2515-2537, doi: https://doi.org/10.1007/s00253-023-12371-0,

part of doctoral dissertation of Katarzyna Pauter-Iwicka, I declare that my contribution included cooperation in the field of methodology development, analysis, interpretation of the results and participation in the review&editing of the manuscripts.

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UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr hab. Małgorzata Szultka-Młyńska, prof. UMK
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Gagarina 7, 87-100

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Wchodzącej w skład rozprawy doktorskiej mgr Katarzyny Pauter-Iwickiej, oświadczam, że mój wkład polegał na współpracy przy opracowaniu koncepcji manuskryptów, napisaniu części manuskryptu oraz współudziale przy korekcie manuskryptów.

Małgorzata Szultka-Młyńska

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr hab. Paweł Pomastowski, prof. UMK
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Wileńska 4, 87-100

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UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr Michał Złoch
Interdyscyplinarne Centrum Nowoczesnych Technologii
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Gagarina 7, 87-100

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UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr Justyna Walczak-Skierska
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Wileńska 4, 87-100

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Wchodzącej w skład rozprawy doktorskiej mgr Katarzyny Pauter-Iwickiej, oświadczam, że mój wkład polegał na przeprowadzeniu badań i napisaniu manuskryptu.

Justyna Walczak-Skierska

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

dr hab. Michał Szumski, prof. UMK
Interdyscyplinarne Centrum Nowoczesnych Technologii
Uniwersytet Mikołaja Kopernika w Toruniu
ul. Wileńska 4, 87-100

OŚWIADCZENIE

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wchodzącej w skład rozprawy doktorskiej mgr Katarzyny Pauter-Iwickiej, oświadczam, że mój wkład polegał na pomocy w doborze warunków elektroforetycznych oraz współudziale przy korekcie manuskryptu.

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr Anna Król-Górniak

OŚWIADCZENIE

Niniejszym oświadczam, że jako współautor następującej publikacji:

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Anne Król-Górniak

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UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

mgr Dominika Błońska
Katedra Chemii Środowiska i Bioanalityki
Wydział Chemii, Uniwersytet Mikołaja Kopernika w Toruniu
ul. Gagarina 7, 87-100

OŚWIADCZENIE

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Wchodzącej w skład rozprawy doktorskiej mgr Katarzyny Pauter-Iwickiej, oświadczam, że mój wkład polegał na współudziale w pozyskaniu próbek biologicznych.

D. Błońska

UNIWERSYTET
MIKOŁAJA KOPERNIKA
W TORUNIU
Wydział Chemii

Toruń, dnia 8 kwietnia 2023 r.

Dr n. med. Wojciech Kupczyk
Katedra Chirurgii Ogólnej, Gastroenterologicznej i Onkologicznej
Wojewódzki Szpital Zespolony im. L. Rydygiera w Toruniu
ul. Św. Józefa 53-59, 87-100

OŚWIADCZENIE

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Mój wkład polegał na pozyskaniu materiału biologicznego od pacjentów oraz na interpretacji medycznej uzyskanych wyników.

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