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MALDI-TOF-ENABLED SUBTYPING AND ANTIMICROBIAL RESISTANCE SCREENING OF THE FOOD- AND WATERBORNE PATHOGEN *CAMPYLOBACTER*

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“If you are working on something exciting that you really care about, you don’t have to be pushed. The vision pulls you.”

Steve Jobs

Affidavit

I hereby confirm that the PhD thesis entitled "MALDI-TOF-ENABLED SUBTYPING AND ANTIMICROBIAL RESISTANCE SCREENING OF THE FOOD- AND WATERBORNE PATHOGEN *CAMPYLOBACTER*" has been written independently and without any other sources than cited.

Luxembourg,19/02/2022 _____

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Summary

For decades, antimicrobial resistance has been considered as a global long-lasting challenge. If no action is taken, antimicrobial resistance-related diseases could give a rise up to 10 million deaths each year by 2050 and 24 million people might end into extreme poverty. The ever-increasing spread and cross-transmission of drug-resistant foodborne pathogens such as *Campylobacter* spp. between reservoirs, such as human, animal and environment are of concern. Indeed, because of the over-exposition and overuse of antibiotics in food-producing animals, the latter could carry multidrug resistant *Campylobacter* that could be transmitted to humans via food sources or from direct animal contacts. One of the solutions to tackle antimicrobial resistances is the development of rapid diagnostics tests to swiftly detect resistances in routine laboratories. By detecting earlier AMR, adapted antibiotherapy might be administrated promptly shifting from empirical to evidence-based practices, conserving effectiveness of antimicrobials. The already implemented cost- and time-efficient MALDI-TOF MS in routine laboratories for the identification of microorganisms based on expressed protein profiles was successfully applied for bacterial typing and detection of specific AMR peak in a research context. In the line of developing rapid tests for diagnostics, MALDI-TOF MS appeared to be an ideal candidate for a powerful and promising "One fits-all" diagnostics tool. Therefore, the present study aimed to get more insights on the ability of MALDI-TOF MS-protein based signal to reflect the AMR and genetic diversity of *Campylobacter* spp.

The groundwork of this research consisted into the phenotypic and genotypic characterization of a One-Health *Campylobacter* collection. Then, isolates were submitted to protein extraction for MALDI-TOF MS analysis. Firstly, mass spectra were investigated to screen AMR to different classes of antibiotics and to retrieve putative biomarkers related to already known AMR mechanisms. The second part evaluated the ability of MALDI-TOF MS to cluster mass spectra according to the genetic relatedness of isolates and congruently compare it to reference genomic-based methods. MALDI-TOF MS protein profiles combined to machine learning displayed promising results for the prediction of the susceptibility and the ciprofloxacin and tetracycline *Campylobacter*'s resistances. Additionally, MALDI-TOF MS *C. jejuni* protein clusters were highly concordant to conventional DNA-based typing methods, such as MLST and cgMLST, when a similarity cut-off of 94% was applied. A similar discriminatory power between 2-20 kDa expressed protein and cgMLST profiles was underlined as well. Finally, putative biomarkers either linked to known or unknown AMR mechanisms, or genetic structural population of *Campylobacter* were identified.

Overall, a single spectrum based on bacterial expressed protein could be used for species identification, AMR screening and potentially as a complete pre-screening for daily surveillance, including genetic diversity and source attribution after further analysis.

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List of abbreviations

2-DE	Two-dimensional gel electrophoresis
AFLP	Amplified Fragment Length Polymorphism
AI	Artificial Intelligence
Amp	Ampicillin
AMR	Antimicrobial resistance
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
API	Analytical Profile Index
AST	Antibiotic Susceptibility Testing
AUPRC	Aera Under the Precision Recall Curve
AUROC	Aera Under the Receiver Operating Curve
BAM	Binary Alignment Map
β-NAD	Beta-Nicotinamide adenine dinucleotide
BTS	Bacterial Test Standard
CA-MRSA	Community-Associated Methicillin Resistant *Staphylococcus Aureus*
CARD	Comprehensive Antibiotic Resistance Database
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
CDx	Companion diagnostics
CFU	Colony-Forming Unit
cgMLST	Core genome MultiLocus Sequence Typing
Cip	Ciprofloxacin
COVID-19	Coronavirus Disease
CT	Complex Type
CWT	Continuous Wavelet Transform
DALYs	Disability-Adjusted Life Years
DDD	Defined Daily Dose
DNA	Deoxyribonucleic Acid
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
Ery	Erythromycin

ESI	Electrospray Ionization
EtOH/ACN	Ethanol and Acetonitrile extraction
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Formic Acid
FAO	Food and Agriculture Organization
FBP	Ferrous Sulfate, Sodium Metabisulfite and Sodium Pyruvate
FDA	Food and Drug Administration
FNR	Luxembourg National Research Fund
FT-IR	Fourier Transform- Infra Red
G+C	Guanine-Cytosine content
GDPR	General Data Protection Regulation
Gent	Gentamycin
GLASS	Global Antimicrobial Resistance and Use Surveillance System
HCCA	α-Cyano- 4-hydroxycinnamic acid
IRBT	Infra-Red Biotyper
ISO	International Organization for Standardization
IVD	In Vitro Diagnostic
Kana	Kanamycin
kDa	Kilo Dalton
K-NN	k-Nearest Neighbors
KPC	Klebsiella Producing Carbapenemase
LIST	Luxembourg Institute of Science and Technology
LNS	National Health Laboratory
LOS	Lipooligosaccharide
M/Z	Mass-to-Charge ratio
MALDI	Matrix Assisted Laser Desorption/Ionization
MBT	MALDI Biotyper
MEE	Multilocus Enzyme Electrophoresis
MH-F	Mueller Hinton Fastidious Agar
MIC	Minimum Inhibitory Concentration
MICROH-DTU	Microbiome In One-Health – Doctoral Training Unit
ML	Machine Learning
MLST	MultiLocus Sequence Typing

MOMP	Major Outer Membrane Porine
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS/MS	Tandem Mass spectrometry
MSPs	Main Spectra Profiles
MSSP	Mass Spectrometry-based phyloproteomics
NB	Naive Bayes
NGS	Next Generation Sequencing
NPV	Negative Predictive Value
OD	Optical Density
OIE	World Organization for Animal Health
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pETN	Phosphoethanolamine
PFGE	Pulsed-Field Gel Electrophoresis
PMF	Peptide Mass Fingerprint
PPV	Positive Predictive Value
PRSA	Penicillin Resistant *Staphylococcus aureus*
RF	Random Forest
rRNA	Ribosomal Ribonucleic Acid
RUO	Research Use Only
SFM	French Society for Microbiology
ST	Sequence Type
Strep	Streptomycin
SVM	Support Vector Machine
SVR	Short Variable Region
Tet	Tetracycline
TOF	Time-Of-Flight
USD	United States Dollard
VISA	Vancomycin Intermediate *Staphylococcus aureus*
wgMLST	Whole genome MultiLocus Sequence Typing
WGS	Whole Genome Sequencing
WHO	World Health Organization

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Part I

Introduction and Research Aims

CHAPTER 1

Fast microbiology: the XXI century challenge

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1. Ongoing challenges in microbiology: the antimicrobial resistances crisis example

Over the two-last century, the anthropic impact on the environment (e.g. urbanization, world migration, food market globalization) led to changes in ecosystem dynamics and diversity, including microbial life. For example, the rise of oceans' level already changed the epidemiology and location of several infectious diseases such as malaria, dengue and cholera (Waldvogel, 2004). Therefore, microbiologists must face new as well as long-lasting challenges, including emergence and re-emergence of pathogens. In the following section, the antimicrobial resistances (AMR) burden will be showcased.

1.1 A global public threat

The World Health Organization (WHO) identifies AMR as one of the top 10 global public health threats currently facing humanity (WHO, 2020). While numerous antibiotics have been almost yearly discovered and commercialized during the 20th century, the golden age of antibiotic

discovery reached an end in the 1960s (Hutchings et al., 2019). Since, the pipeline for new antibiotics is feeble due to loss of interest of pharmaceutical compagnies to develop new ones (Mattar et al., 2020). In 2016, the economist Jim O'Neill chaired a review on the antimicrobial resistances where he highlighted the death of 700,000 people every year due to resistant infections (O'Neill, 2016). As well, he estimated by 2050 a loss of 10 million lives a year and a cumulative 100 trillion USD because of drug resistant infections.

1.2 Causes and consequences

The eventual onset of a "post-antibiotic" era may have important consequences on modern life. In one hand, the decreasing effectiveness of antibiotics will lead to less successful treatment methods (e.g. amputation), which will be longer and invasive in a near future (Michael et al., 2014). In the other hand, it will also have extensive economical and societal impacts, increasing morbidity and mortality (Michael et al., 2014). The rapid evolution and selection of AMR bacterial species stem from numerous factors. Amongst these, human (e.g. increasing population, overuse of antibiotics), clinical miscarriage (e.g. over-prescription), societal misconceptions (e.g. non-prescription purchase) and agricultures (e.g. antibiotherapy for stock and crops) causes should be highlighted (Michael et al., 2014). For instance, *Staphylococcus aureus* developed numerous resistance mechanisms to introduced antibiotics over the last 60 years, including penicillin, methicillin and vancomycin (**Figure 1.1**) (Lowy, 2003; Hardy et al., 2004).

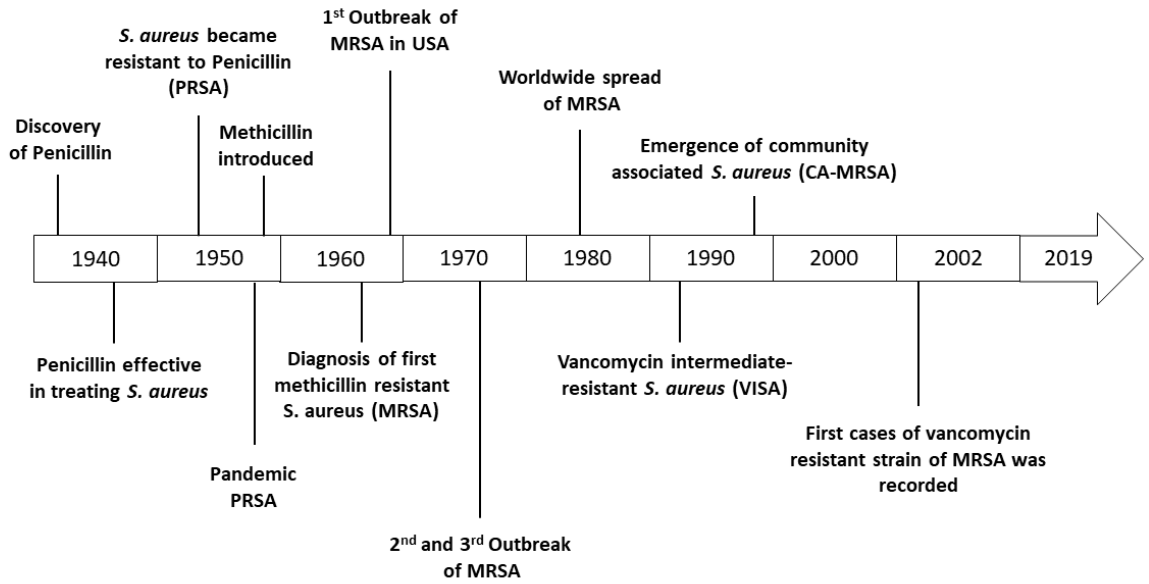

***Figure 1.1**. Antimicrobial resistance timeline from 1940 to 2019 of S. aureus (Adapted from Kumar et al., (2020)).*

The introduction of penicillin in the 1940s offered to treat efficiently bacterial infections. Nevertheless, penicillin resistant *S. aureus* were recognized in 1942 and in the 1960s, 80% of staphylococci were producing penicillinase (Lowy, 2003). Such enzyme is carried by a genetic transposable element located on a plasmid with other AMR genes (e.g. gentamycin). Therefore, *S. aureus* strains non-producing penicillinase were removed from the bacterial population, remaining only the resistant one. Likewise, after the introduction of the methicillin antibiotics in 1961, methicillin resistant *S. aureus* (MRSA) were rapidly counting for a majority of human cases (Jevons, 1961; Parker and Hewitt, 1970). Empiric use of previous antibiotics in human infections treatment is believed to have contributed of the establishment of multidrug (e.g. vancomycin, fluoroquinolones) resistant *S. aureus* (Lowy, 2003). Nowadays, MRSA is considered as global public health threat with 33,110 attributable deaths and 854,541 disability adjusted life years (DALYs) in 2015 in Europe (Cassini et al., 2019). Additionally MRSA associated to livestock were reported and is now considered as an emerging problem worldwide (Anjum et al., 2019). The cause is the use of last generation antibiotics (e.g. cephalosporin) as non-therapeutic use in food industries, which may have contributed to the spread of MRSA in Europe due to pressure of selection (Mehndiratta and Bhalla, 2014). The main problem is that they can be transmitted between different animal species, such as pigs, and humans who have close contact with the latter (e.g. veterinarians, farmers), in both directions (Crespo-Piazuelo and Lawlor, 2021). Antibiotics use in food animals' guidelines and policies emphases the need to reduce non-therapeutic use of antimicrobials, especially the one used in both human and veterinary medicine by implementing different actions (e.g. regulating sales, surveillance monitoring) (Mehndiratta and Bhalla, 2014).

1.3 A lot on the international community plate

Consequently, what have been done or what is envisaged to slow down or overcome the emergence of AMR? International and national measures were taken, leading to the creation of global and European polices to combat AMR (WHO, 2001, 2011; European Commission, 2017). Additionally, in 2015 a global surveillance system was launched by the WHO, better known as Global Antimicrobial Resistance and Use Surveillance System (GLASS) (Agnew et al., 2021). This program aims to "strengthen knowledge through surveillance and research". At the community level, communication actions such as awareness of the rational use of antibiotics or standards of hygiene may be more widely undertaken (Uchil et al., 2014). Several of these actions will eventually end up into a sustainable use of antibiotics in healthcare (Allcock et al., 2017). Nevertheless, considering the previous described MRSA example, a more global vision of the crisis should be considered by including a One-Health vision. One-Health is "a worldwide strategy for expanding interdisciplinary collaborations and communications in all aspect of health care people, animals and

environment" (Gibbs, 2014). Actions like the innovation in new drugs by reviving and reinventing the "dry" antimicrobial pipeline (Luepke and Mohr, 2017; Singer et al., 2019), or the reduction of the usage of antibiotics in live-stocks by the application of regulations such as (EU) 2019/6 and (EU) 2019/4 coming into effect in 2022, for "the prohibition of all form of routine antibiotic use in farming" should be undertaken (Alliance save our antibiotics, 2020).

Additionally, the understanding of AMR mechanisms is important to develop methods to detect resistances (Bergeron and Ouellette, 1998). Development of rapid tools to detect resistance in a routine context is a key step in the choice of the antibiotherapy and therefore in the fight against the AMR. Popularization of molecular tests, including genomics, proteomics and lipidomics, combined to bioinformatics tools will allow a smooth transition from AMR phenotyping to molecular and *in silico* antibiograms (McArthur and Wright, 2015).

2. A fast microbiology: the apogee of molecular biology

In different field of microbiology such as food control or infectiology, the rapid detection or identification of microorganisms is an important step. It exists a broad spectrum of characterization methods, which could be divided into two groups: culture dependant and independent (Yagel and Moran-Gilad, 2021). In one hand, culture independent microbiology used techniques (e.g. polymerase chain reaction (PCR)) directly on samples (e.g. stool or nasal swabs) regardless the culture of microorganisms. On the other hand, culture dependant methods rely on the growth of the investigated microbes from the sample (Yagel and Moran-Gilad, 2021). Both methods are commonly used in diagnostics. Nevertheless, independent culture methods will not be developed further as it is beyond the scope of the current research work. Within the culture dependant techniques two groups could be highlighted: the phenotypic and genotypic one (Sandle, 2016). Developed in 1880's phenotypic techniques regroup conventional culturing methods (e.g. Gram and spore staining), immunological (e.g. ELISA assays) and biochemical (e.g. API test strip) tests. Developed in 2000's genotypic tests regroup all molecular protocols, including omics- powered techniques, such as genomics, proteomics and lipidomics (Ferone et al., 2020). The choice of the methods will depend on the costs, resources, time and the level of identification required (Sandle, 2016). While phenotypic methods are widespread and reference methods for several tests (e.g. antibiograms) due to their low costs, the current culmination and integration of molecular technologies in routine microbiology are slowly replacing it. Molecular methods are considered faster, more accurate, while presenting drawbacks such as the cost or technical issues, including DNA amplification of dead bacteria or the presence enzymatic inhibitors in the case of genomics

(Yagel and Moran-Gilad, 2021). In this section, culture-dependant genomic-, proteomic- and lipidomic-based methods will be discussed.

2.1 Genomics

Firstly, introduced and developed by Sanger in the 1970s, the first generation of DNA sequencing allows the analysis of a single DNA molecule, by using a chain termination method. Nevertheless, it was an expensive, laborious and time-consuming method, with analytical limits, such as being able to read less than one kilobase (Heather and Chain, 2016; Yagel and Moran-Gilad, 2021). Nevertheless, the need of a rapid and high-throughput during a single machine run technology led to the second and third-generation of DNA sequencing, the so-called Next Generation Sequencing (NGS) (Heather and Chain, 2016). Therefore, the NGS term encompass all sequencing technologies (e.g. Whole Genome Sequencing (WGS)) allowing the whole genome analysis of different cells, i.e. eukaryote or prokaryote in one sequence run (Deurenberg et al., 2017).

NGS is mainly used in microbiological research for a bunch of applications. It includes species identification, genotyping for epidemiology investigations, antimicrobial resistance and virulence characterisation (Bertelli and Greub, 2013; Lavezzo et al., 2016). It is undeniable to mention that NGS is a swiss knife for microbiological laboratories. NGS is more efficient than other already implemented techniques, e.g. it has a higher discriminatory power compared to traditional typing methods. Development of standard protocols, such as the ISO/DIS 23418 standard for the application of WGS for typing and genomics characterization of foodborne bacteria, are currently under development (ISO, 2020). Therefore NGS could be consider as a “one test fits all” methodology with an already on-going wide implementation in both routine and research laboratories (Deurenberg et al., 2017)

Its implementation in clinical settings is still challenging due to the need of wet lab, i.e. performed at the laboratory bench, and dry-lab, i.e. data analyses using bioinformatics pipelines, workflow adjustments (Nadon et al., 2017; Couto and Rossen, 2021). On the one hand, such methods require advanced bioinformatics pipelines, swift data processing, and massive data storage capacities (Nadon et al., 2017). While laboratories may have financial resources to acquire NGS equipment, they may not have considered the cost of data management, bioinformaticians, informatics equipment and related analysis (Pereira et al., 2020). For instance, Pereira et *al*. (2020) described that a typical binary alignment map (BAM) file from a single experience consume 30 Gb of storing space, generating significant costs. On the other hand, the following wet-lab limitations could be cited: PCR amplification bias and sequencing errors leading to a weak coverage (Pereira et al., 2020). The final challenge regarding the implementation of NGS in routine laboratories is the

overall cost. Even if current NGS technologies have a lower cost than Sanger sequencing, it is not yet reachable for everyone. Indeed, the initial cost of sequencer is high, as well as reagents and consumables (Kwong et al., 2015). While the sequencing apparatus' cost ranges approximately between 150,000 and 750,000 USD, the cost of WGS in routine microbiology clinical practice, including DNA extraction, library preparation and sequencing was estimated at around 200 euros per sample with an approximate turnaround time of 60h (Kwong et al., 2015; Rossen et al., 2018).

Nevertheless, the arrival of NGS in diagnostics laboratories is a matter of time if the cost of sequencing as well as the preparation time is dropping in the next years.

2.2 Proteomics

While the first proteomics studies happened in 1975, the term "proteomics" was only introduced in 1995. It is traditionally defined as the large scale analysis of proteins (Ames and Nikaido, 1975; Graves and Haystead, 2002). Proteins were first investigated by gel-based methods, such as two-dimensional gel electrophoresis (2-DE). While such techniques have high-resolution protein separation, it presented several limitations, e.g. time consuming, poor reproducibility and poor representation of low abundant proteins (Abdallah et al., 2012; Ning et al., 2016; Marcus et al., 2020). 2-DE-like methods were improved by the direct identification of protein from the gel by gel-free mass spectrometry (MS), giving birth to peptide mass fingerprinting techniques (PMF) (Bowman, 2014). Briefly the protein mass profiles obtained after MS analysis is compared with predicted mass values in databases by search engine (e.g. MASCOT) (Cottrell, 1994). Due to its high-throughput, fast and accuracy characteristics, Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) MS became one of the preferred methods for PMF.

Proteomics became trendy over the last two decades to study infectious pathogens. By looking at protein profiles, i.e. specific or signature of proteins, in clinical sample (e.g. blood), MS-techniques could identify proteins associated with microbial activity, host-pathogen interaction, virulence factors and AMR (Graves and Haystead, 2002; Pérez-Llarena and Bou, 2016; Kathera, 2018). For example, Foudraine et al. (2021) in a multi-omics approach investigated AMR in *E. coli* and *K. pneumonia* to detect mechanisms of resistance by using liquid chromatography tandem mass spectrometry (Foudraine et al., 2021). In their conclusion, the authors highlighted that MS aims to be a rapid and high-throughput method for AMR detection. Other studies reported its application for protein identification and quantification as well as the study of the microbial population of the gut microbiota (Shao et al., 2015; Angel and Aryal, 2020).

Conventionally proteins are isolated in a first place by electrophoresis gel or chromatography and then broken up into peptide by enzymatic digestion (e.g. trypsin), so-called "bottom up" or shotgun proteomics (Armengaud, 2013; Dupree et al., 2020). However, in some cases, these steps

can be skipped resulting in an even faster workflow. In 2010's MALDI-TOF MS was for the first time introduced under clinical settings and is still used as a fast, cost-efficient, reliable technique for the direct microorganisms identification after a whole cell analysis (Singhal et al., 2015). In this case, bacteria are directly applied on a target for testing and identified with a "top down strategy", i.e. analysis of intact proteins (Torres-Sangiao et al., 2021). At the end, sample preparation and machine time is around 25 min, which is much faster than current NGS.

Beside the price of the significant high price of a MS apparatus (e.g. 160.000 euros for a complete MALDI-TOF MS system) and related maintenance (e.g. 25.000 euros per year), which is rapidly amortized by the low-cost of analysis and reagents (e.g. 0.53 euros of reagents and consumable for a 96 sample target), microbial proteomics presents wet-lab and dry-lab drawbacks just like genomics (Tran et al., 2015; Chabriere et al., 2018). In the case of shotgun proteomics, the dynamic range of mass spectrometers as well as protein database used to interpret MS/MS data need to be improved (Armengaud, 2013). Additionally, preparation of samples' homogenization could be tricky due to the existence of a large number of methods and different technologies available (Dupree et al., 2020). In the case of top-down proteomics, low resolution, poor fragmentation and the need of purified samples must be highlighted (Catherman et al., 2014). Finally, the need of efficient bioinformatics tools to handle large datasets still needs to be addressed (Armengaud, 2013).

In a post-genomics world, microbial proteomics will be a foremost complement to other omics-powered technology as protein activity is the most important factor for understanding biological pathway.

2.3 Lipidomics

The development and advances in mass spectrometry permitted the investigation of lipids. In comparison to other presented omics technologies, lipidomics is a newly emerged discipline (Lagarde et al., 2003). It has been defined by Spener et al. (2003) as the "full characterization of lipid molecular species and of their biological roles with respect to expression of proteins involved in lipid metabolism and function, including gene regulation" (Spener et al., 2003). The investigation of such metabolites is relevant to access more information about cellular functions and hence essential to determine genic function (Rolim et al., 2015). Accordingly, lipids are involved in several metabolic pathways, hence perturbations or modifications in lipids will, as a cascade chain, affect other metabolic pathways (Rolim et al., 2015). MS-based shotgun lipidomics, or untargeted lipidomics, is rapid, straightforward and count several advantages, such as high resolution and its ability to maintain intact molecular structure during ionization, to analyse a large variety of lipid classes (e.g. phospholipids, glycolipids, lipoproteins) (Köfeler et al., 2012; Yang and Han, 2016).

On the one hand, lipidomics has mainly be investigated for biomedical sciences, such as investigation of the metabolic syndrome, neurological disorders, cancer and nutrition (Yang and Han, 2016). On the other hand, microbial lipidomics has soared over the last decade with different type of applications (Appala et al., 2020). It has been described likewise genomics and proteomics, for bacterial identification, microbiota investigation, soil microbial ecology and viral mechanisms understanding (Layre et al., 2014; Appala et al., 2020; Ding et al., 2021; Kyle, 2021; Ren et al., 2021). Nevertheless, lipidomics has gained interest the past 3 years with the publication of reports suggesting that resistance to polymyxin antibiotic is possible by observing a modification in the lipid A by using the fast MALDI-TOF mass spectra (Dortet et al., 2018b; Furniss et al., 2019; Dortet et al., 2020). Interestingly, application of different MS-based lipidomics, such as electrospray ionisation (ESI) MS, for AMR study has been known since at least 2013 (Singh et al., 2013). Singh et al. (2013) emphases gradual changes in different lipids classes after exposure to fluconazole antifungals in *Candida albicans.* While lipidomics might still have a stony way before being implemented under clinical settings, manufacturers also started to develop benchtop mass spectrometer combining positive and negative ions mode for lipids detection as well as lipids extraction kit (e.g. MBT Lipid Xtract) for research use only (RUO) (Bruker, 2019a). Therefore, the microbial diagnostics market seems attentive to the future of this technology.

Like microbial lipidomics is currently at its early stage, limitations regarding its implementations in routine diagnostics are still fuzzy. Currently identification and quantification of lipid species and mapping of the whole cellular lipidome is still not fully achievable (Yang and Han, 2016). As for proteomics and genomics, the development of bioinformatic pipelines is needed for the management of large data sets and the construction of metabolic pathways (Yang and Han, 2016). Integration of lipidomics to a multi-omics strategy, might bring deeper insights to understand complex molecular mechanisms (Rolim et al., 2015; Yang and Han, 2016). Lipidomics is still a niche to explore in microbiology, with several exciting venues and benefits to explore in the future. As for WGS it is only a matter of time before lipidomics be added to the physician's toolkit.

Omics-technologies generate innumerable data requiring even more new approaches to deal and extract relevant information from it. Bioinformatics tools have come a long way and became user-friendly for microbiologists with no specific knowledge in data treatment and analysis. Data driven technologies, such as artificial intelligence, were reported to be suitable to analyse clinical big-omics data thanks to their important computational power.

3. A digital microbiology: the artificial intelligence revolution

Nowadays, artificial intelligence (AI) is applied to numerous field of applications from industrial marketing to medicine (Amisha et al., 2019). In the following section the background of AI, the performance and the future of machine learning (ML) in routine microbiology will be discussed.

3.1 Overview and motivations

Firstly described by Alan Turing in the 1950's, AI was coined and defined by John McCarthy as "the science and engineering of making intelligent machines" (Ahuja, 2019). Global AI development knew two important eras of reduced funding and interest leading to fewer significant developments, from the 1970s to the 2000s, the so called AI winters (Kaul et al., 2020). Nevertheless, during this period, life sciences slowly adopted AI for eventual diseases diagnostics. CASNET (1976) and MYCIN (1970s), for the diagnosis/therapy of glaucoma and identification/treatment of several infections respectively, were the two first AI prototypes highlighted for their feasibility in healthcare (Kaul et al., 2020). Nowadays AI is widely and routinely employed on daily basis in healthcare facilities under diverse interfaces.

AI in medical services could be described as an ensemble of technologies, including rule-based expert systems, physical robots, robotic process automation, natural language processing and machine learning (ML) (Davenport and Kalakota, 2019). Expert systems are programs which reason and make judgment based on facts and "If conditions-Then action" rules (Hambali and Jimoh, 2014). Such method was used to diagnose diseases like malaria, typhoid fever or cholera (Hambali and Jimoh, 2014). Nowadays, physical robots are commonly employed and became the new standard of care (Lane, 2018). For example, the U.S food and drug administration (FDA) approved ROBODOC which was designed to improve hip replacement surgery (Lane, 2018). Robotic process automation aims to automatize repetitive numerical tasks or other supply chain processes by using computer software or 'bots' (Soeny et al., 2021). Soeny et al. (2021) demonstrated that robotic process automation accelerates the process of prescription validation and digitalization with less human implication (Soeny et al., 2021). Natural language processing represents all machine program interpreting human language. Such program was successfully used to identify post-operative surgical complications (e.g. renal failure, pneumonia or sepsis) while using medical reports (Murff et al., 2011). Finally, ML could be considered as a mathematical model based on structured data to make predictions (Zhang, 2017). It can be used in the medical imaging field to predict if the image contains benign or malignant tumour (Erickson et al., 2017). ML is currently the most common form of AI applied worldwide (Davenport and Kalakota, 2019). Overall, AI is applied at every stage in healthcare facilities.

Interest for AI in medical applications raised with the digitalization and the growing number of data generated in clinical settings. Indeed, the main motivations for applying AI in health cares was the ability of computer algorithms to derive diagnostics from a larger volume of data (e.g. diagnostic imaging, gene expression, electrodiagnosis or clinical symptoms). From this data, AI models could be trained and hence could assist under clinical settings. Thus, they have a positive impact on healthcare system by improving diagnostic accuracy, clinical operation and most importantly patient management and outcomes (Kaul et al., 2020; Leo et al., 2020). Currently AI is importantly tackling several types of diseases, such as diagnosis of cancer, nervous system and cardiovascular diseases. For example, a patient with quadriplegia retrieved movement thanks the development of intracortical microelectrodes arrays (Bouton et al., 2016). Besides these major fields of study, AI has been used for the investigation of other diseases (e.g. congenital cataract or diabetic retinopathy) and other clinical fields, such as microbiology (Jiang et al., 2017).

3.2 Machine learning: how is it working?

So, what is exactly ML and how does it work? ML could be imaged as an umbrella term referring to several disciplines, e.g. probability theory or statistics, that performs predictions based on a dataset (Qu et al., 2019). Four types of ML exist: supervised, semi-supervised, unsupervised and reinforcement learning (**Figure 1.2**).

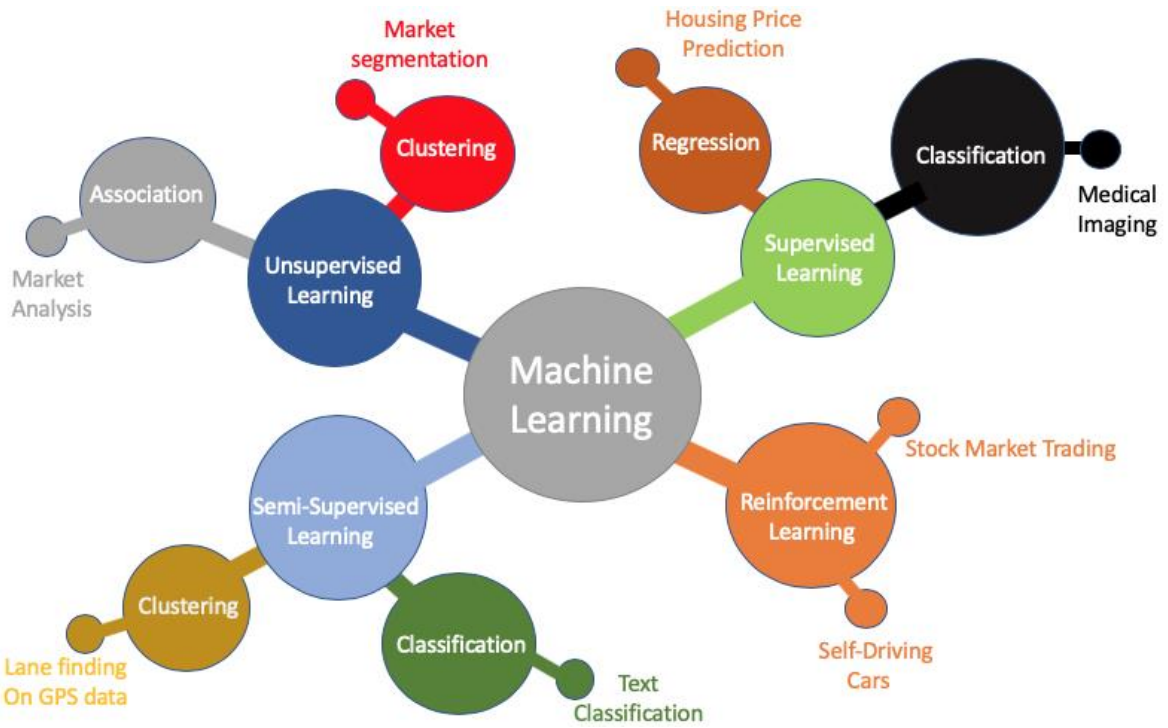

***Figure 1.2**. Machine learning algorithms overview (Omondi Asimba, 2019)*

In this section we will focus mainly of on supervised ML as it is the most commonly model type employed in microbial studies (Goodswen et al., 2021). It can be grouped into two categories of algorithms: classification and regression. Classification algorithms will predict category or a discrete class label (e.g. resistant or susceptible), whereas regression will predict a continuous value (e.g. minimal inhibitory concentration). Therefore, the aim of supervised ML is to predict classification or value of unknown data (Goodswen et al., 2021).

The "supervised" term underlines the need to teach the model. The demand of a ML approach is driven by the necessity to answer a specific question or to reach an industrial objective. In the current section, a ML strategy will be used to answer the following question: is my bacterial protein profile associated to an antibiotic resistant or a susceptible phenotype? This could be answered in five essential steps.

The first one is the data gathering (Goodswen et al., 2021). The dataset is constituted of input data values (features or X), which are associated to an outcome (label or Y) (Nichols et al., 2019). In our example, the dataset could be assimilated to a list of isolates phenotypically characterized by a reference method such as disk-diffusion antibiogram (Y is resistant or susceptible) and associated with their protein profiles obtained by MS (X refers to n protein peaks with intensities values).

The second step is data cleaning and pre-processing. During this stage, missing values, outliers and incorrect formatted data are checked (Nichols et al., 2019). When the dataset is clean, features could be selected, scaled or transformed. Such process is called features engineering, and it is use to prepare data for certain type of algorithms (Goodswen et al., 2021). Then the data is randomly split into two different sets, the training and test sets. The training set encompasses 70-80% of data and is used to build the model, whereas the test set with remaining data is used to evaluate previous model performance. Data included in the test set is never seen by the training one (Nichols et al., 2019; Goodswen et al., 2021).

The third step is to select the best-fitting model to the current data. The general rule is to apply different models supported by diverse algorithms. According to the type of data studied, algorithms might perform in different manners (Goodswen et al., 2021). The most common algorithms in supervised ML are the k-Nearest Neighbors (k-NN), Naïve Bayes (NB), Support Vector Machine (SVM) and Random Forest (RF) (Qu et al., 2019). The training set, including protein profile (X) as well as AMR phenotype (Y), is then used to create a model. During this step, the model will search for statistical patterns using a selected algorithm to detect close patterns in future entries (Goodswen et al., 2021). Once the model is created, it will be used to predict the AMR phenotype (Y) of the test set's isolates regarding their MS protein profiles (X).

The fourth step is the evaluation of the trained model. Test set predictions could be summarized in a confusion matrix, highlighting true positive, true negative, false positive and false negative values. From it, several performance metrics, such as the precision, the recall or the F1-score could be computed (Grandini et al., 2020). The precision or positive predictive value express the portion of predicted isolates as phenotypically resistant by the model, which are actually resistant. Therefore, it underlines how much the model can be trusted when a positive outcome is given. The recall or sensitivity measures the ability of the model to recover all the resistant isolates in the test set. The F1-score is the harmonized means between the precision and recall, and hence assesses model's performance (Grandini et al., 2020). If performance does not meet expectations, the training cycle, including a tuning step of algorithm parameters, could be performed until the model's performance is good enough for the application (Goodswen et al., 2021).

The final step is to do predictions using the refined model on "real world" data. Therefore, when an unseen protein profile will be analysed by the model, the algorithm will associate a "resistant" or "susceptible" label with it. Nonetheless, a wide choice of type of microbial data (e.g. DNA sequence, microscope pictures, AMR or typing profiles) is possible when using a ML approach, opening a tremendous field of applications.

3.3 Machine learning applications in microbiology

Over the last five years ML gained a tremendous interest in microbiology (**Figure 1.3**), due to the facility to collect high-throughput digital data, its economic computing power, its data storage, and its rapid transfer of data.

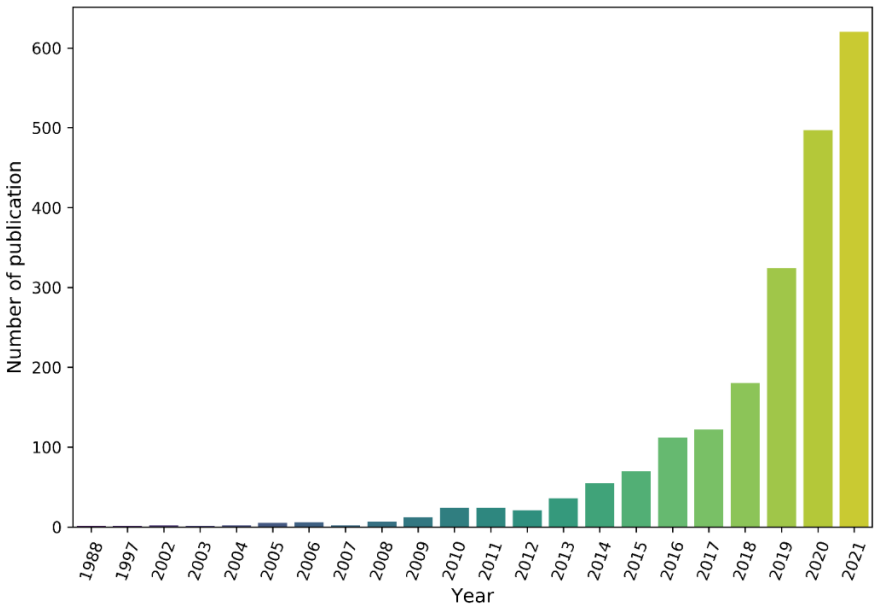

***Figure 1.3**. Machine learning related publications in microbiology with key words "Microbiology" and "Machine Learning" on PubMed (search in December 2021)*

It has been used in all microbiology research fields, including bacteriology, mycology, virology and parasitology (Goodswen et al., 2021). Supervised ML was successfully used for different thematics, such as gut microbiome studies (Chen et al., 2021), optimization of strain typing (Cohen et al., 2021), microorganism identification based on image recognition or motility (Sajedi et al., 2020; Rani et al., 2021; Riekeles et al., 2021) and more recently on the detection of the coronavirus disease 2019 (COVID-19) severity in patients using blood and urine tests (Yao et al., 2020).

To make a parallel with the current AMR crisis and the need to improve the understanding of AMR mechanism and develop rapid technology to screen AMR, the ML strategy was also successfully reported. For example, Pataki et al. (2020) investigated ML to predict ciprofloxacin's minimal inhibitory concentration based on AMR genotype obtained by WGS. The created regression models accurately predicted fluoroquinolone resistance for *E. coli* with 93% (n=264/266) of correctly classified samples with only 4 features, considered as important by the model. These features of importance, i.e. mutations in *gyrA* residues Ser83 and Asp87, mutation in *parC* residue Ser80 and presence of the *qnrS1* gene, were already associated with ciprofloxacin known resistance mechanisms (Pataki et al., 2020). Along the same line, Li et al. (2018) successfully evaluated the SourceTracker classification methods to track antibiotic resistance gene pollution from several sources based on metagenomic datasets (Li et al., 2018). Such study might have an important impact in terms of AMR control strategies.

While Weis et al. suggested that their AMR predictive models based on MALDI-TOF mass spectra could give antibiotherapy guiding 12-72h earlier than traditional methods, there is currently no article describing the use of a ML system in clinical practice or reporting the impact on processes or clinical outcomes (Peiffer-Smadja et al., 2020; Weis et al., 2020a). Also, questions regarding the standardization of data, code sharing and handling, as well as the training of laboratory personnel is still to address (Egli, 2020). Nevertheless, by the digitalization of high-throughput data, implementation of ML and automatization of many diagnostics tests, the microbiology field is entering in a new challenging and exciting era. It is already supported that this new embracement with computational sciences will have a significant impact on the daily routine of laboratories. It is time for a fast and digital microbiology to enhance diagnostics and patient management (Egli et al., 2020).

CHAPTER 2

Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS): a macroscopic and microscopic revolution

Contents

1. Mass spectrometry as a microbiological diagnostic tool

Mass spectrometry (MS) became an analytical key of the "-omics" era (Girolamo et al., 2013). It is widely used in different application fields (e.g. material and biomedical sciences) for different purposes such as, the structural interrogation of new polymer materials or cancer biomarkers discovery (De Bruycker et al., 2020; Macklin et al., 2020). In this section, MS applied to routine and research microbiology will be discussed in depth.

1.1 History

MS has already been introduced in microbiological routine diagnostic for 15 years. However, MS was discovered in the 1900s and was mainly applied to chemical and physics sciences to determine atomic weights of elements (Yates III, 2011). The first application for the analysis of

biomolecules was carried out in the late 1960s (Biemann et al., 1959). The first attempt to use MS for microbial identification happened more than a decade later (Anhalt and Fenselau., 1975). Indeed, Anhalt and Fenselau experimented a combination of pyrolysis and MS and highlighted that spectra obtained from different gram-negative bacteria had compositional differences. These differences were larger for bacteria with a large taxonomic difference than for smaller ones. Matrix-assisted laser desorption ionization (MALDI) MS was first developed and introduced in the late 1980s by Karas and colleagues (**Figure 2.1**)(Karas et al., 1987).

A. B. C.

Figure 2.1*. A. Prof. Michael Karas. B. Prof. Catherine Fenselau. C. Koichi Tanaka*

Simultaneously, in 1988, the same discovery was also reported by Tanaka and colleagues (Tanaka et al., 1988). The latter were rewarded with a shared Nobel Prize in 2002, for the development of "soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules". MALDI method is qualified as a soft ionization method as the energy of the laser is mainly absorbed by the matrix instead of the analyte itself. Hence it caused limited or no fragmentation, allowing the identification of molecular ions (Calderaro et al., 2014).

Since, MALDI-Time-Of-Flight (TOF) MS has been investigated for its application in microbiology (Claydon et al., 1996; Krishnamurthy and Ross, 1996; Jarman et al., 2000; Seng et al., 2009). The technique is rapidly considered as a quick, cost effective and reliable method for the identification of several bacterial phyla (e.g. *Helicobacter*, *Bacillus* or *Staphylococcus*) (Demirev et al., 1999; Nilsson, 1999; V et al., 2000). In 2009, the first MALDI-TOF MS system able to carry out microbial identification was produced by Bruker Daltonics located in Bremen, Germany (Seng et al., 2009). The MS apparatus, was a classical MALDI-TOF mass spectrometer, using an on-site database and a direct transfer of bacterial colony on the target. Nowadays, MALDI-TOF MS is commonly implemented in routine diagnostics. It became the reference method for daily microbial

identifications, benefiting to public health and hospital hygiene through rapid identifications (Rodríguez-Sánchez et al., 2019).

1.2 MALDI-TOF mass spectrometer operating

1.2.1 Principle and Methodology

MALDI-TOF analysis relies on a mass spectrometer apparatus. It could be decomposed in four main components: the sample introduction system, an ionization source, a mass analyser and an ion detector (**Figure 2.2**).

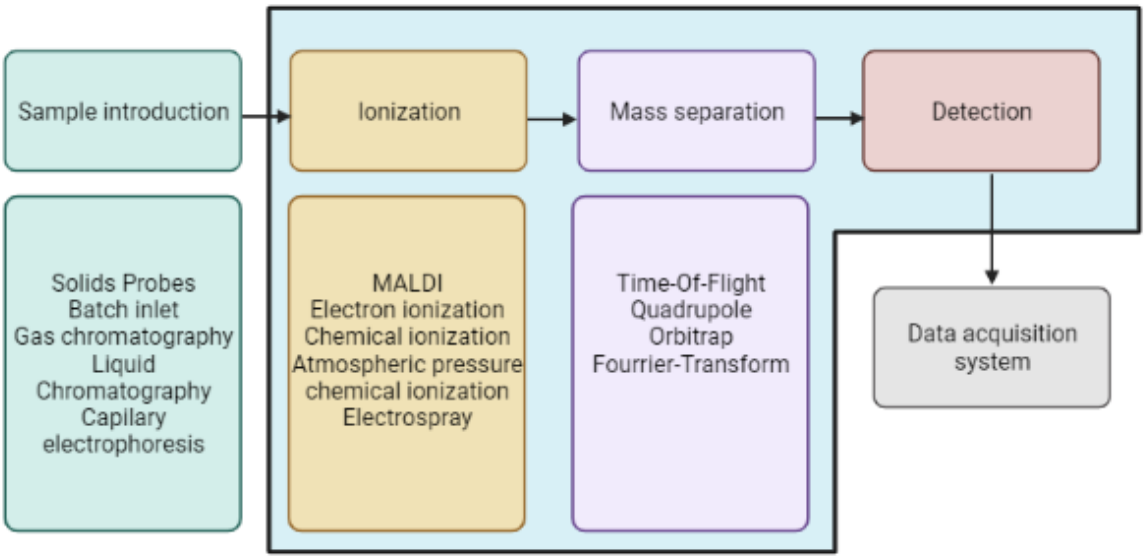

***Figure 2.2**. Configuration overview of commonly used mass spectrometer systems.*

For each component several features exist and are selected depending on the analysed sample (Greaves and Roboz, 2014).

In a case of a MALDI-TOF mass spectrometer, a solid probe (e.g. steel coating or disposable target) will be used as the sample introduction system. In routine diagnostics, targets used are spotted with direct smear of biological material such as bacterial colonies directly removed from agar plates (**Figure 2.3**).

The MALDI methods will be used as the ionization source. In brief, the bacterial smear is co-crystalized with an excess of chemical called matrix (e.g. α-cyano-4-hydroxycinnamic acid or HCCA). When dried, the analyte is submitted inside a vacuum chamber to laser pulses, resulting into the evaporation of the matrix, leading to the formation of reagent ions that protonate the sample. The produced ions are singly charge.

The TOF analysers will be then used, to measure the time that ions are taking to travel through the system depending on their mass-to-charge ratio (m/z).

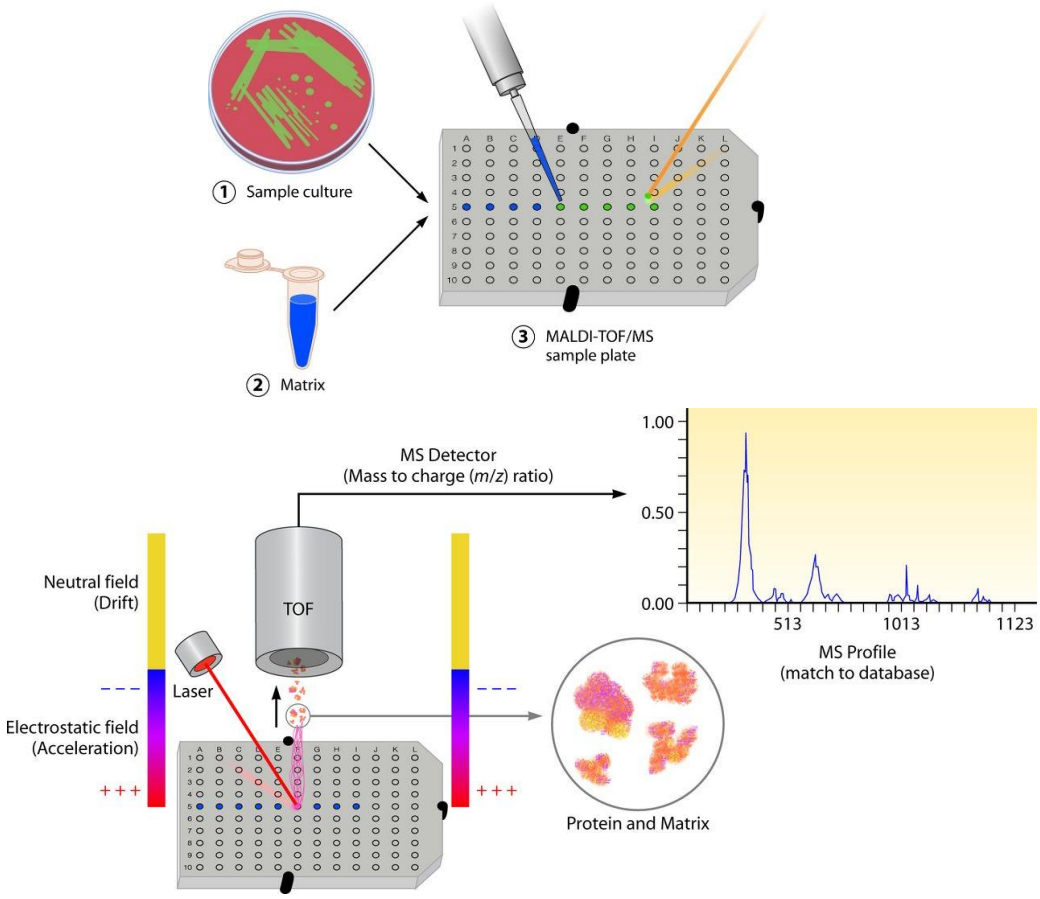

***Figure 2.3**. The process of MALDI-TOF MS for the identification of microorganisms (Adapted from Clark et al., (2013)).*

Finally, ions separated according to their m/z are caught by the detector as they reach the end of the TOF tube. The ions TOF to reach the detector is used to compute the mass of the protein. Finally, the sum of analysed ions is translated by the data system into a mass spectrum or protein mass fingerprint (Carbonnelle and Nassif, 2011). Mass spectra are represented with the m/z ratio on the abscissa axis and the intensity of the signal as ordinate axis. In microbiology the typical investigated m/z range is 2-20 kDa, which represents ribosomal proteins and some housekeeping proteins (Ryzhov and Fenselau, 2001; Murray, 2012; Singhal et al., 2015). Then, identification of microorganisms at the genus or species level can be carried out. Identification relies on the comparison of m/z of the unknown mass spectra and already known microbial isolates mass spectra stored in a commercial or in-house database (Singhal et al., 2015; Seuylemezian et al., 2018). According to the commercial solution used, several libraries exist.

Currently, certain MALDI-TOF systems including reference databases, like the MALDI Biotyper (Bruker Daltonics), cover 567 genus and 3159 species, including 1379 Gram-negative species, 1564 Gram-positive species, 216 yeasts species (Bruker, 2020). Additionally, extra libraries are available

for filamentous fungi, covering 247 species, and mycobacterium, with 182 of the 201 known mycobacteria (Bruker, 2021d, 2021b).

1.2.2 Commercial platforms

While several mass spectrometers for microbial identification exist (e.g. Autof MS 1000 (Autobio Diagnostics, China), AXIMA (Shimadzu, Japan), MicroIDSys (ASTA corp., South Korea)), the current European microbiological MS market is led by two analytical companies (Carbonnelle et al., 2012; Lee et al., 2017b; Yi et al., 2021). The first one is Bruker Daltonics, located in Bremen, Germany and the second one is bioMérieux located in Marcy-Etoile, France. Both companies proposed several benchtop mass spectrometers for rapid microbial identifications (**Figure 2.4**).

MALDI Biotyper® Sirius, Bruker Daltonics
(Bremen, Germany)

MicroIDSys, ASTA corp.
(Suwon, South Korea)

VITEK® MS, bioMérieux
(Marcy l'Etoile, France)

VITEK® MS PRIME, bioMérieux
(Marcy l'Etoile, France)

***Figure 2.4**. Mass spectrometers widely implemented in Europe*

Each system has its own specificity (e.g. databases, extraction methods, cut-off scores) and are often compared together (McElvania TeKippe and Burnham, 2014; Lévesque et al., 2015; Lee et al., 2017a).

On the French company side, the VITEK® MS is commercially available. It is included as a part of the integrated identification/antimicrobial susceptibility test solution of bioMérieux. Indeed, when coupled with the semi-automatic antibiogram device VITEK® 2, the commercial real-time platform reports the identification and the antimicrobial susceptibly profile of the analysed isolates. In 2021, the company announced the release of the new MALDI-TOF MS identification generation system, the VITEK® MS PRIME. In comparison with the already well-known VITEK® MS, it offers a compact benchtop format with new features (e.g. prioritization of urgent samples) (BioMérieux, 2021).

On the German company side, the MALDI Biotyper® (MBT) device was introduced in 2004 as a research tool and in 2009 as an in-vitro diagnostics (IVD) system. Several benchtop format MBT are currently commercialized (e.g. LT/SH, Sirius, Sirius One), based on different technologies, i.e. laser and ion modes. The latest released is the MBT Sirius system offering a laser technology of 200 Hz (smartbeam™) and positive and negative ion modes (Bruker, 2018). Both mass spectrometers systems are working based on the comparison of the different generated spectra to reference databases. Both commercial libraries for microbial identifications are updated with the addition of new or emerging pathogens (e.g. *Candida auris,* VITEK MS V3/KB3.2.0) mainly of clinical interest. However, libraries are built differently according to the platform used. For the MBT system, reference entries are kept as Main Spectra Profiles (MSPs). An MSP is summarizing multiple measurements, from multiple replicate spectra, of a unique strain under the same growing conditions. For the VITEK® MS, the SuperSpectra approach is used. Similarly enough, it is based on the accumulation of spectra of random and reference strains grown under different conditions (Van Belkum et al., 2012). In the end, both approaches do not evaluate the microbial diversity in the same way.

1.3 Pros and Cons

MALDI-TOF MS regard in laboratories may be explained by several aspects. The first one is its polyvalence in a routine context. Indeed, with a single MALDI-TOF MS system, identification of Gram-positive and -negative bacteria, and fungi is possible (Liébana-Martos, 2018). Such flexibility was not possible with classical biochemical differentiation methods. Its friendly user approach does not require any specific trained personnel neither microbiology, nor mass spectrometry knowledge.

Additionally, MS is a robust and reproducible technic. Indeed, the method relies on the peak identification of major structural proteins, i.e. ribosomal proteins, meaning obtained protein

fingerprints are stable (Liébana-Martos, 2018). While comparing MALDI-TOF MS to conventional biochemical based-methods, e.g. API® (bioMérieux) or Phoenix® (Becton-Dickinson) systems, for the identification of clinically relevant bacteria regardless of the operating system, 94% of MALDI-TOF MS identification were the same that those obtained by conventional methods (Benagli et al., 2011). Another report suggested an agreement of 99% and 89% between MALDI-TOF MS and conventional methods for genus and species identification, respectively (El-Bouri et al., 2012). Nowadays, sequencing is considered as the gold standard for bacterial identification. Deng et al. (2014) reported similar results between MALDI-TOF MS and gene-sequencing for the genus identification of clinical enteropathogens, i.e. *Salmonella*, *Aeromonas*, *Plesiomonas*, *Clostridium*, *Campylobacter*, *Yersinia* and *Vibrio* by using the Vitek MS (Deng et al., 2014). While Vitek MS successfully assesses the species identification for *Campylobacter*, *Pleisiomonas*, *Yersinia*, *Clostridium* and *Vibrio*, the identification at the species level was not possible for *Aeromonas* and *Salmonella* (Deng et al., 2014).

The last attractive features of MALDI-TOF MS are its cost-efficiency and rapidness. Indeed, in comparison to other traditional identification methods, MS could provide results at least one and up to six days earlier for regular organisms and fastidious or slow-growing bacteria, respectively (Tan et al., 2012). MALDI-TOF MS is not only presented as an innovative technology for its fast identification of microorganisms, but it also dispenses non-negligible cost-saving. While the acquisition and the annual maintenance of the apparatus is expensive, the analysis cost could be very low (Tran et al., 2015). A net saving of 87.8% and 72.5%, in reagent and direct costs respectively, compared to the use of traditional methods was reported by Tran and colleagues. Also, they estimated per sample an average of 3.14 USD, including reagent, technologist and maintenance, against 6.50 USD for traditional analysis (Tran et al., 2015).

Nevertheless, as all techniques, MS is also known several shortcomings. These limitations are described in the below **Table 2.1**. Several of these well-known drawbacks, i.e. error in reference data, taxonomic discordances or absence of certain reference spectra, are currently being fixed by manufacturers by updating commercial databases regularly. For example, in the last revision (K) of the Bruker library (2020), the names of several of their entries according to the new taxonomy nomenclature were reviewed (e.g. *Candida carpophila* into *Meyerozyma carpophila*) (Bruker, 2020). Along the same line, the upgrade of the database managed to improve identification of certain types of pathogens. Identification of anaerobes used to be challenging due to the absence or number of underrepresented reference isolates (Vega-Castaño et al., 2012; Rodríguez-Sánchez et al., 2016). In a recent study, Alcalá et al. (2021) compared the identification of anaerobic isolates with the latest Brucker commercial database upgrade with previous databases lacking some species

(Alcalá et al., 2021). In 2016, they reported that for the same collection, 85.8% of theses isolates were identified by MALDI-TOF MS at the species level (Rodríguez-Sánchez et al., 2016).

***Table 2.1**. MALDI-TOF MS limitations*

Limitations	Examples	References
Misidentification of closely related species	*Shigella* spp. misidentified as *E. coli*	(Bizzini et al., 2010; Khot and Fisher, 2013; Rychert, 2019)
	Unable to distinguish *Mycobacterium tuberculosis* complex isolates into specific species	(Saleeb et al., 2011; Neuschlova et al., 2017; Akyar et al., 2018; Body et al., 2018)
Limit of detection	Sufficient biomass is a critical factor for successful identification (between 6×10^3 and 1×10^5 CFU/spot)	(Hsieh et al., 2008; Croxatto et al., 2012; Opota et al., 2016; Cuénod et al., 2021)
Database management		
Errors in reference spectra	*Propionibacterium acnes* wrongly identified as *Eubacterium brachy*	(Bizzini et al., 2010)
Taxonomical discordances	*Stenotrophomonas maltophila* misidentified as *Pseudomonas hibiscicola*	(Croxatto et al., 2012)
Absence or lack of reference spectra	Reference spectra mainly limited to clinical strains. Development of in-house databases and therefore database update dependant	(Emami et al., 2012; Seuylemezian et al., 2018; Pinar-Méndez et al., 2021)
Mixed bacterial population	Non-existent mass spectrum generated Identification score hardly reach high confidence threshold score	(Opota et al., 2016; Rahi et al., 2016)
Spectrum quality and normalization	Specific extractions protocols for certain organisms, e.g. *Mycobacteria* spp., *Nocardia* spp., yeasts and fungi (difficulty to lyse the cell wall structure) Fresh colonies must be used for MALDI-TOF MS identification (ribosomal protein degradation)	(Buckwalter et al., 2016; Cuénod et al., 2021)

With the new available database enriched with new anaerobes, they reached 95.7% of correct identification at the species level, with no differences between Gram-positive and Gram-negative (Alcalá et al., 2021).

Also, it is worth noting that for a long-time the need to develop extra reference spectra related to highly pathogenic species (e.g. *Bacillus anthracis*), which were not included in the initial database, was highlighted (Holzmann et al., 2012). Since then, specific libraries have been generated (Rudrik et al., 2017).

Nonetheless, there are still some identification issues to address for several pathogens. Identification of important human pathogen such as *Neisseria meningitidis* is still tricky by the lack of high-quality reference database (Cunningham et al., 2014; Deak et al., 2014). Hong et al. (2019) obtained 52% and 92% of specificity for the diagnosis of *N. meningitidis,* by using Bruker commercial and an in-house database (Hong et al., 2019).

2. Application in routine laboratory

Initially, identification of microorganisms relied on pure cultures, e.g. solid or liquid, performed from various sample origins (e.g. food, stools, urines or blood). From isolated colonies on selected medium or not, primary tests such as, oxygen requirement, Gram-staining, catalase and oxidase tests were performed to give out potential genus or species identification outcomes (Ferone et al., 2020). Nevertheless, confirmatory tests (e.g. API gallery or serology assays) were required to completely characterize the germ (Castro-Escarpulli et al., 2015). Overall, the complete identification workflow was ranging from several days to weeks, for fastidious or slow-growing pathogens.

2.1 Microorganisms identifications

Over the last two decades, identification of microorganisms in diagnostics laboratories has tremendously changed. The advent and the wide implementation of MALDI-TOF MS in laboratories enhanced identification of several microbes, by reducing the patient-physician workflow. Nowadays, MALDI-TOF MS is one the reference method (ISO 16140-6:2019 certification for food microbiology) for routine identification of bacteria and fungi (Singhal et al., 2015). In the case of certain microorganisms such as *Mycobacterium*, specific protein extraction protocols (e.g. MycoEx or VITEK MS Mycobacterium/Nocardia kit) exist, including an inactivation step, which could be a heating step or the utilization of inactivation reagent with mechanical disruption (BioMérieux; Bruker, 2021e). In fact, *Mycobacterium* is manipulated in physical containment (PC) level 3 containment facility making MALDI-TOF MS cumbersome. With the inactivation step, protein extract can be transferred to routine PC level 2 laboratories (Morales et al., 2018).

As described in the previous section (1.2.2 Commercial platforms), databases are included with MALDI-TOF MS commercial platforms. These databases contain average spectra of different cellular organisms such as, bacteria, including aerobes and anaerobes, and fungi. Certain manufacturers developed additional identification modules (e.g. MBT *Mycobacteria* IVD module) or libraries suites (e.g. MBT filamentous fungi) for specific organisms (Torres-sangiao et al., 2021). Nevertheless, not all libraries' packages are currently applicable in an IVD context.

2.2 Identifications from complexes matrices

Like classical microbial identification, mass spectrometry daily analysis relies on pure colonies on solid growth medium obtained from different samples matrices. Among them, blood was one of the matrices of interest for the MALDI-TOF MS development in routine laboratories (Hou et al., 2019). In 2017, 48.9 million incident sepsis cases were recorded, as well as 11.0 million sepsis-related deaths were reported worldwide (Rudd et al., 2020). However, traditional subculturing and biochemical or microscopic methods were time-consuming, delaying the final microbial identification (Chun et al., 2015). Therefore, new detection tools were required for a better patient's management and outcome. Currently several MALDI-TOF MS commercial protocols exist, i.e. MBT Sepsityper® (Bruker Daltonics, Germany), rapidBACpro® (Nittobo Medical, Japan) and Vitek® MS Blood Culture (BioMérieux, France) kit for the fast and direct identification of microorganisms from positive blood cultures (Kayin et al., 2019; Nomura et al., 2020; Oviaño et al., 2021). These kits rely on the neutralization or the elimination of blood and nonmicrobial cells. Overall, such tests give identification results up to 48 hours earlier than classical methods, making MALDI-TOF MS an utmost tool in diagnostics (Morgenthaler and Kostrzewa, 2015).

Urine and cerebrospinal fluids have also been successfully investigated in several research reports (Nyvang Hartmeyer et al., 2010; L et al., 2019). However, there are currently no commercial kit or standardized protocols for its application in routine diagnostics. Along the same line, there is no successful application of MALDI-TOF MS for direct microorganisms' identification from stools. Indeed, a culture step is still required to obtain pure colonies. Nevertheless a comparative study of identification by MALDI-TOF MS and routine phenotypic methods for stool samples highlighted a shortening of 2-3 days with MALDI-TOF MS while using a culture step (He et al., 2010).

Traditionally, routine identification of microorganisms is performed on pure microbial monoculture. Nevertheless, reports suggest that it may be possible to identify mixtures of bi- or ternary bacterial mixtures without a purification step (Mahé et al., 2014; Yang et al., 2018; Mörtelmaier et al., 2019). Yang et al. (2018) established a framework to identify bacterial mixtures by MALDI-TOF MS without purification procedures. While binary and tertiary mixtures reached sensitivity up to 95%, the sensitivity was weaker for quaternary and pentabasic mixtures with 69%

and 63% sensitivity (Yang et al., 2018). Such framework is still at the experimental stage, but it might be a relevant venue to explore to identify polymicrobial infections from complex matrices previously described in routine clinical microbiology practice. Meanwhile, conventional sepsis diagnostics methods seek for the presence of a unique pathogen, a recent metagenomic study highlighted the importance to consider the underestimated aspect of polymicrobial sepsis (Tan et al., 2021). Therefore, the development of polymicrobial identification workflows by MALDI-TOF combined to efficient kits for the direct identification of microorganisms from positive blood culture could breakthroughly make a difference into sepsis diagnostics.

2.3 Selective testing of antibiotic resistances

The antimicrobial susceptibility testing (AST) is a key in diagnostics and orients physicians for antimicrobial treatments. It knew a recent upgrading with the implementation of automated and semi-automated devices combining identification and AST (e.g. Vitek 2®), using an optical system for measuring changes in bacterial growth and determining antimicrobial susceptibility (Mitchell and Alby, 2017; Benkova et al., 2020). Nevertheless, while these phenotypic technics are effective, they are time-consuming, and leading to a lengthening before the choice of the definitive antibiotherapy. Yet, development of "fast microbiology" technologies or rapid diagnostic tests, including MALDI-TOF MS, results in the improvement of the antimicrobial stewardship by decreasing the "patient-physician" workflow before treatment (Bookstaver et al., 2017; Mangioni et al., 2019).

Currently, several MALDI-TOF MS IVD-kits (e.g. MBT STAR-Cepha or -Carba) exist based on the analysis of enzymes activity (e.g. carbapenemase or cephalosporinase) produced by the bacteria (Ota et al., 2021). Briefly, after an incubation of 30 min with the antibiotic, the cleavage of the molecule into an inactive one could be observed by the detection of a mass shift in the MALDI-TOF mass spectrum. Additionally, these kits could be combined upstream with previous mentioned sepsis kits for rapid microbial identification (also called the Bologna workflow)(Cordovana et al., 2018). In the end, bacterial identification and detection of antimicrobial activity on the same system could be performed in a turnaround time from 10 min to 1.5 h (Cordovana et al., 2018). Along the same line, manufacturers developed a typing module under the form of an early warning system. Specific peaks linked to specific AMR (e.g. MRSA, carbapenemase producing *Enterobacteriaceae*, *Bacteroides fragilis* encoding metallo-beta-lactamase enzyme) are recognized under an automated workflow (Bruker, 2018).

Nevertheless, despite the general interest for the development of new MALDI-TOF MS kits or typing modules, and research related to the identification of AMR through mass spectra, there is

still a stony way before it could be applied for all antimicrobials in a routine context. While conventional phenotypic antibiograms remain currently the method of reference for AMR's identification, MS could be an alternative technique to perform rapid AMR screening to lower the application of an empirical antibiotherapy strategy due to the lack of rapid orientation system.

3. Current and future research applications

While MALDI-TOF MS became the reference method for routine microbial identification, it could as well be used for other experimental purposes. In this section, underway applications, such as the improvement of antimicrobial susceptibly workflow, the identification of parasites and bacterial typing will be described.

3.1 Investigations of different microorganisms

As described previously, bacteria, mycobacteria and fungi are currently the only microorganisms identified by MALDI-TOF MS in daily diagnostics routines. However, several research reports highlighted the potential use of protein mass spectra fingerprints to identify a wide diversity of organisms (e.g. viruses, arthropods or protozoa) (Singhal et al., 2016; Iles et al., 2020). Therefore, this first part is committed to investigate the ability of MALDI-TOF MS to identify other organisms, through the helminthology example. In the following systematic review*, we look over the available scientific peer-reviewed literature and expose MALDI-TOF MS potential as a promising future diagnostics tool.

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REVIEW Open Access

MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: a systematic review

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Abstract

Background: Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) has become a widely used technique for the rapid and accurate identification of bacteria, mycobacteria and certain fungal pathogens in the clinical microbiology laboratory. Thus far, only few attempts have been made to apply the technique in clinical parasitology, particularly regarding helminth identification.

Methods: We systematically reviewed the scientific literature on studies pertaining to MALDI-TOF MS as a diagnostic technique for helminths (cestodes, nematodes and trematodes) of medical and veterinary importance. Readily available electronic databases (i.e. PubMed/MEDLINE, ScienceDirect, Cochrane Library, Web of Science and Google Scholar) were searched from inception to 10 October 2018, without restriction on year of publication or language. The titles and abstracts of studies were screened for eligibility by two independent reviewers. Relevant articles were read in full and included in the systematic review.

Results: A total of 84 peer-reviewed articles were considered for the final analysis. Most papers reported on the application of MALDI-TOF for the study of *Caenorhabditis elegans*, and the technique was primarily used for identification of specific proteins rather than entire pathogens. Since 2015, a small number of studies documented the successful use of MALDI-TOF MS for species-specific identification of nematodes of human and veterinary importance, such as *Trichinella* spp. and *Dirofilaria* spp. However, the quality of available data and the number of examined helminth samples was low.

Conclusions: Data on the use of MALDI-TOF MS for the diagnosis of helminths are scarce, but recent evidence suggests a potential role for a reliable identification of nematodes. Future research should explore the diagnostic accuracy of MALDI-TOF MS for identification of (i) adult helminths, larvae and eggs shed in faecal samples; and (ii) helminth-related proteins that are detectable in serum or body fluids of infected individuals.

Keywords: Diagnosis, Helminths, MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight, Neglected tropical diseases, Parasites

Background

In clinical and laboratory diagnostic settings, mass spectrometry (MS) has been utilized for several decades as an approach for protein-centred analysis of samples in medical chemistry [1, 2] and haematology laboratories [3]. In 1975, Anhalt & Fenselau [4] proposed, for the first time, the modification of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS as a method to characterize bacteria. Indeed, it was demonstrated that different bacterial species show specific protein mass spectra, which can be used for rapid identification.

During the past decade, MALDI-TOF MS has been widely introduced as a diagnostic technique in microbiology laboratories, where it has replaced most other tools (e.g. phenotypic tests, biochemical identification and

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agglutination kits) as the first-line pathogen identification method due to its high diagnostic accuracy, robustness, reliability and rapid turn-around time [5]. MALDI-TOF MS is now routinely employed for identification of bacteria [5–8], mycobacteria [5, 9] and some fungi [8]. More recently, MALDI-TOF MS has been applied in research settings for the detection and identification of viruses [10], protozoans and arthropods [11, 12]. In clinical practice, a specific quantity is brought on a target plate (e.g. culture-grown pathogen). Next, the target plate is pre-treated with a chemical reagent (so-called matrix, e.g. α-cyano-4-hydroxycinnamic acid) and subjected to a mass spectrometer for further analysis. The MALDI-TOF apparatus, which is commercially available through different manufacturers [13, 14], uses laser to disperse and ionize the analyte into different molecules, which move through a vacuum driven by an electric field before reaching a detector membrane. The time-of-flight of the various molecules depends on their mass and their electric charge. The specific time-of-flight data are assembled, resulting in specific spectra that are compared to a commercial database, which allows for a rapid identification of the infectious agent and diagnostic accuracy, the latter of which is usually expressed as a score.

MALDI-TOF MS has several strengths if compared to other diagnostic tools, such as polymerase chain reaction (PCR) assays. Once the mass spectrometer and the corresponding databases are available in a laboratory, individual pathogen identification is inexpensive, and the sample preparation procedure does neither require highly skilled technicians nor complex additional laboratory infrastructure. Of note, MALDI-TOF MS is considerably less prone to contamination and results are available within a few minutes. However, constant power supply is a prerequisite, which limits the suitability of the technique in resource-constrained settings. Yet, it should be noted that MALDI-TOF MS is no longer restricted to high-income countries as it is increasingly available in reference laboratories in sub-Saharan Africa and elsewhere [15–19].

MALDI-TOF does not always require culture-grown colonies of a given pathogen. Instead, it can also be employed to identify microorganisms directly from positive blood culture broths [6] with high diagnostic accuracy [7]. Recently, Yang et al. [20] proposed a new framework to analyse MALDI-TOF spectra of bacterial mixtures (instead of only a single pathogen) and to directly characterize each component without purification procedures. Hence, this procedure might become available to be employed directly on other body fluids (e.g. urine, respiratory specimens and faecal samples), which would further increase its relevance in clinical practice [21, 22].

In contrast to clinical bacteriology, little research has been carried out pertaining to the application of MALDI-TOF MS for identification of parasites of human or veterinary importance [23]. Several studies utilized the technique on protozoan parasites such as *Leishmania* spp. [24–26], *Giardia* spp. [27], *Cryptosporidium* spp. [28], *Trypanosoma* spp. [29], *Plasmodium* spp. [30–32] and *Dientamoeba* spp. [33]. These studies used pre-treatment with ethanol and acetonitrile before subjecting the whole pathogens to MALDI-TOF analysis. Additionally, the technique has been used for identification of ectoparasites and vectors, such as ticks [34–37], fleas [38–41] and mosquitoes [42–49]. In contrast to the experiments on protozoans, only selected parts of the ectoparasites and vectors (e.g. legs, thoraxes or wings) were used and subjected to the same extraction method. A further novel approach to apply MALDI-TOF MS in clinical parasitology is the identification of specific serum peptides that are detectable in parasite-infected individuals [50].

Helminth infections caused by nematodes (e.g. *Ascaris lumbricoides*, hookworm, *Strongyloides stercoralis* and *Trichuris trichiura*), cestodes (e.g. *Taenia* spp.) and trematodes (e.g. *Fasciola* spp. and *Schistosoma* spp.) account for a considerable global burden of disease and are among the most common infections in marginalized populations in the tropics and subtropics [51]. Indeed, according to estimates put forth by the Global Burden of Disease (GBD) Study, 3.35 million disability-adjusted life years (DALYs) were attributable to intestinal nematode infections and schistosomiasis in 2017 [52].

Diagnosis is pivotal for effective treatment but requires at least a basic laboratory infrastructure, light microscopes and well-trained laboratory technicians who might not be available in remote areas of tropical and subtropical countries. In high-resource settings, in contrast, knowledge on microscopic identification of helminths is waning in many laboratories. It is surprising that the potential applicability of MALDI-TOF MS as a diagnostic tool for helminths of human and veterinary importance has not yet been systematically assessed, in particular because the technique has been successfully employed for identification of nematode plant pathogens [53–58]. Hence, the goal of this systematic review was to summarize the available data on MALDI-TOF MS application for diagnosis of helminths of medical and veterinary importance, and to provide recommendations for future research needs.

Methods

Search strategy

A systematic literature review was performed to identify all relevant scientific studies pertaining to MALDI-TOF MS as a diagnostic identification technique in medical

and/or veterinary helminthology. The research was performed according to the guidance expressed in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) Statement [59].

The following electronic databases were systematically searched: MEDLINE/PubMed, ScienceDirect-Embase, Cochrane Library, Web of Science and Google Scholar. All studies published from inception to 10 October 2018 were eligible for inclusion without language restrictions. The bibliographies of all eligible documents were hand-searched for additional references. Conference abstracts or book chapters detected through these databases and additional library searches were also considered. The search strategy comprised keywords related to the MALDI-TOF MS technique (e.g. "MALDI-TOF" and "matrix-assisted laser desorption/ionization time-of-flight") and helminthology (e.g. "helminth", "nematode", "cestode" and "trematode"). The full search strategies for every database are provided in Additional file 1 and the PRISMA checklist in Additional file 2.

Eligibility screening

After the systematic literature search, all duplicates were removed. Titles and abstracts of potentially eligible studies were screened to identify manuscripts relevant to the research question. Scientific reports on helminths of either plants or insects as well as studies on symbiotic bacteria of helminths were excluded for this review. However, we kept all publications related to the soil nematode *Caenorhabditis elegans*, as it is used as a model organism for biomedical research. Additionally, studies pertaining to MALDI-TOF/TOF tandem MS were excluded, as this is a different modification of the MALDI-TOF MS technique, which is not routinely employed in clinical microbiology laboratories, but rather in research laboratory use for accurate characterization or sequencing of components like amino acids, metabolites, saccharides, etc. [60–62].

Data extraction and analysis

The literature search was performed by the first author of this manuscript (MF). All titles and abstracts were then independently reviewed by the first and the last author (MF and SLB) for inclusion and any disagreement was discussed until consensus was reached. All extracted manuscripts were analysed using a reference manager software (Mendeley; http://www.mendeley.com).

Results

Search results, number and year of publication of eligible studies

The search procedure and results obtained are shown in Fig. 1. In brief, the initial literature search yielded 329 published studies, with an additional two abstracts identified through further search. Following removal of 142 duplicates, a total of 189 articles were assessed in more detail, of which 66 studies were excluded based on the analysis of the respective titles and abstracts. A full-text analysis was carried out on the remaining 123 studies; 39 articles were finally excluded because their scope was outside the current research question. Hence, 84 articles were included, and these were published between 1997 and 2018. Figure 2 shows the number of publications, stratified by year of publication. The heterogeneity of data reported in the articles precluded any meaningful meta-analysis (Additional file 3).

Specific applications of MALDI-TOF MS

The first two manuscripts published in 1997 described structural analyses of glycosphingolipids found in *Ascaris suum* and *C. elegans* [63, 64]. Indeed, 95% of all eligible studies used MALDI-TOF MS for identification of specific components rather than for the identification of entire pathogens (Fig. 2). It was only in 2015 when a report on MALDI-TOF MS as diagnostic tool for direct identification of *Dirofilaria* spp. became available [65]. Soon thereafter followed a proof-of-concept study utilizing MALDI-TOF MS for identification and differentiation of *Trichinella* spp. and some narrative reviews mentioning the lack of data on MALDI-TOF in helminthology [32, 66, 67]. Yet, most studies focused on distinct analyses of specific components, such as peptides [66–86], proteins [69, 87–114], lipids [61, 62, 115–124], carbohydrates [125–143] and nucleic acids [144] in a research context. Hence, MALDI-TOF was mainly applied to study and compare the proteome or the peptidome of different helminth species, and most reports focused on *C. elegans*. For example, Husson et al. [74] employed a new approach combining liquid chromatography with MALDI-TOF MS to map and differentiate the neuropeptide profiles of *C. elegans* and the closely related species *C. briggsae*.

The two studies aiming at an identification of entire pathogens provided evidence that MALDI-TOF MS could reliably differentiate between species within the genus *Trichinella* [67] and *Dirofilaria* [65], respectively. In the study by Mayer-Scholl et al. [67], nine species and three genotypes of *Trichinella* isolated from mice, domestic pigs, wild boars and guinea pigs were utilized to create an in-house database with 27 raw spectra generated per specimen. All tested isolates could be distinguished with high diagnostic accuracy. The study by Pshenichnaya et al. [65], which had only been published as a conference abstract, investigated five *Dirofilaria repens* and five *D. immitis* specimens, the causative agents of human and

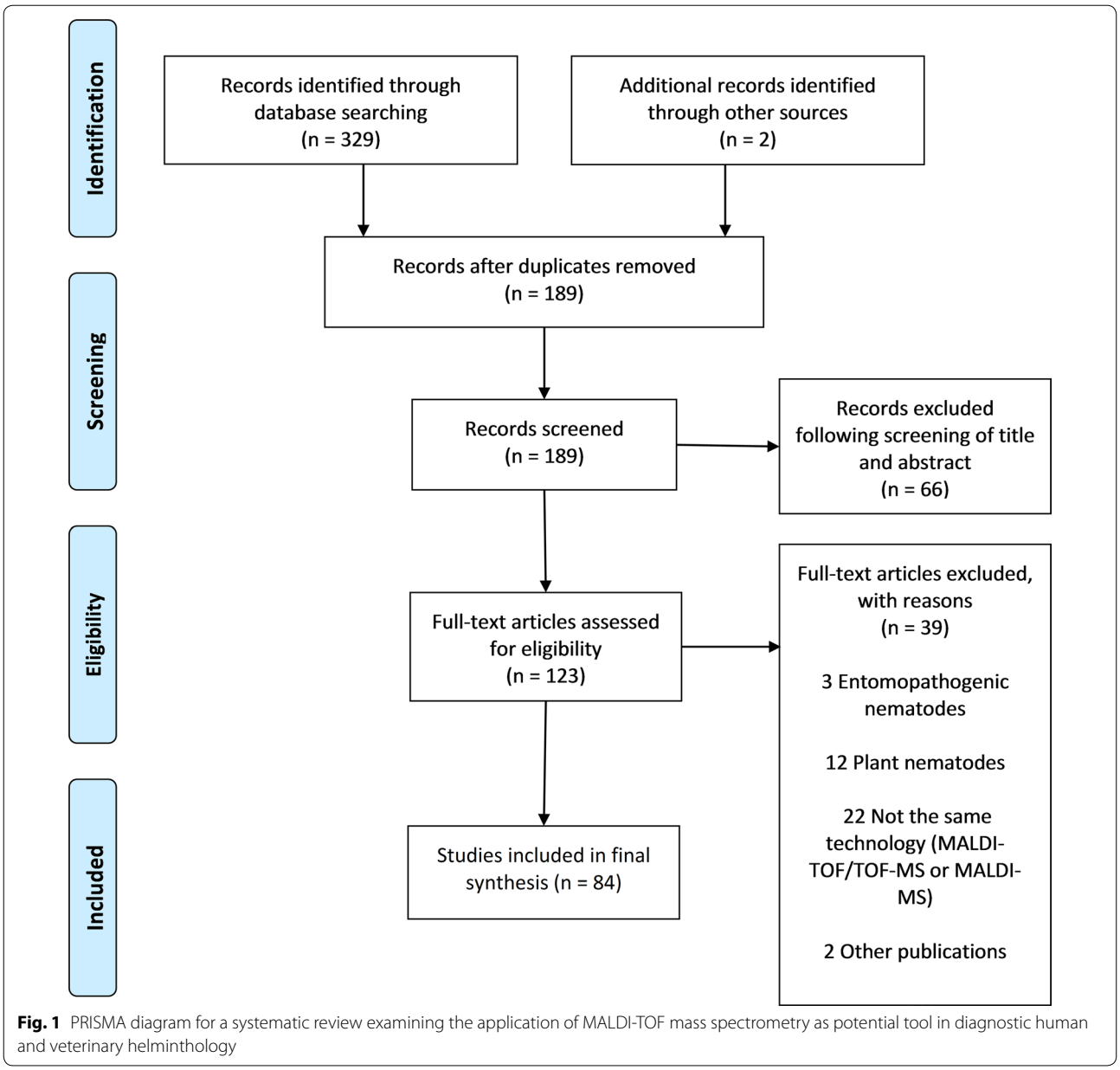

Fig. 1 PRISMA diagram for a systematic review examining the application of MALDI-TOF mass spectrometry as potential tool in diagnostic human and veterinary helminthology

veterinary dirofilariasis, and reported that these could be well differentiated by MALDI-TOF MS. However, data were limited regarding the origin of the study samples, the quality of the spectra obtained by MALDI-TOF and the repeatability of the results. Yet, during the revision of this systematic review, Pshenichnaya et al. [145] published their work on dirofilariasis in a peer-reviewed journal and provided also data for two different species of *Ascaris* (i.e. *A. suum* and *A. lumbricoides*). These helminths could be differentiated by MALDI-TOF based on specific peaks and protein spectra patterns after a cell lysis using the Sepsityper Kit 50 (Bruker Daltonics; Bremen, Germany) and a protein extraction with 70% formic acid and acetonitrile. However, this study has several limitations, and it remains unclear whether calibration steps or assessments of the repeatability and reproducibility of the analyses were performed. An additional paper, published in 2017, reported on MALDI-TOF MS application for cyathostomin helminths, a very diverse group of intestinal parasites infecting horses [66]. These so-called "small strongyles" show a high degree of resistance against benzimidazole anthelminthics and may lead to severe equine enteropathy, colic and death [146]. The study examined several species belonging to the cyathostomin helminths

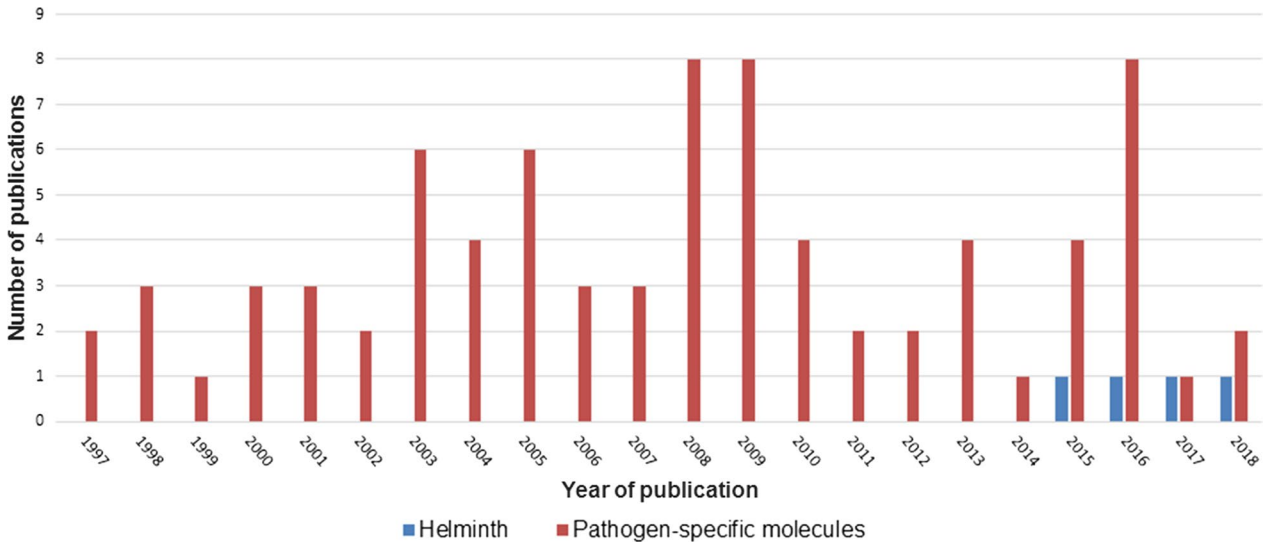

Fig. 2 Publications in the peer-reviewed literature pertaining to the application of MALDI-TOF mass spectrometry for identification of helminths or specific pathogen-related components, as revealed by a systematic review, stratified by year of publication

(e.g. *Coronocyclus coronatus*, *C. labiatus* and *C. labratus*) and found distinct protein spectra among adult helminths of different species [66]. These findings were recently confirmed and substantiated by another study on the application of MALDI-TOF for differentiation of cyathostomins, which was published in April 2019 [147].

Discussion

We systematically reviewed the available literature pertaining to the application of MALDI-TOF MS for identification of helminthic pathogens of human and veterinary importance. While the technique has been successfully employed for many major classes of pathogens (e.g. bacteria, mycobacteria and fungi), data on its use in diagnostic helminthology are scarce. Several studies reported on the differential analysis of specific components, such as proteins, peptides or lipids with MALDI-TOF MS techniques, but only two recent manuscripts and one conference abstract provided 'proof-of-concept' evidence of its potential utility in diagnosing and differentiating helminth species of medical or veterinary relevance.

The majority of articles identified in this systematic review focused on protein-centred analyses of helminth samples. It is important to mention that some of the MALDI-TOF MS devices employed in these studies had been subjected to modifications that are not usually available in routine clinical laboratories. Additionally, these experiments frequently employed a complex sample pretreatment comprising a protein separation by high pressure liquid chromatography (HPLC) or electrophoresis. Yet, some recent proof-of-concept studies have shown that MALDI-TOF MS is also capable of diagnosing entire helminthic pathogens and differentiating similar species within the same genus based on an analysis of their individual protein spectra [66, 67]. Because no helminths are currently included in commercially available MALDI-TOF MS identification databases, individual in-house databases need to be created through generation of main spectra libraries, ideally following established guidelines and protocols that are similar to those employed by the manufacturers of commercially available mass spectrometers [148]. Indeed, previous studies have described the sensitive, reliable and highly reproducible identification of helminths that cause plant infections and have concluded that MALDI-TOF MS should be more widely employed as a 'rapid detection tool' [54–58]. Ahmad et al. [56], for example, reported on the suitability of MALDI-TOF MS to differentiate harmless and juvenile infective stages of single plant nematodes, as these showed unique, characteristic protein peak patterns. These studies should be considered as relevant because plant-parasitic nematodes can sometimes also be found in human stool samples [149, 150]. In Brazil, for example, eggs of the root-knot nematode *Meloidogyne* spp. were detected in human faeces using a microscopic sedimentation method [151]. Future studies should also employ MALDI-TOF on serum, as a recent study reported the detection of specific proteins in serum of mice infected with *Schistosoma japonicum* [50].

While helminth infections pose a considerable burden on human and animal health [152], an accurate diagnosis of these conditions is frequently challenging. Indeed,

simple diagnostic tools such as stool microscopy for soil-transmitted helminth infections are of limited value if the infection intensity is low and highly sensitive diagnostic techniques such as PCR-based assays are only available in selected reference laboratories outside endemic areas [153]. In high-income countries, in contrast, knowledge regarding standard diagnostic parasitology is waning and differentiation of closely related helminth species based on their microscopic morphology requires skilled laboratory technicians [154]. Moreover, some infections cannot be reliably distinguished with standard diagnostic techniques. A prominent example are infections caused by cestodes of the genus *Taenia* [155], which may cause a relatively harmless intestinal infection if cysts of *Taenia saginata* or *T. solium* are orally ingested with meat of cattle or pig. While eggs of *T. saginata* are not infectious to humans, *T. solium* eggs can lead to the potentially fatal disease (neuro-)cysticercosis. While the correct diagnosis has important implications for treatment, patient management and potential contact screening (intestinal carriage of adult *T. solium* worms poses an increased risk of cysticercosis for close contacts, such as family members), it is impossible to distinguish both species based on the identical morphology of their eggs under a microscope. Molecular tools can achieve an accurate differentiation of the two species, but are only available in research settings [155–157]. Sometimes, proglottids of adult worms are also passed in the faeces. While a distinct differentiation is possible based on the uterine branches within a proglottid, misidentification using this approach has been reported in clinical practice [158]. Hence, achieving a species-specific differentiation based on MALDI-TOF MS would contribute to an enhanced, more reliable identification, and future studies should thus address this issue. Similar considerations hold also true for other infective agents that can hardly be differentiated by other methods (e.g. different *Echinococcus* species), novel species (e.g. hybrid species of *Schistosoma* spp., which have recently been reported from Corsica, France [159]) and notoriously difficult-to-detect infections (e.g. strongyloidiasis). An overview of pathogens for which development of MALDI-TOF MS identification protocols would appear particularly promising is summarized in Table 1.

It is important to consider the fixative in which a parasitological sample is stored. Both formaldehyde and ethanol are commonly used to enable a long-term storage of biological specimens, but this may lead to profound changes of the protein structure [160], which is likely to influence on the results of MALDI-TOF examinations carried out on such samples. The virtual impossibility to amplify nucleic acids from formaldehyde-containing solutions [161] due to fragmentation of the single components [162] renders most PCR tests useless on these sample types, but MALDI-TOF analyses of protein spectra might still be possible, albeit with different spectra if compared to native samples. Hence, future studies should evaluate this technique on different kinds of fixatives and on samples that have been stored for prolonged periods.

The present review identified only a few successful studies that employed MALDI-TOF MS to diagnose helminths. Limitations include the complicated pre-treatment procedures employed in some studies and the rather incomplete data presentation in one of the more clinically oriented research projects [65]. New research is needed to determine whether this technique might become a clinically meaningful addendum to the current set of diagnostic options. However, experiences made in clinical bacteriology, mycobacteriology, mycology as well as with ectoparasites (e.g. ticks) and vectors (e.g. mosquitoes) [12, 37, 163] are promising. Whereas MALDI-TOF MS is mainly used on culture-grown colonies for identification of bacteria and mycobacteria, the goal in helminthology will be to provide a species-specific diagnosis based on either macroscopic elements or eggs and larvae that are present in stool samples (or other body fluids and tissue samples). Hence, specific protocols will need to be elaborated to this end, which may include sample preparation, purification and concentration steps, including guidance on the most appropriate sample preservation. However, such protocols have been successfully developed in the past (e.g. for identification of mycobacteria or moulds) [164, 165]. More recently, specific pre-treatment modifications have even allowed to apply MALDI-TOF MS on blood culture broths [166] and fresh urine samples for direct identification of bacteria [167]. Additionally, detection of parasites in complex samples (e.g. blood), should be considered (e.g. as an antigen test for *Wuchereria bancrofti* [168] or for the detection of specific serum peptides [169]).

Yet, much research and rigorous validation is still needed before MALDI-TOF MS might be employed directly on stool samples, and priority should thus be given to (i) the establishment of in-house main spectra library databases to allow for species-specific identification of selected helminths; (ii) the subsequent development of sample treatment protocols; (iii) the validation of this technique on different clinical sample types; and (iv) the elaboration of MALDI-TOF MS to be employed on fixed samples.

Conclusions

The present systematic review elucidated that MALDI-TOF MS, which is now routinely used in many clinical microbiology laboratories for identification of bacteria, fungi and mycobacteria, could potentially also be employed in the context of helminth diagnosis.

Table 1 Current parasitological techniques, related challenges and research needs for a potential application of MALDI-TOF MS as diagnostic tool for major helminths of human and veterinary importance

Characteristics	Nematodes		Cestodes		Trematodes
Helminth species	Soil-transmitted helminths: *Ascaris lumbricoides*; hookworm; *Strongyloides stercoralis*; *Trichuris trichiura*	Tissue nematodes: *Dirofilaria* spp.; *Trichinella* spp.; *Toxocara* spp.; *Wuchereria bancrofti* and other agents causing lymphatic filariasis, *Onchocerca volvulus*, *Loa loa*; animal hookworms causing cutaneous larva migrans	*Taenia* spp.; *Diphyllobothrium latum*	*Echinococcus* spp.	*Schistosoma* spp.; *Fasciola hepatica*; small liver flukes
Type of diagnostic sample	(i) Stool; (ii) Excreted worms	(i) Tissue samples; (ii) Serum; (iii) Extracted worms (e.g. *Loa loa*)	(i) Stool; (ii) Serum (for *T. solium*); (iii) Tissue samples in cysticercosis; (iv) Excreted proglottids	(i) Stool (in animals); (ii) Tissue biopsies, surgical samples; (iii) Serology	(i) Stool; (ii) Urine; (iii) Serum; (iv) Tissue biopsies
Parasitological standard diagnostic techniques	(i) Stool microscopy (e.g. Kato-Katz technique); (ii) PCR in reference laboratories	(i) Direct identification on biopsy samples; (ii) Serology; (iii) PCR in reference laboratories; (iv) Detection of microfilariae in blood; (v) Detection of antigen in blood (*Wuchereria*)	(i) Direct identification of faecally excreted proglottids; (ii) Stool microscopy; (iii) Serology	(i) Microscopy; (ii) PCR of cysts and tissue samples; (iii) Serology	(i) Stool/urine microscopy (dependent on infecting species); (ii) Rapid diagnostic test for circulating cathodic antigen (CCA) in urine; (iii) PCR on serum, stool or urine; (iv) Serology
Difficulties related to currently employed diagnostics	(i) Low sensitivity in light infection intensities; (ii) Specific concentration techniques needed for *S. stercoralis*; (iii) Misidentification of hookworm and *S. stercoralis* larvae possible; (iv) No species differentiation between different hookworm species possible upon microscopy	(i) Serology frequently false-negative; (ii) False-negative PCR results in case of 'new' species; (iii) Lack of expertise outside specialized laboratories	(i) Eggs of related *Taenia* spp. are indistinguishable by microscopy; (ii) Lack of expertise in proglottid differentiation in many laboratories	(i) Serology frequently false-negative in intact cysts; (ii) Microscopy (similar morphology) and serology (cross-reactivity) cannot reliably distinguish between *Echinococcus* spp. (therapeutic implications)	(i) Low sensitivity in light infection intensities; (ii) Microscopy and PCR fail to detect hybrid species

Table 1 (continued)

Characteristics	Nematodes		Cestodes		Trematodes
Research needs for MALDI-TOF application	(i) Establishment of a database for identification of eggs/larvae and adult worms; (ii) Differentiation of microscopically indistinguishable hookworm species; (iii) Protocol development for application on stool samples and bronchial specimens	(i) Establishment of a database for identification of tissue-invasive helminths; (ii) Species differentiation within one genus (e.g. *Dirofilaria*); (iii) Application on biopsy specimens; (iv) Detection of microfilariae in blood; (v) Detection of antigen in blood (e.g. *Wuchereria* spp.)	(i) Establishment of a database for identification of *Taenia* proglottids and eggs; (ii) Differentiation between *T. solium* eggs/proglottids and other *Taenia* spp.; (iii) Protocol development for application on stool specimens	(i) Establishment of a database for species-specific identification of eggs and tissue cysts; (ii) Protocol for application on different sample types	(i) Establishment of a database for species-specific identification; (ii) Protocol for application on different sample types; (iii) Detection of *Schistosoma* antigen(s) in serum; (iv) Detection of products derived from helminth-specific metabolism in serum samples

Preliminary data suggest that MALDI-TOF MS might hold promise as a future diagnostic tool for direct and rapid identification of pathogenic helminths in clinical samples with sufficient diagnostic accuracy. Further studies are needed to evaluate these concepts and to develop specific databases for helminth identification, followed by rigorous validation on well characterised clinical specimens.

Additional files

Additional file 1. Search strategies employed for our systematic review pertaining to the application of MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology.

Additional file 2. PRISMA checklist for a systematic review examining the application of MALDI-TOF mass spectrometry as potential tool in diagnostic human and veterinary helminthology.

Additional file 3. List of references included in the final review ($n = 84$ articles).

Abbreviations

DALY: disability-adjusted life year; GBD: Global Burden of Disease (Study); MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MS: mass spectrometry; PCR: Polymerase chain reaction.

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Authors' contributions

MF conducted the systematic review of the literature and extracted data. MF and SLB independently assessed published articles for eligibility, analysed and discussed data and drafted the first version of the manuscript. SP and JU contributed to data interpretation and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The search strategy and all manuscripts included in this systematic review are available within the article and its additional files.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Tanaka K, Waki H, Ido Y, Akita S, Yoshida Y, Yoshida T, et al. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 1988;2:151–3.
2. Marvin LF, Roberts MA, Fay LB. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry. Clin Chim Acta. 2003;337:11–21.
3. Rees-Unwin KS, Morgan GJ, Davies FE. Proteomics and the haematologist. Clin Lab Haematol. 2004;26:77–86.
4. Anhalt JP, Fenselau C. Identification of bacteria using mass spectrometry techniques. Anal Chem. 1975;47:219–25.
5. Clark AE, Kaleta EJ, Arora A, Wolk DM. Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. Clin Microbiol Rev. 2013;26:547–603.
6. Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, Beretti JL, et al. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem. 2011;44:104–9.
7. Singhal N, Kumar M, Kanaujia PK, Virdi JS. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol. 2015;6:791.
8. Angeletti S. Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology. J Microbiol Methods. 2017;138:20–9.
9. El Khéchine A, Couderc C, Flaudrops C, Raoult D, Drancourt M. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. PLoS One. 2011;6:e24720.
10. Sjöholm MIL, Dillner J, Carlson J. Multiplex detection of human herpesviruses from archival specimens by using matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2008;46:540–5.
11. Yssouf A, Almeras L, Raoult D, Parola P. Emerging tools for identification of arthropod vectors. Future Microbiol. 2016;11:549–66.
12. Vega-Rúa A, Pagès N, Fontaine A, Nuccio C, Hery L, Goindin D, et al. Improvement of mosquito identification by MALDI-TOF MS biotyping using protein signatures from two body parts. Parasit Vectors. 2018;11:574.
13. Faron ML, Buchan BW, Hyke J, Madisen N, Lillie JL, Granato PA, et al. Multicenter evaluation of the Bruker MALDI Biotyper CA System for the identification of clinical aerobic gram-negative bacterial isolates. PLoS One. 2015;10:e0141350.
14. Luo Y, Siu GKH, Yeung ASF, Chen JHK, Ho PL, Leung KW, et al. Performance of the VITEK MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid bacterial identification in two diagnostic centres in China. J Med Microbiol. 2015;64:18–24.
15. Fall B, Lo CI, Samb-Ba B, Perrot N, Diawara S, Gueye MW, et al. The ongoing revolution of MALDI-TOF mass spectrometry for microbiology reaches tropical Africa. Am J Trop Med Hyg. 2015;92:641–7.
16. Lo CI, Fall B, Sambe-Ba B, Flaudrops C, Faye N, Mediannikov O, et al. Value of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry in clinical microbiology and infectious diseases in Africa and tropical areas. Afr J Microbiol Res. 2017;11:1360–70.
17. Diongue K, Kébé O, Faye MD, Samb D, Diallo MA, Ndiaye M, et al. MALDI-TOF MS identification of *Malassezia* species isolated from patients with pityriasis versicolor at the Seafarers' Medical Service in Dakar, Senegal. J Mycol Med. 2018;28:590–3.
18. Taverna CG, Mazza M, Bueno NS, Alvarez C, Amigot S, Andreani M, et al. Development and validation of an extended database for yeast identification by MALDI-TOF MS in Argentina. Med Mycol. 2019;57:215–25.
19. Chabriere E, Bassène H, Drancourt M, Sokhna C. MALDI-TOF-MS and point of care are disruptive diagnostic tools in Africa. New Microbes New Infect. 2018;26:S83–8.
20. Yang Y, Lin Y, Qiao L. Direct MALDI-TOF MS identification of bacterial mixtures. Anal Chem. 2018;90:10400–8.
21. Ferreira L, Sánchez-Juanes F, González-Avila M, Cembrero-Fuciños D, Herrero-Hernández A, González-Buitrago JM, et al. Direct identification of urinary tract pathogens from urine samples by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2010;48:2110–5.

22. Íñigo M, Coello A, Fernández-Rivas G, Rivaya B, Hidalgo J, Quesada MD, et al. Direct identification of urinary tract pathogens from urine samples, combining urine screening methods and matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2016;54:988–93.
23. Singhal N, Kumar M, Virdi JS. MALDI-TOF MS in clinical parasitology: applications, constraints and prospects. Parasitology. 2016;143:1491–500.
24. Cassagne C, Pratlong F, Jeddi F, Benikhlef R, Aoun K, Normand A-C, et al. Identification of *Leishmania* at the species level with matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect. 2014;20:551–7.
25. Culha G, Akyar I, Yildiz Zeyrek F, Kurt Ö, Gündüz C, Özensoy Töz S, et al. Leishmaniasis in Turkey: determination of *Leishmania* species by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Iran J Parasitol. 2014;9:239–48.
26. Lachaud L, Fernández-Arévalo A, Normand AC, Lami P, Nabet C, Donnadieu JL, et al. Identification of *Leishmania* by MALDI-TOF mass spectrometry using a free web-based application and a dedicated mass spectral library. J Clin Microbiol. 2017;55:2914–33.
27. Villegas EN, Glassmeyer ST, Ware MW, Hayes SL, Schaeffer FW. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry-based analysis of *Giardia lamblia* and *Giardia muris*. J Eukaryot Microbiol. 2006;53:S179–81.
28. Magnuson ML, Owens JH, Kelty CA. Characterization of *Cryptosporidium parvum* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol. 2000;66:4720–4.
29. Avila CC, Almeida FG, Palmisano G. Direct identification of trypanosomatids by matrix-assisted laser desorption ionization-time of flight mass spectrometry (DIT MALDI-TOF MS). J Mass Spectrom. 2016;51:549–57.
30. Marks F, Meyer CG, Sievertsen J, Timmann C, Evans J, Horstmann RD, et al. Genotyping of *Plasmodium falciparum* pyrimethamine resistance by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry. Antimicrob Agents Chemother. 2004;48:466–72.
31. Gitau EN, Kokwaro GO, Newton CR, Ward SA. Global proteomic analysis of plasma from mice infected with *Plasmodium berghei* ANKA using two dimensional gel electrophoresis and matrix assisted laser desorption ionization-time of flight mass spectrometry. Malar J. 2011;10:205.
32. de Dios Caballero J, Martin O. Application of MALDI-TOF in parasitology. In: Cobo F, editor. The use of mass spectrometry technology (MALDI-TOF) in clinical microbiology. London: Elsevier Inc.; 2018. p. 235–53.
33. Calderaro A, Buttrini M, Montecchini S, Rossi S, Piccolo G, Arcangeletti MC, et al. MALDI-TOF MS as a new tool for the identification of *Dientamoeba fragilis*. Parasit Vectors. 2018;11:11.
34. Karger A, Kampen H, Bettin B, Dautel H, Ziller M, Hoffmann B, et al. Species determination and characterization of developmental stages of ticks by whole-animal matrix-assisted laser desorption/ionization mass spectrometry. Ticks Tick Borne Dis. 2011;3:78–89.
35. Yssouf A, Flaudrops C, Drali R, Kernif T, Socolovschi C, Berenger JM, et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. J Clin Microbiol. 2013;51:522–8.
36. Diarra AZ, Almeras L, Laroche M, Berenger J-M, Koné AK, Bocoum Z, et al. Molecular and MALDI-TOF identification of ticks and tick-associated bacteria in Mali. PLoS Negl Trop Dis. 2017;11:e0005762.
37. Dib L, Benakhla A, Raoult D, Parola P. MALDI-TOF MS identification of ticks of domestic and wild animals in Algeria and molecular detection of associated microorganisms. Comp Immunol Microbiol Infect Dis. 2018;57:39–49.
38. Dvorak V, Halada P, Hlavackova K, Dokianakis E, Antoniou M, Volf P. Identification of phlebotomine sand flies (*Diptera: Psychodidae*) by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Parasit Vectors. 2014;7:21.
39. Yssouf A, Socolovschi C, Leulmi H, Kernif T, Bitam I, Audoly G, et al. Identification of flea species using MALDI-TOF/MS. Comp Immunol Microbiol Infect Dis. 2014;37:153–7.
40. Lafri I, Almeras L, Bitam I, Caputo A, Yssouf A, Forestier C-L, et al. Identification of Algerian field-caught phlebotomine sand fly vectors by MALDI-TOF MS. PLoS Negl Trop Dis. 2016;10:e0004351.
41. El Hamzaoui B, Laroche M, Almeras L, Bérenger J-M, Raoult D, Parola P. Detection of *Bartonella* spp. in fleas by MALDI-TOF MS. PLoS Negl Trop Dis. 2018;12:e0006189.
42. Dieme C, Yssouf A, Vega-Rúa A, Berenger JM, Failloux AB, Raoult D, et al. Accurate identification of Culicidae at aquatic developmental stages by MALDI-TOF MS profiling. Parasit Vectors. 2014;7:544.
43. Yssouf A, Parola P, Lindström A, Lilja T, L'Ambert G, Bondesson U, et al. Identification of European mosquito species by MALDI-TOF MS. Parasitol Res. 2014;113:2375–8.
44. Niare S, Berenger J-M, Dieme C, Doumbo O, Raoult D, Parola P, et al. Identification of blood meal sources in the main African malaria mosquito vector by MALDI-TOF MS. Malar J. 2016;15:87.
45. Tandina F, Almeras L, Koné AK, Doumbo OK, Raoult D, Parola P. Use of MALDI-TOF MS and culturomics to identify mosquitoes and their midgut microbiota. Parasit Vectors. 2016;9:495.
46. Raharimalala FN, Andrianinarivomanana TM, Rakotondrasoa A, Collard JM, Boyer S. Usefulness and accuracy of MALDI-TOF mass spectrometry as a supplementary tool to identify mosquito vector species and to invest in development of international database. Med Vet Entomol. 2017;31:289–98.
47. Tahir D, Almeras L, Varloud M, Raoult D, Davoust B, Parola P. Assessment of MALDI-TOF mass spectrometry for filariae detection in *Aedes aegypti* mosquitoes. PLoS Negl Trop Dis. 2017;11:e0006093.
48. Niare S, Tandina F, Davoust B, Doumbo O, Raoult D, Parola P, et al. Accurate identification of *Anopheles gambiae* Giles trophic preferences by MALDI-TOF MS. Infect Genet Evol. 2018;63:410–9.
49. Tandina F, Laroche M, Davoust B, Doumbo OK, Parola P. Blood meal identification in the cryptic species *Anopheles gambiae* and *Anopheles coluzzii* using MALDI-TOF MS. Parasite. 2018;25:40.
50. Huang Y, Li W, Liu K, Xiong C, Cao P, Tao J. New detection method in experimental mice for schistosomiasis: ClinProTool and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Parasitol Res. 2016;115:4173–81.
51. Hotez PJ, Brindley PJ, Bethony JM, King CH, Pearce EJ, Jacobson J. Helminth infections: the great neglected tropical diseases. J Clin Invest. 2008;118:1311–21.
52. GBD 2017 DALYs, HALE Collaborators. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392:1859–922.
53. Calvo E, Flores-Romero P, López JA, Navas A. Identification of proteins expressing differences among isolates of *Meloidogyne* spp. (Nematoda: Meloidogynidae) by nano-liquid chromatography coupled to ion-trap mass spectrometry. J Proteome Res. 2005;4:1017–21.
54. Perera MR, Vanstone VA, Jones MGK. A novel approach to identify plant parasitic nematodes using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom. 2005;19:1454–60.
55. Ahmad F, Babalola OO, Tak HI. Potential of MALDI-TOF mass spectrometry as a rapid detection technique in plant pathology: identification of plant-associated microorganisms. Anal Bioanal Chem. 2012;404:1247–55.
56. Ahmad F, Gopal J, Wu H. Rapid and highly sensitive detection of single nematode via direct MALDI mass spectrometry. Talanta. 2012;93:182–5.
57. Ahmad F, Babalola OO. Application of mass spectrometry as rapid detection tool in plant nematology. Appl Spectrosc Rev. 2014;49:1–10.
58. Ahmad F, Babalola OO, Wu H-F. Potential of MALDI-TOF mass spectrometry as a detection tool to identify plant-parasitic nematodes. J Nematol. 2014;46:132.
59. Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. J Clin Epidemiol. 2009;62:1006–12.
60. Yergey AL, Coorssen JR, Backlund PS, Blank PS, Humphrey GA, Zimmerberg J, et al. *De novo* sequencing of peptides using MALDI/TOF-TOF. J Am Soc Mass Spectrom. 2002;13:784–91.
61. Rush MD, Rue EA, Wong A, Kowalski P, Glinski JA, van Breemen RB. Rapid determination of procyanidins using MALDI-TOF/TOF mass spectrometry. J Agric Food Chem. 2018;66:11355–61.

62. Zhan L, Xie X, Li Y, Liu H, Xiong C, Nie Z. Differentiation and relative quantitation of disaccharide isomers by MALDI-TOF/TOF mass spectrometry. Anal Chem. 2018;90:1525–30.
63. Gerdt S, Lochnit G, Dennis RD, Geyer R. Isolation and structural analysis of three neutral glycosphingolipids from a mixed population of *Caenorhabditis elegans* (Nematoda: Rhabditida). Glycobiology. 1997;7:265–75.
64. Lochnit G, Dennis RD, Zähringer U, Geyer R. Structural analysis of neutral glycosphingolipids from *Ascaris suum* adults (Nematoda: Ascaridida). Glycoconj J. 1997;14:389–99.
65. Pshenichnaya N, Nagorny S, Aleshukina A, Ermakova L, Krivorotova E. Paper Poster Session II MALDI-TOF application of matrix-assisted laser desorption/ionization mass spectrometry for the identification of dirofilariasis species. 2015. In: Poster presentation at the 25th European Conference on Clinical Microbiology and Infectious Diseases (ECCMID) in Copenhagen, Denmark on 26 April 2015. https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=22817. Accessed 14 May 2019.
66. Bredtmann CM, Krücken J, Murugaiyan J, Kuzmina T, von Samson-Himmelstjerna G. Nematode species identification—current status, challenges and future perspectives for Cyathostomins. Front Cell Infect Microbiol. 2017;7:283.
67. Mayer-Scholl A, Murugaiyan J, Neumann J, Bahn P, Reckinger S, Nöckler K. Rapid identification of the foodborne pathogen *Trichinella* spp. by matrix-assisted laser desorption/ionization mass spectrometry. PLoS One. 2016;11:e0152062.
68. Marks NJ, Maule AG, Geary TG, Thompson DP, Li C, Halton DW, et al. KSAYMRFamide (PF3/AF8) is present in the free-living nematode, *Caenorhabditis elegans*. Biochem Biophys Res Commun. 1998;248:422–5.
69. Kaji H, Tsuji T, Mawuenyega KG, Wakamiya A, Taoka M, Isobe T. Profiling of *Caenorhabditis elegans* proteins using two-dimensional gel electrophoresis and matrix assisted laser desorption/ionization-time of flight mass spectrometry. Electrophoresis. 2000;21:1755–65.
70. Husson SJ, Clynen E, Baggerman G, Janssen T, Schoofs L. Defective processing of neuropeptide precursors in *Caenorhabditis elegans* lacking proprotein convertase 2 (KPC-2/EGL-3): mutant analysis by mass spectrometry. J Neurochem. 2006;98:1999–2012.
71. Ghaleb AM, Atwood J, Morales-Montor J, Damian RT. A 3 kDa peptide is involved in the chemoattraction *in vitro* of the male *Schistosoma mansoni* to the female. Microbes Infect. 2006;8:2367–75.
72. Yew JY, Davis R, Dikler S, Nanda J, Reinders B, Stretton AO. Peptide products of the afp-6 gene of the nematode *Ascaris suum* have different biological actions. J Comp Neurol. 2007;502:872–82.
73. Nagano I, Wu Z, Takahashi Y. Functional genes and proteins of *Trichinella* spp. Parasitol Res. 2009;104:197–207.
74. Husson SJ, Landuyt B, Nys T, Baggerman G, Boonen K, Clynen E, et al. Comparative peptidomics of *Caenorhabditis elegans* versus *C. briggsae* by LC-MALDI-TOF MS. Peptides. 2009;30:449–57.
75. Weinkopff T, Atwood JA, Punkosdy GA, Moss D, Weatherly DB, Orlando R, et al. Identification of antigenic *Brugia* adult worm proteins by peptide mass fingerprinting. J Parasitol. 2009;95:1429–35.
76. Husson SJ, Clynen E, Boonen K, Janssen T, Lindemans M, Baggerman G, et al. Approaches to identify endogenous peptides in the soil nematode *Caenorhabditis elegans*. Methods Mol Biol. 2010;615:29–47.
77. Laschuk A, Monteiro KM, Vidal NM, Pinto PM, Duran R, Cerveñanski C, et al. Proteomic survey of the cestode *Mesocestoides corti* during the first 24 hours of strobilar development. Parasitol Res. 2011;108:645–56.
78. Rai R, Singh N, Elesela S, Tiwari S, Rathaur S. MALDI mass sequencing and biochemical characterization of *Setaria cervi* protein tyrosine phosphatase. Parasitol Res. 2013;112:147–54.
79. Konop CJ, Knickelbine JJ, Sygulla MS, Vestling MM, Stretton AOW. Different neuropeptides are expressed in different functional subsets of cholinergic excitatory motorneurons in the nematode *Ascaris suum*. ACS Chem Neurosci. 2015;6:855–70.
80. Marks NJ, Shaw C, Halton DW, Thompson DP, Geary TG, Li C, et al. Isolation and preliminary biological assessment of AADGAPLIRFamide and SVPGVLRFamide from *Caenorhabditis elegans*. Biochem Biophys Res Commun. 2001;286:1170–6.
81. Konop CJ, Knickelbine JJ, Sygulla MS, Wruck CD, Vestling MM, Stretton AOW. Mass spectrometry of single GABAergic somatic motorneurons identifies a novel inhibitory peptide, As-NLP-22, in the nematode *Ascaris suum*. J Am Soc Mass Spectrom. 2015;26:2009–23.
82. Mádi A, Mikkat S, Ringel B, Thiesen HJ, Glocker MO. Profiling stage-dependent changes of protein expression in *Caenorhabditis elegans* by mass spectrometric proteome analysis leads to the identification of stage-specific marker proteins. Electrophoresis. 2003;24:1809–17.
83. Yatsuda AP, Krijgsveld J, Cornelissen AWCA, Heck AJR, De Vries E. Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. J Biol Chem. 2003;278:16941–51.
84. Vepřek P, Ježek J, Velek J, Tallima H, Montash M, El Ridi R. Peptides and multiple antigen peptides from *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase: preparation, immunogenicity and immunoprotective capacity in C57BL/6 mice. J Pept Sci. 2004;10:350–62.
85. Gare D, Boyd J, Connolly B. Developmental regulation and secretion of nematode-specific cysteine-glycine domain proteins in *Trichinella spiralis*. Mol Biochem Parasitol. 2004;134:257–66.
86. Yew JY, Kutz KK, Dikler S, Messinger L, Li L, Stretton AO. Mass spectrometric map of neuropeptide expression in *Ascaris suum*. J Comp Neurol. 2005;488:396–413.
87. Quinn GAP, Heymans R, Rondaj F, Shaw C, de Jong-Brink M. *Schistosoma mansoni* dermaseptin-like peptide: structural and functional characterization. J Parasitol. 2005;91:1340–51.
88. Robinson MW, Gare DC, Connolly B. Profiling excretory/secretory proteins of *Trichinella spiralis* muscle larvae by two-dimensional gel electrophoresis and mass spectrometry. Vet Parasitol. 2005;132:37–41.
89. Loukas A, Hintz M, Linder D, Mullin NP, Parkinson J, Tetteh KKA, et al. A family of secreted mucins from the parasitic nematode *Toxocara canis* bears diverse mucin domains but shares similar flanking six-cysteine repeat motifs. J Biol Chem. 2000;275:39600–7.
90. Yamauchi S, Higashitani N, Otani M, Higashitani A, Ogura T, Yamanaka K. Involvement of HMG-12 and CAR-1 in the cdc-48.1 expression of *Caenorhabditis elegans*. Dev Biol. 2008;318:348–59.
91. Monteiro KM, Zaha A, Ferreira HB. Recombinant subunits as tools for the structural and functional characterization of *Echinococcus granulosus* antigen B. Exp Parasitol. 2008;119:490–8.
92. Goldfinch GM, Smith WD, Imrie L, McLean K, Inglis NF, Pemberton AD. The proteome of gastric lymph in normal and nematode infected sheep. Proteomics. 2008;8:1909–18.
93. Ahmad R, Srivastava AK, Walter RD. Purification and biochemical characterization of cytosolic glutathione-S-transferase from filarial worms *Setaria cervi*. Comp Biochem Physiol B Biochem Mol Biol. 2008;151:237–45.
94. Sahoo MK, Sisodia BS, Dixit S, Joseph SK, Gaur RL, Verma SK, et al. Immunization with inflammatory proteome of *Brugia malayi* adult worm induces a Th1/Th2-immune response and confers protection against the filarial infection. Vaccine. 2009;27:4263–71.
95. Yuan SS, Xing XM, Liu JJ, Huang QY, Yang SQ, Peng F. Screening and identification of differentially expressed proteins between adult female and male worms of *Schistosoma japonicum*. Zhonghua Yu Fang Yi Xue Za Zhi. 2009;43:695–9.
96. Pak JH, Moon JH, Hwang SJ, Cho SH, Seo SB, Kim TS. Proteomic analysis of differentially expressed proteins in human cholangiocarcinoma cells treated with *Clonorchis sinensis* excretory-secretory products. J Cell Biochem. 2009;108:1376–88.
97. Sanglas L, Aviles FX, Huber R, Gomis-Rüth FX, Arolas JL. Mammalian metallopeptidase inhibition at the defense barrier of *Ascaris* parasite. Proc Natl Acad Sci USA. 2009;106:1743–7.
98. Yan F, Xu L, Liu L, Yan R, Song X, Li X. Immunoproteomic analysis of whole proteins from male and female adult *Haemonchus contortus*. Vet J. 2010;185:174–9.
99. Jarecki JL, Andersen K, Konop CJ, Knickelbine JJ, Vestling MM, Stretton AO. Mapping neuropeptide expression by mass spectrometry in single dissected identified neurons from the dorsal ganglion of the nematode *Ascaris suum*. ACS Chem Neurosci. 2010;1:505–19.
100. Brophy PM, Jefferies JR. Proteomic identification of glutathione S-transferases from the model nematode *Caenorhabditis elegans*. Proteomics. 2001;1:1463–8.
101. Kawasaki I, Jeong MH, Shim YH. Regulation of sperm-specific proteins by IFE-1, a germline-specific homolog of eIF4E, in *C. elegans*. Mol Cells. 2011;31:191–7.
102. Clegg RA, Bowen LC, Bicknell AV, Tabish M, Prescott MC, Rees HH, et al. Characterisation of the N'1 isoform of the cyclic AMP-dependent

protein kinase (PK-A) catalytic subunit in the nematode, *Caenorhabditis elegans*. Arch Biochem Biophys. 2012;519:38–45.
103. Millares P, LaCourse EJ, Perally S, Ward DA, Prescott MC, Hodgkinson JE, et al. Proteomic profiling and protein identification by MALDI-TOF mass spectrometry in unsequenced parasitic nematodes. PLoS One. 2012;7:e33590.
104. Ondrovics M, Silbermayr K, Mitreva M, Young ND, Razzazi-Fazeli E, Gasser RB, et al. Proteomic analysis of *Oesophagostomum dentatum* (Nematoda) during larval transition, and the effects of hydrolase inhibitors on development. PLoS One. 2013;8:e63955.
105. Tian F, Hou M, Chen L, Gao Y, Zhang X, Ji M, et al. Proteomic analysis of schistosomiasis japonica vaccine candidate antigens recognized by UV-attenuated cercariae-immunized porcine serum IgG2. Parasitol Res. 2013;112:2791–803.
106. JebaMercy G, Durai S, Prithika U, Marudhupandiyan S, Dasauni P, Kundu S, et al. Role of DAF-21protein in *Caenorhabditis elegans* immunity against *Proteus mirabilis* infection. J Proteom. 2016;145:81–90.
107. Henze A, Homann T, Rohn I, Aschner M, Link CD, Kleuser B, et al. *Caenorhabditis elegans* as a model system to study post-translational modifications of human transthyretin. Sci Rep. 2016;6:37346.
108. Timm T, Grabitzki J, Severcan C, Muratoglu S, Ewald L, Yilmaz Y, et al. The PCome of *Ascaris suum* as a model system for intestinal nematodes: identification of phosphorylcholine-substituted proteins and first characterization of the PC-epitope structures. Parasitol Res. 2016;115:1263–74.
109. Wang F, Xu L, Song X, Li X, Yan R. Identification of differentially expressed proteins between free-living and activated third-stage larvae of *Haemonchus contortus*. Vet Parasitol. 2016;215:72–7.
110. Mikeš L, Man P. Purification and characterization of a saccharide-binding protein from penetration glands of *Diplostomum pseudospathaceum*—a bifunctional molecule with cysteine protease activity. Parasitology. 2003;127:69–77.
111. Cheng G-F, Lin J-J, Feng X-G, Fu Z-Q, Jin Y-M, Yuan C-X, et al. Proteomic analysis of differentially expressed proteins between the male and female worm of *Schistosoma japonicum* after pairing. Proteomics. 2005;5:511–21.
112. Robinson MW, Connolly B. Proteomic analysis of the excretory-secretory proteins of the *Trichinella spiralis* L1 larva, a nematode parasite of skeletal muscle. Proteomics. 2005;5:4525–32.
113. Ahn DH, Singaravelu G, Lee S, Ahnn J, Shim YH. Functional and phenotypic relevance of differentially expressed proteins in calcineurin mutants of *Caenorhabditis elegans*. Proteomics. 2006;6:1340–50.
114. Grabitzki J, Ahrend M, Schachter H, Geyer R, Lochnit G. The PCome of *Caenorhabditis elegans* as a prototypic model system for parasitic nematodes: identification of phosphorylcholine-substituted proteins. Mol Biochem Parasitol. 2008;161:101–11.
115. Acosta D, Cancela M, Piacenza L, Roche L, Carmona C, Tort JF. *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. Mol Biochem Parasitol. 2008;158:52–64.
116. Mádi A, Mikkat S, Koy C, Ringel B, Thiesen HJ, Glocker MO. Mass spectrometric proteome analysis suggests anaerobic shift in metabolism of Dauer larvae of *Caenorhabditis elegans*. Biochim Biophys Acta. 2008;1784:1763–70.
117. Wuhrer M, Grimm C, Dennis RD, Idris MA, Geyer R. The parasitic trematode *Fasciola hepatica* exhibits mammalian-type glycolipids as well as Gal(**B**1-6)Gal-terminating glycolipids that account for cestode serological cross-reactivity. Glycobiology. 2004;14:115–26.
118. Mika A, Gołębiowski M, Szafranek J, Rokicki J, Stepnowski P. Identification of lipids in the cuticle of the parasitic nematode *Anisakis simplex* and the somatic tissues of the Atlantic cod *Gadus morhua*. Exp Parasitol. 2010;124:334–40.
119. Lochnit G, Nispel S, Dennis RD, Geyer R. Structural analysis and immunohistochemical localization of tow acidic glycosphingolipids from the porcine, parasitic nematode, *Ascaris suum*. Glycobiology. 1998;8:891–9.
120. Lochnit G, Dennis RD, Ulmer AJ, Geyer R. Structural elucidation and monokine-inducing activity of two biologically active zwitterionie glycosphingolipids derived from the porcine parasitic nematode *Ascaris suum*. J Biol Chem. 1998;273:466–74.
121. Gerdt S, Dennis RD, Borgonie G, Schnabel R, Geyer R. Isolation, characterization and immunolocalization of phosphorylcholine-substituted glycolipids in developmental stages of *Caenorhabditis elegans*. Eur J Biochem. 1999;266:952–63.
122. Wuhrer M, Rickhoff S, Dennis RD, Lochnit G, Soboslay PT, Baumeister S, et al. Phosphocholine-containing, zwitterionic glycosphingolipids of adult *Onchocerca volvulus* as highly conserved antigenic structures of parasitic nematodes. Biochem J. 2000;348(Pt2):417–23.
123. Wuhrer M, Berkefeld C, Dennis RD, Idris MA, Geyer R. The liver flukes *Fasciola gigantica* and *Fasciola hepatica* express the leucocyte cluster of differentiation marker CD77 (globotriaosylceramide) in their tegument. Biol Chem. 2001;382:195–207.
124. López-Marín LM, Montrozier H, Lemassu A, García E, Segura E, Daffé M. Structure and antigenicity of the major glycolipid from *Taenia solium* cysticerci. Mol Biochem Parasitol. 2002;119:33–42.
125. Iriko H, Nakamura K, Kojima H, Iida-Tanaka N, Kasama T, Kawakami Y, et al. Chemical structures and immunolocalization of glycosphingolipids isolated from *Diphyllobothrium hottai* adult worms and plerocercoids. Eur J Biochem. 2002;269:3549–59.
126. Wuhrer M, Grimm C, Zähringer U, Dennis RD, Berkefeld CM, Idris MA, et al. A novel GlcNAcα1-HPO3-6Gal(1-1)ceramide antigen and alkylated inositol-phosphoglycerolipids expressed by the liver fluke *Fasciola hepatica*. Glycobiology. 2003;13:129–37.
127. Friedl CH, Lochnit G, Zähringer U, Bahr U, Geyer R. Structural elucidation of zwitterionic carbohydrates derived from glycosphingolipids of the porcine parasitic nematode *Ascaris suum*. Biochem J. 2003;369:89–102.
128. Paschinger K, Rendić D, Lochnit G, Jantsch V, Wilson IBH. Molecular basis of anti-horseradish peroxidase staining in *Caenorhabditis elegans*. J Biol Chem. 2004;279:49588–98.
129. Paschinger K, Wilson IBH. Two types of galactosylated fucose motifs are present on N-glycans of *Haemonchus contortus*. Glycobiology. 2015;25:585–90.
130. Hewitson JP, Nguyen DL, van Diepen A, Smit CH, Koeleman CA, McSorley HJ, et al. Novel O-linked methylated glycan antigens decorate secreted immunodominant glycoproteins from the intestinal nematode *Heligmosomoides polygyrus*. Int J Parasitol. 2016;46:157–70.
131. Veríssimo CM, Morassutti AL, von Itzstein M, Sutov G, Hartley-Tassell L, McAtamney S, et al. Characterization of the N-glycans of female *Angiostrongylus cantonensis* worms. Exp Parasitol. 2016;166:137–43.
132. Paschinger K, Wilson IBH. Analysis of zwitterionic and anionic N-linked glycans from invertebrates and protists by mass spectrometry. Glycoconj J. 2016;33:273–83.
133. Jiménez-Castells C, Vanbeselaere J, Kohlhuber S, Ruttkowski B, Joachim A, Paschinger K. Gender and developmental specific N-glycomes of the porcine parasite *Oesophagostomum dentatum*. Biochim Biophys Acta. 2017;1861:418–30.
134. Yan S, Wang H, Schachter H, Jin C, Wilson IBH, Paschinger K. Ablation of N-acetylglucosaminyltransferases in *Caenorhabditis* induces expression of unusual intersected and bisected N-glycans. Biochim Biophys Acta. 2018;1862:2191–203.
135. Yan S, Vanbeselaere J, Jin C, Blaukopf M, Wöls F, Wilson IBH, et al. Core richness of N-glycans of *Caenorhabditis elegans*: a case study on chemical and enzymatic release. Anal Chem. 2018;90:928–35.
136. Cipollo JF, Awad AM, Costello CE, Hirschberg CB. N-glycans of *Caenorhabditis elegans* are specific to developmental stages. J Biol Chem. 2005;280:26063–72.
137. Robijn MLM, Koeleman CAM, Hokke CH, Deelder AM. *Schistosoma mansoni* eggs excrete specific free oligosaccharides that are detectable in the urine of the human host. Mol Biochem Parasitol. 2007;151:162–72.
138. Robijn MLM, Koeleman CAM, Wuhrer M, Royle L, Geyer R, Dwek RA, et al. Targeted identification of a unique glycan epitope of *Schistosoma mansoni* egg antigens using a diagnostic antibody. Mol Biochem Parasitol. 2007;151:148–61.
139. Kaneiwa T, Yamada S, Mizumoto S, Montaño AM, Mitani S, Sugahara K. Identification of a novel chondroitin hydrolase in *Caenorhabditis elegans*. J Biol Chem. 2008;283:14971–9.
140. Tefsen B, van Stijn CMW, van den Broek M, Kalay H, Knol JC, Jimenez CR, et al. Chemoenzymatic synthesis of multivalent neoglycoconjugates carrying the helminth glycan antigen LDNF. Carbohydr Res. 2009;344:1501–7.
141. Borloo J, De Graef J, Peelaers I, Nguyen DL, Mitreva M, Devreese B, et al. In-depth proteomic and glycomic analysis of the adult-stage *Cooperia oncophora* excretome/secretome. J Proteome Res. 2013;12:3900–11.

142. Parsons LM, Mizanur RM, Jankowska E, Hodgkin J, O'Rourke D, Stroud D, et al. *Caenorhabditis elegans* bacterial pathogen resistant bus-4 mutants produce altered mucins. PLoS One. 2014;9:e107250.
143. Yan S, Jin C, Wilson IBH, Paschinger K. Comparisons of *Caenorhabditis* fucosyltransferase mutants reveal a multiplicity of isomeric N-glycan structures. J Proteome Res. 2015;14:5291–305.
144. Alim MA, Fu Y, Wu Z, Zhao S, Cao J. Single nucleotide polymorphisms of toll-like receptors and association with *Haemonchus contortus* infection in goats. Pak Vet J. 2016;36:286–91.
145. Nagorny SA, Aleshukina AV, Aleshukina IS, Ermakova LA, Pshenichnaya NY. The application of proteomic methods (MALDI-toff MS) for studying protein profiles of some nematodes (*Dirofilaria* and *Ascaris*) for differentiating species. Int J Infect Dis. 2019;S1201–9712(19):30113–4.
146. Lyons E, Tolliver S, Drudge J. Historical perspective of cyathostomes: prevalence, treatment and control programs. Vet Parasitol. 1999;85:97–112.
147. Bredtmann CM, Krücken J, Murugaiyan J, Balard A, Hofer H, Kuzmina TA, et al. Concurrent proteomic fingerprinting and molecular analysis of cyathostomins. Proteomics. 2019;19:e1800290.
148. To G, Maldi E, Table LT, Fume V, Eppendorf M, Analytical R, et al. Generating new MSP with Bruker Microflex LT. https://spectra.folkhalsomyndigheten.se/spectra/. Accessed 14 May 2019.
149. Yajima F, Kurono Y, Igarashi K, Uchida A, Ozeki Y. On the unknown nematoda eggs found in human stool which are confusing with the egg of human parasites. J Jpn Assoc Rural Med. 1959;8:57–61.
150. Bradbury RS, Speare R. Passage of *Meloidogyne* eggs in human stool: forgotten, but not gone. J Clin Microbiol. 2015;53:1458–9.
151. Santos FLN, de Souza AMGC, Dantas-Torres F. *Meloidogyne* eggs in human stool in Northeastern Brazil. Rev Soc Bras Med Trop. 2016;49:802.
152. Utzinger J, Becker SL, Knopp S, Blum J, Neumayr AL, Keiser J, et al. Neglected tropical diseases: diagnosis, clinical management, treatment and control. Swiss Med Wkly. 2012;142:w13727.
153. Becker SL, Liwanag HJ, Snyder JS, Akogun O, Belizario V, Freeman MC, et al. Toward the 2020 goal of soil-transmitted helminthiasis control and elimination. PLoS Negl Trop Dis. 2018;12:e0006606.
154. van Lieshout L, Roestenberg M. Clinical consequences of new diagnostic tools for intestinal parasites. Clin Microbiol Infect. 2015;21:520–8.
155. González LM, Montero E, Harrison LJS, Parkhouse RME, Garate T. Differential diagnosis of *Taenia saginata* and *Taenia solium* infection by PCR. J Clin Microbiol. 2000;38:737–44.
156. Flisser A, Viniegra A-E, Aguilar-Vega L, Garza-Rodriguez A, Maravilla P, Avila G. Portrait of human tapeworms. J Parasitol. 2004;90:914–6.
157. Mayta H, Gilman RH, Prendergast E, Castillo JP, Tinoco YO, Garcia HH, et al. Nested PCR for specific diagnosis of *Taenia solium* taeniasis. J Clin Microbiol. 2008;46:286–9.
158. Mayta H, Talley A, Gilman RH, Jimenez J, Verastegui M, Ruiz M, et al. Differentiating *Taenia solium* and *Taenia saginata* infections by simple hematoxylin-eosin staining and PCR-restriction enzyme analysis. J Clin Microbiol. 2000;38:133–7.
159. Boissier J, Grech-Angelini S, Webster BL, Allienne JF, Huyse T, Mas-Coma S, et al. Outbreak of urogenital schistosomiasis in Corsica (France): an epidemiological case study. Lancet Infect Dis. 2016;16:971–9.
160. Klopfleisch R, Weiss ATA, Gruber AD. Excavation of a buried treasure—DNA, mRNA, miRNA and protein analysis in formalin fixed, paraffin embedded tissues. Histol Histopathol. 2011;26:797–810.
161. Lehmann U, Kreipe H. Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods. 2001;25:409–18.
162. Bonin S. PCR analysis in archival postmortem tissues. Mol Pathol. 2003;56:184–6.
163. Laroche M, Bérenger JM, Gazelle G, Blanchet D, Raoult D, Parola P. Corrigendum: MALDI-TOF MS protein profiling for the rapid identification of chagas disease triatomine vectors and application to the triatomine fauna of French Guiana. Parasitology. 2018;145:676.
164. Neuschlova M, Vladarova M, Kompanikova J, Sadlonova V, Novakova E. Identification of *Mycobacterium* species by MALDI-TOF mass spectrometry. Adv Exp Med Biol. 2017;1021:37–42.
165. Sanguinetti M, Posteraro B. Identification of molds by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2017;55:369–79.
166. Lagace-Wiens PRS, Adam HJ, Karlowsky JA, Nichol KA, Pang PF, Guenther J, et al. Identification of blood culture isolates directly from positive blood cultures by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry and a commercial extraction system: analysis of performance, cost, and turnaround time. J Clin Microbiol. 2012;50:3324–8.
167. Huang B, Zhang L, Zhang W, Liao K, Zhang S, Zhang Z, et al. Direct detection and identification of bacterial pathogens from urine with optimized specimen processing and enhanced testing algorithm. J Clin Microbiol. 2017;55:1488–95.
168. Steel C, Golden A, Kubofcik J, LaRue N, De los Santos T, Domingo GJ, et al. Rapid *Wuchereria bancrofti*-specific antigen Wb123-based IgG4 immunoassays as tools for surveillance following mass drug administration programs on lymphatic filariasis. Clin Vaccine Immunol. 2013;20:1155–61.
169. Sparbier K, Wenzel T, Dihazi H, Blaschke S, Müller GA, Deelder A, et al. Immuno-MALDI-TOF MS: new perspectives for clinical applications of mass spectrometry. Proteomics. 2009;9:1442–50.

Publisher's Note

3.2 Antimicrobial resistances screening improvement

In this section, the current and future's applications of mass spectrometry for foodborne pathogens antimicrobial resistance screening is investigated under the form of a mini review*.

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Review

MALDI-TOF Mass Spectrometry and Specific Biomarkers: Potential New Key for Swift Identification of Antimicrobial Resistance in Foodborne Pathogens

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Abstract: Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is today the reference method for direct identification of microorganisms in diagnostic laboratories, as it is notably time- and cost-efficient. In the context of increasing cases of enteric diseases with emerging multi-drug resistance patterns, there is an urgent need to adopt an efficient workflow to characterize antimicrobial resistance (AMR). Current approaches, such as antibiograms, are time-consuming and directly impact the "patient-physician" workflow. Through this mini-review, we summarize how the detection of specific patterns by MALDI-TOF MS, as well as bioinformatics, become more and more essential in research, and how these approaches will help diagnostics in the future. Along the same lines, the idea to export more precise biomarker identification steps by MALDI-TOF(/TOF) MS data towards AMR identification pipelines is discussed. The study also critically points out that there is currently still a lack of research data and knowledge on different foodborne pathogens as well as several antibiotics families such as macrolides and quinolones, and many questions are still remaining. Finally, the innovative combination of whole-genome sequencing and MALDI-TOF MS could be soon the future for diagnosis of antimicrobial resistance in foodborne pathogens.

Keywords: MALDI-TOF MS; biomarkers; foodborne pathogens; antimicrobial resistance; diagnostics

1. The Burden of Antimicrobial Resistances Worldwide: The Case of Foodborne Pathogens

For decades, antibiotics have been increasingly used in human and veterinary medicine, to treat bacterial infections such as gastrointestinal, respiratory or urinary tract infections and septicemia [1]. Drugs of veterinary importance are not only used for therapeutic purposes, but also as a preventive measure (metaphylaxis and prophylaxis) and growth promoter [2]. Hence, selected resistances within pathogens appear along the food chain with most often humans as the final hosts. Likewise, antibiotics overuse and inappropriate prescribing are other main reasons for bacterial genetic adaptation and exchange facing selective pressure [3]. These mechanisms are naturally present in microbial communities among various ecosystems, such as aquatic systems [4]. Nowadays, antimicrobial resistance (AMR) is considered a major threat to global public health by its influence on human health and the related economic issues. According to a report from the Organization for Economic Cooperation

and Development (OECD), infections by resistant microorganisms will cause 2.4 million deaths in Europe, North America and Australia in the next 30 years and cost up to $3.5 billion per year [5]. As well, a World Health Organization (WHO) report highlighted a total of 349 million registered foodborne illnesses and 187,285 deaths caused by bacteria worldwide in 2010 [6]. Among these pathogens, *Acinetobacter* spp., *Bacillus* spp., *Campylobacter* spp., *Citrobacter* spp., *Clostridium* spp., *Enterobacter* spp., *Escherichia* spp., *Klebsiella* spp., *Listeria* spp., *Salmonella* spp., *Shigella* spp., *Staphylococcus* spp., *Vibrio* spp. and *Yersinia* spp are the main causes of such diseases [7]. Specifically, foodborne pathogens are in an ever-increasing focus due to the emergence of multi-drug resistance patterns worldwide. Studying and understanding interfaces between human health, animal health and the environment seems to be a requirement to understand the circulation of AMR among the food chain [8]. The "One Health" approach combines various disciplines to ensure optimal health for humans, animals, wildlife, plants and the environment on the local, national and global levels [8]. This concept is not new but is experiencing an upsurge and has become increasingly popular within the past few years [9]. According to Robinson and colleagues, AMR is the quintessential "One Health" issue, as it is linked to all domains of life, especially with microbiology as its core [10]. *Campylobacter* spp. for example, is highly relevant in a "One Health" approach. Campylobacteriosis is the first cause of bacterial gastroenteritis in humans worldwide [11,12], where it occurs more frequently than infections caused by *Salmonella* spp., *Shigella* spp. and *Escherichia coli* O157:H57 [13,14]. Since the introduction of fluoroquinolones and macrolides as drugs of choice for the treatment of human gastroenteritis in the 1980s, many reports highlighted the emergence of resistance patterns within the *Campylobacter* genus. Likewise, recent studies reported the emergence of multi-resistant *Campylobacter* spp., to different classes of antibiotics from different sources [15–18]. Gölz and colleagues point out that a better understanding of the sources and pathways at the different stages of the food chain, thanks to a "One Health" approach, should allow better control and prevention of the *Campylobacter* burden in humans [19]. The overall understanding of the co-evolution dynamics between the three compartments is urgently needed to develop novel approaches to study AMR [9,10]. Mangioni and colleagues already highlighted the important need for the development of a "fast microbiology" era in diagnostics and especially in antimicrobial stewardship policies, resulting in a more rapid optimization of antimicrobial therapy, in order to improve patients handling and care [20]. The surveillance or quantification of AMR in all the different reservoirs is a challenging task as it requires complex tools [21]. In 2015, WHO launched a new surveillance program, called GLASS, for AMR monitoring of bacteria by regions, giving established guidelines to collect data for several restricted clinical pathogens and antibacterial classes [22]. Collecting data will be an important issue through antimicrobial susceptibility tests (AST) from diagnostic laboratories involved in the program. Hence, diagnostic laboratories are on the frontline for the detection of AMR, and they require fast and cost-effective tools for analysis. During the last decade, diagnostics underwent a real revolution with the advent of molecular biology techniques (e.g., DNA based-methods or proteomics), reducing the turn-around time [20]. However, the current "patient–physician" workflow (Figure 1) is still relatively long depending on the type of primary sample (e.g., blood, urine, stool or cerebrospinal fluid) and of the requirement for the full characterization of the pathogen, i.e., species/subspecies and AMR identification. Mass spectrometry may be considered as one of the main actors in the development of future fast microbiology technologies, as the method is already implemented in a majority of health care infrastructures for routine identification of microorganisms.

The aim of this mini-review is to show how matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) could be handful for a fast combined species and AMR identification in enteric pathogens, by detecting specific biomarkers within protein spectra generated by MALDI-TOF MS. Likewise, the use of tandem mass spectrometry and bioinformatics as support tools for advanced identification of AMR will be discussed.

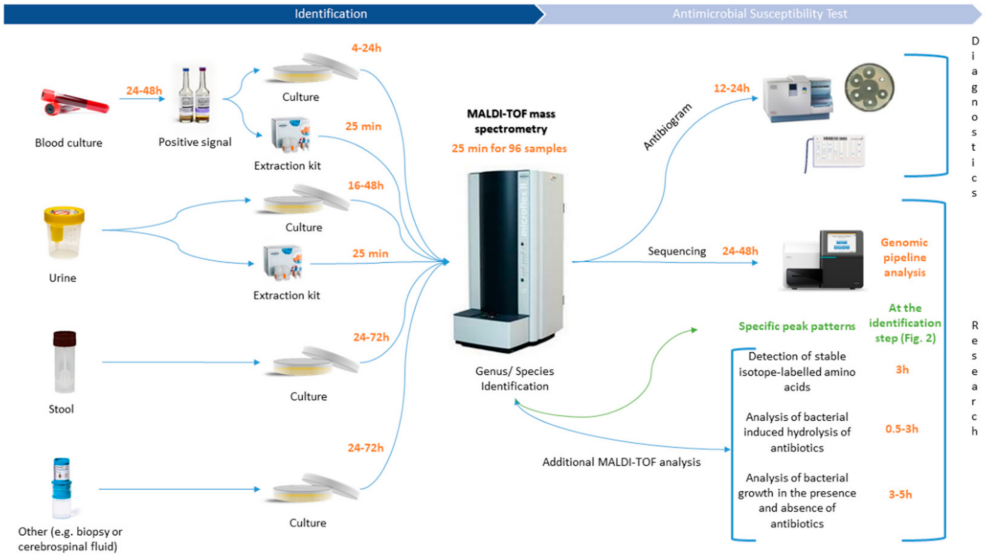

Figure 1. MALDI-TOF MS related analysis workflow in clinical routine diagnostic and research laboratories.

2. MALDI-TOF MS: A New Era for the Diagnostic Field

Current reference methods in routine laboratories for detection and identification of AMR, consist of antibiogram disk diffusion or microdilution tests and automated antibiograms (e.g., VITEK® 2 apparatus from Biomérieux©). These approaches are time-consuming and require an incubation time between 12–24 h before the physician is able to prescribe the right cohort of antibiotics to the patient. In clinical research, molecular methods such as next-generation sequencing (e.g., whole-genome sequencing (WGS)) or nucleic acid based methods (e.g., polymerase chain reaction (PCR) techniques) are also used to detect and identify AMR genes [23]. However, even if PCR methods are already implemented in many clinical diagnostic and reference laboratories and there is a notable decrease of per-sample cost for WGS, their application in routine AMR surveillance especially in resource-limited countries is restricted [24].

In the field of biology, soft ionization mass spectrometry, such as MALDI-TOF MS, has been established for decades for the analysis of important biological molecules, such as proteins, peptides, oligonucleotides, lipids or glycans [25]. In 1975, Anhalt and Fenselau proved that mass spectrometry, coupled with pyrolysis, produced characteristic mass spectra for gram-negative bacteria [26]. The MALDI method was first introduced in biology in 1987 by Karas and colleagues, and followed by Tanaka and colleagues who were awarded a Nobel prize in chemistry "for their development of soft desorption ionization methods, for mass spectrometric analysis of biological macromolecules" [27–29]. With these findings and outcomes, growing interest in mass spectrometry and its application as a screening and diagnostic research tool has emerged [30]. In the last decade, MALDI-TOF MS has become popular in routine diagnostic laboratories and is now considered the new gold standard for the direct identification of microorganisms, and somehow revolutionized the microbiology field by progressively replacing all the biochemical (e.g., API gallery) and phenotypic tests [31] for species characterization. Despite the price of the MALDI-TOF MS apparatus, analyzing a full 96 MALDI target is virtually costless and only requires around 0.50 € of chemicals and consumables [32], and only requires a maximum time of 25 min to give 96 reliable species identifications. Commercial databases included with the device cover a large panel of bacteria [33], mycobacteria [29,34] and also fungi [35] of medical interest. In addition, several reports highlighted its successful application in other microbiology areas, for the identification of viruses [36], ectoparasites [37], protozoa [38] and helminths [39,40]. In clinical application, organisms isolated from different matrices (e.g., blood, urine, stool and cerebrospinal fluid), are applied directly on the target and covered by an acid reagent. Then the target is subjected

to mass spectrometry for analysis, where a laser will shoot and ionize proteins that are separated by their mass-to-charge ratio (*m*/*z*) and analyzed by a detector. The signal will be translated into spectra, which will be compared with commercial or in-house databases and provide a rapid and reliable identification at a low cost and high precision (e.g., relevance score) [41].

Since the introduction of mass spectrometry in the field of microbiology, the speed of pathogen identification has tremendously increased, thereby improving antimicrobial therapy, infection prevention and leading to a major impact in public health and epidemiology [42]. Today, direct antimicrobial resistance detection in the acquired mass spectra is one of the most suggested and asked about applications in specialized reviews [43–47]. Four main uses have been successfully tested: (1) the detection and expression of antibiotic resistance mechanisms (e.g., β-Lactamase, rRNA methyl-transferase activity), (2) specific mass peak profiles within spectra, (3) the detection of stable isotope-labeled biomarkers and (4) the estimation of the effect of antibiotics on microorganism growth. On one hand, the detection of antibiotic resistance mechanisms is the most explored method so far, as the degradation of antibiotics produces intracellular metabolites that generate specific peaks on spectra [48,49]. These peaks are directly visible on the spectra during analysis of the latter (Figure 2A,B). Nevertheless, those investigations still imply supplementary incubation time, yet less than for antibiograms, but are inherently further postponing the diagnosis to setting up an optimal antibiotherapy. Hence, the "patient–physician" workflow requires a concrete optimization for AMR detection with novel MALDI-TOF MS approaches, which is a special scope of this review. Identification of specific biomarkers within the protein spectra presents obvious advantages compared to other techniques (Figure 2C,D). Indeed, thanks to a unique spectrum, it will be possible to couple an accurate identification at the species/subspecies level as well as antimicrobial resistances only after a 25 min run of the MALDI-TOF (Figures 1 and 2). It will drastically decrease workflow time, cost for diagnosis and hence, allow the physician to apply the effective cohort of antibiotics in an optimized time to the patient.

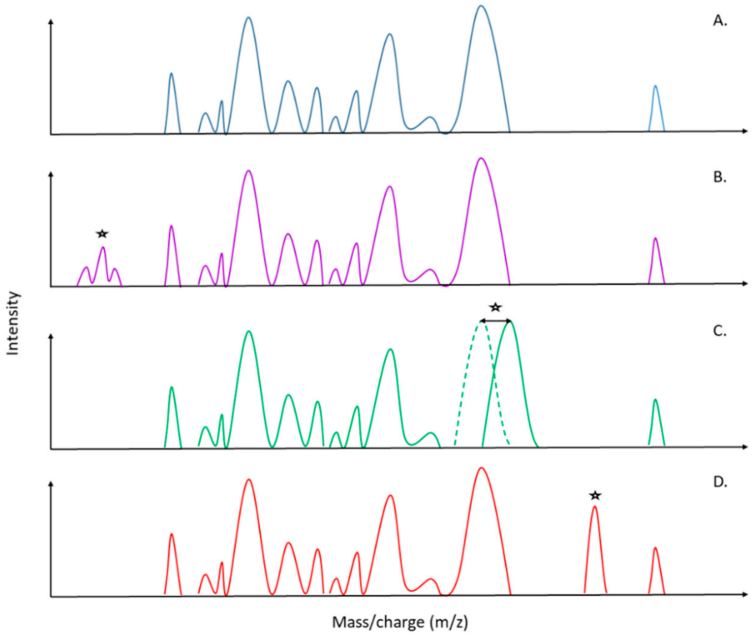

Figure 2. Schematic representation of possible MALDI-TOF MS spectra patterns for direct determination and identification of antimicrobial resistance. (**A**) Sensitive strain. (**B**) Detection of antimicrobial resistance by the detection of metabolites related to the degradation of the antibiotic. (**C**) Detection of antimicrobial resistance by the detection of a peak shift, which could be related to a mutation in the biomarker gene that confers antimicrobial resistance (AMR). (**D**) Detection of antimicrobial resistance by the detection of unique biomarkers, which could be related to the production of a specific molecule (e.g., enzymes, porins). (*) Peak differences in comparison with the sensitive strain spectra (**A**).

3. Specific Biomarkers as a Future Key for the Detection of AMR

In the clinical field, biomarkers are defined as biomolecules that are determined in a tissue or body fluid of a patient to identify a disease at the molecular level [50]. Developments of protein biomarker descriptions have been done for biological fluids, cell lines and solid tissues for many purposes like diagnosis, treatment, follow-up, etc. [50]. In mass spectrometry, a biomarker could be defined and identified as a specific unique peak, numerous peaks or a shift in the mass-to-charge ratio. Since the application of MALDI-TOF MS for the identification of microorganisms, only several publications remarked on its potential usefulness in detecting and characterizing antimicrobial resistances through specific biomarker(s) (Table 1). In 2000, Edwards-Jones and colleagues carried out the first work on the subject by noticing specific biomarkers, allowing the distinction between methicillin-sensitive (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) by intact cell mass spectrometry (ICMS), and concluded that ICMS could have the capacity to identify and perform typing of MRSA [51]. Their results were validated two years later by another group working on *S. aureus* [52], by also demonstrating a variation between the spectral profiles in the mass range of *m*/*z* 500–3500 Da.

Table 1. Specific whole-cell MALDI-TOF MS spectra patterns literature for identification of antimicrobial resistance in enteric bacteria.

Organism	Antibiotic Classes Tested	Biomarkers	Year	Reference
Staphylococcus aureus	β-lactams	MRSA: 891, 1140, 1165, 1229 and 2127 *m*/*z* MSSA: 2548 and 2647 *m*/*z*	2000	[51]
Staphylococcus aureus	β-lactams	Variation between in the spectral profiles in the mass range of *m*/*z* 500–3500 Da	2002	[52]
Lactococcus lactis *Bacillus coagulans* *Escherichia coli*	Bacteriocins (lantibiotic)	Lacticin 481: 2902, 2924,2940 *m*/*z* Nisin A: 3392 *m*/*z* Coagulin: 4650 *m*/*z*	2003	[53]
Escherichia coli	β-lactams	Ampicillin: 29.000 *m*/*z*	2007	[54]
Bacteroides fragilis	Carbapenems	cfiA negative: 4711, 4817, 5017, 5204, 5268 *m*/*z* cfiA positive: 4688, 4826, 5002, 5189, 5282 *m*/*z*	2011	[55]
Klebsiella spp.	Carbapenems	OmpK36 porin: 38000, 19000 *m*/*z*	2012	[56]
Enterococcus faecium	Glycopeptides	VanA/B: 6603 *m*/*z*	2012	[57,58]
Enterobacteriaceae	Carbapenems	bla_{KPC}: 11109 *m*/*z*	2014	[59]
Campylobacter jejuni	β-lactams Tetracyclines Glycopeptides	Spectrum processing parameters increased the resistance detection	2016	[60]
Staphylococcus aureus *Staphylococcus epidermidis*	β-lactams	mecA: 2415 *m*/*z*	2016	[61]
Escherichia coli *Klebsiella pneumonia* *Enterobacter cloacae*	Polymyxin	Lipid A modification: 1919 *m*/*z*	2018	[62]
Escherichia coli *Serratia marcescens* *Citrobacter braakii,* *Pseudomonas aeruginosa*	Carbapenems	KPC-2: 28544 *m*/*z*	2019	[63]
Bacteroides fragilis	Carbapenems	Identification of *B. fragilis* with the validated "cfiA library" [55]	2019	[64]

Hindre and colleagues showed that it was possible to detect bacteriocins without specific purification from bacterial colonies, as lacticin, nisin and coagulin producing bacteria generate specific

mass to charge ratio peaks for each molecule [53]. Additionally, Camara and Hays [54] differentiated wild-type *E. coli* from ampicillin-resistant plasmid-transformed *E. coli* strains by direct visualization of β-lactamase in the spectra. In 2011, another team reported the successful application of MALDI-TOF MS to differentiate between cfiA-positive and cfiA-negative *Bacteroides fragilis*, and hence their capacity to be potentially resistant to carbapenems, by the observation of a protein profile shift between the two different classes [55]. Currently the major avenue with MALDI-TOF MS is seeking specific peaks linked to porins [56], enzymes (e.g., VanA/B, mecA, KPC-2) [57–59,61,63] or even lipid modifications [62]. Furthermore, number of listed studies settle not only on the detection of specific biomarkers, but focus on processing parameters and creation of in-house databases, and therefore bioinformatics.

4. Bioinformatics: A Powerful Tool to Reinforce Diagnostics

Early automatic typing methods were mainly of a phenotypic nature (e.g., serotype or biochemical characteristics). However, with the advent of molecular biology, bioinformatics became unmissable and hence, a must in research to proceed and analyze genomic data in research. Bioinformatics can be defined as an interdisciplinary field developing methods and software tools for a better understanding of biological systems.

In diagnostics, dilution- or diffusion-based antibiograms are still currently the reference methods for phenotypic detection of AMR. With the emergence of new sequencing technologies, such as whole-genome sequencing (WGS), genomic data are more and more used for the identification and prediction of AMR thanks to the detection of specific sequences. Nowadays, different online user-friendly platforms able to use whole-genome data to extract relevant information, such as AMR genes, exist. The real advantage of these tools is that they are intended for scientists who do not necessarily have advanced bioinformatic skills. Many pipelines that are able to predict AMR patterns, such as Resfinder [65], AMRFinder [66], ARGS-OAP [67], SEAR [68] or ARGminer [69] are today online. Historically, Resfinder, developed by the Center for Genomic Epidemiology, was one of the first types of platforms of this kind, and it is a widely used AMR determinant detection program [65]. It is a web server that uses data for identifying acquired AMR genes and/or chromosomal mutations in total or partial sequenced isolates of bacteria, referring to nucleotide sequences from the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/nuccore/). Recently, NCBI developed a new tool, AMRFinder, using either protein annotations or nucleotide sequences to identify AMR genes. A first report comparing AMRFinder and Resfinder performance, using bacterial isolates from a collection from the U.S. AMR surveillance system program (NARMS) [66], highlights that incomplete or incorrect databases can lead to AMR misidentification. As an example, in some cases, where Resfinder generates a high scoring for an identification, the latter was incorrect due to the absence of a specific sequence in the database. However, the database issue is currently the same with MALDI-TOF MS for the identification of different species, with the results depending on the quality of the used database. Hence, even if online AMR detection platforms are useful to give a first glimpse of which AMR could be present, there is still a need to improve and implement databases with new and reliable sequences. For now these bioinformatics tools should be combined with phenotypic methods.

Mass spectrometers manufacturers, such as Bruker Daltonics© (https://www.bruker.com/) propose software platforms (e.g., FlexControl™, FlexAnalysis™, Maldi Biotyper Compass Explorer™ and Clinpro Tools™) allowing the acquisition, processing of spectra and the creation of customized databases, and together with other bioinformatics pipelines provide new performant tools to the MALDI-TOF MS community [70,71]. Applied Maths NV© (http://www.applied-maths.com/bionumerics), notably, proposes BioNumerics™, a pipeline platform for advanced analysis of spectra. It offers a large panel of competitive analysis applications, including fingerprinting, typing, MALDI spectrum processing and the creation of in-house databases, by the utilization of different default or customized modules [72]. Among the publications listed in Table 1, reports highlighted that spectrum-processing parameters (e.g., baseline subtraction and curve smoothing) increased the detection of AMR from *Campylobacter jejuni* [60]. Indeed, by applying optimized processing parameters, beta-lactam resistances detection

was increased by 34%. Spectrum processing parameters should not be neglected and indeed enhance screening performance. Several other MALDI-TOF MS studies used BioNumerics™ as their main tool for analysis [73–75]. However, even if previously mentioned software suggests a high capacity to customize and optimize spectra, it is also important to highlight the fact that it is also possible to carry it out during the acquisition step by modifying MALDI-TOF parameters. Variables such as acquisition range (e.g., 2–20 kDa or 300 Da for the detection of antibiotic hydrolysis products), laser intensity, spectrum evaluation (e.g., peaks limit intensity) or ion source modifications (e.g., increase the resolution for low- and high-weight molecules), might be modified and adjusted. The combination of appropriate acquisition parameters and processing/optimization steps is key for MALDI-TOF spectra analysis and exploitation.

Various other software gives the opportunity to create and perform in-house databases. Jeverica and colleagues have successfully screened routine clinical *B. fragilis* isolates and determined their division (e.g., I or II), hence their potentiality to be resistant to carbapenems, thanks to the created in-house database of Nagy and colleagues [55,64]. Therefore, the creation of in-house databases, ideally sharing close experimental conditions and spectrum processing parameters should be the main avenues to be explored in the future, for the full optimization of the application of MALDI-TOF MS to detect AMR. In complement to commercial libraries, in-house, online or external databases exist and allow the comparison of user spectra. For example, the Centers for Disease Control and Prevention (CDC) curates a platform: MicrobeNet (https://microbenet.cdc.gov/), which is a free online database launched in 2013 with the goal to help clinical laboratories to improve their diagnostics. Moreover, they developed a collaboration with Bruker©, allowing users to search the database directly from the generated MALDI-TOF mass spectra. It is yet possible to match unknown acquired spectra to find out if someone else already identified it. As an example, a recent study [76] showed the application of external databases, such as SARAMIS™ (Spectral Archive and Microbial Identification System database) and PAPMID™ (Putative Assigned Protein Masses for Identification Database), and the 5800 TOF/TOF MALDI research instrument from Absciex©, as an efficient tool for the identification of 26 bacterial strains, with comparable accuracy to a commercial system. If the primary use of this online-database is widened to AMR thematics, it will be possible to share freshly discovered AMR biomarkers far more easily. In brief, bioinformatics offers a wide range of tools for the detection and identification of AMR, easily practicable in combination with MALDI-TOF MS.

5. MALDI-TOF/TOF Tandem Mass Spectrometry: To Infinity and Beyond

The development of soft-ionization methods such as MALDI or electrospray ionization (ESI) were important discoveries, as it was preserving the integrity of larger molecular weight compounds like proteins, carbohydrates or lipids [77]. MALDI-TOF MS would detect mainly ribosomal proteins, housekeeping proteins and structural proteins that are abundant in the cell, relatively independent of the growth state of the microorganism, in a mass range between 2 to 20 kDa [78]. However, this type of mass spectrometry is somehow self-limiting in its efficiency, depending on the mode used to give primary information, such as the mass of the analyzed compound [79]. Indeed, mass spectrometry technology presents different possible parameter adjustments, such as the linear (i.e., ion moves in a straight line from the source to detector) and reflectron (i.e., ion mirrors increasing the time of flight and the resolution) modes, or the investigation of positive and/or negative ions, to increase the resolution and selectivity of generated spectra [79]. The desire to know more than the mass of molecules brought up the development of complex mass spectrometers combining two analyzers (e.g., quadrupole, ion trap and TOF), called multi-analyzer systems or MS/MS [79]. The association of two identical types of analyzers is a tandem instrument. Among these tandem mass spectrometers, MALDI-TOF/TOF MS is commonly used in proteomic research, for the sequencing of peptides [80]. The first TOF analyzer serves as a mass filter [81], to select an ion of interest, whose corresponding fragment is communicated (or not) to the second analyzer [81]. High resolution and mass selectivity enable the identification of peptides, i.e., an individual biomarker from the protein, essential for the

analysis of closely related species (or strains) or gene expression patterns [77]. However, fragmentation is only feasible for low mass weights (up to approximately 3 kDa), and if identified biomarkers have a higher mass, there will be a need to process through other MS approaches. As mentioned in previous sections, antimicrobial resistance can be targeted thanks to the presence of a specific peak related to the presence of enzymes, by peak shifting due to chromosomic mutation(s), and/or also by the presence of degradation molecules (Figure 2). The standard MALDI-TOF MS is able to detect such mechanisms. However, to know in precision which enzyme or mutation is involved in these specific mass-to-charge ratios, advanced analysis is required. In 2006, Pieper and colleagues carried out proteomic analyses of a sub-cellular fraction of *S. aureus* isolate VP32 with different resistances to the cell-wall targeting compound vancomycin [82,83]. They analyzed and determined significant protein abundance differences for 65 proteins by MALDI-TOF/TOF MS and liquid chromatography-MS/MS. Among these proteins, several enzymes involved in the biosynthesis of purines, peptidoglycan hydrolases and penicillin-binding proteins were identified. They concluded that different expression levels of these proteins might be responsible for structural changes of the peptidoglycan and hence conferring resistance to glycopeptide antibiotics. Such studies largely support the idea to link, in a close future, specific biomarkers detected by MALDI-TOF MS spectra to characteristic and often well-known biological phenotypic mechanisms.

However, until a MALDI-TOF MS spectrum could be able to give the utmost information at once, there is still a long way to go and issues can already be identified. First of all, before carrying out MALDI-TOF/TOF MS analysis, there is the need to identify a specific antimicrobial resistance biomarker. Nevertheless, if the biomolecules of interest, here an enzyme, is expressed in a low quantity by the cell, there are three possible limiting scenarios. The first one will be that MALDI-TOF MS does not detect it, due to too low intensity and hence no appearance on the spectra. The second one, the peak exists but the intensity is that low that during spectrum processing it could disappear. The last one, the specific peak will go through all the steps but would still have a too low intensity to be explored. An important point to mention is the resolution of the device itself. Indeed, manufacturers do not propose all the same resolution for their mass spectrometers. Most of the software used for the identification of spectra are working with three different components: (1) mode forward: How many peaks of the spectrum to be identified are present in the reference spectrum, (2) reverse mode: How many peaks in the spectrum of reference are present in the sample and (3) symmetry: Count the common peaks, and sum the intensity ratios. In this configuration, intensity is an important factor, whereas the frequency of apparition of peaks is not taken into account. As a suggestion, identification software should consider integrating into their algorithm a special mode dedicated to the calculus of peak frequency between the different analyzed spectra. Finally, there remains the question of the transition between the MALDI-TOF/TOF and MALDI-TOF spectra: Will it be possible to integrate specific biomarkers data from the MALDI-TOF/TOF spectra into a MALDI-TOF database? Indeed, the main objective for routine diagnostic laboratories will be to couple species identification, subtyping and antimicrobial resistance identification after the generation of one single spectrum. However, the detection of shifts due to the mutation of one or two bases in the genome requires high sensitivity and resolution. The integration of tandem TOF/TOF MS data will be ideal for the detection of such shifts, as the tandem technology has a higher setting than single MALDI-TOF MS. Straightaway, there is no report of a successful transfer of MALDI-TOF/TOF data through a MALDI-TOF system so far, which means there is still a specific need for further scientific and technological development. In the same line of thought, the cost of such a device and the development of specific skills for spectra analysis are currently still a serious stumbling block for its concrete implementation in diagnostics.

6. Outlook and Future Challenges for MALDI-TOF MS and AMR in the Diagnostic Field

During the last decade, antimicrobial resistance obviously became a serious issue for public health. However, international projects (e.g., EU-JAMRAI, EFFORT, JPIAMR, etc.) and challenging competitions (e.g., Antimicrobial Resistance Rapid, Point-of-Need Diagnostic Test-Challenge) have

surfaced to find a way to reduce and/or optimize the use of antibiotics. Amongst others, the Longitude prize launched in 2014, with the aim to reward teams that can develop a cheap, accurate, rapid and easy-to-use point-of-care diagnostic test for bacterial infection, with a focus on antimicrobial resistance. In the context of developing a fast technology for diagnostics, much effort has been directed toward finding new alternatives for the detection of antimicrobial resistances implying MALDI-TOF MS as a new potential reference tool, and has now largely gone beyond the proof-of-concept stages [84]. The diagnostics mass spectrometry stage is mainly represented by the two manufacturers Bruker© and Biomérieux©, which have largely contributed to the most recent innovation in terms of AMR detection by mass spectrometry. In one hand, Biomérieux© proposes a complete automated identification (ID) /AST system, i.e., the VITEK® SOLUTION (https://www.biomerieux-diagnostics.com/vitek-solutions), by coupling two of their devices: the MALDI-TOF VITEK® MS, which furnishes the ID, and the VITEK® 2 for AST. The ID/AST complex is supposed to give a result to clinicians within 14–20 h. On the other hand, during the ASM Microbe conference 2019 (www.asm.org), Bruker© announced the launch of the MALDI Biotyper® Sirius system [85], a versatile MALDI-TOF system for research purposes. It supports a novel negative/positive-ion switch mode assay for research and clinical studies in fast antibiotic resistance testing, such as colistin resistance in gram-negative bacteria [85]. Simultaneously, they introduced the MBT-STAR assay kit for detection of carbapenem and cephalosporin resistance. It measures the level of hydrolysis of the β-lactam ring after a 30 min incubation, thus providing a result within 60 min, after analysis by the MBT STAR-BL software module [85]. Finally Bruker© developed a software module for subtyping antimicrobial resistances such as KPC-producing *K. pneumonia*, MRSA, and *B. fragilis* cfiA [86], inspired by the previously described studies in Table 1. According to the manufacturer, after a simple direct transfer on the target from the agar plate, the software will be able, after a high confidence identification, to process an automated typing (e.g., "presumptive KPC", "presumptive PSM positive MRSA") thanks to the detection of specific biomarkers [87]. However, much work still needs to be accomplished before exporting this technology to diagnostic and reference laboratories [84]. The detection of specific biomarkers in foodborne pathogens should give an advantage to obtaining the three-fold information within a single spectrum: species identification, sub-typing and antimicrobial susceptibility, to efficiently treat foodborne infections. The elaboration of in-house databases and processing parameters should be considered a key step to make MALDI-TOF MS a potential new gold standard for AMR detection.

The successful detection of specific antimicrobial resistance biomarkers on MALDI spectra within the same bacterial genus has been described in previous sections. However, a question still remains: could a specific AMR biomarker from one bacterial genus be applied and steadily transferred to another one? A working group detected the presence of biomarkers for the protein pKpQIL_p019, conferring carbapenem resistances in the *Enterobacteriaceae* family, in three different bacteria: *K. pneumoniae*, *E. coli* and *E. gergoviae*, at a mass-to-charge ratio of 11,109 m/z [87,88]. They specified the implementation of screening and analysis in the routine clinical workflow of their laboratory, with all spectra scanned by the automated script for peaks within a window of 11,109 ± 15 Da using Bruker©-provided platform software. By the creation of specific peak scripts peculiar for specific antimicrobial resistance, it is possible to detect antimicrobial mechanisms or resistances for different bacteria and to integrate these in a diagnosis workflow. However, this technique still needs to be explored for more antibiotics classes such as β-lactams, glycopeptides or macrolides.

Nowadays, WGS is considered as the current approach with the highest levels of discrimination in terms of subtyping, and studies have already reported its application as being effective to predict antimicrobial resistance in bacteria [89–91], and making it a valuable tool for antimicrobial resistance surveillance [23]. However, even if the sequencing price has significantly decreased during the past decade, this technology is not implemented in every diagnosis laboratory, and the analysis requires much more time than mass spectrometry. Yet still, very few studies show the tandem utilization of WGS and MALDI-TOF MS [92,93]. Both techniques present advantages and disadvantages but seem to show a particular complementarity. As an example, colony identification of *Elizabethkingia*

spp., a ubiquitous bacteria found both in the environment and hospital settings, was carried out by MALDI-TOF MS [93]. WGS was used for the detection of antimicrobial resistance genes and to confirm MALDI-TOF MS identification. WGS showed a better identification rate than MALDI-TOF MS, due to the lack of reference spectra for *Elizabethkingia* spp. in MALDI-TOF MS commercial databases at the time of the study. They concluded that MALDI-TOF MS databases should be continuously updated and upgraded, while WGS proved to be a valuable tool for species identification confirmation and quite detailed characterization of multidrug-resistance. Further, a report [92] studied the usefulness of MALDI-TOF MS in an outbreak of vancomycin-resistant *Enterococcus faecium* in a hospital in comparison to WGS. They reported, due to multiple cluster types involved in the outbreak, that the cohort showed discrepancies between the two techniques. The authors highlighted MALDI-TOF MS limitations in this situation and suggested to study results carefully, while WGS can be used for determination of evolutionary distance between isolates. However, another important point to highlight, which is not mentioned in the latter studies, is that WGS is certainly able to accurately spot resistance genes, but it does not give any information on gene expression, while phenotype-based MALDI-TOF MS generates a spectrum based on protein expression and hence, gene expression. As MALDI-TOF MS is mainly used as a frontline tool in diagnostic laboratories, first results, such as species identification or AMR in the future, could be obtained rapidly, while species confirmation and antimicrobial resistance detection on the genome side could be obtained in a more delayed second step by WGS. MALDI-TOF MS and WGS should be seriously considered as complementary tandem tools and more studies should be led on this dual application.

Escherichia coli, *Staphylococcus aureus* and *Bacteroides fragilis* are the most MALDI-TOF MS studied enterobacteria according to Table 1. However, other enteric pathogens with a high impact incidence on human and animal health exist, which were not included in research reports so far. Lately, Batz and colleagues in their "Ranking the risk report" [94], list the three first bacterial foodborne pathogens as *Campylobacter* spp., *Salmonella* spp. and *Listeria monocytogenes*. Zautner and colleagues already reported the ability of MALDI-TOF MS to subtype *Campylobacter* spp. by shifts in biomarker masses, due to amino acid substitutions caused by single-point mutations in the respective biomarker gene [95], and they further described proteotyping as a promising tool for microbial typing at the species, subspecies, and even below subspecies levels [96–98]. These last studies show how generated spectra are exploitable and accurate enough to detect various AMR biomarkers in important pathogens such as *Salmonella* spp. or *Listeria* spp. Along the same line, carbapenems and β-lactams antibiotics families were the most tested and studied. However, gastroenteritis is the main end-up of a foodborne pathogen, and quinolones (e.g., ciprofloxacin) and macrolides (e.g., azithromycin and erythromycin) are the first frontline antibiotics used to treat such diseases [99]. Moreover, WHO categorized these two antibiotics as critically important [100] due to a high resistance prevalence concerning pathogens such as *Campylobacter* spp., *E. coli*, or non-typhoidal *Salmonella* spp. Nevertheless, at the moment there are no reports highlighting potential biomarkers for AMR to quinolones and macrolides. In a context where emerging multiple antimicrobial resistances are a critical issue, there is a need to collect data at least on these two antibiotic classes in order to ensure the collection, within one spectrum, of all the needed information.

Regarding our review on the detection of AMR by specific MALDI-TOF spectra patterns, there is still a lot to accomplish before MALDI-TOF MS could be considered the new reference method for the detection of antimicrobial resistance in routine diagnostics. Many questions still remain open and more studies should specifically be led on foodborne pathogens. Exploration on critical important antibiotics such as quinolones or macrolides, which are widely used for the treatment of foodborne illnesses, but unfortunately with no available data on it, should be of major interest for the scientific community. Finally, the dual combination of WGS and MALDI-TOF MS should soon become the main approach for the utmost reliable and fast identification of AMR in foodborne pathogens.

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References

1. Centers for Disease Control and Prevention. Antibiotic Use in the United States, 2017: Progress and Opportunities. Available online: https://www.cdc.gov/antibiotic-use/stewardship-report/index.html (accessed on 9 October 2019).
2. Schwarz, S.; Kehrenberg, C.; Walsh, T.R. Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents* **2001**, *17*, 431–437. [CrossRef]
3. Ventola, C.L. The Antibiotic Resistance: Part 1: Causes and Threats. *P T a Peer Rev. J. Formul. Manag.* **2015**, *40*, 277–283.
4. Orruño, M.; Garaizabal, I.; Arana, I.; Barcina, I. Persistence and dissemination of antimicrobial resistances in aquatic systems. In *Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education*; Formatex Research Center: Badajoz, Spain, 2013; pp. 624–629.
5. The Organisation for Economic Co-operation Development. *Stemming the Superbug Tide*; OECD Health Policy Studies: Paris, France, 2018.
6. World Health Organization. WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group 2007–2015. Available online: https://apps.who.int/iris/handle/10665/199350 (accessed on 9 October 2019).
7. Priyanka, B.; Patil, R.K.; Dwarakanath, S. A review on detection methods used for foodborne pathogens. *Indian J. Med. Res.* **2016**, *144*, 327–338. [CrossRef] [PubMed]
8. Lammie, S.L.; Hughes, J.M. Antimicrobial Resistance, Food Safety, and One Health: The Need for Convergence. *Annu. Rev. Food Sci. Technol.* **2016**, *7*, 287–312. [CrossRef] [PubMed]
9. Destoumieux-Garzón, D.; Mavingui, P.; Boetsch, G.; Boissier, J.; Darriet, F.; Duboz, P.; Fritsch, C.; Giraudoux, P.; Le Roux, F.; Morand, S.; et al. The one Health Concept: 10 years old and a long road ahead. *Front. Vet. Sci.* **2018**, *5*, 14. [CrossRef] [PubMed]
10. Robinson, T.P.; Bu, D.P.; Carrique-Mas, J.; Fèvre, E.M.; Gilbert, M.; Grace, D.; Hay, S.I.; Jiwakanon, J.; Kakkar, M.; Kariuki, S. Antibiotic resistance is the quintessential One Health issue. *Trans. R. Soc. Trop. Med. Hyg.* **2016**, *110*, 377–380. [CrossRef]
11. European Food Safety Authority (EFSA). The European union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011. *EFSA J.* **2013**, *11*, 3129. [CrossRef]
12. European Food Safety Authority (EFSA). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* **2017**, *15*, 5077.
13. Allos, B.M. *Campylobacter jejuni* Infections: Update on Emerging Issues and Trends. *Clin. Infect. Dis.* **2002**, *32*, 1201–1206.
14. Chlebicz, A.; Śliżewska, K. Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *Int. J. Environ. Res. Public Health* **2018**, *15*, 863. [CrossRef]
15. Szczepanska, B.; Andrzejewska, M.; Spica, D.; Klawe, J.J. Prevalence and antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolated from children and environmental sources in urban and suburban areas. *BMC Microbiol.* **2017**, *17*, 80. [CrossRef] [PubMed]
16. García-Fernández, A.; Dionisi, A.M.; Arena, S.; Iglesias-Torrens, Y.; Carattoli, A.; Luzzi, I. Human campylobacteriosis in Italy: Emergence of multi-drug resistance to ciprofloxacin, tetracycline, and erythromycin. *Front. Microbiol.* **2018**, *9*, 1906. [CrossRef] [PubMed]

17. Karikari, A.B.; Obiri-Danso, K.; Frimpong, E.H.; Krogfelt, K.A. Antibiotic resistance of *Campylobacter* recovered from faeces and carcasses of healthy livestock. *Biomed. Res. Int.* **2017**, *2017*, 4091856. [CrossRef] [PubMed]
18. Maktabi, S.; Ghorbanpoor, M.; Hossaini, M.; Motavalibashi, A.; Dvm, S.M. Detection of multi-antibiotic resistant *Campylobacter coli* and *Campylobacter jejuni* in beef, mutton, chicken and water buffalo meat in Ahvaz, Iran. *Artic. Vet. Res. Forum* **2019**, *10*, 37–42.
19. Gölz, G.; Schielke, A.; Josenhans, C.; Rosner, B.; Löwenstein, A.; Alter, T.; Wieler, L.H.; Kreienbrock, L.; Stark, K.; Suerbaum, S.; et al. Relevance of *Campylobacter* to public health—The need for a One Health approach. *Int. J. Med. Microbiol.* **2014**, *304*, 817–823. [CrossRef]
20. Mangioni, D.; Viaggi, B.; Giani, T.; Arena, F.; D'Arienzo, S.; Forni, S.; Tulli, G.; Rossolini, G.M. Diagnostic stewardship for sepsis: The need for risk stratification to triage patients for fast microbiology workflows. *Future Microbiol.* **2019**, *14*, 169–174. [CrossRef]
21. Hendriksen, R.S.; Munk, P.; Njage, P.; van Bunnik, B.; McNally, L.; Lukjancenko, O.; Röder, T.; Nieuwenhuijse, D.; Pedersen, S.K.; Kjeldgaard, J.; et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage. *Nat. Commun.* **2019**, *10*, 1124. [CrossRef]
22. World Health Organization. Global Antimicrobial Resistance Surveillance System. Manual for Early Implementation. Available online: https://www.who.int/antimicrobial-resistance/publications/surveillance-system-manual/en/ (accessed on 9 October 2019).
23. Whitehouse, C.A.; Young, S.; Li, C.; Hsu, C.H.; Martin, G.; Zhao, S. Use of whole-genome sequencing for *Campylobacter* surveillance from NARMS retail poultry in the United States in 2015. *Food Microbiol.* **2018**, *73*, 122–128. [CrossRef]
24. Anjum, M.F.; Zankari, E.; Hasman, H. Molecular methods for detection of antimicrobial resistance. *Microbiol. Spectr.* **2017**, *5*, 1–17. [CrossRef]
25. Vestal, M.L. Evolution of quantitative MALDI-TOF mass spectrometry for clinical applications. *Clin. Chem.* **2016**, *62*, 20–23. [CrossRef]
26. Anhalt, J.P.; Fenselau, C. Identification of bacteria using mass spectrometry. *Anal. Chem.* **1975**, *47*, 219–225. [CrossRef]
27. Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Process.* **1987**, *78*, 53–68. [CrossRef]
28. Tanaka, K.; Waki, H.; Ido, Y.; Akita, S.; Yoshida, Y.; Yoshida, T.; Matsuo, T. Protein and polymer analyses up to m/z 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1988**, *2*, 151–153. [CrossRef]
29. Clark, A.E.; Kaleta, E.J.; Arora, A.; Wolk, D.M. Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* **2013**, *26*, 547–603. [CrossRef] [PubMed]
30. Pusch, W.; Kostrzewa, M. Application of MALDI-TOF mass spectrometry in screening and diagnostic research. *Curr. Pharm. Des.* **2005**, *11*, 2577–2591. [CrossRef]
31. Hou, T.Y.; Chiang-Ni, C.; Teng, S.H. Current status of MALDI-TOF mass spectrometry in clinical microbiology. *J. Food Drug Anal.* **2019**, *27*, 404–414. [CrossRef]
32. Chabriere, E.; Bassène, H.; Drancourt, M.; Sokhna, C. MALDI-TOF-MS and point of care are disruptive diagnostic tools in Africa. *New Microbes New Infect.* **2018**, *26*, S83–S88. [CrossRef]
33. Carbonnelle, E.; Mesquita, C.; Bille, E.; Day, N.; Dauphin, B.; Beretti, J.L.; Ferroni, A.; Gutmann, L.; Nassif, X. MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. *Clin. Biochem.* **2011**, *44*, 104–109. [CrossRef]
34. Rotcheewaphan, S.; Lemon, J.K.; Desai, U.U.; Henderson, C.M.; Zelazny, A.M. Rapid one-step protein extraction method for the identification of mycobacteria using MALDI-TOF MS. *Diagn. Microbiol. Infect. Dis.* **2019**, *94*, 355–360. [CrossRef]
35. L'Ollivier, C.; Ranque, S. MALDI-TOF-Based Dermatophyte Identification. *Mycopathologia* **2017**, *182*, 183–192. [CrossRef]
36. Sjöholm, M.I.L.; Dillner, J.; Carlson, J. Multiplex detection of human herpesviruses from archival specimens by using matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* **2008**, *46*, 540–545. [CrossRef] [PubMed]

37. Boucheikhchoukh, M.; Laroche, M.; Aouadi, A.; Dib, L.; Benakhla, A.; Raoult, D.; Parola, P. MALDI-TOF MS identification of ticks of domestic and wild animals in Algeria and molecular detection of associated microorganisms. *Comp. Immunol. Microbiol. Infect. Dis.* **2018**, *57*, 39–49. [CrossRef] [PubMed]
38. Calderaro, A.; Buttrini, M.; Montecchini, S.; Rossi, S.; Piccolo, G.; Arcangeletti, M.C.; Medici, M.C.; Chezzi, C.; De Conto, F. MALDI-TOF MS as a new tool for the identification of *Dientamoeba fragilis*. *Parasites Vectors* **2018**, *11*, 11. [CrossRef] [PubMed]
39. Mayer-Scholl, A.; Murugaiyan, J.; Neumann, J.; Bahn, P.; Reckinger, S.; Nöckler, K. Rapid identification of the foodborne pathogen *Trichinella* spp. by matrix-assisted laser desorption/ionization mass spectrometry. *PLoS ONE* **2016**, *11*, e0152062. [CrossRef]
40. Feucherolles, M.; Poppert, S.; Utzinger, J.; Becker, S.L. MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: A systematic review. *Parasite Vectors* **2019**, *12*, 245. [CrossRef]
41. Singhal, N.; Kumar, M.; Kanaujia, P.K.; Virdi, J.S. MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. *Front. Microbiol.* **2015**, *6*, 791. [CrossRef]
42. Rodríguez-Sánchez, B.; Cercenado, E.; Coste, A.T.; Greub, G. Review of the impact of MALDI-TOF MS in public health and hospital hygiene, 2018. *Eurosurveillance* **2019**, *24*, 1800193. [CrossRef]
43. Pliakos, E.E.; Andreatos, N.; Shehadeh, F.; Ziakas, P.D.; Mylonakis, E. The cost-effectiveness of rapid diagnostic testing for the diagnosis of bloodstream infections with or without antimicrobial stewardship. *Clin. Microbiol. Rev.* **2018**, *31*, e00095-17. [CrossRef]
44. Florio, W.; Tavanti, A.; Ghelardi, E.; Lupetti, A. MALDI-TOF MS applications to the detection of antifungal resistance: State of the art and future perspectives. *Front. Microbiol.* **2018**, *9*, 2577. [CrossRef]
45. Florio, W.; Tavanti, A.; Barnini, S.; Ghelardi, E.; Lupetti, A. Recent advances and ongoing challenges in the diagnosis of microbial infections by MALDI-TOF mass spectrometry. *Front. Microbiol.* **2018**, *9*, 1097. [CrossRef]
46. Vrioni, G.; Tsiamis, C.; Oikonomidis, G.; Theodoridou, K.; Kapsimali, V.; Tsakris, A. MALDI-TOF mass spectrometry technology for detecting biomarkers of antimicrobial resistance: Current achievements and future perspectives. *Ann. Transl. Med.* **2018**, *6*, 240. [CrossRef] [PubMed]
47. Oviaño, M.; Bou, G. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the rapid detection of antimicrobial resistance mechanisms and beyond. *Clin. Microbiol. Rev.* **2019**, *32*, e00037-18. [CrossRef] [PubMed]
48. Sparbier, K.; Schubert, S.; Kostrzewa, M. MBT-ASTRA: A suitable tool for fast antibiotic susceptibility testing? *Methods* **2016**, *104*, 48–54. [CrossRef] [PubMed]
49. Kostrzewa, M.; Sparbier, K.; Maier, T.; Schubert, S. MALDI-TOF MS: An upcoming tool for rapid detection of antibiotic resistance in microorganisms. *Proteomics-Clin. Appl.* **2013**, *7*, 767–778. [CrossRef] [PubMed]
50. Ballesté, R.N. Proteomics: Technology and Applications. In *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*; Academic Press: London, UK, 2018; pp. 1–17.
51. Edwards-Jones, V.; Claydon, M.A.; Evason, D.J.; Walker, J.; Fox, A.J.; Gordon, D.B. Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *J. Med. Microbiol.* **2000**, *49*, 295–300. [CrossRef] [PubMed]
52. Du, Z.; Song, Y.; Wang, J.; Yang, R.; Guo, Z. Identification of *Staphylococcus aureus* and determination of its methicillin resistance by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* **2002**, *74*, 5487–5491. [CrossRef]
53. Hindre, T.; Didelot, S.; Le Pennec, J.-P.; Haras, D.; Dufour, A.; Vallee-Rehel, K. Bacteriocin detection from whole bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *Appl. Environ. Microbiol.* **2003**, *69*, 1051–1058. [CrossRef]
54. Camara, J.E.; Hays, F.A. Discrimination between wild-type and ampicillin-resistant *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Bioanal. Chem.* **2007**, *389*, 1633–1638. [CrossRef]
55. Nagy, E.; Becker, S.; Sóki, J.; Urbán, E.; Kostrzewa, M. Differentiation of division I (cfiA-negative) and division II (cfiA-positive) *Bacteroides fragilis* strains by matrix-assisted laser desorption/ionization time of-flight mass spectrometry. *J. Med. Microbiol.* **2011**, *60*, 1584–1590. [CrossRef]
56. Cai, J.C.; Hu, Y.Y.; Zhang, R.; Zhou, H.W.; Chen, G.-X. Detection of OmpK36 porin loss in *Klebsiella* spp. by matrix-assisted laser desorption ionization time of flight mass spectrometry. *J. Clin. Microbiol.* **2012**, *50*, 2179–2182. [CrossRef]

57. Griffin, P.M.; Price, G.R.; Schooneveldt, J.M.; Schlebusch, S.; Tilse, M.H.; Urbanski, T.; Hamilton, B.; Venter, D. Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. *J. Clin. Microbiol.* **2012**, *50*, 2918–2931. [CrossRef] [PubMed]
58. Griffin, P.M.; Price, G.R.; Schooneveldt, J.M.; Schlebusch, S.; Urbanski, T.; Hamilton, B.; Venter, D. Rapid identification of VRE with MALDI-TOF MS. *Pathology* **2013**, *45*, S57. [CrossRef]
59. Lau, A.F.; Wang, H.; Weingarten, R.A.; Drake, S.K.; Suffredini, A.F.; Garfield, M.K.; Chen, Y.; Gucek, M.; Youn, J.H.; Stock, F.; et al. A rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for single-plasmid tracking in an outbreak of carbapenem-resistant *Enterobacteriaceae*. *J. Clin. Microbiol.* **2014**, *52*, 2804–2812. [CrossRef] [PubMed]
60. Penny, C.; Grothendick, B.; Zhang, L.; Borror, C.M.; Barbano, D.; Cornelius, A.J.; Gilpin, B.J.; Fagerquist, C.K.; Zaragoza, W.J.; Jay-Russell, M.T. A designed experiments approach to optimizing MALDI-TOF MS spectrum processing parameters enhances detection of antibiotic resistance in *Campylobacter jejuni*. *Front. Microbiol.* **2016**, *7*, 818. [CrossRef]
61. Rhoads, D.D.; Wang, H.; Karichu, J.; Richter, S.S. The presence of a single MALDI-TOF mass spectral peak predicts methicillin resistance in *staphylococci*. *Diagn. Microbiol. Infect. Dis.* **2016**, *86*, 257–261. [CrossRef]
62. Dortet, L.; Bonnin, R.A.; Pennisi, I.; Gauthier, L.; Jousset, A.B.; Dabos, L.; Furniss, R.C.D.; Mavridou, D.A.I.; Bogaerts, P.; Glupczynski, Y.; et al. Rapid detection and discrimination of chromosome- and MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in *Escherichia coli*: The MALDIxin test. *J. Antimicrob. Chemother.* **2018**, *73*, 3359–3367. [CrossRef]
63. Figueroa-Espinosa, R.; Costa, A.; Cejas, D.; Barrios, R.; Vay, C.; Radice, M.; Gutkind, G.; Conza, J. Di MALDI-TOF MS based procedure to detect KPC-2 directly from positive blood culture bottles and colonies. *J. Microbiol. Methods* **2019**, *159*, 120–127. [CrossRef]
64. Jeverica, S.; Sóki, J.; Premru, M.M.; Nagy, E.; Papst, L. High prevalence of division II (cfiA positive) isolates among blood stream *Bacteroides fragilis* in Slovenia as determined by MALDI-TOF MS. *Anaerobe* **2019**, *58*, 30–34. [CrossRef]
65. Zankari, E.; Hasman, H.; Cosentino, S.; Vestergaard, M.; Rasmussen, S.; Lund, O.; Aarestrup, F.M.; Larsen, M.V. Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* **2012**, *67*, 2640–2644. [CrossRef]
66. Feldgarden, M.; Brover, V.; Haft, D.H.; Prasad, A.B.; Slotta, D.J.; Tolstoy, I.; Tyson, G.H.; Zhao, S.; Hsu, C.-H.; McDermott, P.F. Validating the NCBI AMRFinder tool and resistance gene database using antimicrobial resistance genotype-phenotype correlation in a collection of NARMS isolates. *Antimicrob. Agents Chemother.* **2019**, *63*, 550707. [CrossRef]
67. Yang, Y.; Jiang, X.; Chai, B.; Ma, L.; Li, B.; Zhang, A.; Cole, J.R.; Tiedje, J.M.; Zhang, T. ARGs-OAP: Online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. *Bioinformatics* **2016**, *32*, 2346–2351. [CrossRef] [PubMed]
68. Rowe, W.; Baker, K.S.; Verner-Jeffreys, D.; Baker-Austin, C.; Ryan, J.J.; Maskell, D.; Pearce, G. Search engine for antimicrobial resistance: A cloud compatible pipeline and web interface for rapidly detecting antimicrobial resistance genes directly from sequence data. *PLoS ONE* **2015**, *10*, e0133492. [CrossRef] [PubMed]
69. Argoty, G.A.A.; Guron, G.K.; Garner, E.; Riquelme, M.V.; Heath, L.; Pruden, A.; Vikesland, P.; Zhang, L. ARG-miner: A web platform for crowdsourcing-based curation of antibiotic resistance genes. *BioRxiv* **2018**, 274282. [CrossRef]
70. Huang, Y.; Li, W.; Liu, K.; Xiong, C.; Cao, P.; Tao, J. New detection method in experimental mice for *schistosomiasis*: ClinProTool and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Parasitol. Res.* **2016**, *115*, 4173–4181. [CrossRef]
71. Hoyos-Mallecot, Y.; Riazzo, C.; Miranda-Casas, C.; Rojo-Martín, M.D.; Gutiérrez-Fernández, J.; Navarro-Marí, J.M. Rapid detection and identification of strains carrying carbapenemases directly from positive blood cultures using MALDI-TOF MS. *J. Microbiol. Methods* **2014**, *105*, 98–101. [CrossRef]
72. Vranckx, K.; De Bruyne, K.; Pot, B. Analysis of MALDI-TOF MS Spectra using the BioNumerics Software. In *MALDI-TOF and Tandem MS for Clinical Microbiology, First Edition*; Gharbia, H.N.S.S.E., Ed.; John Wiley & Sons Ltd.: Oxford, UK, 2017; pp. 539–562.

73. Tracz, D.M.; Tyler, A.D.; Cunningham, I.; Antonation, K.S.; Corbett, C.R. Custom database development and biomarker discovery methods for MALDI-TOF mass spectrometry-based identification of high-consequence bacterial pathogens. *J. Microbiol. Methods* **2017**, *134*, 54–57. [CrossRef]
74. Rodrigues, C.; Passet, V.; Brisse, S.; Brisse, S. Identification of *Klebsiella pneumoniae* complex members using MALDI-TOF mass spectrometry. *Front. Microbiol.* **2018**. [CrossRef]
75. Pinto, T.C.A.; Costa, N.S.; Castro, L.F.S.; Ribeiro, R.L.; Botelho, A.C.N.; Neves, F.P.G.; Peralta, J.M.; Teixeira, L.M. Potential of MALDI-TOF MS as an alternative approach for capsular typing *Streptococcus pneumoniae* isolates. *Sci. Rep.* **2017**, *7*, 45572. [CrossRef]
76. Huber, C.A.; Pflüger, V.; Reed, S.; Cottrell, K.; Sidjabat, H.E.; Ranasinghe, A.; Zowawi, H.M.; Harris, P.; Paterson, D.L. Bacterial identification using a SCIEX 5800 TOF/TOF MALDI research instrument and an external database. *J. Microbiol. Methods* **2019**, *164*, 105685. [CrossRef]
77. Karlsson, R.; Gonzales-Siles, L.; Boulund, F.; Lindgren, Å.; Svensson-Stadler, L.; Karlsson, A.; Kristiansson, E.; Moore, E.R.B. Proteotyping: Tandem mass spectrometry shotgun proteomic characterization and typing of pathogenic microorganisms. In *MALDI-TOF and Tandem MS for Clinical Microbiology*; John Wiley & Sons Ltd.: Oxford, UK, 2017; pp. 419–450.
78. Welker, M.; Moore, E.R.B. Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Syst. Appl. Microbiol.* **2011**, *34*, 2–11. [CrossRef]
79. Greaves, J.; Roboz, J.; Roboz, J. *Mass Spectrometry for the Novice, First Edition*; CRC Press: Boca Raton, FL, USA, 2013.
80. Yergey, A.L.; Coorssen, J.R.; Backlund, P.S.; Blank, P.S.; Humphrey, G.A.; Zimmerberg, J.; Campbell, J.M.; Vestal, M.L. De novo sequencing of peptides using MALDI/TOF-TOF. *J. Am. Soc. Mass Spectrom.* **2002**, *13*, 784–791. [CrossRef]
81. Pusch, W.; Flocco, M.T.; Leung, S.-M.; Thiele, H.; Kostrzewa, M. Mass spectrometry-based clinical proteomics. *Pharmacogenomics* **2003**, *4*, 463–476. [CrossRef] [PubMed]
82. Pieper, R.; Gatlin-Bunai, C.L.; Mongodin, E.F.; Parmar, P.P.; Huang, S.T.; Clark, D.J.; Fleischmann, R.D.; Gill, S.R.; Peterson, S.N. Comparative proteomic analysis of *Staphylococcus aureus* strains with differences in resistance to the cell wall-targeting antibiotic vancomycin. *Proteomics* **2006**, *6*, 4246–4258. [CrossRef] [PubMed]
83. Shah, A.J.; Mkrtchyan, H.V.; Xu, Z.; Serafim, V.; Shah, H.N. Determination of antimicrobial resistance using tandem mass spectrometry. In *MALDI-TOF and Tandem MS for Clinical Microbiology*; John Wiley & Sons Ltd.: Oxford, UK, 2017; pp. 399–418.
84. Welker, M.; van Belkum, A.; Girard, V.; Charrier, J.-P.; Pincus, D. An Update on the Routine Application of MALDI-TOF MS in Clinical Microbiology. *Expert Rev Proteomics* **2019**, *8*, 695–710. [CrossRef]
85. Bruker. Bruker Launches MALDI Biotyper Sirius at ASM Microbe Conference. Available online: https://www.prnewswire.com/news-releases/bruker-launches-maldi-biotyper-sirius-at-asm-microbe-conference-300871836.html (accessed on 22 June 2019).
86. Bruker. Innovation with Integrity Changing Microbiology MALDI Biotyper ® Subtyping Module. Available online: https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1851663_MBT_Subtyping_brochure_04-2017_ebook.pdf (accessed on 10 September 2019).
87. Cordovana, M.; Pranada, A.B.; Ambretti, S.; Kostrzewa, M. MALDI-TOF bacterial subtyping to detect antibiotic resistance. *Clin. Mass Spectrom.* **2019**, in press. [CrossRef]
88. Youn, J.-H.; Drake, S.K.; Weingarten, R.A.; Frank, K.M.; Dekker, J.P.; Lau, A.F. Clinical performance of a matrix-assisted laser desorption ionization time of flight mass spectrometry method for detection of certain bla KPC -containing plasmids. *J. Clin. Microbiol.* **2016**, *54*, 35–42. [CrossRef]
89. Sydenham, T.V.; Sóki, J.; Hasman, H.; Wang, M.; Justesen, U.S. Identification of antimicrobial resistance genes in multidrug-resistant clinical *Bacteroides fragilis* isolates by whole genome shotgun sequencing. *Anaerobe* **2015**, *31*, 59–64. [CrossRef]
90. Metcalf, B.J.; Chochua, S.; Gertz, R.E.; Li, Z.; Walker, H.; Tran, T.; Hawkins, P.A.; Glennen, A.; Lynfield, R.; Li, Y.; et al. Using whole genome sequencing to identify resistance determinants and predict antimicrobial resistance phenotypes for year 2015 invasive pneumococcal disease isolates recovered in the United States. *Clin. Microbiol. Infect.* **2016**, *22*, 1002.e1–1002.e8. [CrossRef]

91. Tyson, G.H.; McDermott, P.F.; Li, C.; Chen, Y.; Tadesse, D.A.; Mukherjee, S.; Bodeis-Jones, S.; Kabera, C.; Gaines, S.A.; Loneragan, G.H.; et al. WGS accurately predicts antimicrobial resistance in *Escherichia coli*. *J. Antimicrob. Chemother.* **2015**, *70*, 2763–2769. [CrossRef]
92. Schlebusch, S.; Price, G.R.; Gallagher, R.L.; Horton-Szar, V.; Elbourne, L.D.H.; Griffin, P.; Venter, D.J.; Jensen, S.O.; Van Hal, S.J. MALDI-TOF MS meets WGS in a VRE outbreak investigation. *Eur. J. Clin. Microbiol. Infect. Dis.* **2017**, *36*, 495–499. [CrossRef]
93. Eriksen, H.B.; Gumpert, H.; Faurholt, C.H.; Westh, H. Determination of *Elizabethkingia* diversity by MALDI-TOF mass spectrometry and whole-genome sequencing. *Emerg. Infect. Dis.* **2017**, *23*, 320–323. [CrossRef] [PubMed]
94. Batz, M.B.; Hoffmann, S.A.; Morris, J.G. Ranking the Risks: The 10 Pathogen-Food Combinations with the Greatest Burden on Public Health. Available online: https://folio.iupui.edu/bitstream/handle/10244/1022/72267report.pdf?sequence=1 (accessed on 10 September 2019).
95. Zautner, A.E.; Masanta, W.O.; Tareen, A.M.; Weig, M.; Lugert, R.; Groß, U.; Bader, O. Discrimination of multilocus sequence typing-based *Campylobacter jejuni* subgroups by MALDI-TOF mass spectrometry. *BMC Microbiol.* **2013**, *13*, 247. [CrossRef] [PubMed]
96. Emele, M.F.; Karg, M.; Hotzel, H.; Graaf-van Bloois, L.; Groß, U.; Bader, O.; Zautner, A.E. Differentiation of *Campylobacter fetus* subspecies by proteotyping. *Eur. J. Microbiol. Immunol.* **2019**, *9*, 62–71. [CrossRef] [PubMed]
97. Emele, M.F.; Možina, S.S.; Lugert, R.; Bohne, W.; Masanta, W.O.; Riedel, T.; Groß, U.; Bader, O.; Zautner, A.E. Proteotyping as alternate typing method to differentiate *Campylobacter coli* clades. *Sci. Rep.* **2019**, *9*, 4244. [CrossRef] [PubMed]
98. Zautner, A.E.; Lugert, R.; Masanta, W.O.; Weig, M.; Groß, U.; Bader, O. Subtyping of *Campylobacter jejuni* ssp. doylei isolates using mass spectrometry-based phyloproteomics (MSPP). *J. Vis. Exp.* **2016**, *116*, e54165.
99. Guarino, A.; Bruzzese, E.; Giannattasio, A. Antibiotic treatment of acute gastroenteritis in children. *F1000Research* **2018**, *7*, 193.
100. World Health Organization. Critically Important Antimicrobials for Human Medicine 6th Revision 2018. Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use. Available online: https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/ (accessed on 5 September 2019).

3.3 Bacterial typing and detection of virulence factors

The subtyping could be defined as a method to identify different strains of a specific bacterial species. Fine-drawn divergence in the genome could distinguish strains stemming from a common source from an unrelated one (Ann Luna, 2016). DNA-based methods relying on amplification or restriction such as multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) are the major reference methods for bacterial typing at the strain level (i.e. genetic variant or subtype of a microorganism) (Opota et al., 2016). Investigation at the subpopulation level is essential for molecular epidemiology and routine surveillance in order to track outbreaks (Ann Luna, 2016).

While these methods are fairly discriminant and efficient, the total cost and time-consuming workflow have led scientists to find more straightforward, rapid and cost-effective methods. The versatile MALDI-TOF MS has been successfully investigated in several reports for the typing of several bacterial species such as, methicillin resistant *Staphylococcus aureus* (MRSA), *Enterobacteriaceae, Mycobacterium* spp. or other bacteria (e.g. *Pseudomonas aeruginosa*, *Acinetobacter baumannii* or *Campylobacter jejuni*) (Zautner et al., 2013; Sauget et al., 2017). Likewise MALDI-TOF MS AMR screening, bacterial profiling at the strain level relies on the investigation of specific peaks or signature of peaks identified by either visual or bioinformatics inspections (Culebras, 2018). Although MALDI-TOF MS is a promising tool for bacterial typing, there is still numerous questions remaining, such as the level of assignation and technical settings used in routine laboratories settings.

By using the same process as previously described for AMR screening and subtyping, MALDI-TOF MS also has been investigated for the detection of bacterial virulence factors (e.g. toxins and antigens) (Rojo-Martín, 2018). For example, healthcare-associated infection caused by hypervirulent *Clostridium difficile* NAP1/ribotype 027 is associated with high-mortality rate (Valiente et al., 2014). Recently, two biomarkers, i.e. 6,654 Da and 6,712 Da, with an overall good performance to discriminate 027 from non-027 ribotype *C. difficile* was highlighted (Flores-Treviño et al., 2019). Therefore, MALDI-TOF MS may be also a promising tool for the fast detection of virulent strains in diagnostics.

CHAPTER 3

The *Campylobacter* spp. burden

Contents

1. Campylobacter: An irksome foodborne pathogen

Firstly reported as a non-culturable spiral-shaped bacteria by Theodore Escherich (Silva et al., 2011), *Campylobacter,* at this time known as *Vibrio fetus*, was identified for the first time in 1906 and was associated as the "vibrionic abortion" (Skirrow, 2006). It is only in 1963, that the genus "*Campylobacter"* was proposed by Sebald and Véron (On, 2001). Since the apogee of molecular DNA-based methods, the taxonomic structure and ecological comprehensive aspects of the *Campylobacter* genus have tremendously evolved. In this section, an emphasis on the *Campylobacter* landscape will be presented.

1.1 Overview of the *Campylobacter* genus

The *Campylobacteraceae* family comprises three different taxa, including *Campylobacter*, *Arcobacter* and *Sulfurospirillum* (Lastovica et al., 2014). The genus *Campylobacter* presents an important diversity with currently at least 35 validly described taxa (https://www.bacterio.net/genus/campylobacter, accessed in November 2021) more or less related, and 11 subspecies (**Figure 3.1**). *Campylobacter* spp. are small (0.5-5 µm long and 0.2-0.9 µm wide), curved or spiralled gram-negative rods (**Figure 3.2**), that could form coccoid under certain form of stress (e.g. lack of nutriments) (Fitzgerald, 2015; Facciolà et al., 2017). Special growth requirements are needed in terms of atmosphere and temperature of incubation making their daily culture fastidious in vitro. On the one hand, the majority of *Campylobacter* spp., such as *C. fetus* or *C. lari* are growing at 37°C. On the other hand, relevant *Campylobacter* spp. in human infections, such as *C. jejuni* and *C. coli*, are thermophilic organisms, with an optimum growth temperature among 40-42°C (Silvan and Martinez-Rodriguez, 2018; Costa and Iraola, 2019). As well, they are microaerophilic organisms, meaning a reduced oxygen, i.e. 5-8%, and elevated dioxide carbon i.e. 3-10% concentrations are compulsory (Silvan and Martinez-Rodriguez, 2018). However, certain strains could grow and survive under anaerobic or aerobic conditions (Fitzgerald, 2015). *Campylobacter* spp. are highly motile due to the presence of one or two flagella, which are required for the colonization of the gastrointestinal tract mucus lining (Guerry, 2007; Devi, 2019). Nevertheless their flagella could be useful for other pathogenesis purposes such as the secretion of non-flagellar proteins modulating virulence (e.g CiaB, FspA or FlaC) or biofilm formation (Guerry, 2007). Regarding the genome size, *Campylobacter* spp. have a small genome around 1.6 Mb with a 30.6 % G+C. It is predicted to encode 1,654 proteins and 54 RNA species in the *C. jejuni* case (Parkhill et al., 2000; Pearson et al., 2007).

1.2 Occurrence and burden

The EFSA defines zoonoses as infections or diseases that can be transmitted directly or indirectly between animals and humans. Zoonoses can occur by consuming food or drinking-water (indirect), or touching food (direct) contaminated by pathogenic agents (e.g. bacteria, parasites or viruses) (EFSA, 2014). Over the 13 zoonoses monitored in 2019, campylobacteriosis was the most commonly reported zoonosis in the EU and this since 2005 (EFSA and ECDC, 2021a). While *C. jejuni* and *C. coli* counted for more than 93% of reported cases, other *Campylobacter* spp. such as *C. lari, C. fetus* or *C. upsaliensis* were also less frequently detected (EFSA and ECDC, 2021a). While looking for *Campylobacter*, most laboratories incubate their cultures at 42°C, and thus miss *Campylobacter* spp. growing at 37°C, such as *C. lari* or *C. fetus*. Nevertheless, the implementation

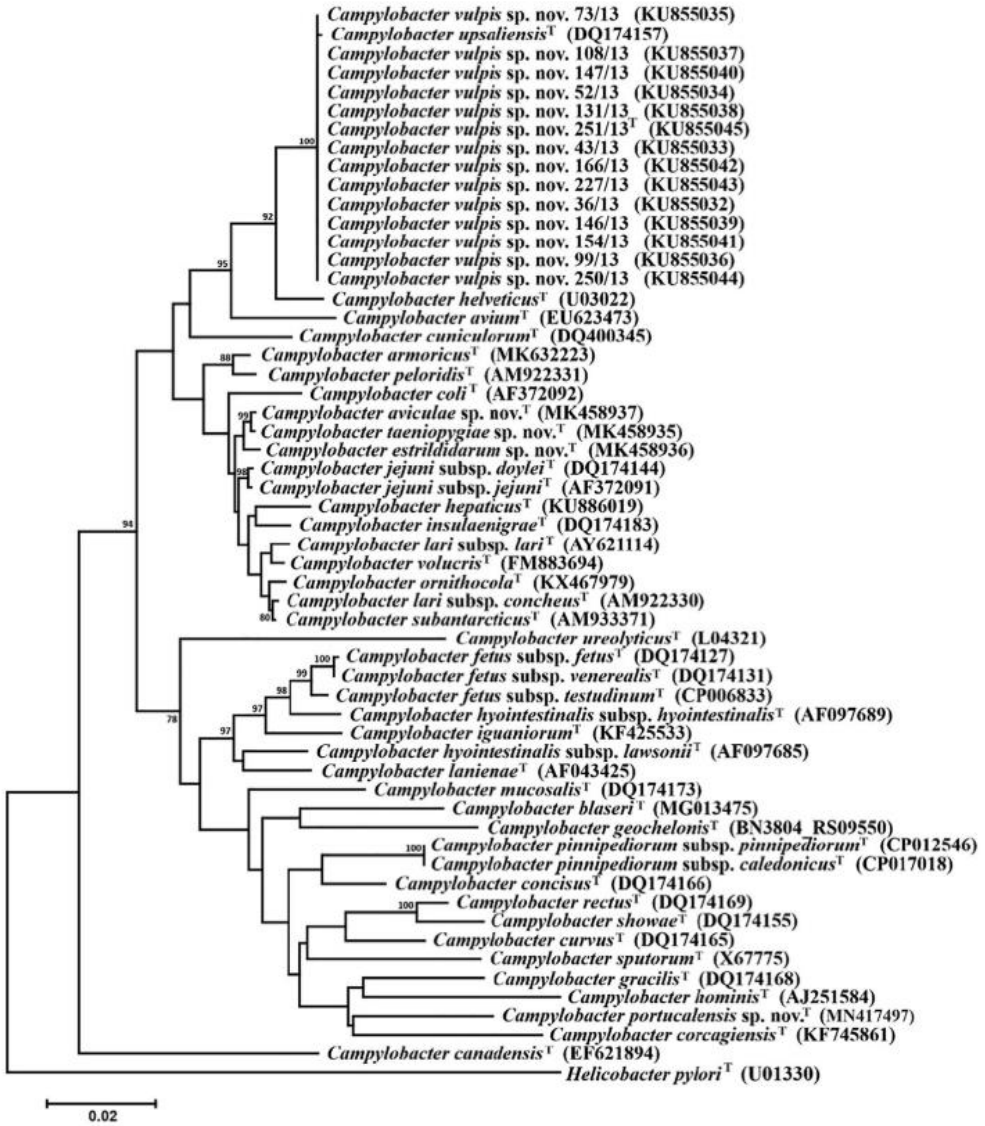


***Figure 3.1**. 16S phylogenetic tree representing Campylobacter type strains. Adapted from* Parisi et al., (2021)

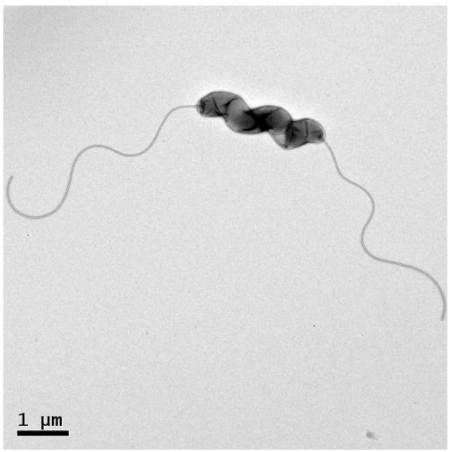


***Figure 3.2**. Transmission electron micrograph of* Campylobacter jejuni *(x2200 magnification). Image taken by Dr Aidan Taylor of the University of Sheffield*

of non-culture-based methods (e.g. PCR) in private laboratories for the identification of *Campylobacter*, like in Luxembourg, might increase *C. lari* and *C. fetus* detection in the future (EFSA and ECDC, 2021a).

The average EU notification rate in 2019 was 59.7 per 100,000 population (**Table 3.1**).

***Table 3.1**. Top 5 of highest country-specific notification rate and cases in Europe in 2019* (EFSA and ECDC, 2021a).

Rank	European member states	Confirmed cases	Notification rate (cases per 100,000 population)
1	Czech Republic	22,894	215.0
2	Slovakia	7,829	141.1
3	Denmark	5,402	93.0
4	United Kingdom	58,718	88.1
5	Finland	4,382	79.4
…	…	…	…
14	Luxembourg	271	44.1

Nevertheless, each notification rate should be cautiously compared as Campylobacteriosis notification is not mandatory in all member states of the EU. While reporting is compulsory in 26 EU countries, France and the United Kingdom use a voluntary system (EFSA and ECDC, 2021a).

As well, the health and economic burden of *Campylobacter* must be mentioned. Indeed, according to the last Global Burden of Diseases study published in 2010, it represented 7.5 million DALYs (time-based measure comprising years lost due to premature mortality and years of healthy life lost due to disability) and an important cost-of-illness (e.g. 82 Mio EUR in Netherland in 2011) (Murray et al., 2012; Devleesschauwer et al., 2016). Interestingly a clear seasonality phenomenon for Campylobacteriosis cases exist, where a peak is reached during summer months (EFSA and ECDC, 2021a). Nevertheless, such event is still not fully understood and remains unclear. In the EU campylobacteriosis cases are mainly sporadic with few recognized foodborne outbreaks (Silva et al., 2011). In 2019, 0.6% of human campylobacteriosis cases in EU would be linked to foodborne outbreaks (EFSA and ECDC, 2021a).

1.3 Sources of infections

In the EU most of the recognized or suspected human outbreaks are caused by the consumption of contaminated broiler meat and milk (EFSA and ECDC, 2021a). On the one hand, *Campylobacter* spp. are commensals found in live stocks (e.g. bovine, sheep and poultry) or wild animals (e.g. swine), considered as reservoirs or amplifying hosts (**Figure 3.3**) (Silva et al., 2011;

Wagenaar et al., 2014). Poultry and more specifically chickens are the main reservoir infecting humans worldwide

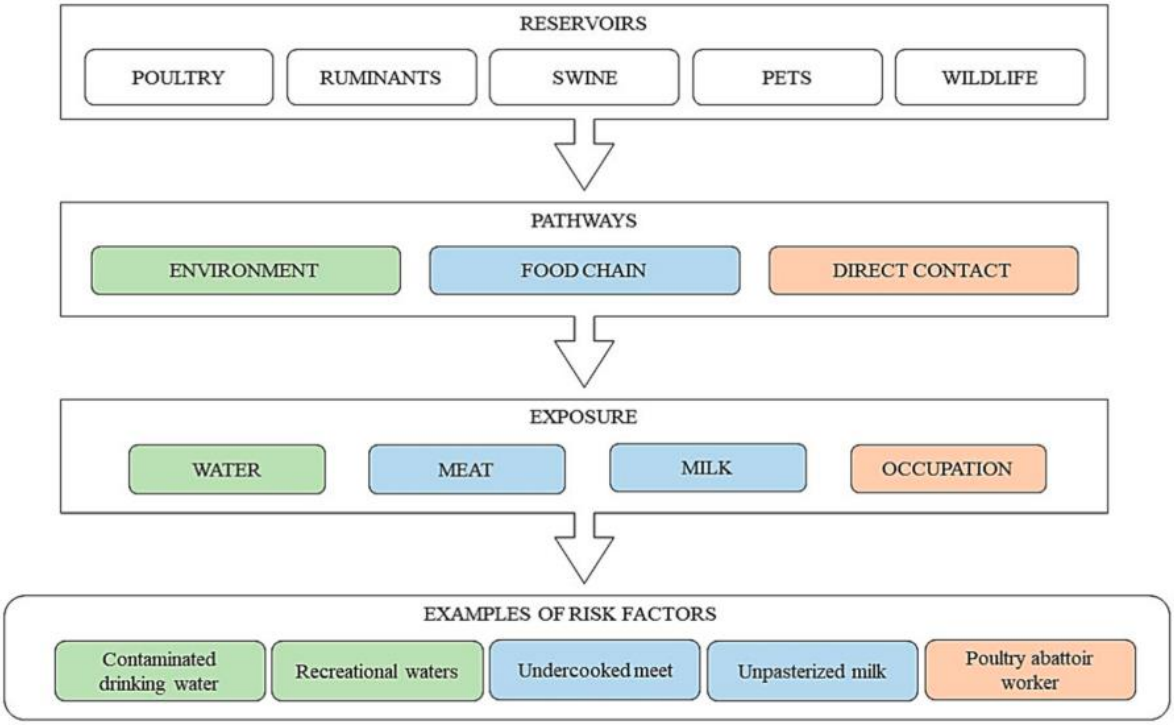


Figure 3.3. *Route of transmission of* Campylobacter *infections. Adapted from* Chlebicz and Śliżewska (2018)

and are often associated with outbreaks (Wagenaar et al., 2014; Wensley et al., 2020). Studies highlighted that specific subgroups of *C. jejuni* were frequently associated with specific hosts (Sheppard et al., 2010, 2011, 2014; Epping et al., 2021).

On the other hand, *Campylobacter* spp. excreted by specific hosts could be then transported by different pathways, such as the food chain, water cycle (e.g. surface or recreational water) or insects (e.g. flies), or less frequently by direct contact with infected organisms (Hald et al., 2008; Wagenaar et al., 2014). In this context, in the USA a *C. jejuni* outbreak was identified after people got direct contact with sick puppies in a pet store (Watkins et al., 2021). These different pathways result in the potential contamination of edible goods (e.g. meat or milk) that could contaminate humans or other susceptible hosts.

While consuming contaminated food is recognized as the main source of human infection (EFSA and ECDC, 2017), cross-contamination (e.g. rinsing chicken) and poor hygiene (e.g. proper hand washing) in the kitchen is also a major key in its transmission (Cardoso et al., 2021). While 50% to 80% human cases could be attributed to the poultry reservoir, up to 30% of human campylobacteriosis cases could be attributed to the handling, preparation and consumption of broiler meat (EFSA, 2010). Luber et al. (2006) estimated that the *Campylobacter* spp. mean transfer

from hands or kitchen utensils to foods were ranging from 3% to 28% (Luber et al., 2006). Along the same line, while preparing naturally contaminated poultry, cross-contamination can occur in the kitchen. Indeed, Gorman et al. (2002) highlighted contamination of surfaces, such as oven handle, counter-top and draining board (Gorman et al., 2002).

Therefore, consuming contaminated drinking/untreated water (e.g. well water), not washing hands before/after food preparation, or not cleaning a knife after using it for raw meat represent a pathorisk to be infected by *Campylobacter* spp. (Kapperud et al., 2003; Mughini Gras et al., 2012). In 2015, a risk factor analysis for *Campylobacter* infections in Norway between 2010 and 2011 was performed. Drinking water directly from river or lake (odds ratio: 2.96) was more likely to increased *Campylobacter* infections risk than eating chicken (odds ratio:1.69) (MacDonald et al., 2015). A similar study was conducted in Luxembourg for the 2010-2013 frame time (Mossong et al., 2016). Consumption of chicken at both home and outside (odd ratio: 4.77) was one of the significant risk factors for human campylobacteriosis.

1.4 Campylobacteriosis & associated diseases

Recognized to have a role in sheep's abortion from 1906 to nowadays, *Campylobacter* spp. were only identified as a major agent enteritis disease in the 1970's (Butzler et al., 1973; Skirrow, 1977). The majority of campylobacteriosis are significant self-limiting zoonotic foodborne diseases in humans, resulting in watery and/or bloody diarrhea, cramps and sometimes fever and last around 6 days (Kaakoush et al., 2015). Reports suggested that the dose to get illness was ranging from 100 to 800 *Campylobacter* cells (Igwaran and Okoh, 2019). While most of human infections are caused by *C. jejuni* and *C. coli*, other emergent species (e.g. *C. concisus*, *C. hepaticus*, *C. hyoinstestinalis*, *C. ureolyticus*, *C. upsaliensis)* were sporadically associated with humans and animals illness (Costa and Iraola, 2019). Beside the diarrheic side of *Campylobacter* infections, other gastrointestinal and extra-gastrointestinal post-infection sequelae could be highlighted (Kaakoush et al., 2015). Inflammatory bowel, oesophageal and celiac diseases could be cited for gastrointestinal infections. Extra-gastrointestinal infections could be defined as complications outside the gastrointestinal tract. This includes Guillain Barré syndrome, reactive arthritis, meningitis as well as bacteremia in the *Campylobacter* case (Kaakoush et al., 2015; Igwaran and Okoh, 2019).

1.5 *Campylobacter* in a One World – One Health

The majority of human campylobacteriosis cases are linked to the consumption of contaminated food products, including poultry. While in some cases the establishment of a direct link to a specific source is missing, several studies have pointed out the importance of non-poultry

(e.g. wild-life and surface water) sources or vectors for human campylobacteriosis or colonization of poultry flocks (**Figure 3.4**)(Gölz et al., 2014; Cody et al., 2015; Mossong et al., 2016; Mughini-Gras et al., 2016).

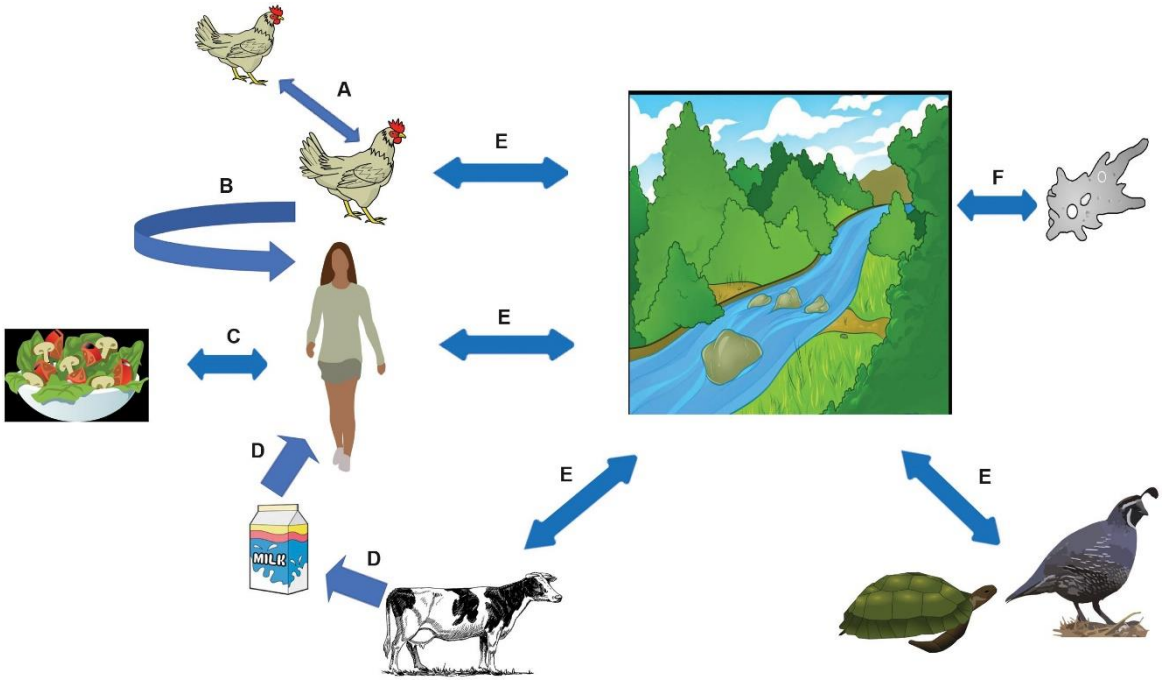


Figure 3.4. *Overview of* Campylobacter *spp. sources and transmission* Campylobacter *spp. reside in large numbers in the gastrointestinal tract of chickens, where bacteria are spread throughout the flock via the fecal-oral route (A). In the developed world,* Campylobacter *is usually acquired by consuming under-cooked poultry (B). However, outbreaks have been associated with different types of fresh produce (C) and dairy products (D).* Campylobacter *spp. is frequently found in surface water, usually from contamination from animal feces, and has been known to infect humans (E)). It has also been postulated that Campylobacter may be able to infect amoebae, which may serve as a reservoir (F) (Adapted from Johnson et al. (2017)*

Thermophilic *Campylobacter*, including *C. jejuni* and *C. coli*, are ubiquitously and commensally found in the intestinal tract of animals. *C. jejuni* was identified in numerous avian species, including wild birds (e.g. feral pigeon, house sparrow, common blackbird) and poultry, as well as cattle and pigs (Hald et al., 2016). In 2018, a *Campylobacter* prevalence of 26% in broiler chicken and 71.6% in turkeys was reported, where *C. jejuni* and *C. coli* were highly represented (Backert, 2021). While the vertical transmission, i.e. from breeders to their progeny, of *Campylobacter* spp. is supposed to be negligible, horizontal transmission is considered as the principal introduction pathway of *Campylobacter* in broilers flocks at the farm level (Agunos et al., 2014; Ingresa-Capaccioni et al., 2016). The presence of the wild or domestic animals (e.g. insects, adjacent broilers or pets) or human equipment (e.g. transport crates were suggested as *Campylobacter* source (Kittler et al., 2021). Therefore livestock farms could have an important role in *Campylobacter* spread in the environment and in humans (Backert, 2021).

Campylobacter infections in human are principally self-limiting and do not require antibiotherapy. In case of persistent, invasive cases, immunocompromised patients or severe infections, the prescription of antibiotics may be needed to treat it. Over the last decades, the excessive administration of antibiotics to food animals for nontherapeutic (e.g. growth promotion) purposes was linked to significant bacterial AMR (Marshall and Levy, 2011). Actually, numerous critically important antibiotics considered as essential in human medicine, is also used in veterinary medicine (WHO, 2019b). However, the overuse and misuse of such molecules for animal production is to be expected to rapidly increase the emergence of AMR in pathogens (Agyare et al., 2019). Such situation might lead to inefficient treatment, economic losses and human transmission. While macrolides and fluoroquinolones are used in poultry, such molecules are considered to be the first- and second-line drugs for *Campylobacter* human infections when antibiotherapy is required (Luangtongkum et al., 2009; Roth et al., 2019). Nevertheless, according the last EFSA report approximately 61% and up to 86.7% for both *C. coli* and *C. jejuni* isolates from human and poultry respectively were resistant to fluroquinolones (EFSA and ECDC, 2021b). Therefore, the increase of fluroquinolones resistance among *Campylobacter* had limited treatment options for patients (CDC, 2019). Even more worrisome is the emergence of multi-resistant *Campylobacter* spp. (Du et al., 2019; Noreen et al., 2019).

2. Antimicrobial resistance mechanisms

Ever-increasing multi-drug resistance patterns among *Campylobacter* in Europe is a concerning public health issue. While up to 85% of isolates are already resistant to at least one or two antimicrobial classes, 26.9% *C. coli* food isolates were multi-resistant to four antimicrobials in 2019 (EFSA and ECDC, 2021b). It underlines the need for enhanced effort to obtain straightforward AMR assessment information. As a first step toward it, a better knowledge on AMR mechanisms is required. In this section, *Campylobacter*'s antimicrobial known and emergent resistance mechanisms will be discussed. **Table 3.2** summarizes identified AMR mechanisms of *Campylobacter*.

2.1 Resistances due to target mutations

Microorganisms can reach AMR by different genetic events. Alteration of the antibiotics target site is one of the most common mechanism of resistance (Lambert, 2005).

(Fluoro)quinolone molecules belong to a broad spectrum of antibiotics, including ciprofloxacin and nalidixic acid. They target DNA gyrase and topoisomerase IV and thus, impede DNA replication (Higgins et al., 2005). Mutations within GyrA, GyrB and ParC and ParE are responsible of bacterial fluoroquinolone resistances (Shen et al., 2018). The main *Campylobacter* fluoroquinolone

resistance mechanism is an amino acid substitution in the quinolone resistance determining region (QRDR) involved in the DNA binding domain of the GyrA (Payot et al., 2006). The single point mutation C257T in the *gyr*A gene leads to the Thr86Ile amino acid substitution and corresponds to the main molecular mechanism conferring a high level to fluoroquinolone resistance. Other amino acid substitutions, such as Asp90Asn, Thr86Lys, Thr86Ala, Thr86Val, and Asp90Tyr are associated with a lower level of resistance (Tang et al., 2017b).

***Table 3.2**. Summary of antibiotic resistance mechanisms of* Campylobacter *(adapted from Iovine, 2013). MOMP: Major outer membrane protein.*

Inhibition mechanism	Classification	Antibiotics	Resistance mechanisms
Cell wall synthesis	β-Lactams	ampicillin, amoxicillin	▪ Enzymatic inactivation (β-lactamase OXA-61) ▪ Membrane permeability due to MOMP ▪ Efflux through the (RE)-CmeABC pump
DNA topoisomerase	(Fluoro)quinolones	ciprofloxacin, nalidixic acid	▪ DNA gyrase single point mutation (e.g. Thr-86-ile; Asp-90-Asn, Ala-70-Thr) ▪ Efflux through the (RE)-CmeABC pump
Protein synthesis	Macrolides	erythromycin, azithromycin	▪ 23S rRNA single point mutation (e.g. A2075G, A2074G, A2074C) ▪ Mutations in L4/L22 ribosomal protein ▪ Horizontal transferable *erm*(B) gene ▪ Efflux through the (RE)-CmeABC pump ▪ Membrane permeability due to MOMP
	Tetracyclines	tetracycline	▪ Modification of the ribosomal target A by ribosomal protection protein (e.g. Tet(O)) ▪ Efflux through the (RE)-CmeABC pump ▪ Membrane permeability due to MOMP
	Aminoglycosides	gentamycin, kanamycin, streptomycin	▪ Contribution of modifying enzymes (e.g. Apha, AadE) ▪ Mutation in the *rpsL* gene (streptomycin)
	Phenicol	chloramphenicol	▪ Conjugative plasmid born *cfr*(C)

Macrolide antimicrobials, including erythromycin and azithromycin, are drugs of choice for the treatment of *Campylobacter* infections, when antibiotherapy is required (Yang et al., 2019). These agents inhibit the protein synthesis by targeting the 50S subunit of the ribosome (Iovine, 2013). The main mechanism of macrolide resistance in *Campylobacter* is the modification of the ribosome target. Modifications include point mutations in the 23S rRNA, which is the most common mechanism for high-level macrolide resistance. Changes in the nucleotide sequence mainly occur at positions A2075G, A2074G, A2074C, and to a lesser extend are associated with A2074T 23S rRNA substitutions (Bolinger and Kathariou, 2017). Low-intermediate macrolide resistances are rather related to changes in the L4 and L22 ribosomal proteins and substitutions and insertions in the in *rpl*D and *rpl*V of the ribosomal proteins (Bolinger and Kathariou, 2017).

2.2 Resistance due to acquired genes

Acquisition or interchanging genes, so called horizontal gene transfer, is one of the numerous mechanisms deployed by microorganisms to become resistant to antimicrobials (Van Hoek et al., 2011).

Tetracyclines is one of the most important and used class of antibiotics, targeting the inhibition of the protein synthesis by interacting with the 30S ribosomal subunit (Tang et al., 2017b). While it has been successfully used in human and veterinary medicine, its heavy use lead to a widespread of resistances, limiting its use nowadays (Iovine, 2013). In 2019, between 47.2% and 66.9% of *C. jejuni* and *C. coli* human isolates, respectively, were resistant to tetracyclines (EFSA and ECDC, 2021b). Tetracycline resistances are mainly due to the expression of *tet*(O) gene encoding ribosomal protection proteins (Wieczorek and Osek, 2013). Tet(O) binds to an unoccupied ribosomal A site and initiates a conformation change of the molecular structure, resulting in the release of the bounded antimicrobials (Roberts, 2005). While the A site configuration is different, the site is still stable and functional for protein elongation (Roberts, 2005). The *tet*(O) gene could be located on the chromosomal DNA or on a plasmid, such as pTet in *C. jejuni* and pCC31 in *C. coli* (Shen et al., 2018).

Developed in 1944, the aminoglycosides class, including gentamycin, streptomycin or kanamycin antibiotics, is one of the oldest developed class of antibiotics (Falagas et al., 2008). Elaboration of new compound classes as well as adverse events (e.g. nephro- and ototoxicity for the gentamycin) associated to its consumption, resulted into a decrease in the use of aminoglycosides (Tang et al., 2017b). Nevertheless, global AMR increased obliged clinicians to revaluate its use (Falagas et al., 2008). Like tetracyclines, aminoglycosides interact with the decoding region of the A site of 30S ribosomal proteins, leading to the biosynthesis of aberrant proteins (Wieczorek and Osek, 2013). In *Campylobacter* aminoglycoside resistance genes

producing modifying enzymes such as aminoglycoside phosphor-transferase (e.g. AphA-3) and adenyl-transferase (e.g. AadE) were described (Wieczorek and Osek, 2013). *Campylobacter*'s AphA-3 phospho-transferases is the main transforming enzyme in *Campylobacter* spp., conferring a phosphorylation to aminoglycoside molecules (Iovine, 2013). While the majority of these resistance genes are plasmid-borne, several studies report the existence of chromosomally encoded aminoglycoside resistance genes (e.g. *aph(2")-If*) and transferable multidrug resistance genomic island (e.g. *aadE–sat4–aphA-3* cluster, *aacA-aphD*, *aac*, and *aadE)* (Qin et al., 2012; Tang et al., 2017b; Yao et al., 2017). The potential emergence and the dispersion worldwide of such genomic structure constitute a direct threat to public health. While aminoglycosides resistances are mainly driven by specific resistance genes chromosomally encoded, it could be less frequently associated with a single mutation. Indeed, the Lys43Arg single point mutation in the *rpsL* gene has been shown to be involved in the streptomycin resistance in *C. coli* and *C. jejuni* (Olkkola et al., 2010; Hormeño et al., 2018; Ocejo et al., 2021).

β-lactams are the most widely used antimicrobials class worldwide (Bush and Bradford, 2016). In the European Union, consumption of β-lactams for systematic use in the community reached 8 defined daily dose (DDD) per 1000 inhabitants per day in 2019, which is fourfold higher for tetracyclines or macrolides (ECDC, 2020). β-lactams class regroups different subclasses: penicillin, cephalosporins, cephamycin, monobactams and carbapenems. Such antimicrobials interrupt bacterial cell wall synthesis by binding to penicillin-binding proteins, which are involved in the final cell wall biosynthesis (Bush and Bradford, 2016; Shen et al., 2018). The main gene responsible of producing β-lactamase in *Campylobacter* is $bla_{OXA\text{-}61}$ chromosomally encoded. Nevertheless, it was reported that a majority of *Campylobacter* harbouring the OXA-61 enzyme were in fact susceptible to ampicillin (Griggs et al., 2009; Shen et al., 2018). Therefore, the level expression of this gene regulates *Campylobacter* final resistance phenotype (Zeng et al., 2014). Ocejo et al. (2021) analysed by WGS 70 *Campylobacter* strains. 57 were harbouring genes coding for β-lactamases. Nevertheless, over the 57 identified strains, only 25 isolates were phenotypically resistant to ampicillin. Interestingly, single nucleotide G-T transversion in the $bla_{OXA\text{-}61}$-like promoter area, was associated with high level of ampicillin resistance in 24 isolates (Ocejo et al., 2021). Therefore, the presence of the guanine is associated with ampicillin susceptible phenotype, whereas the presence of the thymine is associated with resistant phenotype. The authors suggested that the combination of the bla_{OXA} gene combined with the thymine mutation could provide a performant WGS-based resistance prediction (Ocejo et al., 2021).

2.3 Intrinsic resistances

In addition to resistance obtained after the acquisition of specific resistance genes or single point mutations, intrinsic resistances in both *C. jejuni* and *C. coli* were described.

Efflux pumps are commonly found in Gram- positive and -negative bacteria as well as in eukaryotic cells (Webber and Piddock, 2003). While specific substrate pumps exist, other ones could transport large range of molecules, which could include antimicrobials. In *Campylobacter*, 14 possible efflux pumps were reported (Iovine, 2013). The best-described one is the tripartite CmeABC multidrug efflux pump (Lin et al., 2002). CmeC is an outer membrane protein, CmeB is an inner membrane drug transporter and CmeA is periplasmic protein bridging CmeB and CmeC (Lin et al., 2002). This efflux pump is both implied in acquired and intrinsic resistances to a broad spectrum of antimicrobials (e.g. fluoroquinolones, β-lactams, tetracyclines) and to bactericidal detergents such as bile (Shen et al., 2018). In fact, the recurring pattern in *Campylobacter* resistances is the synergy between antibiotic efflux and another AMR mechanism (Iovine, 2013). It is worth noting that two other multidrug efflux pumps also exist in *Campylobacter*, i.e. CmeDEF and CmeG, which will not be described in this section. Antibiotics exclusion via the major outer membrane porin (MOMP) also confers resistance (Iovine, 2013).

As well, *Campylobacter* exhibits natural resistance to several antimicrobials, hypothetically due to the absence of specific targets or poor affinity to binding targets (Iovine, 2013). Intrinsic resistances to novobiocin, bacitracin, vancomycin, polymyxin/colistin, sulfamethoxazole, trimethoprim and rifampicin for both *C. jejuni* and *C. coli* were described (Iovine, 2013; Wieczorek and Osek, 2013). Nevertheless, intrinsic resistance mechanisms still remain unclear but active efflux by previously described multidrug-efflux pumps may play an key role in it (Luangtongkum et al., 2009).

2.4 Emergent resistance mechanisms

Previous described *Campylobacter* AMR mechanisms are linked to the resistance of critically important antimicrobials, such as fluoroquinolones or macrolides. While such resistances were already threatening for the global public health, variants or new AMR genes recently emerged in *Campylobacter*.

A variant of the CmeABC efflux pump, so called resistance-enhancing (RE)-CmeABC, was firstly described in China (Yao et al., 2016). The RE-*cme*ABC operon has a specific *cme*B sequence with only 80% amino acid identity to other *cme*B *Campylobacter* (Shen et al., 2018). This sequence variation confers a high-level resistance to fluoroquinolones, phenicols, macrolides, and tetracyclines and reduces intracellular antibiotics accumulation (Liu et al., 2020). While this

new efflux pump is highly prevalent in China, it has not been yet encountered in Europe (Shen et al., 2018).

Additionally, two new emergent resistance genes were recently described. A rRNA methyltransferase encoded by the *cfr* gene confers the resistance to several antimicrobial classes such as phenicols or lincosamides (Tang et al., 2017a). For the first time, Tang and colleagues reported in 2017, a novel plasmid born harbouring a *cfr*-gene like, called *cfr*(C), in the foodborne pathogen *Campylobacter*. Likewise to other known *cfr* genes, *cfr*(C) confers transferable multi-drugs resistance, i.e. oxazolidinones, phenicols, lincosamides and pleuromutilins (Tang et al., 2017a). Along the same line, the Erm(B) rRNA methylase may confer to *Campylobacter* a high-level resistance to macrolides, lincosamides and streptogramin B antimicrobials (Qin et al., 2014). It is the first transferable horizontal macrolides resistance mechanism described in *Campylobacter* (Qin et al., 2014). Originally described in Asia, this rRNA methylase has now also been detected in animal isolates in Europe and overseas (Florez-Cuadrado et al., 2016; Chen et al., 2018).

The emergence of such new AMR mechanisms and their potential dispersion worldwide would be a significant threat to the global public health. To watch it, the EFSA and ECDC highlighted the need of a better detection of "emerging and threatening" AMR mechanisms as well as their genetic supports (e.g. plasmids or multi-drug resistance genomic island) in *Campylobacter*. Finally, they recommended to apply NGS to highlight genes, clones and compare animal to human isolates, leading to a better understanding of *Campylobacter* resistance dynamics (EFSA and ECDC, 2021b).

3. Typing technics

Campylobacter cases occur sporadically and are rarely associated with outbreaks (Taboada et al., 2013). Additionally, infected people may have issues to remember the potential ingested contaminated food because of incubation time between infection and first clinical manifestation, which could take up to 4.3 days on average (Awofisayo-Okuyelu et al., 2017). Thus it is making hard to trace the route and source of transmission (Eberle and Kiess, 2012). Typing methods are helpful to compare strains at the species and subspecies level. Characterization of these isolates enables to study several parameters such as the investigation or the detection of potential human clusters and support *Campylobacter* surveillance (Eberle and Kiess, 2012). In this section, traditional and emergent typing tools will be presented. **Table 3.3** summarizes the advantages and the drawbacks of the presented tools.

3.1 Phenotyping

The presence or absence of biological traits or activities could be used to differentiate isolates and could be referred as phenotyping. Biotyping, serotyping and multilocus enzyme electrophoresis

(MEE) used to be the most popular phenotypic methods to type *Campylobacter* due to their low-cost and friendly use to perform. However, the discriminatory power and reproducibility was poor

***Table 3.3**. Advantages and disadvantages of typing methods for* Campylobacter *spp. (Adapted from Eberle and Kiess (2012))*

Method	Advantage	Disadvantage
Phenotypic		
Biotyping	Cost and easy procedures	Low discriminatory power and reproducibility
Serotyping	Reproducibility and typeability	Discriminatory power, cost, tedious procedures, and time
Multilocus enzyme electrophoresis (MEE)	Discriminatory power	Exchange of results and reproducibility
Genotypic		
Polymerase Chain reaction (PCR)	Discriminatory power, equipment availability, and reproducibility	Characterisation of a limited size of the genome, optimization of reaction conditions can be tedious, time-consuming
Pulse-field gel electrophoresis (PFGE)	Discriminatory power	Cost, Time-consuming, reproducibility of results across different laboratories
Ribotyping	Large percentage of strains assigned to a type (typeability)	Discriminatory power, cost, and time
Flagellin typing	Discriminatory power and time	Instability of the marker, accuracy of results due to intra- and intergenomic recombination of genes
Amplified fragment length polymorphism (AFLP)	Discriminatory power, reproducibility, and typeability	Complex analysis and pure culture required to prevent misinterpretation of results due to foreign DNA
Multilocus sequence type (MLST)	Easy reproduction, interpretation, and transfer of results, population structure study	Cost, Time-consuming, complexity of the technique, no difference between genetically highly related isolates (clones), not for traceability
Core genome MLST (cgMLST)	Resolution and reproducibility	Time-consuming, cost and complexity to perform technique, dry-lab part, i.e. bioinformatics pipelines and data storage
Whole genome MLST (wgMLST)	Sensitive, high resolution to differentiate isolates from a same outbreak, from a same source	
Proteomic		
Mass spectrometry-based phyloproteomics (MSSP)	Fast, straightforward, cost-efficient and possibility to combine it with MLST	No common nomenclatures, no database Number of sequence data available is decisive for the quality of the typing scheme

(Eberle and Kiess, 2012). Biotyping refers at the characterization of bacterial isolates by studying biological activities, i.e. colonies morphology, environmental tolerance or biochemical reactions (Eberle and Kiess, 2012). Beside conventional tests to identify *Campylobacter* genus, such as the utilization of selective growth medium, i.e. blood agar combined with antibiotics, or biochemicals tests, such as Gram-staining, oxidase and catalase, biotyping schemes were developed (Skirrow, 1977; Leaper and Owen, 1981). In 1980, a first *Campylobacter* biotyping scheme relying on Hippurate hydrolysis and rapid H_2S tests, was able to differentiate not only *C. coli* from *C. jejuni*, but also to subtype *C. jejuni* into two groups, so-called biotypes on Hippurate hydrolysis, rapid H_2S tests and DNA hydrolysis for the distinction between *C. jejuni*, *C. coli* and "*C. laridis*" (now *C. lari*), resulting in four biotypes (Lior, 1984). While they are cost-effective and easy to perform, such methods had poor reproducibility and discriminatory power due to biochemical variability between isolates (Eberle and Kiess, 2012). Nevertheless, biotyping efficiency may be increased by the utilization of other phenotypic methods such as serotyping.

The study of microbial surface differences, so called serotyping, plays an important role in subdividing isolate from a same species and subspecies of various bacteria based on the use of antisera and antibodies. In the case of *C. jejuni*, surfaces structures such as lipopolysaccharides or membrane proteins could be cited (Logan and Trust, 1982). Since 1971, different serotyping scheme for *Campylobacter* were developed (Berg et al., 1971; Penner and Hennessy, 1980; Lior et al., 1982). While similar efficiency was observed for the different schemes, serotyping remains a tedious and time-consuming method (Patton et al., 1985). While serotyping is currently not the method of choice for *Campylobacter*, it is still applied for bacteria such as *Salmonella* spp. in routine laboratories. Nevertheless, *in silico* genome-based serotyping (e.g. SeqSero2 or *Salmonella* TypeFinder) could become the new reference standard for *Salmonella* in a close future (Zhang et al., 2015; Longo et al., 2019; Banerji et al., 2020).

Finally, MEE represented the first method used for epidemiological studies and was firstly applied in 1989 to characterize *Campylobacter* isolates from human and non-human sources (Aeschbacher and Piffaretti, 1989). It investigated the protein polymorphism by using a gel electrophoresis. Alteration in the amino acid sequence resulted into different pattern of mobility or electromorph type. Nevertheless, several nucleotides substitutions resulted into silent mutations in the amino acid sequence leading to low-resolution power (Araujo and Sampaio-Maia, 2018).

3.2 Genotyping

In comparison to phenotyping methods, molecular DNA-based typing tools have a higher reproducibility, typeability, i.e. portion of the population strains that can be assigned to a type, and

discriminatory power but they are time-consuming and expensive (Hunter, 1990; Eberle and Kiess, 2012). They have enhanced epidemiological surveillance, outbreak identification and furnished important insights into isolates from different reservoirs, i.e. human, environment and animal (Sheppard et al., 2012; Magana et al., 2017). Numerous genotypic methods for *Campylobacter* could be cited: the amplified fragment-length polymorphism (AFLP), the ribotyping, the polymerase chain reaction (PCR), the flagellin typing, the pulsed-field gel electrophoresis (PFGE), the multilocus sequence typing (MLST), the core genome MLST (cgMLST) and whole-genome MLST (wgMLST). Among these techniques, PFGE and MLST are the more common methods used for studying the epidemiology of *Campylobacter* infection (Magana et al., 2017).

Developed in 1984, the PFGE is the first DNA-based typing method widely applied for bacteria, including *Camyplobacter* spp (Schwartz and Cantor, 1984; Eberle and Kiess, 2012). The fingerprint is obtained after the separation of large bacterial DNA molecules by applying a periodical electric field to the electrophoresis gel. It was assumed to be the reference standard for bacterial typing and online molecular surveillance network (e.g. PulseNet) of several bacterial genus, such as *Salmonella, E. coli, Shigella* and *Listeria* (Tolar et al., 2019). While such approach is valid for *Campylobacter* typing in a determined space time (e.g. slaughterhouse study), PFGE used for routine surveillance was considered as controversial, due to the small number outbreaks and numerous isolates to investigate (Hedberg et al., 2001). Nowadays, WGS-based methods are validated for *Campylobacter* typing and surveillance national programs started the transition toward the age of the NGS for surveillance (Ribot et al., 2019; Tong et al., 2021).

MLST is another widely used method for the typing of *Campylobacter*. Thanks to the cost-accessibility of whole genome sequencing (WGS) and improvement in bioinformatics, MLST, cgMLST or wgMLST became affordable over the last few years. Developed on the principle of MEE, MLST is a gene-by-gene approach looking into the DNA sequencing of several housekeeping genes (Maiden et al., 1998). Housekeeping genes are constitutive genes required for the maintenance of basic cellular functions and essential for the existence of the cell (Keim, 2005). The first *Campylobacter* MLST scheme was developed for *C. jejuni* based on seven genes, i.e. *asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt*, *unc*A (Dingle et al., 2001). Additionally, extended MLST schemes were developed for species such as *C. coli*, *C. lari*, *C. upsaliensis* or *C. helveticus* (Miller et al., 2005). As a result of MLST, a unique Sequence Type (ST) is assigned to a unique combination of alleles (for details please see http://pubmlst.org/campylobacter/). Therefore, it is reflecting the *Campylobacter* population structure and host specificity. MLST method improved the understanding on *Campylobacter* spp. transmission route causing human infections as well as the identification of ecological niches (Sheppard et al., 2011; Magana et al., 2017). In terms of

resolution, MLST has a higher resolution than 16S rRNA sequencing. While 16S rRNA (1 locus) identifies bacteria to the genus level, MLST (7 loci) goes to the species by categorizing isolates in lineages or clonal complex (Maiden and Harrison, 2016). Interestingly by including *porA* and *gyrA* to the traditional MLST scheme the resolution scale can be refined, resulting into a so-called extended MLST typing method (Nennig et al., 2021). The 9-loci method can define different lineages and human clusters (Dingle et al., 2008; Ragimbeau et al., 2014).

cgMLST could be consider as the extension of the MLST concept to the genome level through the combination of hundred to thousand core genes, which could be defined as conserved genes throughout a group of genomes from a same species (Segata and Huttenhower, 2011). Alternatively, wgMLST is also a gene-by-gene approach which utilized all identified *C. jejuni* and *C. coli* loci of absolute presence (Cody et al., 2013). These methods show a higher resolution and discriminatory power than classical MLST typing schemes and can determine relationships between isolates up to the clone level (Maiden and Harrison, 2016). Likewise, it could give additional information (e.g. resistome) for the full characterization of the strain, with the condition that the typing scheme include acquired genes for resistances. However, the main goal of cgMLST and wgMLST is to determine the genetic distance between different isolates (Jamin et al., 2021). As an example, studies successfully evaluated cgMLST techniques for source attributions of human pathogenic strains of *C. coli* and *C. jejuni* (Hsu et al., 2020; Harrison et al., 2021).

It is now clear that WGS-based typing methods will have an important role and benefits into its integration for the routine monitoring and outbreak investigation of *Campylobacter* (Llarena et al., 2017).

3.3 Proteotyping

The term “Proteotyping” refers to a typing method based on protein mass spectra analysis (Karlsson et al., 2015). It is used to characterize microbial communities viruses and bacteria such as *Salmonella* or *Staphylococcus aureus* based on the expressed proteins (Hugo et al., 2012; Kuhns et al., 2012; Nguyen and Downard, 2013; Kondori et al., 2021). For example, Kuhns et al. (2012) tested the ability of MALDI-TOF MS to discriminate *S. enterica* subsp. *enterica* serovar Typhi from other serovars (Kuhns et al., 2012). They suggested that even by using a direct bacterial smear for analysis, MALDI-TOF MS was able to discriminate clinically important serovars.

In 2013, Zautner and colleagues used whole cell MALDI-TOF MS for the first time to discriminate *C. jejuni* clinically relevant and less relevant isolates, based on protein biomarker shifts and PCA-clustering. They suggested that this technic may be “a more meaningful typing approach than MLST” (Zautner et al., 2013). Later the name mass spectrometry-based phyloproteomics (MSPP) was highlighted and suggested as a novel microbial typing method (Zautner et al., 2015,

2016). While the first study was based on clustering approaches where the presence and absence of a peak is used, MSPP investigated mass changes in specific set of allelic isoforms of the same protein. Additionally MSPP was investigated for other *Campylobacter* species such as *C. fetus* or *C. coli*, isolated from diverse sources (e.g. blood culture, preputial washing, faeces or chicken) (Emele et al., 2019a, 2019b).

Historically, several techniques were developed for the subtyping of *Campylobacter*. Nowadays, only a few of them, such as MLST-based methods, are still applied in laboratories for this purpose. The existence of these numerous different typing methods underlines the fact that there is not a unique reference method methodology to subtype *Campylobacter* spp.

CHAPTER 4

Research aims, objectives and methodology

The current AMR crisis is one of the most important public health challenges of the 21st century. While the emergence of multidrug resistant pathogens is growing, there are few new antimicrobials under development. Nevertheless, there are alternative actions to initiate an effective management of AMR. The improvement of laboratory testing, for the rapid and reliable detection of resistances and their related epidemiology, is one of them.

While current bacterial infection investigation, including identification and antibiotic susceptibility testing, could take between 2-3 days, an antimicrobial empiric treatment strategy is established without the diagnosis of the potential pathogen. In 50% of cases, the latter results in an inappropriate prescription, leading to an overuse and overexposure of antimicrobials (Vasala et al., 2020). The development of rapid, simple to use, low-cost and with short results time, i.e. 1-2 hours, diagnostic tests could improve the use of antibiotics by determining an appropriate tailor-made antibiotherapy. By answering these everyday questions from a single cell monitoring method: what kind of bacteria is causing the infection? Are the bacteria causing the infection resistant or susceptible? What is the best antibiotic to fight against the infection? Rapid diagnostic tests will reduce unnecessary prescription, improve infection control and restrain potential spread of multidrug pathogens (Health First Europe, 2017; Sykes, 2018).

On the one hand, swift and accurate tool such as the whole-cell MALDI-TOF MS has been successful applied in routine laboratories for the identification of microorganisms. On the other hand, this technology has been also successfully used for the typing of several bacterial genera and the detection of specific AMR in a research context. Nevertheless, it was only mainly investigated for several clinical pathogens and few antibiotic families. Since 2005, *Campylobacter* is considered as the major cause of foodborne gastrointestinal diseases worldwide. As well, ever-growing *Campylobacter*'s resistances to critically important antibiotics, such as quinolones or macrolides, for human and veterinary medicine is particularly of concern. While MALDI-TOF MS was partially investigated for the typing of several *Campylobacter* species, identification of resistances by MALDI-TOF MS within foodborne pathogens is poorly documented.

Considering that MALDI-TOF MS signals are mainly based on ionised ribosomal proteins released from bacterial lysis, the aim of this research is to answer the following questions:

1. Could those proteins reflect the AMR profile of *Campylobacter* spp.? If yes, how could it been explained?

2. Could the same protein profile be used to have an insight on the genetic diversity and population structure of *Campylobacter* spp.?

These hypotheses were based on facts that MALDI-TOF MS successfully allows the identification of variations within the genomic structures, such as the presence of antimicrobial resistance genes or specific allelic profiles, resulting in the presence of related protein biomarkers. Additionally, the combination of MALDI-TOF MS and Machine Learning (ML) enhances the research of biomarkers by recognizing specific peak patterns. Therefore, these questions will be answered through the development of MS-based workflows, WGS and on a ML approach.

The groundwork of this research will consist into the characterization of a One-Health *Campylobacter* collection (Chapter 5). Isolates will be selected from previous and on-going projects located in the Greater Region. AMR profiles will be phenotypically assessed and genotypically based on the whole genome sequence. Every isolate will be analysed by MALDI-TOF MS after different types of protein extraction, i.e. direct bacterial colonies smear, off-plate and on-plate extractions. Firstly, MALDI-TOF mass spectra will be investigated to screen AMR to different class of antibiotics and to retrieve putative biomarkers related to already known AMR mechanisms (Chapter 6). The second part will evaluate the ability of MALDI-TOF MS to cluster mass spectra according the genetic relatedness of isolates and congruently compare it to reference genomic-based methods (Chapter 7).

Part II

Results

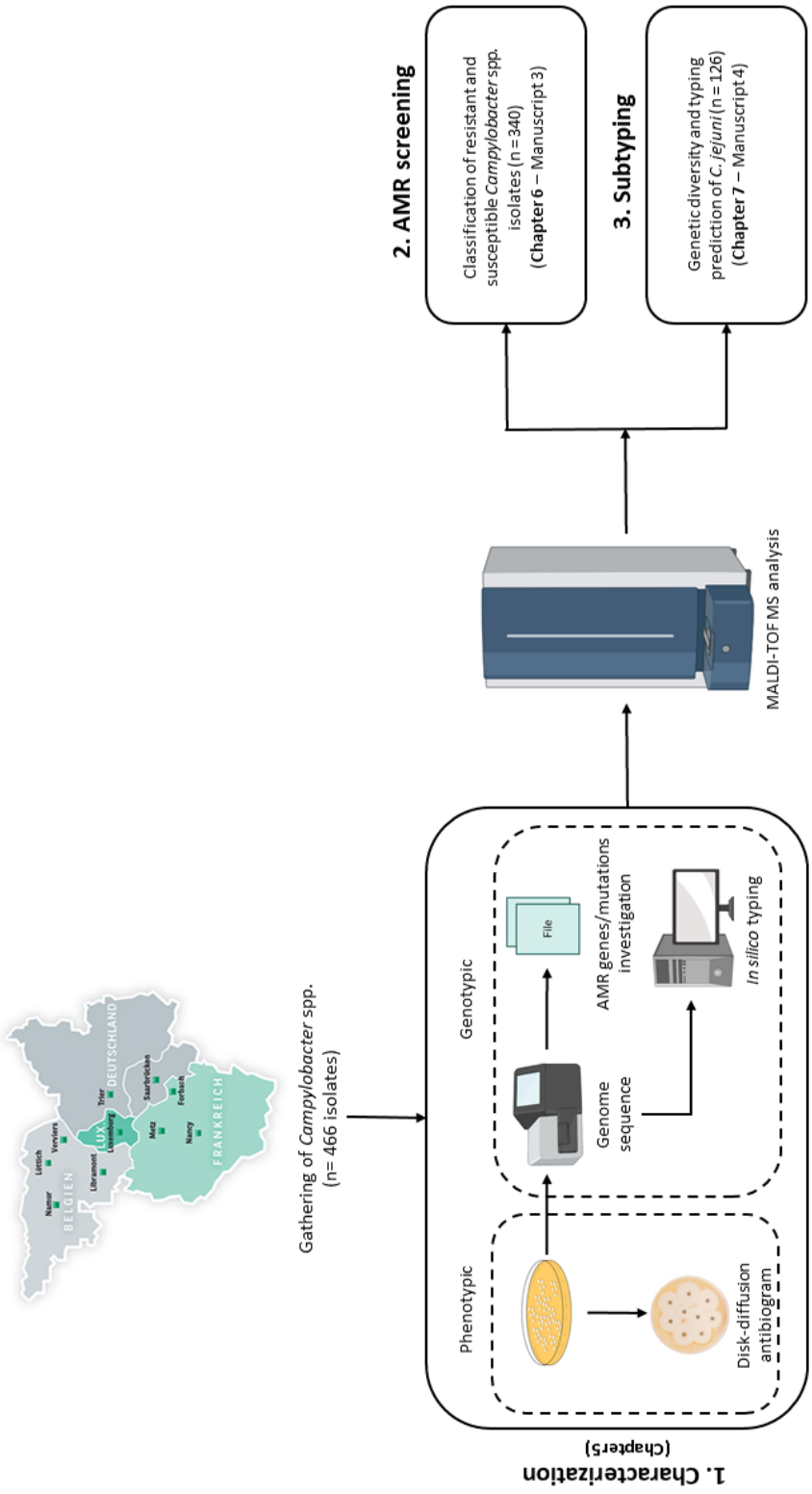


***Figure 5.1**. Organization of the results part*

CHAPTER 5

The One-Health *Campylobacter* spp. collection

The current collection is the result of the gathering of isolates of two different research projects and routine identifications of different institutions in the Greater Region, i.e. area covering Saarland and Rhineland-Palatinate in Germany, the Grand Duchy of Luxembourg, the French region of Lorraine as well as Belgian Wallonia with its French and German-speaking communities. 234 isolates stemming from two FNR funded main Luxembourgish research projects running over the last decade were selected. The first one is the HypoCamp project (C09/BM/09 - 2010-2013), which aimed to investigate environmental contamination sources of *Campylobacter* infections in the Grand Duchy of Luxembourg. The second one is the still on-going CampylOmic project (C17/BM/11684203 - 2018-2022), which aims to explore the phenomenon of recurring genotypes by using genomics. The cited projects were led by the National Health Laboratory of Luxembourg (LNS) and were conducted in collaboration with the LIST. Additionally, strains isolated and identified in routine or monitoring settings (n = 232) at the LNS, the Luxembourg veterinary governmental laboratory, the medical university of Saarland and the Belgium national *Campylobacter* reference centre were picked out based on their phenotypic AMR profiles and genetic diversity. Therefore, a total of 466 isolates, including 116 *C. coli* and 350 *C. jejuni* based on their phenotypic AMR profiles and genetic diversity were retained for the collection.

The collected isolates were sampled from various origins, including humans (n = 309), cattle (n = 97) and environment (n = 60) reservoirs. Details of the origins are listed in the following **Table 5.1**. Isolates were grown on chocolate agar plates with a loopful of a -80°C stock suspension stored in a combination of ferrous sulphate, sodium metabisulfite and sodium pyruvate (FBP) medium complemented with a *Campylobacter* growth supplement. Then, agar plates were incubated for 48h ± 2H at 42°C under microaerophilia condition using a gas pack.

***Table 5.1**. Samples' origins.*

Type of samples	**n**
Humans	309
Poultry	56
Bovine	39
Pig	1
Ovine	1
Surface water	35
Wild birds	17
Wildlife	8

Two independent isolate panels, to explore the AMR (*Chapter 6*) and typing side (*Chapter 7*) of the research work, were established. On the one hand, a dataset of 224 *C. jejuni* and 116 *C. coli* was characterized by disk-diffusion antibiograms. Seven antibiotics, i.e. ampicillin, ciprofloxacin, tetracycline, kanamycin, streptomycin, gentamycin and erythromycin were tested. These were explored because C. jejuni and C. coli are known to have resistance mechanisms to these antibiotics but also because some of these antibiotics' families, e.g. fluoroquinolones, macrolides, tetracyclines and aminoglycosides are used in both veterinary and human medicine (*Chapter 3*). Thus, for each isolate a suspension of 0.2 OD_{600} (eq. 0.5 McFarland) was streaked on a Mueller-Hinton agar combined with 5% horse blood and 20mg/L of B-NAD (MH-F). Agar plates were incubated during 24h ± 2h at 42°C under microaerophilia (5% O_2) conditions. Interpretation of the inhibition halo was performed by using the French Microbiology Society (SFM, recommendations 2020 v1.1 April) based on EUCAST recommendations resulting in patterns addressed in **Figure 5.2**

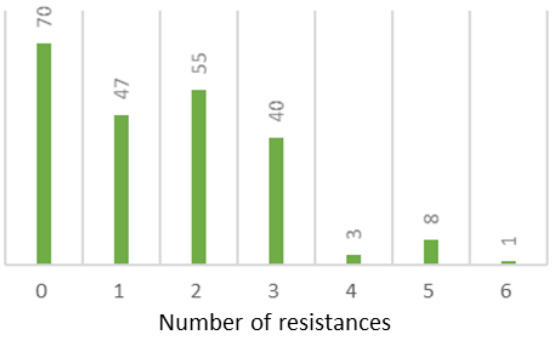
A.

Antimicrobial resistances	Total
Ampicillin	90
Ciprofloxacin	123
Susceptible	70
Tetracycline	90
Kanamycin	18
Streptomycin	11
Gentamycin	1
Erythromycin	2

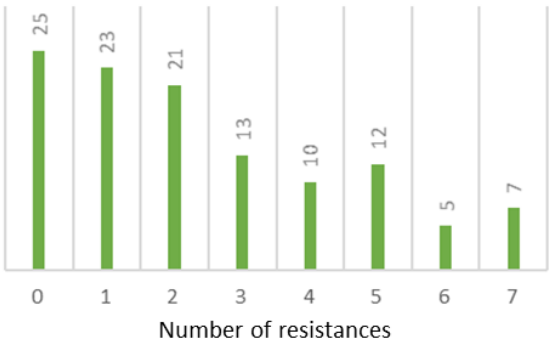
C.

Antimicrobial resistances	Total
Ampicillin	58
Ciprofloxacin	60
Susceptible	25
Tetracycline	70
Kanamycin	18
Streptomycin	35
Gentamycin	11
Erythromycin	31

B.



D.



(CASFM, 2020).

Figure 5.2. Campylobacter *resistances and susceptibility characteristics for* C. jejuni *(A-B) and* C. coli *(C-D).*

For antibiotics not described for *Campylobacter* spp., i.e. kanamycin and streptomycin, EUCAST recommendation for the *Enterobacteriaceae* group was applied. An isolate is considered susceptible when it is susceptible to the seven tested antibiotics.

On the other hand, a subset of 126 *C. jejuni* was selected from the Luxembourg national molecular monitoring program, performed between 2005 and 2021. Each strain isolated from various origins (e.g. food samples, human and environment) was subjected by WGS and characterized by *in silico* MLST and cgMLST by using the Ridom SeqSphere+ software platform resulting in 10 CCs, 21 STs and 42 CTs. The detail of the typing collection is summarized in the following **Table 5.2**. Among those strains, 74 were assigned to four different lineages, i.e. A (n = 34), B (n = 15), C (n = 15) and D (n = 10) by Nennig et al. (2021) (Nennig et al., 2021). The classification was initially based on their extensive ST-*gyrA*-*porA* combination and their frequency in human infection over time. Actually, the four lineages were confirmed to share the same core genome by using 3 cgMLST schemes i.e. SeqSphere+ (n = 637 loci), Oxford (n = 1,343 loci), INNUENDO (n = 678 loci). Additionally, most of the isolates classified in lineages A, B and D were identified as clonal by sharing a same pangenome within each subgroup respectively (same profile in wgMLST with less than 9 differences in alleles out 2795 targets screened and compared).

***Table 5.2**. Details of the typing collection.*

Clonal complex (CC)	Sequence Type (ST)	Complex Type (CT)	Total (n =126)	Lineages	Clones
21	19	82	34	A (n = 34)	n = 31
		588	1		
		1300	1		
		1333	1		
		1355	3		
		2474	1		
	21	46	5		
		50	1		
		681	2		
		1648	1		
	50	364	1		
		441	1		
		606	1		
		1377	1		
		2249	1		
		2383	1		
	104	1643	1		
	336	1650	1		
	861	1652	1		
	883	2477	1		
	3574	1639	2		
	3633	2542	1		
	6175	543	10	D (n = 10)	n = 9
	10298	2149	3		
42	42	1644	1		
45	5503	1649	1		
48	48	660	1		
		1646	1		
		1661	1		
	5173	1642	1		
206	122	1640	1		
257	2254	51	15	B (n = 15)	n = 12
353	2882	1641	1		
354	354	772	1		
464	464	75	16	C (n = 15)	
		596	3		
		1428	1		
		1514	1		
		1668	2		
		2130	1		
607	607	1645	1		

CHAPTER 6

MALDI-TOF MS as a fast and straightforward routine screening tool for the detection of antimicrobial resistances

In this chapter*, *C. jejuni* and *C. coli* protein profiles generated by MALDI-TOF MS were explored by ML to predict resistances to different class of antimicrobials, i.e. quinolones, macrolides, β-lactams, tetracyclines and aminoglycosides. Firstly, AMR predictions based on mass spectra were investigated at the species- and antibiotic resistances level. Secondly, the impact of the different protein extraction methods, i.e. on- and off-plate extraction, on resistance predictions was performed. Finally, features tagged as relevant for the prediction of specific AMR were probed, to identify known and unknown AMR biological mechanisms.

Highlights:

- High performance was observed for classifiers detecting susceptible as well as ciprofloxacin and tetracycline resistant isolates.
- A maximum sensitivity and a precision of 92.3% and 81.2%, respectively, were reached.
- No significant prediction performance differences were observed between on- and off-plate types of protein extractions.
- Three putative AMR biomarkers for fluoroquinolones, tetracyclines and aminoglycosides were identified during the current study.
- Combination of MALDI-TOF MS and machine learning could be an efficient and inexpensive tool to swiftly screen certain AMR in foodborne pathogens, which may enable a rapid initiation of a precise, targeted antibiotic treatment.

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Combination of MALDI-TOF Mass Spectrometry and Machine Learning for Rapid Antimicrobial Resistance Screening: The Case of *Campylobacter* spp.

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While MALDI-TOF mass spectrometry (MS) is widely considered as the reference method for the rapid and inexpensive identification of microorganisms in routine laboratories, less attention has been addressed to its ability for detection of antimicrobial resistance (AMR). Recently, some studies assessed its potential application together with machine learning for the detection of AMR in clinical pathogens. The scope of this study was to investigate MALDI-TOF MS protein mass spectra combined with a prediction approach as an AMR screening tool for relevant foodborne pathogens, such as *Campylobacter coli* and *Campylobacter jejuni*. A One-Health panel of 224 *C. jejuni* and 116 *C. coli* strains was phenotypically tested for seven antimicrobial resistances, i.e., ciprofloxacin, erythromycin, tetracycline, gentamycin, kanamycin, streptomycin, and ampicillin, independently, and were submitted, after an on- and off-plate protein extraction, to MALDI Biotyper analysis, which yielded one average spectra per isolate and type of extraction. Overall, high performance was observed for classifiers detecting susceptible as well as ciprofloxacin- and tetracycline-resistant isolates. A maximum sensitivity and a precision of 92.3 and 81.2%, respectively, were reached. No significant prediction performance differences were observed between on- and off-plate types of protein extractions. Finally, three putative AMR biomarkers for fluoroquinolones, tetracyclines, and aminoglycosides were identified during the current study. Combination of MALDI-TOF MS and machine learning could be an efficient and inexpensive tool to swiftly screen certain AMR in foodborne pathogens, which may enable a rapid initiation of a precise, targeted antibiotic treatment.

Keywords: MALDI-TOF MS, antimicrobial resistance screening, AMR, machine learning, *Campylobacter*, diagnostics

INTRODUCTION

Antimicrobial susceptibility testing (AST) is a key technology in diagnostic microbiology and is essential for a targeted treatment and to limit the widespread use of broad-spectrum antibiotics. Over the past decades, many improvements have helped to accelerate, standardize, and harmonize testing facilities, e.g., through the implementation of automated and semi-automated devices combining identification and AST (e.g., Vitek 2®), using optical systems for measuring changes in bacterial growth and determining antimicrobial susceptibility, and using rapid diagnostic tests for same-day AST results (Mitchell and Alby, 2017; Benkova et al., 2020; Roth et al., 2021). In a concern for harmonization, disk-diffusion and microdilution antibiograms, recommended by the European committee on antimicrobial susceptibility testing (EUCAST, human medicine) or the European food safety authority (EFSA, veterinary medicine), are still the reference methods for determination of antimicrobial resistances (AMR). These tests are based on bacterial growth, requiring between 16 and 24 h for rapid growing pathogens and longer for fastidious pathogens (e.g., mycobacteria and *Helicobacter pylori*) (Barlam et al., 2016; Arena et al., 2017). Results are usually qualitative and classed into categories, i.e., susceptible or resistant, depending on the breakpoint calibrated by the EUCAST, or expressed as minimum inhibitory concentration (MIC) (Benkova et al., 2020). While these conventional methods are effective, they are cumbersome, time-consuming, and do not enable the rapid choice of an effective targeted anti-infective treatment. Yet, development of "fast microbiology" technologies or rapid diagnostic tests, including Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), results in the improvement of the antimicrobial stewardship by decreasing the "patient–physician" workflow before treatment (Bookstaver et al., 2017; Mangioni et al., 2019).

MALDI-TOF MS is a soft-ionized mass spectrometry method developed as an analytical tool to identify and understand the structure of unknown biomolecules (Gibson and Costello, 2000). In an evolving field, this automatic technique became the reference method for identifying microorganisms such as bacteria (Clark et al., 2013; Singhal et al., 2015), mycobacteria (Rodriguez-Granger et al., 2018; Rotcheewaphan et al., 2019) and fungi (Florio et al., 2018; Robert et al., 2021). The resolution power of the system operates at the species level and even at sub-species level for a number of pathogens in clinical microbiology (Fall et al., 2015; Feucherolles et al., 2021). It is a fast and cost-efficient process, with a positive impact on public health analytical pipelines (Ge et al., 2017; Rodríguez-Sánchez et al., 2019). Identification of other organisms, like protozoa (Del Chierico et al., 2016), helminths (Bredtmann et al., 2017; Feucherolles et al., 2019b; Sy et al., 2021; Wendel et al., 2021), viruses (Iles et al., 2020; Rybicka et al., 2021), and arthropods (Tahir et al., 2017; Boucheikhchoukh et al., 2018; Tandina et al., 2018), is also feasible in a research context. However, only the routine identification part of the diagnostics workflow is currently carried out by MALDI-TOF MS.

Over the last 5 years, machine learning (ML), a subset of artificial intelligence, has gained interest in many areas of research pertaining to an improved diagnosis of diseases (e.g., cancer detection, infectious diseases, etc.) (Caballé et al., 2020; Goodswen et al., 2021; Nami et al., 2021). This popularity is greatly explained by the current era, where large daily amounts of data are being collected digitally, known as big data, which are requiring new approaches to investigate it. Mass spectra are routinely generated by MALDI-TOF MS and most of the time not exploited for additional analysis beyond the sole identification of microorganisms. Even if several reports highlighted successful applications of MALDI-TOF MS for detection of bacterial AMR, by the presence of specific biomarkers (Feucherolles et al., 2019a; Oviaño and Bou, 2019; Yoon and Jeong, 2021) identified by classical statistical methods, there is still a mine of information encrypted in the mass spectra. More recently, a growing number of reports combining MALDI-TOF mass spectrometry and ML have shown promising results for clinical big data problems, such as AMR screening (Weis et al., 2020a,b). The majority of these studies used pathogens such as *Staphylococcus aureus* and the β-lactam antibiotic family (Sogawa et al., 2017; Wang et al., 2018; Tang et al., 2019). Therefore, there are very few published data concerning other relevant clinical or foodborne pathogens or antimicrobials such as the quinolones (e.g., ciprofloxacin) and macrolides (e.g., erythromycin and azithromycin) (Sabença et al., 2020; Sousa et al., 2020). However, macrolides and quinolones are frontline antibiotics used to treat severe infectious gastroenteritis and categorized by the World Health Organization (WHO) as critically important in human medicine (WHO, 2019).

Campylobacteriosis, mainly caused by *C. jejuni* and *C. coli*, is the main global cause of bacterial gastroenteritis in humans (Chlebicz and Śliżewska, 2018). Likewise, 10.9 and 0.6% of *C. coli* and *C. jejuni,* respectively, isolated from humans were multi-resistant to ciprofloxacin, erythromycin, tetracycline, and gentamycin in 2019 (EFSA and ECDC, 2021). In food-producing animals, 26.9% of *C. coli* isolated from calves were resistant to at least three of the previously cited antimicrobials. MALDI-TOF MS already has been applied for proteo-typing of *C. coli*, *C. fetus*, and more recently for *C. concisus* genomospecies (Emele et al., 2019a,b; On et al., 2021). Also, its ability to distinguish β-lactam-resistant strains from sensitive ones by pre-processing mass spectra before analysis was reported (Penny et al., 2016). However, there are no published reports concerning the direct application of the mass spectrometry and ML for direct prediction of AMR in *Campylobacter* spp.

Therefore, the aim of this study is to show that MALDI-TOF MS combined with an ML approach could be a useful tool for a fast and precise AMR screening of relevant foodborne pathogens, such as *C. coli* and *C. jejuni*. While campylobacteriosis is mainly self-limiting and do not require specific antibiotherapy, such a combination strategy may aid to swiftly prescribe a definitive antimicrobial therapy and therefore limit an empirical broad-spectrum strategy for other pathogens. ML prediction based on protein mass spectra will be investigated at the species-specific and antibiotic resistance level. The impact of different protein extraction methods, i.e., on- and off-plate extraction, on resistance predictions will also be considered.

MATERIALS AND METHODS

Campylobacter Collection

Strains

A One-Health collection of 224 *C. jejuni* and 116 *C. coli* isolates, obtained from humans (n = 226), in environmental samples, i.e., surface water (n = 33), and animals including wild life: raccoons (n = 8), wild birds (n = 17), and cattle, i.e., bovine (n = 20), pig (n = 1), and poultry (n = 35), were used in the current study.

Antimicrobial resistances patterns were established by disk diffusion antibiograms for fluroquinolones [ciprofloxacin (Cip, 5 μg)], macrolides [erythromycin (Ery, 15 μg)], tetracyclines [tetracycline (Tet, 30 μg)], aminoglycosides [gentamycin (Gent, 10 μg), kanamycin (Kana, 30 μg), Streptomycin (Strep, 10 μg)], and β-lactams [ampicillin (Amp, 10 μg)] following the French Microbiology Society (SFM) and EUCAST recommendations (Recommendations 2020 v1.1 April) resulting in patterns addressed in **Table 1**. For antibiotics not described for *Campylobacter* spp., i.e., kanamycin and streptomycin, EUCAST recommendation for the *Enterobacterales* group was applied. The latter was added to the study based on ResFinder analysis by using Whole Genome Sequencing (WGS) data (Bortolaia et al., 2020). The Lys43Arg mutation in the *rspL* gene as well as *ant*(6) and *aadE* genes and conferring the streptomycin resistance were detected (Olkkola et al., 2010; Fabre et al., 2018). Likewise, the *aph*(3) gene conferring among other kanamycin resistance was detected (Fabre et al., 2018). The phenotypic details of the collection are described in **Supplementary File 1**.

Growth Conditions

All strains were inoculated on chocolate agar plates (Thermo Scientific, Waltham, MA, United States) with -80°C stock suspension stored in FBP medium complemented with *Campylobacter* growth supplement (Thermo Fisher Scientific), and incubated for 48 ± 2 h at 42°C under micro aerobic conditions using CampyGen 2.5 L gas packs (Thermo Fisher Scientific).

Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry Analysis

Sample Preparation

For every biological assay, an off- and on-plate extraction and a direct deposit were performed. For the off-plate or also known as ethanol/formic acid protein extraction (EtOH/ACN), bacteria were suspended in 300 μl milliQ water and 900 μl absolute ethanol (Merck, Darmstadt, Germany). The mix was centrifuged for a further 2 min and the residual ethanol was discarded. A total of 25 μl for both 70% (v/v) formic acid (Merck, Darmstadt, Germany) and acetonitrile (Merck) were mixed up to the dry pellet. A final centrifugation was performed, and then 1 μl of supernatant was spotted onto a one-use MALDI Biotarget (96 targets; Bruker Daltonics GmbH, Bremen, Germany). For the formic acid on-plate extraction (FA), a smear of a bacteria colony is directly carried out on the biotarget and then overlayed with a 1 μl 70% formic acid. For the direct deposit, a bacteria colony is directly streaked on the biotarget. For all deposits and extractions, as soon as the sample was dried, the spot was overlaid with 1 μl of portioned HCCA matrix solution (Bruker Daltonics GmbH) prepared with standardized acetonitrile 50%, water 47.5%, and trifluoroacetic acid 2.5% solution (Sigma-Aldrich, Saint Louis, MO, United States). Bruker bacterial test standard (BTS) was used for an external calibration of the apparatus.

For each method of extraction, three independent cultures (biological replicates) on three different days (reproducibility) were performed. Each biological replicate was spotted thrice (technical replicates) on the same day (repeatability), resulting in nine spectra per isolate.

Data Acquisition

MALDI-TOF MS analysis was performed using a Biotyper Microflex LT/SH (Bruker Daltonics GmbH) by using the AutoXecute acquisition method (MBT_AutoX) in FlexControl software v3.4., with a 2–20 kDa mass-to-charge ratio (m/z) range in a positive linear mode. Before measurement, the system was calibrated using the automatic calibration feature with the BTS. For each sample spot, an automatic acquisition with 240 laser shots was performed.

Mass Spectra Analysis

All protein spectra were identified by using the BDAL Bruker database (n = 8,468 MSPs), containing at least 3,000 different bacterial and fungi species, through the MBT Compass Explorer interface (v.4.1). The software attributed a log score value between 0 and 3.00. A score between 0 and 1.69 was considered as a not reliable identification. A score between 1.70 and 1.99 was considered as probable genus identification and scores from 2.00 to 2.29 as reliable genus identification and a probable species identification. Finally, a score between 2.30 and 3.00 was deemed as highly probable species identification.

Then, spectra were uploaded on FlexAnalysis v3.0 (Bruker Daltonics GmbH) and an internal calibration was carried out on the 4,365 m/z peak, identified as a 50 S ribosomal protein L36 by Zautner et al. (2016) in *Campylobacter*, which is shared by all samples and the BTS. Mass spectra were converted into mzML files and imported into BioNumerics v7.6 software platform (BioMérieux, Craponne, France). Spectra were pre-processed using the workflow described by Penny and collaborators [binned baseline (size = 77), Kaiser Window (size = 33), Moving bar (width = 129)], with a sound-to-noise ratio threshold of 10 (Penny et al., 2016). The peak detection parameters were the following: Continuous wavelet transformation (CWT) ridges, double peaks, and a relative intensity of 2%. Biological replicate spectra were summarized to create an average spectrum, or Main Spectra Profile (MSP), per isolate and extraction. Finally, a peak matching was performed on MSPs, resulting in 91 peaks.

Machine Learning Analysis

Pre-processing

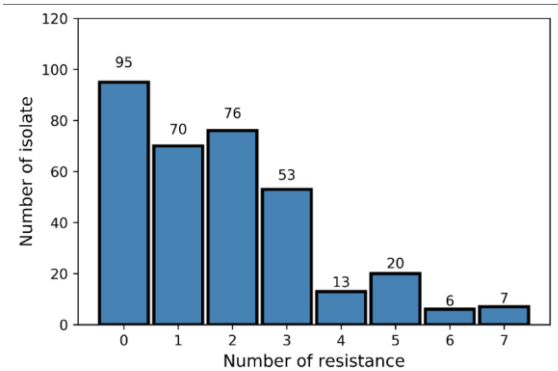
Tables including intensity values of the peak matching MSPs for the three types of extraction were exported into csv files (**Supplementary File 2**) for ML analysis using Python programming language (v3.7.6) and Scikit-learn package

TABLE 1 |

(A) Antimicrobial susceptibility patterns of *Campylobacter* isolates used in the present study.

		Resistant isolates	
Antibiotic classes	**Antibiotics**	***C. jejuni* (*n* = 224)**	***C. coli* (*n* = 116)**
	*Susceptible (S)**	70 (31.2%)	25 (21.6%)
Fluroquinolones	*Ciprofloxacin (Cip)*	123 (54.9%)	60 (51.7%)
Macrolides	*Erythromycin (Ery)*	2 (0.9%)	31 (26.7%)
Tetracyclines	*Tetracycline (Tet)*	90 (40.2%)	70 (60.3%)
Aminoglycosides	*Gentamycin (Gent)*	1 (0.4%)	11 (9.5%)
	Kanamycin (Kana)	18 (8.0%)	18 (15.5%)
	Streptomycin (Strep)	11 (4.9%)	35 (30.2%)
Beta-Lactams	*Ampicillin (Amp)*	90 (40.2%)	58 (50.0%)

(B) Diversity of antimicrobial resistance pattern in the collection.



Susceptible to all tested antimicrobials.

(v0.22.1) in Jupyter NoteBook (v6.0.3). Then, MSPs were grouped by their AMR profiles and eight distinct files have been created according their AMR classes and susceptibility, i.e., S, Cip^R, Tet^R, Amp^R, Ery^R, $Gent^R$, $Strep^R$, and $Kana^R$ (**Figure 1**). Category names (e.g., S and R) were binarized, where 0 and 1 represented MSPs susceptible and resistant to the AMR class studied, respectively. All peaks, here called features, were transformed using a Min-Max scaler which transformed values into the (0,1) range. Such a step is necessary to bring different variables at the same level, as variables that are measured at different scales may not contribute equally to the model fitting.

Feature's Selection

Dataset with many features, which could be redundant or irrelevant, may lead to an overcomplicated algorithm with low prediction accuracy and long training time. Feature selection is the process of choosing relevant features, to use in a classification model construction, either to improve accuracy scores or to boost performance. For this purpose, a meta-transformer based on a Random Forest estimator, implemented into scikit-learn library, was used to discard irrelevant features.

Model Selection

MSPs were randomly split into 70% training and 30% test datasets, with a stratification based on their binarized AMR profiles. The training dataset is implemented to build up a prediction model, while the test dataset is used as an external validation step of the trained model. For each studied AMR classes, Random Forests (RF), Logistic Regression (LR), and Naïve Bayes (NB) models were built, as they are common algorithms used in microbiology (Goodswen et al., 2021). RF is currently among the most used ML methods due to its robustness. It is essentially a collection of independent decision trees, where each tree could be different from the others, as the algorithm will make completely different random choices to make sure trees are distinct. Such algorithms make aggregated predictions using a group of decision trees. LR is a linear classifier, which predicts the probabilities of success and failure event. It is easy to implement and interpret and efficient to train. NB classifier assumes that the presence of a particular feature is not related to the presence of another feature. It is easy to interpret and is often applied for many medical applications. The area under the precision recall curve (AUPRC) was investigated to determine the most performant model (data not shown).

Tuning

Upon selection of the best performing model, it was optimized by looking for the best combination of hyper-parameters according to the F1-score, described in the metrics section. Hyper-parameters for each selected model were tuned by using an instance which generates candidates from a grid of given

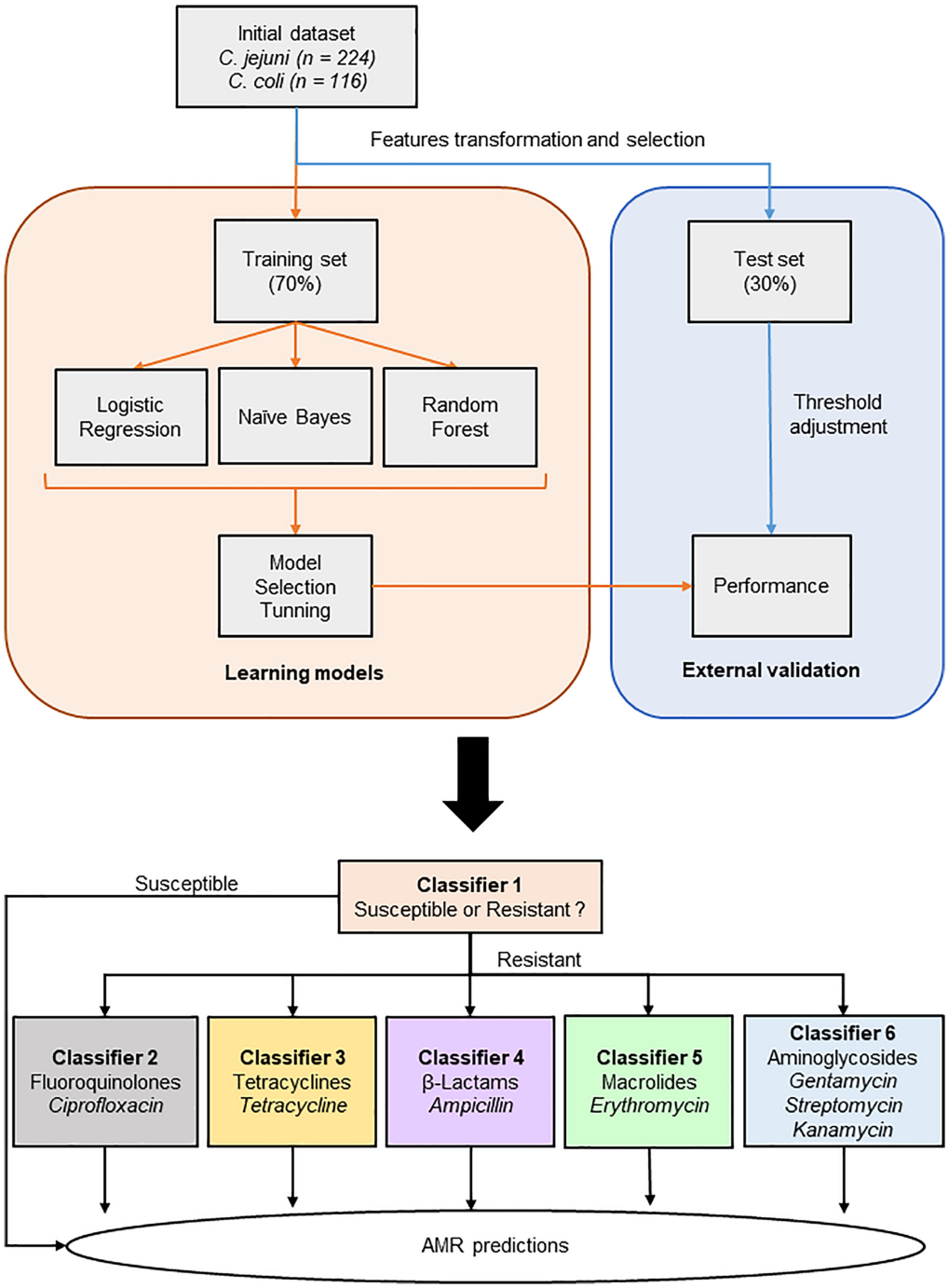


FIGURE 1 | Schematic overview of the machine learning workflow.

parameter values, a grid search, with a 10-fold cross validation, with a scoring method looking for the more optimized F1-score. K-fold cross validation is a resampling method, which estimates the performance of the ML model.

The 0.5 default probability score threshold may not represent an optimal interpretation and can result in poor performance. Therefore, a threshold adjustment was investigated to bring a higher predictive performance (Weis et al., 2020a). A threshold selection, for each classifier, based on their precision recall curve (PRC) was applied, according to the best F1-score. In the case of imbalance classes, like the current dataset, PRC can suggest an optimal threshold (Saito and Rehmsmeier, 2015). In this study, detection of resistant isolates (true positives) is the key point of the study. PRC is based on true positive values,

i.e., true positive and positive predictive values, among positive prediction. Hence, PRC relies on positive classes regardless of true negative value, making it a tool of choice for the study threshold selection. In the end, values less than the custom threshold are assigned to class 0, or susceptible, while value greater than or equal to the custom threshold are assigned to class 1, or resistant.

Performance and Metrics

As a next step, performance of the selected classifier needed to be assessed on data not yet seen by the model. For this, an external validation has been carried out by using the test dataset. Classification of spectra was summarized in a confusion matrix. From it, several performance metrics, such as the specificity, the recall, the precision or the positive predictive value (PPV), the negative predictive value (NPV), and area under the receiver operating characteristic curve (AUROC) and PRC were calculated. The PPV tells us how much we can trust the model when a resistant result is predicted, and in the other way, the NPV tells us how much we can trust the model when a sensitive result is predicted. The recall, also called sensitivity, measures how the model can find all positive units. The specificity refers to the model's ability to give a negative result when an isolate is susceptible. The ROC curve is a graphical way to represent the performance of the classifier for all threshold classifications, with the false-positive rate and true-positive rate as axis. Therefore, the AUROC can be used to measure the model's discriminative ability. Usually, an AUC of 0.5 is assimilated to a non-discriminative model, while 0.7–0.8 is considered acceptable, 0.8–0.9 is excellent, and more than 0.9 is considered outstanding (Hosmer et al., 2013). Along the same line, the PRC is a graphical visualization that combines the precision and the recall. The higher curve on the *y*-axis, the better the performance. Therefore, the AUPRC returns a value between 0 and 1, where 0 is the worst and 1 is the best. Finally, the F1-score is calculated from the precision and the recall. It conveys balance between the precision (PPV) and the recall (sensitivity).

Detailed information on ML analysis is shown in **Supplementary File 3**.

Biomarker Identification

Features of importance, based on RF algorithm trained on the whole dataset, were investigated to potentially identify already known antimicrobial resistance mechanisms or new antimicrobial targets. It rates how important each feature is for the decision tree. A score based on between 0 and 1 for each feature is calculated, where 0 means "Not used" and 1 highlighted a "perfect biomarker." Score for features of importance is computed as the mean and standard deviation of accumulation of the impurity decrease within each tree. Therefore, it describes the relevancy of a peak and, hence, can help to understand the biological problem. The five first features with the higher importance were checked in on Uniprot[1] according their mass in Da. Average theoretical masses were calculated using the online Expasy portal tool[2] based on Uniprot amino acid sequence.

Statistical Analysis

Effects of extraction methods on AMR predictions were analyzed based on analysis of variance (ANOVA) of the sum of AUPRCs of the different antimicrobial classifiers. ANOVA assumptions were verified with a Shapiro-Wilks and Levene tests. Shapiro-Wilks test determines if your data are normally distributed. The Leven test evaluates the equality of the variance. Differences were considered significant at $p < 0.05$.

RESULTS

Spectra Quality and Reproducibility

A total of 9,180 mass spectra were generated. An average identification log score of 2.0 was obtained for all spectra. Outlines, flatlines, and spectra not identified at the *Campylobacter* genus level were discarded for the analysis, resulting into 9,173 spectra. The latter was transformed into 1,020 MSPs, including 672 and 348 MSPs for *C. jejuni* and *C. coli,* respectively. Three different types of extractions, i.e., off-plate ethanol/acetonitrile extraction, direct deposit, and on-plate acid formic extraction, were carried out for both species. Hence, reproducibility was tested for the three biological replicates. Average similarities in percentage between the type of extraction and species are provided in **Figure 2**. For both species, no significant differences were observed between off- and on-plate extractions. Average similarity of means ranged from 77.1 to 92.7% between biological replicates for *C. jejuni* and *C. coli*, respectively.

Antimicrobial-Specific Screening

As a first step, different ML models, i.e., RF, LR, and NB, were trained for specific antimicrobials from different classes, regardless of the species identification to evaluate the potential of fast AM-screening without knowing the microbial identification. For this purpose, 1,020 MSPs, combining the three types of extractions and the two species, were split into a training and a validation set. The training set served to build the model, and the test set, to evaluate the performance of the model. Seven classifiers were built with RF and one with an LR algorithm. ROC and PR curves were computed to investigate the model's performance for each antibiotic (**Figure 3**), as well as other evaluation metrics such as sensitivity, specificity, PPV, and NPV summarized in **Table 2**.

Among the eight antimicrobials tested, three models performed better than the other considering both AUROC and AUPR curves. The best-performing model was the classifier allowing the distinction between resistant and completely susceptible isolates, with an area of 0.80 and 0.89 under the ROC and PR curves, respectively. The ciprofloxacin and tetracycline classifiers were the two other performant models according to their AUROC and the AUPR curves,

[1]https://www.uniprot.org/

[2]http://web.expasy.org/compute_pi/

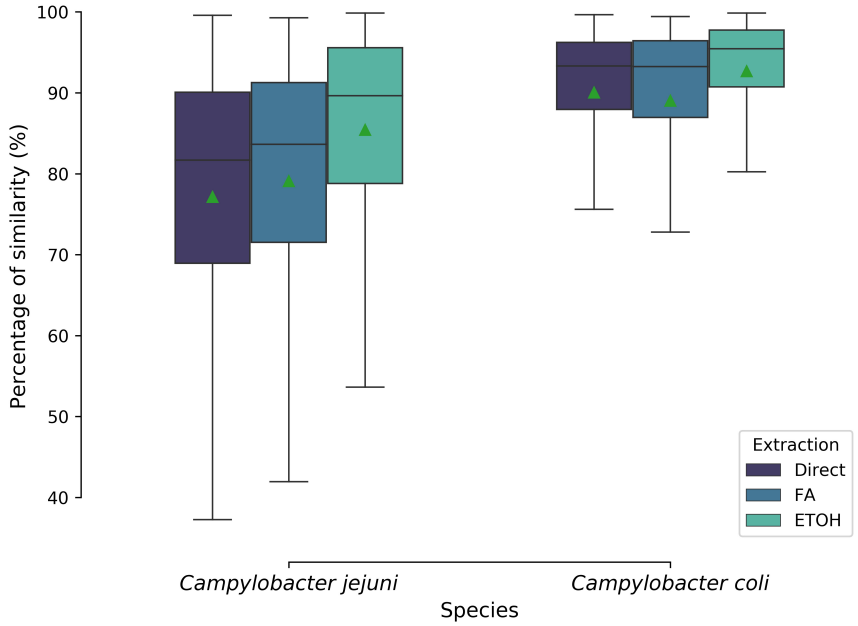


FIGURE 2 | Biological reproducibility of MALDI-TOF mass spectra based on their protein extraction type and species level. Boxplots show the isolates average similarities in percentage. Green triangle represented the mean. Direct, direct deposit; FA, formic acid extraction; EtOH, ethanol/acetonitrile extraction.

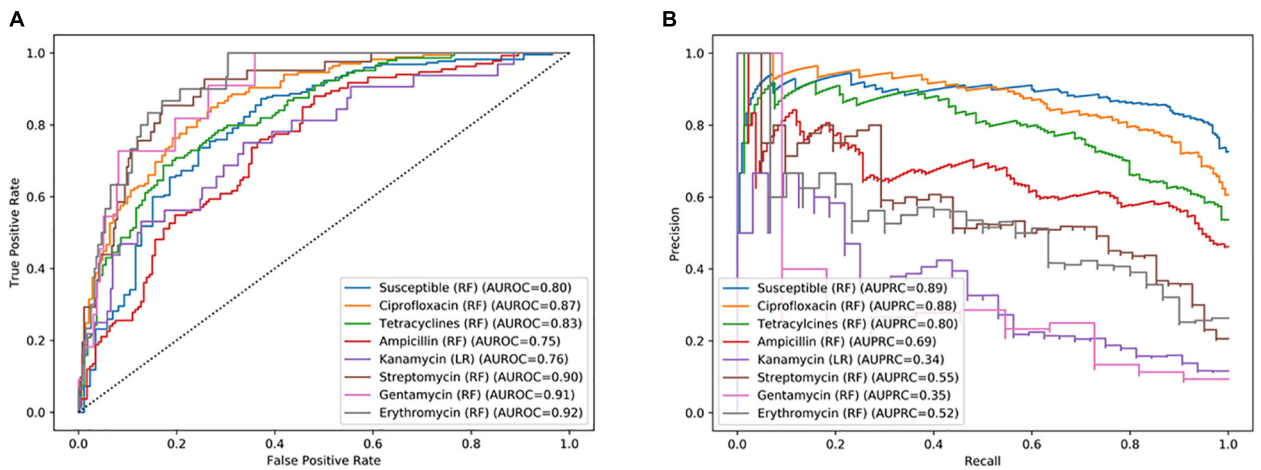


FIGURE 3 | (A) Receiver operating characteristic (ROC) curve and **(B)** recall–precision (PR) curves, and their related area under the curve, of specific antimicrobials based on combined *C. jejuni* and *C. coli* MALDI-TOF main protein spectra profiles (MSPs) of the test set (30%, *n* = 306). RF, Random Forest algorithm; LR, Logistic Regression algorithm; AUROC, Area Under the ROC Curve; AUPRC, Area Under the Precision Recall Curve.

an area of 0.87, 0.83, and 0.88, 0.80 under the AUROC and AUPRC, respectively (**Figure 3**). While the specificity was low for the three models, with a maximum of 63.8%, a sensitivity ranging from 87.5 and 92.3% was obtained (**Table 2**). Additionally, 74.6 and 85.7% of predicted values of the ciprofloxacin classifier could be reliable for resistant and susceptible values, respectively.

Remaining models had an AUROC of up to 0.92. However, considering the precision and the recall, they performed poorly. Indeed, the AUPR curve was between 0.34 and 0.69. Sensitivity and specificity may be high, but PPVs were low, e.g., 80.0, 88.4, and 42.8%, respectively, for the erythromycin model.

Species-Specific Screening

In a second phase, *C. coli* and *C. jejuni* MSPs were investigated separately to look over potential differences between tested antimicrobials. Previously, ROC and PR curves and their respective area under the curve have been computed, based on 202 and 105 MSPs, for the *C. jejuni* and *C. coli* test sets, respectively (**Figure 4**). As well, performance metrics were

TABLE 2 | Performance of retained machine learning classifier using combined *C. jejuni* and *C. coli* MALDI-TOF main protein spectra profiles (MSPs) of the test set (30%, *n* = 306 MSPs), grouped by the resistance profile.

Species	Antibiotics	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
C. jejuni and *C. coli* (*n* = 306 MSPs)	Susceptible* (*n* = 86)	92.3	45.3	81.2	69.6
	Ciprofloxacin (*n* = 165)	90.9	63.8	74.6	85.7
	Erythromycin (*n* = 30)	80.0	88.4	42.8	97.6
	Tetracycline (*n* = 144)	87.5	62.3	67.4	84.9
	Ampicillin (*n* = 133)	90.2	47.4	56.9	86.3
	Kanamycin (*n* = 32)	43.8	91.6	37.8	93.3
	Streptomycin (*n* = 41)	78.0	87.2	48.5	96.3
	Gentamycin (*n* = 11)	72.7	93.6	29.6	98.9

*Threshold applied for metrics calculation is based on the best F1-scores. PPV, positive predictive value; NPV, negative predictive value. *Susceptible to all tested antimicrobials.*

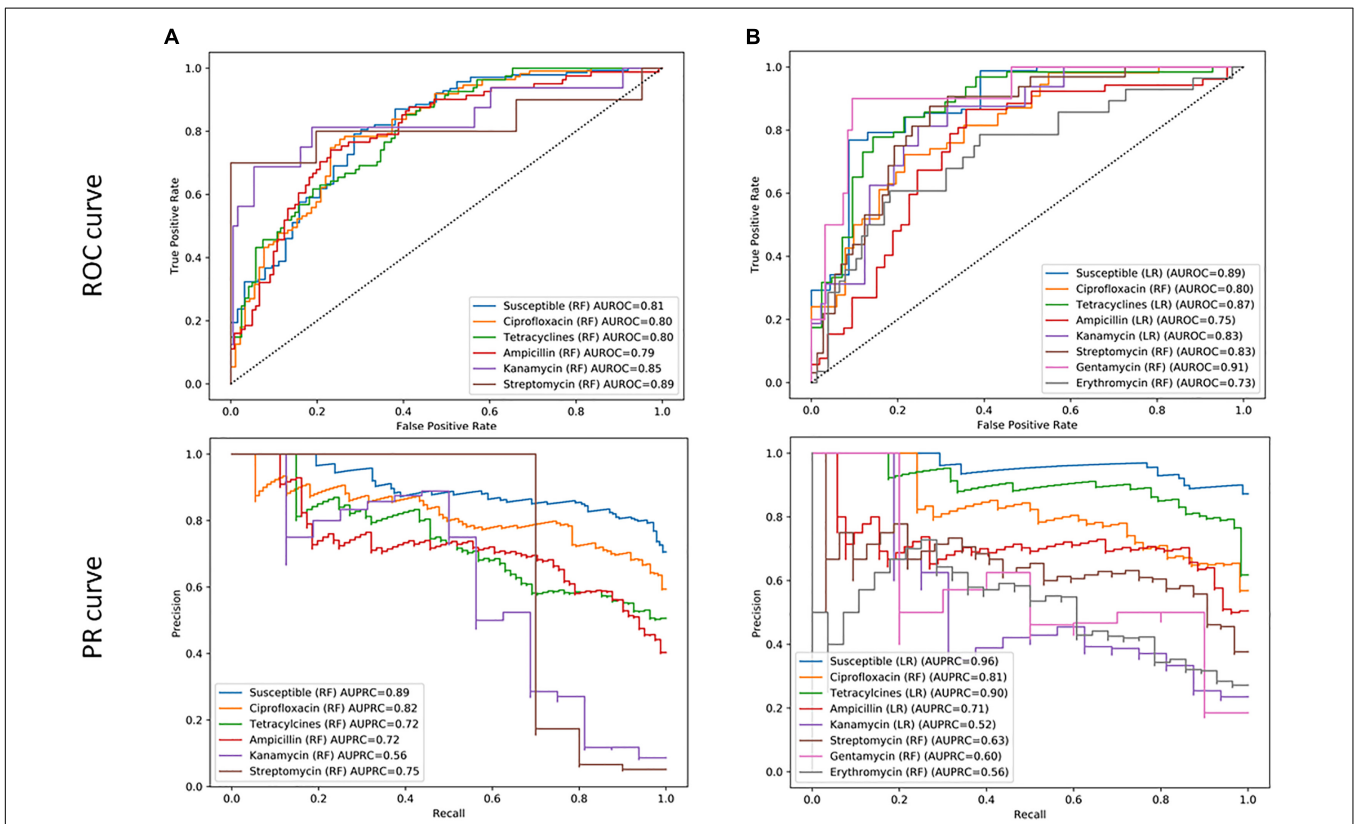


FIGURE 4 | Receiver operating characteristic (ROC) curve and recall–precision (PR) curves, and their related area under the curve, of specific antimicrobials based on 202 *C. jejuni* **(A)** and 105 *C. coli* **(B)** MALDI-TOF main protein spectra profiles (MSPs) of the test set (30%). RF, Random Forest algorithm; LR, Logistic regression algorithm; AUROC, Area under the ROC curve; AUPRC, Area under the precision–recall curve.

calculated (**Table 3**). Due to few gentamycin- and erythromycin-resistant isolates for *C. jejuni* in the initial collection (one and two, respectively), no model was built for these two antibiotics. RF and LR were once again fitting the best data. All six *C. jejuni* models were based on RF algorithms. Four models were built using LR and the remaining four were built using RF algorithms for *C. coli*.

As described in the specific antimicrobial section, the susceptible, ciprofloxacin, and tetracycline classifiers were the three best-performing models in both species, with an AUROC and AURP curve ranging from 0.80 to 0.89 and from 0.72 to 0.96, respectively (**Figure 4**). The susceptible classifier was the more performant model in both *C. jejuni* and *C. coli*. Tetracycline classifier was the second more effective model for *C. coli*, with an AUROC of 0.87 and AUPRC of 0.90, while it was the ciprofloxacin classifier for *C. jejuni*, with an AUROC of 0.80 and AUPRC of 0.82. Overall, sensitivity values up to 98.8% were obtained for these models. High PPVs and NPVs were obtained for susceptible classifiers. *C. coli* tetracycline classifier also performed well with a 79.2 and 92.9%

TABLE 3 | Performance of retained machine learning classifier using *C. jejuni* (*n* = 202 MSPs) and *C. coli* (*n* = 105 MSPs) MALDI-TOF main protein spectra profiles (MSPs) of the test set (30%), grouped by the resistance profile.

Species	Antibiotics	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
C. jejuni (*n* = 202 MSPs)	Susceptible* (*n* = 63)	92.8	55.6	82.2	77.8
	Ciprofloxacin (*n* = 111)	96.4	41.8	66.9	90.5
	Erythromycin (*n* = 2)	NA	NA	NA	NA
	Tetracycline (*n* = 81)	92.6	47.1	53.9	90.5
	Ampicillin (*n* = 81)	77.7	70.3	63.6	82.5
	Kanamycin (*n* = 16)	62.5	97.9	71.4	96.8
	Streptomycin (*n* = 10)	70.0	100.0	100.0	98.5
	Gentamycin (*n* = 1)	NA	NA	NA	NA
C. coli (*n* = 105 MSPs)	Susceptible* (*n* = 23)	98.8	60.9	90.0	93.3
	Ciprofloxacin (*n* = 54)	98.2	45.1	65.4	95.8
	Erythromycin (*n* = 28)	71.4	70.1	46.5	87.1
	Tetracycline (*n* = 63)	96.8	61.9	79.2	92.9
	Ampicillin (*n* = 52)	86.5	64.1	70.3	82.9
	Kanamycin (*n* = 16)	62.5	86.5	45.5	92.7
	Streptomycin (*n* = 32)	84.3	75.3	60.0	91.7
	Gentamycin (*n* = 10)	70.0	93.7	53.8	96.7

*Threshold applied for metrics calculation is based on the best F1-scores. PPV, positive predictive value; NPV, negative predictive value. *Susceptible to all tested antimicrobials. NA, Not applicable due to few isolates in the category.*

for PPV and NPV, respectively. Surprisingly, the ciprofloxacin classifier was less efficient in both species. Indeed, a lower PPV was obtained, i.e., 10% differences, in comparison with previous results where the microbial identification was not taken into consideration. For erythromycin, kanamycin, and gentamycin classifiers, observations described in the previous section could be assessed.

Differences were observed for the ampicillin and streptomycin classifier for *C. coli* and *C. jejuni*. *C. jejuni* streptomycin's classifier performed more efficiently than the one of *C. coli*. PPVs and NPVs of 100 and 98.5%, against 60.0 and 91.7%, were calculated, respectively. *C. coli* ampicillin's classifier was more performant than that of *C. jejuni*, while similar AUROC and AUPR curves were found. Indeed, PPVs and NPVs of 70.3 and 82.9% against 63.6 and 82.5% were calculated for *C. coli* and *C. jejuni*, respectively (**Table 3**).

Protein Extraction Impact on Resistance Predictions

Thirdly, methods of extraction, i.e., direct deposit, FA on-plate, and EtOH/ACN off-plate extraction, were investigated to check potential variation for specific antimicrobials. Thereby, MSPs acquired for each extraction for both *C. jejuni* (n = 224 MSPs) and *C. coli* (n = 116 MSPs) were used to build a specific ML model per antimicrobial. Models are compared in **Figure 5**. The ANOVA resulted in 0.976 and 0.936 ($p > 0.05$) values for *C. jejuni* and *C. coli,* respectively. Therefore, the null hypothesis, i.e., there is no difference between extraction methods, is retained.

Nevertheless, in the case of the *C. coli* gentamycin's classifier, while the performance is low for the EtOH/ACN extraction (AUPRC = 0.23), the classifier for the direct deposit is more efficient (AUPRC = 0.92). Features of extractions for both classifiers were investigated. For the EtOH/ACN classifier, 2,356.29 Da was the more important feature. For the direct deposit classifier, 10,323.79 Da was the more important feature. While these features in a model were particularly important, they were the less important features in the other model. The 10,323.79 Da peak was detected in both extractions, while softly shifting for the EtOH/ACN, i.e., 10,333.67 Da. The 2,356.29 peak was not detected in the direct deposit (**Figure 6**).

Biomarkers: Antimicrobial Resistance Mechanisms

RF classifiers performing the best, i.e., susceptible, ciprofloxacin, and tetracycline, while microbial species is not known, were used to retrieve features of importance. Then, the Uniprot database was investigated to potentially identify each feature according their mass in Dalton, regardless post-translational modifications. **Table 4** summarizes the top five features for each classifier. When several proteins had the same mass, proteins with the most probable function linked to AMR were retained. No protein for *C. jejuni* or *C. coli* was identified at 6,436.22 Da. The DNA methyltransferase at 6,436 Da was in *Helicobacter pylori*, a closely related genus of *Campylobacter*.

DISCUSSION

Several reports described MALDI-TOF MS as a more time- and cost-effective alternative approach to current classic AST methods (Hrabák et al., 2013; Oviaño and Bou, 2019). Being combined with ML, such an approach may be even more relevant for AST in routine diagnostics (Weis et al., 2020b). However, to our knowledge, no study implying relevant foodborne pathogens for AMR screening has been published yet. Therefore, the scope of this study was to consider whether a mass spectrometry

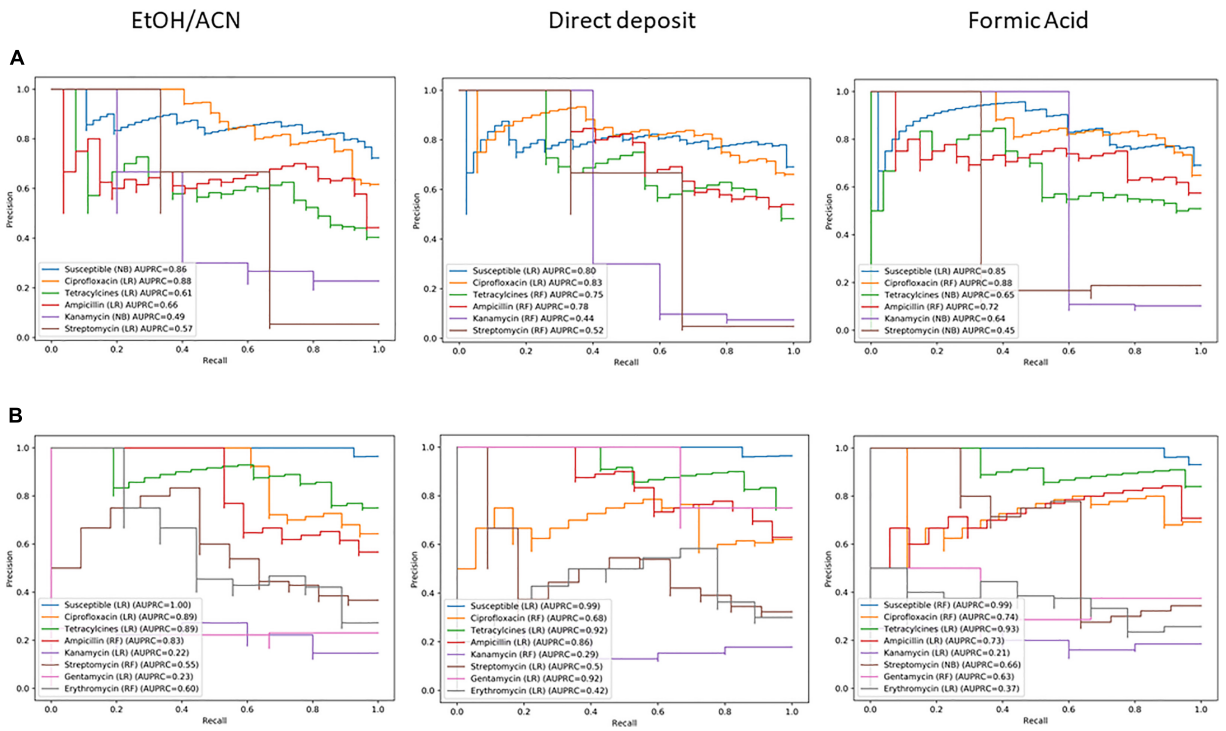


FIGURE 5 | Comparison of precision-recall curves for the three-extraction tested on **(A)** *C. jejuni* (*n* = 68 MSPs) and **(B)** *C. coli* (*n* = 35 MSPs) of the test set (30%). EtOH/ACN: complete ethanol/acetonitrile-based proteins extraction. RF, Random Forest algorithm; LR, Logistic Regression algorithm; NB, Nave Bayes algorithm.

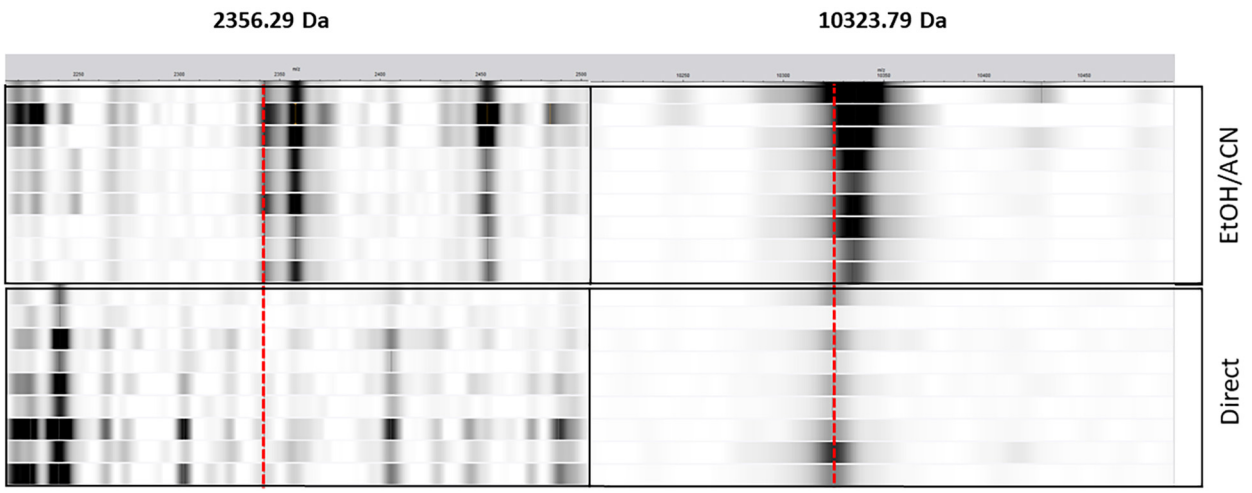


FIGURE 6 | Pseudogel view representation of mass spectra from *C. coli* from the direct deposit (direct, *n* = 8) and the ethanol/acetonitrile off-plate extraction (EtOH/ACN, *n* = 8). The *x*-axis represents the mass-to-charge (m/z) ratio in Da. Strips intensities is function of the peak intensity. The red dashed lines represent the observed peaks, i.e., 2,356.29 and 10,323.79 Da.

technique combined with an ML approach could be utilized for a combined rapid species identification and AMR screening for foodborne pathogens.

The main result of this study was to observe whether mass spectra with 91 protein peaks selected by automatic peak-matching could predict with a high average sensitivity and precision the strains' susceptibility and resistance to ciprofloxacin and tetracycline, independent of the microbial species identification. Therefore, these models were missing very few resistant isolates. Similarly, Weis and colleagues, computed an AUROC for 42 different antibiotics on a large "real-world" clinical dataset by combining multiple species

TABLE 4 | Top five ranking of Random Forest features of importance.

Classifier	Rank	Features (Da)	Average theoretical mass (Da)	Protein	UniProt ID
Susceptible	1	8460.76	8460.07	Transcriptional regulator	A0A1T1ZLP8
	2	3257.41	3256.98	GNAT family N-acetyltransferase	A0A6N3Q833
	3	5867.81	5867.86	ATP-binding protein	A0A2A5MAC7
	4	2766.98	2767.13	Poly(A) polymerase	A0A5T1K937
	5	4365.25	4364.39	50 S ribosomal protein L36	A0A1E7P1M9
Ciprofloxacin	1	6436.22	6435.55	DNA methyltransferase*	A0A438RVN3*
	2	2766.98	2767.13	Poly(A) polymerase	A0A5T1K937
	3	2241.84	2241.67	Type II toxin-antitoxin system HicB family antitoxin	A0A691V648
	4	3257.41	3256.98	GNAT family N-acetyltransferase	A0A6N3Q833
	5	7083.30	7083.03	MmgE/PrpD family protein	A0A4Y8C2R1
Tetracycline	1	4365.25	4364.39	50 S ribosomal protein L36	A0A1E7P1M9
	2	2766.98	2767.13	Poly(A) polymerase	A0A5T1K937
	3	7083.30	7083.03	MmgE/PrpD family protein	A0A4Y8C2R1
	4	6436.22	6435.55	DNA methyltransferase*	A0A438RVN3*
	5	2713.95	2713.06	Superoxide dismutase	A0A431FY74

*Da, Dalton. *Identified in the closely related genus Helicobacter pylori (former Campylobacter pylori).*

(Weis et al., 2020a). They pointed out that they reached AUROC values above 0.90 for 23 of the tested antibiotics. Such results support the idea that mass spectra could provide far more than simple species information. Nevertheless, in the literature, most of the publications focused on specific species such as *S. aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. Additionally, they mainly analyzed one type of antimicrobial classes, e.g., glycopeptides such as vancomycin (Mather et al., 2016; Asakura et al., 2018; Wang et al., 2018; Candela et al., 2021). For example, Asakura et al. (2018) obtained a sensitivity of 99.0% and a specificity of 88.0% while comparing vancomycin-susceptible and heterogeneous vancomycin intermediately resistant *S. aureus*. Wang et al. (2018) obtained similar results with a 77.0 and 81.4% sensitivity and specificity, respectively, for the same comparison. When comparing *C. jejuni* and *C. coli* separately and for different antimicrobials, we found that susceptible, ciprofloxacin, and tetracycline classifiers were the three best-performing models in both species, while the others performed less accurately. Similarly to other studies, a sensitivity ranging from 92.6 to 98.8% was obtained for both species and the three performant classifiers. Weis et al. (2020a) also looked at species-specific antimicrobial resistance prediction for *S. aureus*, *E. coli*, and *K. pneumoniae*. They reported an AUROC ranging from 0.77 to 0.81, and an AUPRC ranging from 0.52 to 0.70 for ciprofloxacin predictions. In the current study, similar AUROC values were found but a higher AUPRC was observed with 0.82 and 0.81 for *C. jejuni* and *C. coli*, respectively, meaning that the current model may accurately predict ciprofloxacin-resistant isolates. Considered as a critically important antimicrobial, ciprofloxacin is widely used for the treatment of broad human bacterial infections, including enteric ones (WHO, 2019). Therefore, early screening of its resistance may play an essential role for the administration of the definitive antimicrobial therapy. Nevertheless, the comparison between the different studies is intricate to perform due to the number of isolates, the genus analyzed, the type of extraction, as well as the type of algorithm used. In the current study, classifiers performing poorly, i.e., kanamycin, streptomycin, gentamycin, and erythromycin, were subject to a highly imbalanced dataset, with an average of 10/90 resistant/susceptible ratio, instead of a close 50/50 ratio one (e.g., 36 gentamycin-resistant MSPs for 984 gentamycin-susceptible MSPs). Precision disparities were observed for the ciprofloxacin, ampicillin, and streptomycin classifiers of both species, in comparison to classifiers not considering the species level. While such differences could be attributed to the unbalanced number of resistant isolates for ampicillin and streptomycin, the ciprofloxacin classifier was in contrast well balanced. The ciprofloxacin classifier may be less effective for predictions, while looking specifically at the species level. In the end, prediction based on protein mass spectra grouped by AMR, regardless of bacterial species, may be the best option for an efficient and swift AMR-screening. Such observations might also be explained by average similarity differences obtained between *C. jejuni* and *C. coli*. Cuénod and Egli (2021), Cuénod et al. (2021) reported that the preparation protocol used, the duration of incubation, maintenance of the device, for example, could potentially impact the quality of the spectra. Inevitably it may have influenced the final prediction for both species. Hypothetically, such observations may also show that AMR screening by MALDI-TOF MS is going beyond the bacterial genus or species and might be directly linked to the resistance mechanism and protein/metabolite expression itself. To our knowledge, this is the first study establishing that ML and MALDI-TOF MS could be applied for AMR screening of foodborne pathogens, such as *Campylobacter* spp.

Nevertheless, in the current study, the specificity was not as high as the specificity described by the previously mentioned studies. While creating the ML pipeline, sensitivity was chosen as the most important parameter to adjust the threshold score during the tuning part. Hence, the optimal threshold was selected based on the F1-score, meaning the

best compromise between higher sensitivity and precision, specific to each classifier. Classifiers guiding antibiotic therapy decision must have high sensitivity (Weis et al., 2020a). On the one hand, assuming an isolate is susceptible, while it is resistant, may lead to an ineffective treatment and eventually have an important impact on patient management. On the other hand, assuming an isolate is resistant, while it is susceptible, may still lead to an effective treatment. However, while seeking and picking to have high sensitivity, it will inevitably decrease the specificity, by decreasing it. In the previously cited reports, threshold adjustments were not mentioned. Therefore, threshold adjustment may be a key step while elaborating ML pipeline for routine laboratories based on MALDI-TOF mass spectra.

The impact of protein extraction methods was also evaluated. Indeed, the EtOH/ACN extraction is the most popular extraction protocol when it comes to research investigations. However, the direct deposit and the on-plate FA extractions are the most straightforward methods used in routine laboratories. No significant differences were observed between the direct deposit, the FA on-plate, and the EtOH/ACN extraction. Therefore, in order to rapidly obtain straightforward AMR assessment information, the application of the direct deposit method could be applied for species identification as well as AMR screening in *Campylobacter*. Interestingly, *C. coli* gentamycin classifier performance was different between EtOH/ACN extraction and the direct deposit. Indeed, with a simple biological smear on the MALDI-TOF target, gentamycin's prediction was more precise. Surprisingly, the absence of the 2,356.29 Da peak resulted in a higher AUPRC for the direct deposit classifier. In the literature, the loss of a specific peak between different types have already been described (Josten et al., 2014). However, in their case, the loss of a protein happened during the ethanol washing step of the EtOH/ACN extraction. Thus, the peptide was only present during a direct deposit measurement. However, to confirm our observation, additional gentamycin-resistant isolates should be analyzed as currently too few gentamycin isolates are present in the current dataset.

Along the same line, putative biomarkers have been identified for each class of studied antibiotics by looking into RF algorithm features of importance. Majority of these proteins, such as transcriptional regulator, ATP-binding, GCN5-related N-acetyltransferase, DNA-methyltransferase, toxin-antitoxin system, PrpD, and superoxide dismutase proteins had a direct or indirect link with already known antibiotic resistance, tolerance, or spread mechanisms in different genera of bacteria (e.g., *Salmonella*, *Enterococcus*, *Escherichia*, *Mycobacterium*, and *Pseudomonas*) (Draker and Wright, 2004; Yugendran and Harish, 2016; Hicks et al., 2018; Kang et al., 2018; Martins et al., 2018; Su et al., 2018; Shaheen et al., 2020). Nevertheless, *Campylobacter*'s AMR mechanisms are either chromosomal mutations, such as the single mutation C257T in the *gyrA* gene or the A207G mutation in the 23 S rRNA gene for ciprofloxacin and erythromycin, respectively, or acquired genes, such as *tet(O)*, bla_{OXA}*-61* and *aph(3')-III* for tetracycline, ampicillin, and gentamycin resistances, respectively (Payot et al., 2006; Iovine, 2013). Overall, these mechanisms are working in synergy with the *cmeABC* efflux pump or porines, such the Major-Out-Membrane Porines (MOMP) (Lin et al., 2002). Over the biomarkers identified as relevant by RF susceptible classifier, the GCN5-related N-acetyltransferase and the 50 S ribosomal protein L36 may be linked to already known aminoglycosides or tetracyclines resistance mechanisms of *Campylobacter*, respectively. On one hand, aminoglycoside-modifying enzymes, such as acetyltransferase [e.g., *aac(6′)-Ie–aph(2″)-If2]* were already detected in gentamycin-resistant *Campylobacter* isolates (Zhao et al., 2016). On the other hand, the Tet(O) ribosomal protection protein is known to bind on both 30S and 50S subunits, conferring tetracycline resistance (Li et al., 2013). Interestingly, the L36 proteins were the first feature of importance highlighted for the tetracycline classifier. Identification of specific proteins directly implied to AMR mechanisms, while using MALDI-TOF MS within the 2–20 kDa range, could be problematic (Welker and Van Belkum, 2019). Indeed, proteins responsible for resistances are large proteins (e.g., GyrA = 96,974 Da). Therefore, in case an indicative biomarker is identified, it may not be a necessary protein conferring the resistance itself, but it may be a protein or peptide co-coded on the plasmid of the protein responsible of the resistance (Lau et al., 2014). Therefore, the 4,365.25 m/z peak may be a biosignature linked to the presence of the *tet(O)* gene. In the literature, two protein biomarkers, i.e., 3,665.79 *m/z* and 6,036.59 *m/z*, have been reported to be a potential biomarker of the tetracycline resistance in other bacterial genera (Sabença et al., 2020; Sousa et al., 2020). However, these biomarkers were not observed here. Along the same line, the 6,436.22 Da protein was considered as the most important feature for the ciprofloxacin's classifier. The protein was identified as a DNA methylase in *H. pylori*, formerly related to the *Campylobacter* genus. Yugendran and Harish put in light the hypothesis that ciprofloxacin-resistance in *E. coli* may be induced by DNA methylation, leading to the possible involvement of some mechanism other than the quinolone-resistance determining region (QRDR) capable of inducing fluoroquinolone resistance (Yugendran and Harish, 2016). While the single point mutation in *gyrA* represents the major fluoroquinolones resistance mechanism in *Campylobacter*, such venue may be worth exploring in the future. Other potential ciprofloxacin biomarkers, neighboring 6,300 Da, were put recently in light for other *E. coli* (Sousa et al., 2020) and *Enterococcus* (Sabença et al., 2020; Sousa et al., 2020). Nevertheless, interpretation on the biological role of features may be cautiously interpreted, and a peptide sequencing by tandem mass spectrometry should be performed to assess the real biological function of these biomarkers.

Little is known on the impact of such approaches as described here on the health management potential cost savings in clinical practice. Weis and colleagues affirmed in their study that the application of such workflow provided a treatment guidance 12–72 h earlier than classical approaches and to have a significant impact on the physician–patient workflow (Weis et al., 2020a). It is worth mentioning that the ML is intended for supporting the decision making process. Therefore, it is a

support giving guidance on possible resistance outcomes that lead early antibiotherapy in a specific direction. ML may be used as an AMR screening tool, displaying an alert message on the MALDI-TOF MS microbial identification report, when the isolate is classified as a positive category value. It is already the case for several Bruker subtyping modules (e.g., MRSA, cfiA positive or bla$_{KPC}$ modules). Therefore, instead of giving an empirical treatment until the AMR confirmation by reference AST, the patient's antibiotherapy may be defined faster (e.g., 24 h earlier).

Phenotypic antibiogram should still follow up to establish the AMR profile and, in case, reorient the antibiotherapy. Additionally, 2025 AMR monitoring of food-producing isolates, such as ESBL/AmpC/carbapenemase-producing *E. coli*, will be done by WGS (Aerts et al., 2019). Therefore, a combination of MALDI-TOF MS, ML, and WGS could be an interesting monitoring tool with a relevant impact on the control of the emergence of AMR in the European Union. As well, the application of MALDI-TOF MS in microbiology for lipid investigation has conceptualized several breakthroughs for AMR screening (Bruker, 2019; Furniss et al., 2019; Dortet et al., 2020). In case of the ability of such method to distinct microbial lipids directly from body fluids such as serum, blood, and urine, there will be no need of a culture step (Solntceva et al., 2021). So far, only the last-line treatment for multidrug-resistant Gram-negative bacteria, i.e., polymyxin, has been investigated without a ML approach. Lipidomics combined to artificial intelligence may be a new venue to explore AMR problem cases that proteomics could not solve. However, there is still a stony way before the long-term implementation of ML in routine laboratories for AMR screening. Nevertheless, a single protein mass spectra may be used in the future as an utmost "One-fits all" diagnostics tool for: species identification, AMR screening, and genetic diversity (Feucherolles et al., 2021).

Several limitations of our study are offered for consideration. First, the employed dataset might be considered as relatively small to train an ML algorithm properly. Indeed, lack of data could lead a model to overfit or underfit the data. Several models (e.g., gentamycin or kanamycin) were trained on heavy unbalanced classes, which is not recommended to build a robust and reliable tool for AMR predictions. Therefore, extra isolates resistant to these antimicrobials should be added to the current dataset. Additionally, only three ML algorithms, i.e., RF, LR, and NB, were tested. The support vector machine algorithm was not included in the study, while it is also a widely used algorithm for AMR predictions. Another limitation of the study is the use of disk-diffusion antibiograms, which—while being a valid and highly reproducible method to characterize an isolate as resistant or susceptible—do not allow quantifying the minimal inhibitory concentration (MIC) of a given antibiotic. Additionally, it would have been possible to test for further antibiotics, e.g., carbapenems. The final limitation of this study could be the fact that the RF model, used for putative biomarkers identification, was trained on the whole dataset. Indeed, under these settings, there is no proof that these biomarkers could work in a given analysis. For such investigations, the model should have been trained on a split dataset, including a training and test set, with a 70/30% ratio, respectively.

CONCLUSION

On the one hand, MALDI-TOF MS in combination with supervised ML may be a powerful tool for the fast screening of foodborne pathogens such as *C. coli* and *C. jejuni*, which might be susceptible, ciprofloxacin, or tetracycline resistant. On the other hand, other antimicrobials tested, i.e., ampicillin, gentamycin, kanamycin, streptomycin, and erythromycin, did not provide good results to reach a conclusion for its application under clinical settings, due to unbalance datasets. Nonetheless, this work could serve as a proof-of-concept, and future research should include other important foodborne pathogens such as *Salmonella* spp. Our approach has the potential to obtain the following information from one single protein spectrum analysis: species identification, antimicrobial susceptibility patterns, and genetic diversity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MF carried out MALDI-TOF MS, machine learning, and data analysis, and drafted the manuscript with MN and CR. MN and CR isolated and performed the identification and AMR characterization of the *Campylobacter* collection. SB and DM supplied extra *Campylobacter* strains from their respective clinical laboratories. CR, SB, and DM provided their expert critical point of view on the current work. SL gave access to his lab for all mass spectrometry analysis. H-MC supervised the project. CP wrote the project proposal and obtained funding. All authors contributed to the formal analysis, writing, review, and editing of the manuscript, read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.804484/full#supplementary-material

Supplementary File 1 | Details of the *Campylobacter* spp. collection used for the study.

Supplementary File 2 | *C. jejuni* and C. coli peak matching table for the EtOH/ACN, FA extraction, and direct deposit.

Supplementary File 3 | Example of one of the supervised machine learning Python workflows.

REFERENCES

Aerts, M., Battisti, A., Hendriksen, R., Kempf, I., Teale, C., Tenhagen, B. A., et al. (2019). Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. *EFSA J.* 17:5709. doi: 10.2903/j.efsa.2019.5709

Arena, F., Giani, T., Pollini, S., Viaggi, B., Pecile, P., and Rossolini, G. M. (2017). Molecular antibiogram in diagnostic clinical microbiology: advantages and challenges. *Future Microbiol.* 12, 361–364. doi: 10.2217/fmb-2017-0019

Asakura, K., Azechi, T., Sasano, H., Matsui, H., Hanaki, H., Miyazaki, M., et al. (2018). Rapid and easy detection of low-level resistance to vancomycin in methicillin-resistant *Staphylococcus aureus* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *PLoS One* 13:e0194212. doi: 10.1371/journal.pone.0194212

Barlam, T. F., Cosgrove, S. E., Abbo, L. M., Macdougall, C., Schuetz, A. N., Septimus, E. J., et al. (2016). Implementing an antibiotic stewardship program: guidelines by the infectious diseases society of America and the society for healthcare epidemiology of America. *Clin. Infect. Dis.* 62, e51–e77. doi: 10.1093/cid/ciw118

Benkova, M., Soukup, O., and Marek, J. (2020). Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *J. Appl. Microbiol.* 129, 806–822. doi: 10.1111/jam.14704

Bookstaver, P. B., Nimmich, E. B., Smith, T. J., Justo, J. A., Kohn, J., Hammer, K. L., et al. (2017). Cumulative effect of an antimicrobial stewardship and rapid diagnostic testing bundle on early streamlining of antimicrobial therapy in Gram-negative bloodstream infections. *Antimicrob. Agents Chemother.* 61, 1–10. doi: 10.1128/AAC.00189-17

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi: 10.1093/jac/dkaa345

Boucheikhchoukh, M., Laroche, M., Aouadi, A., Dib, L., Benakhla, A., Raoult, D., et al. (2018). MALDI-TOF MS identification of ticks of domestic and wild animals in algeria and molecular detection of associated microorganisms. *Comput. Immunol. Microbiol. Infect. Dis.* 57, 39–49. doi: 10.1016/j.cimid.2018.05.002

Bredtmann, C. M., Krücken, J., Murugaiyan, J., Kuzmina, T., and von Samson-Himmelstjerna, G. (2017). Nematode species identification—current status, challenges and future perspectives for cyathostomins. *Front. Cell Infect. Microbiol.* 7:1–8. doi: 10.3389/fcimb.2017.00283

Bruker (2019). *Bruker Launches MALDI Biotyper Sirius at ASM Microbe Conference*. Available online at: www.asm.org (accessed July 8, 2019)

Caballé, N. C., Castillo-Sequera, J. L., Gómez-Pulido, J. A., Gómez-Pulido, J. M., and Polo-Luque, M. L. (2020). Machine learning applied to diagnosis of human diseases: a systematic review. *Appl. Sci.* 10, 1–27. doi: 10.3390/app10155135

Candela, A., Arroyo, M. J., Mancera, L., Microbiology, C., General, H., Software, C. B., et al. (2021). Rapid and reproducible MALDI-TOF-based method for detection vancomycin- resistant *Enterococcus faecium* using classifying algorithms. *bioRxiv* [preprint]. doi: 10.1101/2021.06.23.449689

Chlebicz, A., and Śliżewska, K. (2018). Campylobacteriosis, salmonellosis, yersiniosis, and listeriosis as zoonotic foodborne diseases: a review. *Int. J. Environ. Res. Public Health* 15, 1–29. doi: 10.3390/ijerph15050863

Clark, A. E., Kaleta, E. J., Arora, A., and Wolk, D. M. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* 26, 547–603. doi: 10.1128/CMR.00072-12

Cuénod, A., and Egli, A. (2021). "Advanced applicationsof maldi-tof ms—typing and beyond," in *Application and Integration of Omics-powered Diagnostics in Clinical and Public Health Microbiology*, eds J. Moran-Gilad and Y. Yagel (Heidelebrg/Berlin, Germany: Springer), 153–173. doi: 10.1007/978-3-030-62155-1_9

Cuénod, A., Foucault, F., Pflüger, V., and Egli, A. (2021). Factors associated with MALDI-TOF mass spectral quality of species identification in clinical routine diagnostics. *Front Cell Infect Microbiol.* 16:1–15. doi: 10.3389/fcimb.2021.646648

Del Chierico, F., Di Cave, D., Accardi, C., Santoro, M., Masotti, A., D'Alfonso, R., et al. (2016). Identification and typing of free-living *Acanthamoeba* spp. by MALDI-TOF MS biotyper. *Exp. Parasitol.* 170, 82–89. doi: 10.1016/j.exppara.2016.09.007

Dortet, L., Bonnin, R. A., Le Hello, S., Fabre, L., Bonnet, R., Kostrzewa, M., et al. (2020). Detection of colistin resistance in *Salmonella enterica* using MALDIxin test on the routine MALDI biotyper sirius mass spectrometer. *Front. Microbiol.* 11:1–6. doi: 10.3389/fmicb.2020.01141

Draker, K. A., and Wright, G. D. (2004). Molecular mechanism of the enterococcal aminoglycoside 6' -N-acetyltransferase': role of GNAT-conserved residues in the chemistry of antibiotic inactivation. *Biochemistry* 43, 446–454. doi: 10.1021/bi035667n

EFSA, and ECDC (2021). The european union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA J.* 19, 1–179. doi: 10.2903/j.efsa.2021.6490

Emele, M. F., Karg, M., Hotzel, H., Graaf-van Bloois, L., Groß, U., Bader, O., et al. (2019a). Differentiation of campylobacter fetus subspecies by proteotyping. *Eur. J. Microbiol. Immunol.* 9, 62–71. doi: 10.1556/1886.2019.00006

Emele, M. F., Možina, S. S., Lugert, R., Bohne, W., Masanta, W. O., Riedel, T., et al. (2019b). Proteotyping as alternate typing method to differentiate campylobacter coli clades. *Sci. Rep.* 9, 1–11. doi: 10.1038/s41598-019-40842-w

Fabre, A., Oleastro, M., Nunes, A., Santos, A., Sifré, E., Ducournau, A., et al. (2018). Whole-genome sequence analysis of multidrug-resistant campylobacter isolates: a focus on aminoglycoside resistance determinants. *J. Clin. Microbiol.* 56, 1–12. doi: 10.1128/jcm.00390-18

Fall, B., Lo, C. I., Samb-Ba, B., Perrot, N., Diawara, S., Gueye, M. W., et al. (2015). The ongoing revolution of MALDI-TOF mass spectrometry for microbiology reaches tropical Africa. *Am. J. Trop. Med. Hyg.* 92, 641–647. doi: 10.4269/ajtmh.14-0406

Feucherolles, M., Cauchie, H., and Penny, C. (2019a). MALDI-TOF mass spectrometry and specific biomarkers: potential new key for swift identification of antimicrobial resistance in foodborne pathogens. *Microorganisms* 7, 1–16. doi: 10.3390/microorganisms7120593

Feucherolles, M., Poppert, S., Utzinger, J., and Becker, S. L. (2019b). MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: a systematic review. *Parasit. Vectors* 12, 1–13. doi: 10.1186/s13071-019-3493-9

Feucherolles, M., Nennig, M., Becker, S. L., Martiny, D., Losch, S., Penny, C., et al. (2021). Investigation of MALDI - TOF mass spectrometry for assessing the molecular diversity of *Campylobacter jejuni* and comparison with MLST and cgMLST: a luxembourg one - health study. *Diagnostics* 11, 1–17. doi: 10.3390/diagnostics11111949

Florio, W., Tavanti, A., Ghelardi, E., and Lupetti, A. (2018). MALDI-TOF MS applications to the detection of antifungal resistance: state of the art and future perspectives. *Front. Microbiol.* 9:1–7. doi: 10.3389/fmicb.2018.02577

Furniss, R. C. D., Dortet, L., Bolland, W., Drews, O., Sparbier, K., Bonnin, R. A., et al. (2019). Detection of colistin resistance in *Escherichia coli* by use of the MALDI biotyper sirius mass spectrometry system. *J. Clin. Microbiol.* 57, 1–7. doi: 10.1128/JCM.01427-19

Ge, M. C., Kuo, A. J., Liu, K. L., Wen, Y. H., Chia, J. H., Chang, P. Y., et al. (2017). Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: success rate, economic analysis, and clinical outcome. *J. Microbiol. Immunol. Infect.* 50, 662–668. doi: 10.1016/j.jmii.2016.06.002

Gibson, D., and Costello, C. E. (2000). Mass spectrometry of biomolecules. *Separation Sci. Technol.* 2, 299–327. doi: 10.1016/S0149-6395(00)80053-7

Goodswen, S. J., Barratt, J. L. N., Kennedy, P. J., Kaufer, A., Calarco, L., and Ellis, J. T. (2021). Machine learning and applications in microbiology. *FEMS Microbiol. Rev.* 015, 1–19. doi: 10.1093/femsre/fuab015

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi: 10.1038/s41564-018-0218-3

Hosmer, D. W., Lemeshow, S., and Sturdivant, R. X. (2013). "Assessing the fit of the model," in *Applied Logistic Regression*, (John Willey & Sons, Inc.), 177. doi: 10.1002/9781118548387

Hrabák, J., Chudác ková, E., and Walková, R. (2013). Matrix-assisted laser desorption ionization-time of flight (MALDITOF) mass spectrometry for detection of antibiotic resistance mechanisms: from research to routine diagnosis. *Clin. Microbiol. Rev.* 26, 103–114. doi: 10.1128/CMR.00058-12

Iles, R. K., Zmuidinaite, R., Iles, J. K., Carnell, G., Sampson, A., and Heeney, J. L. (2020). A clinical MALDI-ToF mass spectrometry assay for SARS-CoV-2: rational design and multi-disciplinary team work. *Diagnostics* 10, 1–15. doi: 10.1101/2020.08.22.20176669

Iovine, N. M. (2013). Resistance mechanisms in campylobacter jejuni. *Virulence* 4, 230–240. doi: 10.4161/viru.23753

Josten, M., Dischinger, J., Szekat, C., Reif, M., Al-Sabti, N., Sahl, H. G., et al. (2014). Identification of agr-positive methicillin-resistant *Staphylococcus aureus* harbouring the class a mec complex by MALDI-TOF mass spectrometry. *Int. J. Med. Microbiol.* 304, 1018–1023. doi: 10.1016/J.IJMM.2014.07.005

Kang, S. M., Kim, D. H., Jin, C., and Lee, B. J. (2018). A systematic overview of type II and III toxin-antitoxin systems with a focus on druggability. *Toxins* 10, 1–21. doi: 10.3390/toxins10120515

Lau, A. F., Wang, H., Weingarten, R. A., Drake, S. K., Suffredini, A. F., Garfield, M. K., et al. (2014). A rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for single-plasmid tracking in an outbreak of carbapenem-resistant *Enterobacteriaceae*. *J. Clin. Microbiol.* 52, 2804–2812. doi: 10.1128/JCM.00694-14

Li, W., Atkinson, G. C., Thakor, N. S., Allas, U., Lu, C. C., Yan Chan, K., et al. (2013). Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). *Nat. Commun.* 4, 1–8. doi: 10.1038/ncomms2470

Lin, J., Overbye Michel, L., and Zhang, Q. (2002). CmeABC functions as a multidrug efflux system in campylobacter jejuni. *Antimicrob. Agents Chemother.* 46, 2124–2131. doi: 10.1128/AAC.46.7.2124-2131.2002

Mangioni, D., Viaggi, B., Giani, T., Arena, F., D'Arienzo, S., Forni, S., et al. (2019). Diagnostic stewardship for sepsis: the need for risk stratification to triage patients for fast microbiology workflows. *Future Microbiol.* 14, 169–174. doi: 10.2217/fmb-2018-0329

Martins, D., McKay, G., Sampathkumar, G., Khakimova, M., English, A. M., and Nguyen, D. (2018). Superoxide dismutase activity confers (p)ppgppmediated antibiotic tolerance to stationary-phase *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 115, 9797–9802. doi: 10.1073/pnas.1804525115

Mather, C. A., Werth, B. J., Sivagnanam, S., SenGupta, D. J., and Butler-Wu, S. M. (2016). Rapid detection of vancomycin-intermediate *Staphylococcus aureus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 54, 883–890. doi: 10.1128/JCM.02428-15

Mitchell, S. L., and Alby, K. (2017). Performance of microbial identification by MALDI-TOF MS and susceptibility testing by VITEK 2 from positive blood cultures after minimal incubation on solid media. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 2201–2206. doi: 10.1007/s10096-017-3046-0

Nami, Y., Imeni, N., and Panahi, B. (2021). Application of machine learning in bacteriophage research. *BMC Microbiol.* 21:1–8. doi: 10.1186/s12866-021-02256-5

Olkkola, S., Juntunen, P., Heiska, H., Hyytiäinen, H., and Hänninen, M. L. (2010). Mutations in the rpsL gene are involved in streptomycin resistance in campylobacter coli. *Microb. Drug Resist.* 16, 105–110. doi: 10.1089/mdr.2009.0128

On, S. L. W., Zhang, J., Cornelius, A. J., and Anderson, T. P. (2021). Markers for discriminating campylobacter concisus genomospecies using MALDI-TOF analysis. *Curr. Res. Microb. Sci.* 2, 1–5. doi: 10.1016/j.crmicr.2020.100019

Oviaño, M., and Bou, G. (2019). Matrix-assisted laser desorption ionization–time of flight mass spectrometry for the rapid detection of antimicrobial resistance mechanisms and beyond. *Clin. Microbiol. Rev.* 32, 1–29. doi: 10.1128/CMR.00037-18

Payot, S., Bolla, J. M., Corcoran, D., Fanning, S., Mégraud, F., and Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in campylobacter spp. *Microbes Infect.* 8, 1967–1971. doi: 10.1016/j.micinf.2005.12.032

Penny, C., Grothendick, B., Zhang, L., Borror, C. M., Barbano, D., Cornelius, A. J., et al. (2016). A designed experiments approach to optimizing MALDI-TOF MS spectrum processing parameters enhances detection of antibiotic resistance in *Campylobacter jejuni*. *Front. Microbiol.* 7:1–9. doi: 10.3389/fmicb.2016.00818

Robert, M. G., Cornet, M., Hennebique, A., Rasamoelina, T., Caspar, Y., Pondérand, L., et al. (2021). Maldi-tof ms in a medical mycology laboratory: on stage and backstage. *Microorganisms* 9, 1–16. doi: 10.3390/microorganisms9061283

Rodriguez-Granger, J., Mendiola, J. C., and Martínez, L. A. (2018). "Identification of mycobacteria by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry," in *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*, ed. F. Cobo (Academic Press), 181–195. doi: 10.1016/B978-0-12-814451-0.00013-7

Rodríguez-Sánchez, B., Cercenado, E., Coste, A. T., and Greub, G. (2019). Review of the impact of MALDI-TOF MS in public health and hospital hygiene, 2018. *Euro Surveill* 24, 1–12. doi: 10.2807/1560-7917.ES.2019.24.4.1800193

Rotcheewaphan, S., Lemon, J. K., Desai, U. U., Henderson, C. M., and Zelazny, A. M. (2019). Rapid one-step protein extraction method for the identification of mycobacteria using MALDI-TOF MS. *Diagn. Microbiol. Infect. Dis.* 94, 355–360. doi: 10.1016/j.diagmicrobio.2019.03.004

Roth, S., Berger, F. K., Link, A., Nimmesgern, A., Lepper, P. M., Murawski, N., et al. (2021). Application and clinical impact of the RESIST-4 O.K.N.V. rapid diagnostic test for carbapenemase detection in blood cultures and clinical samples. *Eur. J. Clin. Microbiol. Infect. Dis.* 40, 423–428. doi: 10.1007/s10096-020-04021-4

Rybicka, M., Miłosz, E., and Bielawski, K. P. (2021). Superiority of MALDI-TOF mass spectrometry over real-time PCR for SARS-CoV-2 RNA detection. *Viruses* 13, 1–11. doi: 10.3390/v13050730

Sabença, C., de Sousa, T., Oliveira, S., Viala, D., Théron, L., Chambon, C., et al. (2020). Next-generation sequencing and maldi mass spectrometry in the study of multiresistant processed meat vancomycin-resistant enterococci (VRE). *Biology (Basel).* 9, 1–17. doi: 10.3390/biology9050089

Saito, T., and Rehmsmeier, M. (2015). The precision-recall plot is more informative than the ROC plot when evaluating binary classifiers on imbalanced datasets. *PLoS One* 10:1–21. doi: 10.1371/journal.pone.0118432

Shaheen, A., Tariq, A., Shehzad, A., Iqbal, M., Mirza, O., Maslov, D. A., et al. (2020). Transcriptional regulation of drug resistance mechanisms in *Salmonella*: where we stand and what we need to know. *World J. Microbiol. Biotechnol.* 36, 1–8. doi: 10.1007/s11274-020-02862-x

Singhal, N., Kumar, M., Kanaujia, P. K., and Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. *Front. Microbiol.* 6:1–16. doi: 10.3389/fmicb.2015.00791

Sogawa, K., Watanabe, M., Ishige, T., Segawa, S., Miyabe, A., Murata, S., et al. (2017). Rapid discrimination between methicillin-sensitive and

methicillin-resistant *Staphylococcus aureus* using MALDI-TOF mass spectrometry. *Biocontrol Sci.* 22, 163–169. doi: 10.4265/bio.22.163

Solntceva, V., Kostrzewa, M., and Larrouy-Maumus, G. (2021). Detection of species-specific lipids by routine MALDI TOF mass spectrometry to unlock the challenges of microbial identification and antimicrobial susceptibility testing. *Front. Cell Infect. Microbiol.* 10:1–12. doi: 10.3389/fcimb.2020.621452

Sousa, T., De, Viala, D., Th, L., and Chambon, C. (2020). Putative protein biomarkers of *Escherichia coli* antibiotic multiresistance identified by MALDI mass spectrometry. *Biology (Basel)* 9, 1–14. doi: 10.3390/biology9030056

Su, W., Kumar, V., Ding, Y., Ero, R., Serra, A., Lee, B. S. T., et al. (2018). Ribosome protection by antibiotic resistance ATP-binding cassette protein. *Proc. Natl. Acad. Sci. U.S.A.* 115, 5157–5162. doi: 10.1073/pnas.1803313115

Sy, I., Margardt, L., Ngbede, E. O., Adah, M. I., Yusuf, S. T., Keiser, J., et al. (2021). Identification of adult *Fasciola* spp. using matrix-assisted laser/desorption ionization time-of-flight (maldi-tof) mass spectrometry. *Microorganisms* 9, 1–11. doi: 10.3390/microorganisms9010082

Tahir, D., Almeras, L., Varloud, M., Raoult, D., Davoust, B., and Parola, P. (2017). Assessment of MALDI-TOF mass spectrometry for filariae detection in *Aedes aegypti* mosquitoes. *PLoS Negl. Trop. Dis.* 11:1–18. doi: 10.1371/journal.pntd.0006093

Tandina, F., Laroche, M., Davoust, B., Doumbo, K., and Parola, P. (2018). Blood meal identification in the cryptic species *Anopheles gambiae* and *Anopheles coluzzii* using MALDI-TOF MS. *Parasite* 25, 1–7. doi: 10.1051/parasite/2018041

Tang, W., Ranganathan, N., Shahrezaei, V., and Larrouy-Maumus, G. (2019). MALDI-TOF mass spectrometry on intact bacteria combined with a refined analysis framework allows accurate classification of MSSA and MRSA. *PLoS One* 14:1–16. doi: 10.1371/journal.pone.0218951

Wang, H. Y., Chen, C. H., Lee, T. Y., Horng, J. T., Liu, T. P., Tseng, Y. J., et al. (2018). Rapid detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* based on matrix-assisted laser desorption ionization time-of-flight: using a machine learning approach and unbiased validation. *Front. Microbiol.* 9:1–10. doi: 10.3389/fmicb.2018.02393

Weis, C. V., Cuénod, A., Rieck, B., Llinares-López, F., Dubuis, O., Graf, S., et al. (2020a). Direct antimicrobial resistance prediction from MALDI-TOF mass spectra profile in clinical isolates through machine learning. *bioRxiv* 1, 1–35. doi: 10.1101/2020.07.30.228411

Weis, C. V., Jutzeler, C. R., and Borgwardt, K. (2020b). Machine learning for microbial identification and antimicrobial susceptibility testing on MALDI-TOF mass spectra: a systematic review. *Clin. Microbiol. Infect.* 26, 1310–1317. doi: 10.1016/j.cmi.2020.03.014

Welker, M., and Van Belkum, A. (2019). One system for all: is mass spectrometry a future alternative for conventional antibiotic susceptibility testing? *Front. Microbiol.* 10:1–12. doi: 10.3389/fmicb.2019.02711

Wendel, T., Feucherolles, M., Rehner, J., Keiser, J., Poppert, S., Utzinger, J., et al. (2021). Evaluating different storage media for identification of *Taenia saginata* proglottids using MALDI-TOF mass spectrometry. *Microorganisms* 9:2006. doi: 10.3390/microorganisms9102006

WHO (2019). *Critically Important Antimicrobials for Human Medicine 6th Re.Vision 2018. Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use*. Available online at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1 (accessed May 15, 2019).

Yoon, E., and Jeong, S. H. (2021). MALDI-TOF mass spectrometry technology as a tool for the rapid diagnosis of antimicrobial resistance in bacteria. *Antibiotics* 10, 1–13. doi: 10.3390/antibiotics10080982

Yugendran, T., and Harish, B. N. (2016). Global DNA methylation level among ciprofloxacin-resistant clinical isolates of *Escherichia coli*. *J. Clin. Diagnostic Res.* 10, 27–30. doi: 10.7860/JCDR/2016/19034.7830

Zautner, A. E., Lugert, R., Masanta, W. O., Weig, M., Groß, U., and Bader, O. (2016). Subtyping of *Campylobacter jejuni* ssp. doylei isolates using mass spectrometry-based phyloproteomics (MSPP). *J. Visual. Exp. JoVE* 116, 1–9. doi: 10.3791/54165

Zhao, S., Tyson, G. H., Chen, Y., Li, C., Mukherjee, S., Young, S., et al. (2016). Whole-genome sequencing analysis accurately predicts antimicrobial resistance phenotypes in campylobacter spp. *Appl. Environ. Microbiol.* 82, 459–466. doi: 10.1128/AEM.02873-15

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In this section, protein mass spectra were only investigated at the antibiotic resistance level. While associated with species identification, MALDI-TOF MS could already have an added value for routine diagnostics. Nevertheless, *Campylobacter* spp. diversity, which may also display specific biomarkers, was not taken into consideration. Therefore, *Campylobacter* diversity will be explored in the following chapter.

CHAPTER 7

MALDI-TOF MS as a complementary tool for the daily surveillance of *Campylobacter jejuni*

In this chapter*, the ability of the MALDI-TOF MS is investigated as an alternative and straightforward surveillance tool to assess *C. jejuni* genetic diversity and population structure through two stages. In a first section, *C. jejuni* mass spectra were congruently compared to MLST and cgMLST genomic classification. In a second one, supervised machine learning is explored to automatically subtype *C. jejuni* strains and identify putative biomarkers linked to *Campylobacter* genetic structure.

Highlights:

- As AMR characterization introduced in the Chapter 6, protein profiles generated by MALDI-TOF MS were successfully used for assessing genetic relatedness of *C. jejuni* isolates.
- During this specific phase of the research project, it was observed that isolates clustered together were belonging to the same ST.
- As well, a similar discriminatory power and high concordance to the cgMLST method was highlighted.
- While performing Random Forest machine learning analysis, the model was able to unambiguously predict four different STs based on protein profiles and related features intensities. Finally, the single 4174.19 and 4159.99 m/z peak shift, assimilated to the flagellin subunit protein, was able to distinguish ST-6175 and ST-2254 isolates.

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Investigation of MALDI-TOF Mass Spectrometry for Assessing the Molecular Diversity of *Campylobacter jejuni* and Comparison with MLST and cgMLST: A Luxembourg One-Health Study

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Abstract: There is a need for active molecular surveillance of human and veterinary *Campylobacter* infections. However, sequencing of all isolates is associated with high costs and a considerable workload. Thus, there is a need for a straightforward complementary tool to prioritize isolates to sequence. In this study, we proposed to investigate the ability of MALDI-TOF MS to pre-screen *C. jejuni* genetic diversity in comparison to MLST and cgMLST. A panel of 126 isolates, with 10 clonal complexes (CC), 21 sequence types (ST) and 42 different complex types (CT) determined by the SeqSphere+ cgMLST, were analysed by a MALDI Biotyper, resulting into one average spectra per isolate. Concordance and discriminating ability were evaluated based on protein profiles and different cut-offs. A random forest algorithm was trained to predict STs. With a 94% similarity cut-off, an AWC of 1.000, 0.933 and 0.851 was obtained for $MLST_{CC}$, $MLST_{ST}$ and cgMLST profile, respectively. The random forest classifier showed a sensitivity and specificity up to 97.5% to predict four different STs. Protein profiles allowed to predict *C. jejuni* CCs, STs and CTs at 100%, 93% and 85%, respectively. Machine learning and MALDI-TOF MS could be a fast and inexpensive complementary tool to give an early signal of recurrent *C. jejuni* on a routine basis.

Keywords: *Campylobacter*; MALDI-TOF MS; subtyping; MLST; cgMLST; machine learning

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1. Introduction

Campylobacter spp. was recognized as an important human pathogen in the 1970s even if it had been previously described at the end of the 19th century by Escherich in the colons of children [1]. It has emerged as being the main cause of enteritis in humans and the most common foodborne bacterial zoonosis, superseding *Salmonella* spp. infections worldwide. Since 2005, campylobacteriosis is the most prevalent bacterial zoonosis in Europe with an underestimated incidence of 59.7 per 100,000 population in 2019 [2]. It is frequently mentioned as an important health and economic burden [3], which represented

7.5 million disability-adjusted life years (DALYs) in the 2010 Global Burden of Disease Study [4]. According to the European Food Safety Authority (EFSA) and European Centre for Disease Prevention and Control (ECDC) 2019 zoonoses report, *C. jejuni* represented 83.1% of the confirmed cases of campylobacteriosis in Europe [2]. Therefore, *C. jejuni* plays a key-role in the overall campylobacteriosis cases.

The genomic surveillance of *C. jejuni* infections is only applied in few European countries [5], despite the proven applicability of advanced molecular methods (e.g., next generation sequencing (NGS)) in routine surveillance [6] and following standard protocols (cf. ISO/DIS 23418 standard under development [7]). On the other hand, EFSA will request the use of whole genome sequencing (WGS) for the harmonisation of the monitoring of antimicrobial resistances in food-producing animals and derived meat by 2026 [8]. Driven by a high incidence over the last decade (i.e., 103.8 per 100,000 inhabitants in 2018), Luxembourg has the molecular monitoring of *Campylobacter* stemming from patients, food, animal reservoirs and environmental samples at a national level [9–11].

Multi-locus sequence typing (MLST) consists of the analysis of internal fragments of seven housekeeping genes, i.e., *asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt*, *unc*A, resulting in an allelic profile. It was the first proposed and widely used "gene-by-gene" method to classify *Campylobacter* isolates into genotypes, revealing an unexpected semi-clonal population structure through its application [12,13]. A unique sequence type (ST) is assigned to a unique combination of alleles. Alternatively, core-genome MLST (cgMLST), which is an improvement of the MLST, contains a hundred to a thousand of core genes, and therefore show a higher discriminatory power than classical MLST typing scheme. Genomics may determine the clonal relationships between isolates with an unprecedented resolution [14,15]. For *C. jejuni*, three main cgMLST typing schemes were developed, i.e., the Oxford scheme with 1343 loci [16]; the one from Ridom SeqSphere+ software (Ridom GmbH, Münster, Germany) with 637 loci, resulting into complex type (CT), and the INNUENDO scheme with 678 loci [17,18]. All showed high concordance when compared together [19]. However, the existence of different typing methods with different typing schemes underlines there is not a unique standard subtyping methodology for *Campylobacter* [20] and the lack of a common nomenclature.

Over the past 15 years, the diagnostics field took a new turn with the development of cheaper molecular tests, such as DNA-based assays (e.g., polymerase chain reaction) or proteomic analyses. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS), based on protein fingerprints, has become a popular technique in clinical microbiology and is now the reference method for the fast, reliable, and cost-efficient identification of microorganisms. On one hand, it has been successfully applied in routine for the identification of various microorganisms at the species level including aerobic and anaerobic bacteria, mycobacteria and yeasts including mycobacterium, and fungi in diagnostics [21–23]. On the other hand, researches in taxonomy usefulness extended to a wider range of organisms have suggested new perspectives, such as for helminths [24–26], for ectoparasites (e.g., ticks, fleas, mosquitoes), protozoa, and even more recently for the screening of the SARS-CoV-2 [27–30]. Further, MALDI-TOF MS has been used for other research proposes such as antimicrobial resistance screening [31–33].

Several reports highlighted the ability of MALDI-TOF MS to subtype different microorganisms at the sequence type (ST) level and even single clones, by the identification of specific peaks [34–36]. For example, Meng et al. (2019) investigated the molecular epidemiology of carbapenem-resistant *Klebsiella pneumoniae* by using MALDI-TOF MS and MLST [37]. Giacometti et al. (2018) evaluated the ability of MALDI-TOF MS to characterize *Arcobacter butzleri* strains according their peak patterns and performed a comparative analysis with MLST and pulsed field gel electrophoresis (PFGE) [38]. Along the same line, several reports showed it was possible to differentiate allelic isoforms within *Campylobacter* spp. spectra [39–42]. Indeed, thanks to the presence of specific peak shift in the 2–20 kDa range, Zaunter and colleagues developed the mass spectrometry-based phyloproteomics (MSPP), with the creation of a scheme including 14 different biomarkers, enabling the

subtyping as well as sub-grouping *C. jejuni* ssp. *doylei* [43]. Nevertheless, most of the cited studies rely on empirical observations or statistical methods for the identification of discriminatory peaks.

Important breakthroughs have been possible thanks to the optimization of analysis of mass spectra with machine learning methods [44]. Conventional mass spectra analysis relies on few features, such as peak height or area under the peak, whereas machine learning algorithms are able to extract and analyse useful information which are embedded in mass spectra, that conventional approaches cannot detect, making it a powerful and promising tool for further applications [44]. Studies combining mass spectrometry and machine learning algorithms are focusing on antimicrobial susceptibility testing in both bacteria and fungi [45,46]; on the differentiation of close related species (e.g., *Escherichia coli* and *Shigella* spp.) [47] and on serotyping [48]. Moreover, such prediction approach has also been employed for the differentiation of clonal lineages of relevant clinical pathogens, such as methicillin-resistant *Staphylococcus aureus* [49,50].

As highlighted earlier, campylobacteriosis is the most reported bacterial zoonosis worldwide. The actual problem with *Campylobacter* surveillance is the numerous amounts of isolates to sequence daily and its generated high cost. While it is already implemented in routine at the Luxembourg's reference national center level, many European member states and routine laboratories may not be able to assume such routine for financial and staff reasons. Thus, there is a need of a straightforward and faster alternative/complementary tool to current surveillance methods. Such a tool should give an early signal putting forward related cases of campylobacteriosis, and hence making easier strain sorting for sequencing. Therefore, the aim of this study was to figure out whether, the widely implemented MALDI-TOF MS, best-known for its analysis of speed and cost-efficiency, was able to assess the genetic diversity and the population structure of a selected Luxembourg One-Health *C. jejuni* collection, congruently to genomic classification by MLST and cgMLST. Additionally, an exploration of the potential of machine learning for making subtyping swift and automatic is also considered to look over its potential for future routine application.

2. Materials and Methods

2.1. Collection

A set of 126 strains of *C. jejuni* was selected from the national molecular monitoring program, carried out between 2005 and 2021, in Luxembourg. Strains were isolated from food samples (e.g., bovine, ovine and poultry) (n = 41), human (n = 83) and environment (e.g., surface water, n = 2) sources. All strains were subjected to WGS and characterized by MLST (n = 7 loci) and cgMLST (n = 637 loci) by using the Ridom SeqSphere+ software platform (Ridom GmbH, Münster, Germany) resulting in 10 Clonal Complex (CC, MLST), 21 Sequence Type (ST, MLST) and 42 Complex Type (CT, cgMLST).

Among these isolates, a total of 74 were identified in a previous study, Nennig et al. (2021), as belonging to four different lineages, i.e., A (n = 34), B (n = 15), C (n = 15) and D (n = 10), based on their ST-*gyr*A-*por*A combination and their frequency in human infection over time. Three clones, defined as a set of independent isolated bacteria with similar genotypic characteristic, were identified in isolates (Lineage A (n = 31), B (n = 12) and D (n = 9)), by complete genomic analysis, including 3 cgMLST schemes and whole genome MLST (wgMLST). Concerning the rest of the collection, no other clones were identified. Details of the collection are available in the Supplementary File S1.

2.2. MALDI-TOF MS Analysis

2.2.1. Sample Preparation

Each strain was streaked on chocolate agar plates (Thermo Scientific, Waltham, MA, USA) with a loopful using a −80 °C stock suspension stored in FBP medium complemented with *Campylobacter* growth supplement (Thermo Scientific, Waltham, MA, USA), and incubated for 48 ± 2 h at 42 °C under micro-aerobic conditions (5% O_2, 10% CO_2, 85% N_2) using CampyGen 2.5 L gas packs (Thermo Scientific, Waltham, MA, USA).

For each biological assay, a standardized ethanol/acetonitrile protein-based extraction was performed. Each strain was suspended in 300 µL milliQ water and 900 µL absolute ethanol (Merck, Darmstadt, Germany). The mix was centrifuged for 2 min and the residual ethanol supernatant was discarded. A total of 25 µL of both 70% formic acid (Merck, Darmstadt, Germany) and acetonitrile (Merck, Darmstadt, Germany) were added up to the dry pellet. A final centrifugation was performed, and then 1 µL of supernatant was spotted thrice onto a one-use MALDI Biotarget 96 targets (Bruker Daltonics GmbH, Bremen, Germany). As soon as the samples were dried, the spots were overlaid with 1 µL of portioned HCCA matrix solution (Bruker Daltonics GmbH, Bremen, Germany) prepared with standardized acetonitrile (50% *v*/*v*), water (47.5%) and trifluoroacetic acid (2.5%) solution (Sigma-Aldrich, Saint Louis, MO, USA). Bruker Bacterial Test Standard (BTS), which is a mix of *Escherichia coli* proteins supplemented with RNAse A and myoglobin, was used for external calibration of the apparatus.

2.2.2. Data Acquisition

MALDI-TOF MS analyses were fulfilled with a Biotyper Microflex LT/SH (Bruker Daltonics GmbH, Bremen, Germany) by using the AutoXecute acquisition method (MBT_AutoX) in FlexControl software v3.4., with a 2–20 kDa mass-to-charge ratio (m/z) range in a positive linear mode. Before measurement, the system was calibrated using the automatic calibration feature with the BTS. For each sample spot, an automatic acquisition with 240 laser shots was performed.

The workflow was performed on three different days (reproducibility) with three technical replicates on the same day (repeatability), resulting in nine spectra per isolate.

2.2.3. Mass Spectra Analysis

Spectra were uploaded on FlexAnalysis v3.0 (Bruker Daltonics GmbH, Bremen, Germany) and an internal calibration was carried out on the 4365.00 m/z peak, which is shared by all samples and the BTS, with no shift observed in *C. jejuni* [43]. Then, mass spectra were converted into mzML files and imported into BioNumerics v7.6 software platform (BioMérieux, Craponne, France). Spectra were pre-processed using the strict program template (rolling disc: 50 points, CWT noise, Kaiser window: 20 points/beta = 10, rolling disc: 200 points) with a sound-to-noise ratio threshold of 20. Spectra of technical replicates were summarized to create an average spectra or main spectra profile (MSP) per isolate.

MSP were used to calculate an unweighted pair group method with arithmetic mean (UPGMA) dendrogram using a curve based ranked Pearson correlation similarity coefficient, as it is less sensitive to outliers. The corresponding ST has been indicated using a colour code, a same ST can be classified in different CTs. Three cut-offs of, 92%, 93% and 94% of similarity, have been selected to have a close number of clusters than CC, ST and CT respectively defined by cgMLST analysis. Threshold choice was made by investigating the similarity-cluster size plot (Supplementary File S2).

For each similarity-based cluster identified, a MALDI-profile number was attributed to each MSP, allowing partitions mapping. Specific peak matching parameters were applied: constant tolerance: 1 m/z, linear tolerance: 300 ppm, peak detection rate: 20%, on all peak classes. Therefore, peaks within this range were appraised to belong to the same peak group.

2.3. Typing Methods Concordance

Concordance and discrimination power of the three typing methods, i.e., MLST, cgMLST and MALDI-TOF MS, were estimated by using the adjusted Wallace coefficient (AWC) [51] and the Simpson's index of diversity (SID) [52], respectively, using the online comparing partitions tool (http://www.comparingpartitions.info/ accessed on 10 August 2021). AWC is the probability that two strains with the same typing profile are classified together through a given method while using another typing method. SID

translates the probability that two different strains will be placed into different typing groups. Both values were estimated with their 95% confidence interval (CI).

2.4. Machine Learning Approach

2.4.1. Data Pre-Processing

A character table showing peaks intensity values of the peak matching table was exported into a csv. file and was labelled with the respective ST profiles. ST groups with less than 5 representatives were excluded from this part of the study, resulting into 91 MSPs to analyse. Such criteria of selection have been applied to avoid having less than two representatives during the validation phase. All features were standardized using a min–max scaler, which transformed values into the (0, 1) range, where 0 and 1 will be the minimum and the maximum respectively. Such a step is performed as variables that are measured at different scales may not contribute equally to the model fitting, thus creating a bias in the end. MSPs were randomly split into 80% (n = 63 MSPs) training and 20% (n = 28 MSPs) test datasets, with a stratification based on their ST. The training dataset is implemented to build up a prediction model, while the test panel is used to validate the trained model.

2.4.2. Prediction Models and Evaluation

A random forest model was trained. A 10-fold cross validation was performed to establish the overall accuracy of each model. K-fold cross validation is a resampling method which estimates the performance of the machine learning model. Once the best performing model has been chosen based on metrics described below, performance on data not yet seen by the model, has been carried out by using the test dataset.

2.4.3. Evaluation Metrics

To evaluate the different and final models, a multiclass confusion matrix was carried out. Different metrics for multiclass classification, such as the model's precision, recall, macro F1-score and balanced accuracy will be calculated as they are not affected by the number of cases of each class in case of an imbalanced dataset [53]. The precision, also called positive predictive value, reflects the reliability of the model when a positive value is predicted. The recall, also called sensitivity, measures how the model can find all true positive values. The accuracy computes how much the model is correctly predicting on the entire dataset. In the case of a balanced accuracy, a mean of the recall for each class is calculated, therefore, every class has the same importance and weight. The F1-score measures the model accuracy by aggregating the precision and the recall into a harmonic mean, where 1 is the best score whereas 0 is the worst. In case of a macro F1-score, classes with different size are equally weighted.

2.4.4. Retro-Engineering

To go further in the analysis, algorithms such as decision tree (DT) based on the dataset, showed features of importance, meaning the peaks that the algorithm used to classify spectra based on their ST. DT is a widely used supervised machine learning algorithm, represented under the shape of a tree with nodes and branches. Here, each branch depends on the intensity of each mass spectra peak. Inside each node, information about the feature name, impurity, i.e., the Gini ratio, the number of isolates per nodes and categories, and the class gave at each node. The Gini index measures the probability of an isolate to be wrongly classified when it is randomly chosen where 0 denotes that all isolates belong to a certain class and 1 denotes all elements are randomly distributed. In biology such algorithms may be helpful to potentially understand biological mechanisms. In our case, it will be to understand which protein may be associated with a specific MLST or cgMLST profiles. All biomarkers retained by the algorithm were checked on Uniprot (https://www.uniprot.org/ accessed on 13 August 2021) according to their mass in Da. Average theoretical masses

were calculated using the online Expasy portal tool (http://web.expasy.org/compute_pi/ accessed on 13 August 2021) based on Uniprot amino acid sequence.

The machine learning workflows were carried out using Python programming language (v3.7.6) and the Scikit-learn package (v0.22.1) in Jupyter Notebook (v6.0.3). Detailed information on data analysis is shown in Supplementary File S3.

3. Results

3.1. Spectra Quality

A total of 1134 spectra acquired after an ethanol/acetonitrile extraction were identified by the Bruker BDAL database (n = 8468 spectra) on MBT compass explorer (v4.1). All isolates were identified as *C. jejuni* with a score average $\geq$ 2.00 and all BTS were identified as *E. coli* with a score average $\geq$ 2.00. A score of $\geq$ 2.30 represents reliable species level identification; score 2.00–2.29, probable species level identification; score 1.70–1.90, probable genus level identification, and score $\leq$ 1.70 is considered an unreliable identification. Then, the reproducibility of MSPs based on spectra similarity, using a Pearson correlation coefficient, was established. Inter-spectra similarity average was 85.6% with a standard deviation of 12.9%.

3.2. Classification

As a first step, the clustering of MSPs was investigated in relation to their ST and CT determined by cgMLST. A dendrogram was generated using the 126 MSPs (n = 1134 spectra) with all peak classes (n = 91 peaks) (Figure 1). Consequently, strains associated to ST-464 (n = 24) were subdivided into two main clusters, one grouping a majority of CT-75 (n = 14/16) and another one grouping other CTs such as CT-596 or CT-1514. Overall, several isolates which were clustered together belongs to the same ST. For example, 86.7% of ST-2254 (n = 13/15), 90.0% ST-6175 (n = 9/10), 100.0% ST-10298 (n = 3/3) and 100.0% ST-3574 (n = 2/2) were clustered together.

Then similarity threshold according to the number of CC, ST and CT's clusters were selected. Each MSP, sharing more than 92%, 93% and 94% similarity, were assigned to a same MALDI profile number. This resulted in 12, 20 and 40 distinct clusters. A partition mapping has been carried out for STs and CTs grouped by their MALDI profiles, resulting in a contingency table available in Supplementary File S4. The discriminatory ability between proteomics and genomics methods was tested. For this, a SID was calculated for the three methods, i.e., MALDI-TOF MS, including the three different similarity thresholds, cgMLST and MLST from the SeqSphere+ software platform (Table 1).

Table 1. Simpson's index diversity (CI 95%) for typing schemes comparison.

	Clusters	SID	CI (95%)
Complex Clonal (CC)	10	0.579	0.495–0.664
Sequence Type (ST)	21	0.829	0.785–0.873
Complex Type (CT)	42	0.887	0.849–0.926
MALDI-TOF MS (Cut-off = 92%)	12	0.830	0.800–0.861
MALDI-TOF MS (Cut-off = 93%)	20	0.862	0.828–0.897
MALDI-TOF MS (Cut-off = 94%)	40	0.939	0.918–0.960

SID of MALDI-TOF profiles with a threshold of 92%, 93% and 94% were compared to CC, ST, and CT respectively. On one hand, mass spectrometry had a significant higher discriminatory power than $MLST_{CC}$, i.e., 0.830 versus 0.579 respectively. On the other hand, with a SID of 0.862 and 0.939, mass spectrometry had a similar discriminatory power than $MLST_{ST}$ and cgMLST, with a SID of 0.829 and 0.887.

MALDI-TOF MS profiles (threshold = 94%) were investigated for the three clones, identified in a previous study (Supplementary File S1). Clone belonging to the Lineage A (n = 31/34) was represented by four different MALDI-TOF MS profiles: 19 (n = 9/31), 20 (n = 1/31), 30 (n = 1/31), which were specific to the clone, while the MALDI-TOF

MS Profile 22 (n = 20/31) also referred to three other isolates of the Lineage A. Clone belonging to the Lineage B (n = 11/13) was assimilated to three MALDI-TOF MS profiles: 14 (n = 1/11), 15 (n = 1/11), 40 (n= 1/11), which were specific to the clone, while the MALDI-TOF MS Profile 13 (n = 9/11) is found in the two other isolates of the Lineage B. Clone belonging to the Lineage D (n = 9/10) was linked to four MALDI-TOF MS profiles: 2 (n = 1/9), 3 (n = 1/9), 34 (n = 1/9), which were specific to the clone, while MALDI-TOF MS Profile 1 (n = 6/9) also referred to another isolates of the Lineage D. In the end, those MALDI-TOF MS profiles were only found in Lineages A, B and D. As well MALDI-TOF MS profiles 10 (n = 8/14), 11 (n = 1/14) and 12 (n = 5/14) were only linked to Lineage C. Average similarity between specific lineage MALDI-TOF MS profiles was close to the defined cut-off (94%). For example, for Lineage A, when the MALDI-TOF MS Profile 19 is compared to the Profiles 20 and 22, the average similarity was 93.6% and 93.3% respectively. The MALDI-TOF MS Profile 30 was less close to the MALDI-TOF MS Profile 19 (90.0%).

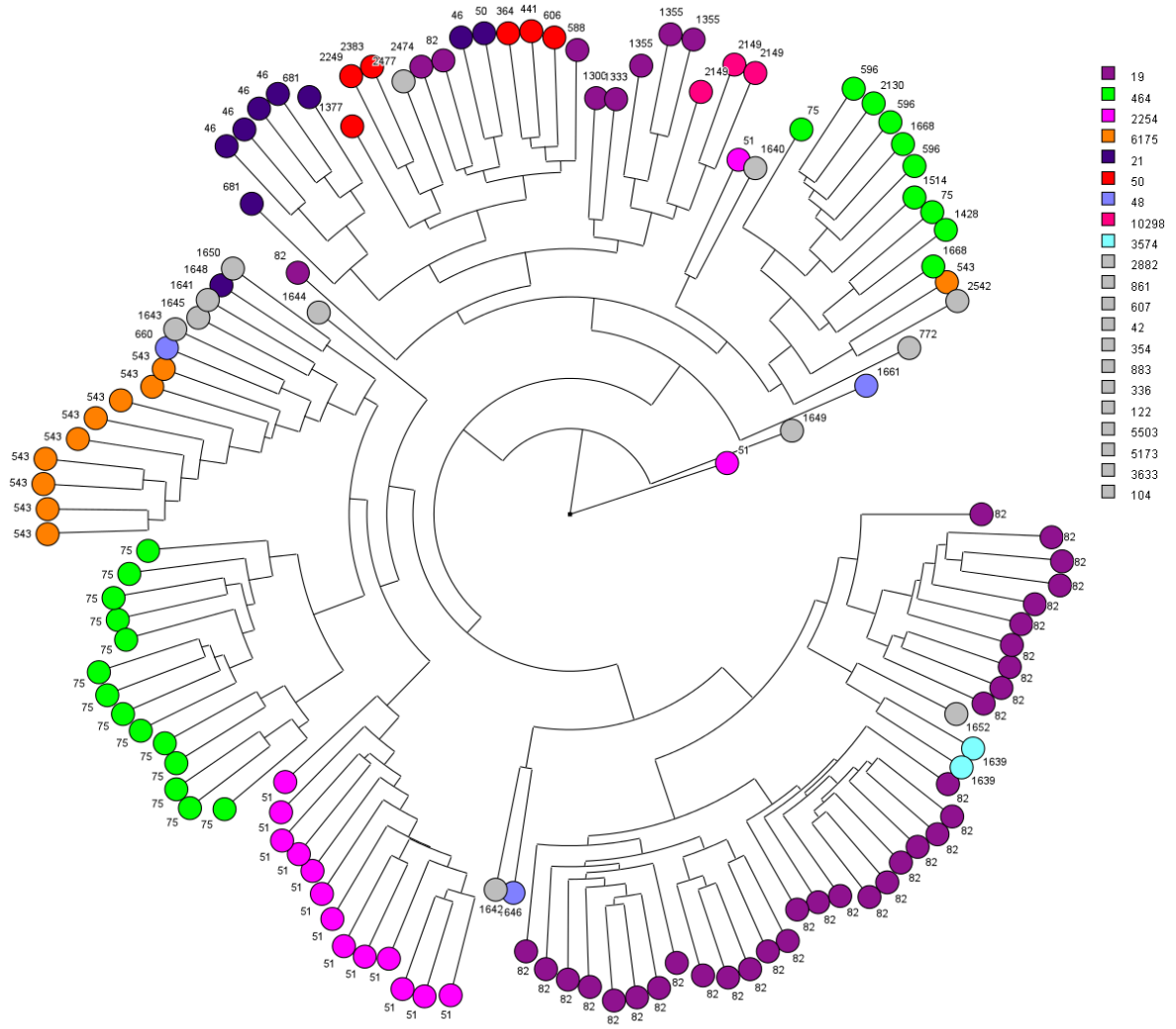

Figure 1. Circular top-score UPGMA dendrogram (ranked Pearson correlation coefficient) based on MSPs grouped by their sequence type (ST) profiles using logarithmic scaling. The whole spectra have been used to compute the figure (n = 91 peaks). Colours represents specific ST and outside numbers are CT profiles. Gray colour represents groups with one representant per ST.

3.3. Congruency: Proteomics vs. Genomics

According to previously described results, MALDI-TOF MS spectra may be clustered with spectra related to the same genotype, as defined as specific combination of alleles. Therefore, we looked over for the congruency between proteomics and genomics methods.

For this, an AWC has been calculated for the three methods, i.e., MALDI-TOF MS, cgMLST and MLST (ST and CC) from the SeqSphere+ software platform (Table 2) by using MALDI-TOF MS profiles with the three different thresholds, i.e., 92%, 93% and 94%, STs and CTs. Overall, MALDI-TOF MS with a 94% similarity threshold shown a high concordance for both MLST and cgMLST typing scheme.

Table 2. Adjusted Wallace coefficient (CI 95%) for typing schemes comparison.

Adjusted Wallace Coefficient	MLST (CC)	MLST (ST)	cgMLST (CT)	MALDI (94%)	MALDI (93%)	MALDI (92%)
MLST (CC)		0.284 (0.171–0.396)	0.175 (0.079–0.270)	0.090 (0.040–0.140)	0.212 (0.132–0.293)	0.248 (0.179–0.317)
MLST (ST)	1.000 (1.000–1.000)		0.616 (0.474–0.758)	0.297 (0.197–0.396)	0.563 (0.427–0.699)	0.567 (0.447–0.686)
cgMLST (CT)	1.000 (1.000–1.000)	1.000 (1.000–1.000)		0.439 (0.317–0.561)	0.829 (0.703–0.955)	0.824 (0.696–0.951)
MALDI-TOF MS (94%)	1.000 (1.000–1.000)	0.933 (0.916–0.949)	0.851 (0.830–0.872)		1.000 (1.000–1.000)	1.000 (1.000–1.000)
MALDI-TOF MS (93%)	0.965 (0.934–0.996)	0.725 (0.608–0.843)	0.658 (0.551–0.765)	0.410 (0.309–0.511)		1.000 (1.000–1.000)
MALDI-TOF MS (92%)	0.881 (0.830–0.932)	0.572 (0.470–0.673)	0.512 (0.423–0.602)	0.321 (0.236–0.406)	0.783 (0.724–0.841)	

MALDI-TOF MS profiles with a threshold of 92%, 93% and 94% were compared to CC, ST, and CT respectively. When the threshold was settled according to the CCs, mass spectrometry was able to predict 88.1% of CCs. As well, when the ST's threshold was applied, mass spectrometry could predict 72.5% of STs. Finally, when the CT's threshold was settled, MALDI-TOF MS was able to predict 85.1% of CT. Overall if the last threshold (94%) was kept for analysis, MALDI-TOF MS could predict 100.0%, 93.3% and 85.1% of CCs, STs, and CTs, respectively.

3.4. Machine Learning for Automatic Attribution of ST

MALDI-TOF MS has a high concordance for the MLST method, so a supervised Machine Learning approach was applied to swiftly predicted STs of unknow spectra. In this context, a total of 91 MSPs were examined, associated to ST-19 (n = 42); ST-464 (n = 24); ST-2254 (n = 15) and ST-6175 (n = 10).

A random forest has been trained and evaluated by using the training dataset. Metrics such as balanced accuracy, precision, recall and F1-score have been calculated with for this purpose. Results are described in Figure 2A. Overall, the trained model had a high performance for the training set (n = 63 MSPs), used to build up the prediction model, used to build the model, with values ranging from 96.6% to 97.5%. Therefore, this model was evaluated by performing an external validation by using the test dataset (n = 28 MSPs), to appreciate how the model will performed when encountering data, it has not been trained on (Figure 2B). A high performance was obtained for the test set with scores between 95.0% and 97.5%. According to the confusion matrix, the trained random forest classifier could correctly classify studied STs, except for ST-19 where one mismatch was observed. In the end, an average sensitivity and specificity of 98.1% and 100% respectively, were obtained for the current classification.

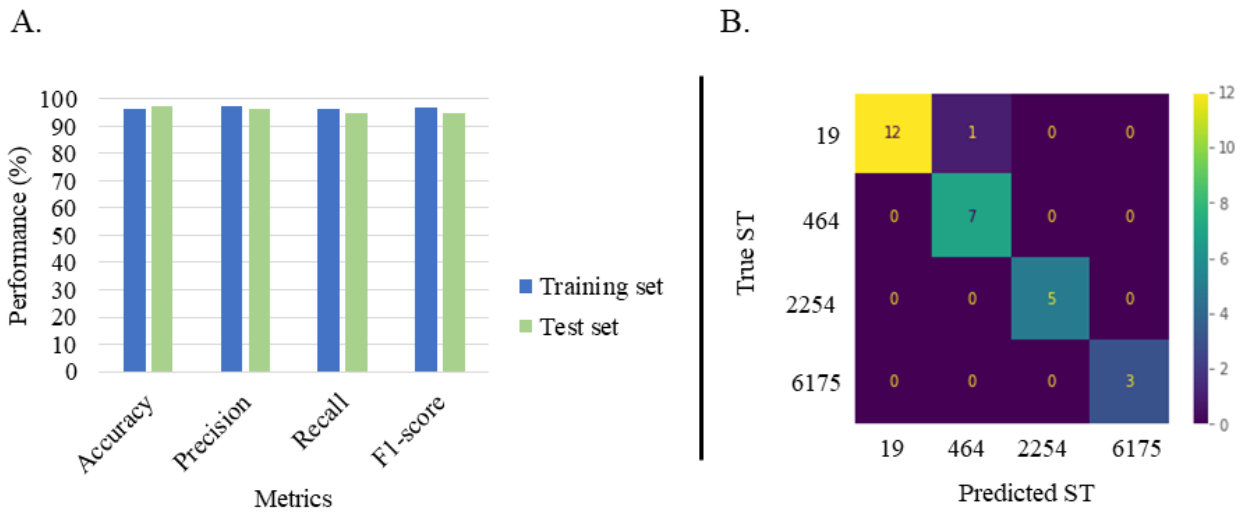

Figure 2. (**A**): Performance metrics for the random forest classifier for both training and test dataset. The horizontal axis was the four metrics balanced accuracy, precision, recall and F1-score, which were averaged over 10-fold cross validations. (**B**): Confusion matrix obtained for the classification of the test set using the trained random forest classifier.

3.5. Features of Importance: Beyond Biomarkers

Certain machine learning algorithms, such as DT, do not only predict a result based on a probabilistic score, but it may also give a new venue to visualize pattern of features, here proteins, which may be linked to biological mechanisms. In this context, a DT model has been trained on the previous dataset with ST groups with at least five representatives. The related tree was plotted in Figure 3.

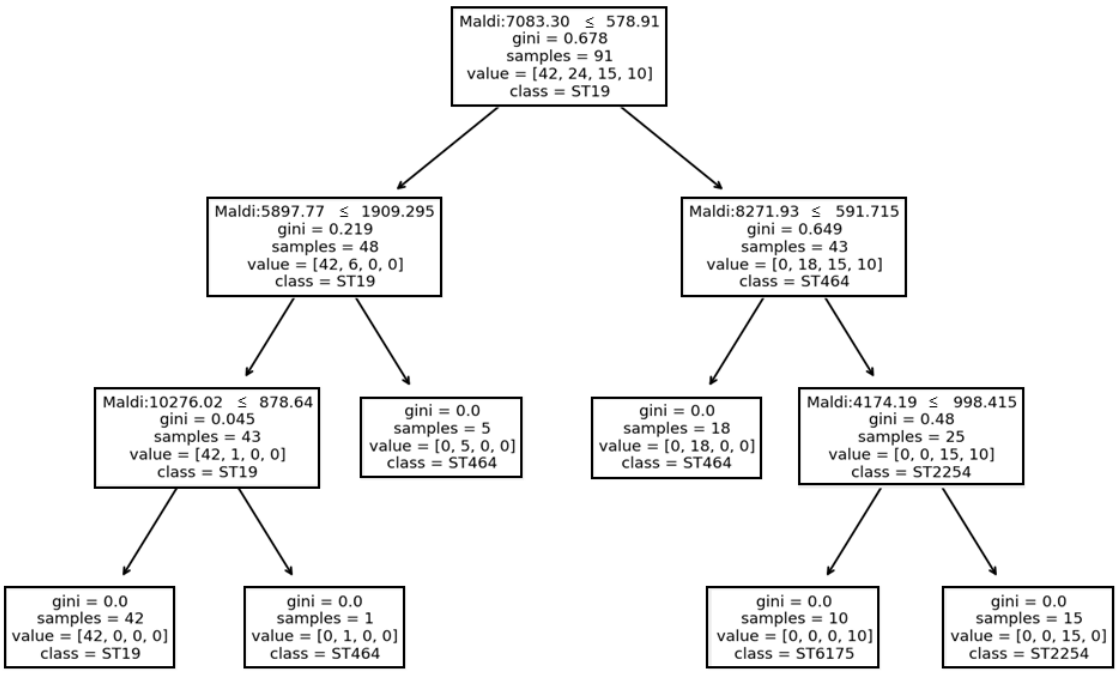

Figure 3. Decision tree trained on the peak matching table with intensity values from the complete dataset (n = 91 MSPs) grouped by their ST profiles. Maldi: peak feature; Gini: measures the probability of misclassifying an observation; samples: illustrates how many samples the node contains; value: refers to how many samples at the node fall into each ST category.

In the latter, patterns of protein peaks, based on their intensities, retained by the algorithms for each class could be observed (Figure 4). Overall, for the classification into four different STs, the DT algorithm was considering only to five proteins over the

91 initially identified by the peak matching. A combination of three peaks was enough for the algorithm to distinguish the three different STs: ST-19, ST-2254 and ST-6175. However, the identification of ST-464 seemed a bit trickier with the involvement of several biomarkers, which may be linked the genetic diversity of isolates classified in six different CTs (75, 596, 1428, 1514, 1668 and 2130) (Figure 1). Interestingly, the 4174.19, 5897.77 and 8271.93 Da peaks are associated with 14.20 Da, 30.17 Da, 15.27 Da shifts respectively, while the 7083.30 and 10,276.02 Da peaks were linked to the intensity's level. Therefore, those proteins are putatively related to the genetic diversity of *C. jejuni*. The Uniprot database has been investigated to give a potential identification of these latter, regardless potential post-translational modifications. Identifications are summarized in the Table 3.

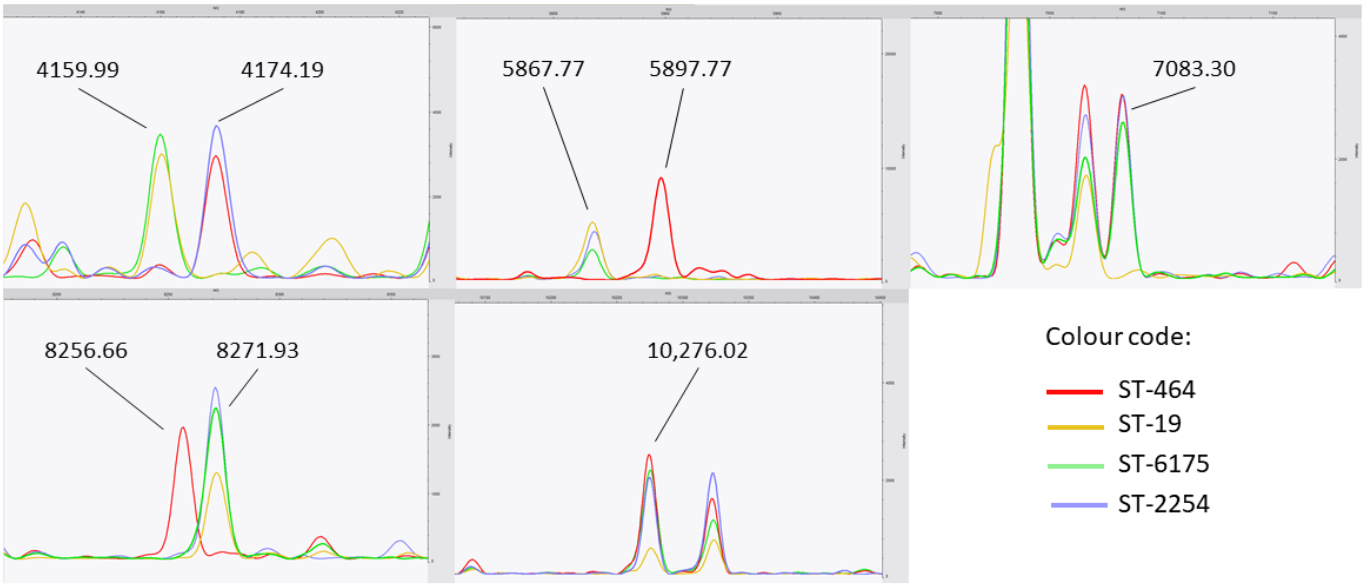

Figure 4. Biomarker mass peak of *C. jejuni* identified by machine learning decision tree algorithm. *X*-axes: mass-to-charge ratio (m/z). *Y*-axes: intensity in arbitrary unit.

Table 3. Putative biomarkers linked to the *Campylobacter* type and their identification through the Uniprot database.

Mass Observed (Da)	Theoretical Average Molecular Weight (Da)	Gene Names	Protein Name	UniProt ID
4159.99	4158.55	APU78_09005	Flagellin subunit protein FlaA	A0A690Z7F7
4174.19	4173.56	CDX23_07240, FQZ36_04085, FV854_03335	Uncharacterized protein	A0A5Z0CYS5
5867.60	5868.02	FH034_10320	Sulfurtransferase-like selenium metabolism protein YedF	A0A5C4YC48
	5867.95	F1576_10330	Magnesium transporter CorA family protein	A0A698D3Z1
	5867.84	FDW21_07355		A0A3Z8JXU3
5897.77	5897.85	GSG42_09710	Motility accessory factor Polysaccharide deacetylase	A0A7I9U468
	5895.96	FXB36_09400	DNA adenine methylase	A0A7I9S1R5
7083.30	7081.66	B9Q65_09070, E7P40_09640, F0N82_09625, FC283_09220, FW424_09040	Uncharacterized protein	A0A400EER0
8256.66	8256.27	JJD26997_1194	Conserved domain protein	A7H434
	8256.39	GD714_06815	Uncharacterized protein	A0A6W1IK17
	8255.77	EJC82_07015	Uncharacterized protein	A0A6C7UKG7
	8256.96	TM42_09010	Membrane protein	A0A0D7V4A9

Table 3. *Cont.*

Mass Observed (Da)	Theoretical Average Molecular Weight (Da)	Gene Names	Protein Name	UniProt ID
8271.93	8270.56	N/A	Uncharacterized protein	Q4VRA4
	8271.37	FW192_09775	Integrase	A0A7I9QCT5
	8271.52	B7Q70_09720	Terminase small subunit	A0A5T0PDL9
10,276.02	10,276.22	JJD26997_0928 AT778_09125, B7Q70_06195,	Uncharacterized protein	A7H3G2
	10,276.17	C3H43_07780,	Uncharacterized protein	A0A2U0QNA2
	10,274.87	C3H69_07590, C3H86_07890 A2E15_06760	Glycosyltransferase Family 9 protein	A0A5T0CX51

4. Discussion

Nowadays, WGS is established as a successful and highly discriminating typing method, providing opportunities for the surveillance and outbreak investigation of foodborne pathogens, such as *Campylobacter* spp. [54]. The main drawback of *Campylobacter* surveillance is the important number of isolates to sequence, due to its status as first bacterial human zoonosis. A high-throughput and cost-efficient method, such as MALDI-TOF MS, could be an efficient pre-screening tool to relevant isolates that warrant further sequencing. By coupling WGS with mass spectrometry, it could increase typing's ability and therefore, elucidate genotypes circulating in human infections, animal production and environment. The aim of this study was to investigate the ability of MALDI-TOF MS, increasingly implemented in routine laboratories, to assess *C. jejuni* genetic diversity and to compare its congruency to MLST and cgMLST methods as gold standards for epidemiologic surveillance.

The main result of this study was to observe that a mass spectrometry approach on 91 automatically generated peaks had a higher discriminatory power than the classical MLST scheme with seven loci for attribution of CCs ($SID_{MLST\text{-}CC}$ = 0.579, $SID_{MALDI\text{-}92\%}$ = 0.830). However, similar discriminatory power has been found for attribution of STs ($SID_{MLST\text{-}CC}$ = 0.829, $SID_{MALDI\text{-}93\%}$ = 0.862). As well, proteomics was compared to the cgMLST scheme, which is more discriminant than MLST typing methods. The discriminatory ability of MALDI-TOF MS was comparable to SeqSphere+ cgMLST scheme based on 637 loci (SID_{cgMLST} = 0.889, $SID_{MALDI\text{-}94\%}$ = 0.939). Sequence based methods, such as MLST, are known to reflect the population genetics and where STs are often related to ecological niches [13,55]. As an outlook of the present study, mass spectra should be investigated to check the potential link between protein profile and host specificity, barely described in the literature for *Campylobacter* spp. [56]. Lawton et al. (2018) reported that MALDI-TOF MS was non-congruent to *Campylobacter* clade identified by either 16S rDNA or WGS and therefore unlikely to be useful for assessing genetic relationship among *C. jejuni* isolates [57]. Nevertheless, in the current study high concordance between genomic and proteomic typing methods was found. MALDI-TOF MS could predict 100.0%, 93.3% and 85.1% of CCs, STs and CTs, respectively. To our knowledge this is the first time that this was demonstrated for cgMLST. There are few reports highlighting the ability of MALDI-TOF MS to subtype bacterial species at the ST level. During a *Klebsiella pneumoniae* outbreak in central China, the vast majority of the epidemic ST11 strains were associated with similar MALDI-TOF MS profiles [37]. MALDI-TOF MS was explored for the subtyping of *Arcobacter butzleri* and compared with MLST. MALDI-TOF MS was less discriminant (SID_{MLST} = 0.920, SID_{MALDI} = 0.863) but still comparable to MLST. Therefore, the possibility of subtyping by MALDI-TOF MS displayed variability in performance according to bacterial species. In addition, it may be explained by the quality, pre-processing steps and chosen similarity cut-off, depending on the level of concordance intended, of mass spectra. Indeed in the study by Meng et al. (2019), a similarity cut-off of 70% was applied according to the ST assignment of *K. pneumoniae*, whereas a 93% one has selected for the current study, based on the number of ST clusters. This difference may be explained by the type of extraction, such as the off-plate procedure,

used in this study, and the on-plate protocol. Standardisation of protocols for such analysis should be as well essential. Indeed, it has been pointed out that growing medium type and conditions could influence MALDI-TOF MS data and congruence with the PFGE typing method [58]. Additionally, other parameters such as preparation protocol used, duration of incubation, maintenance of the device and so on, could impacted the quality of the spectra and hence the MALDI-TOF MS discriminatory power [59]. Additionally, fastidious growing conditions (e.g., microaerophilia), such as the one encountered for *Campylobacter* spp., may be impacting for the spectra.

Analysis of the decision tree pointed out several protein peaks or biomarkers, which may be associated to specific STs. Since 2011, pioneering studies highlighted the extended application of MALDI-TOF MS as a tool to discriminate several genera (e.g., *Clostridium*, *Salmonella* and *Staphylococcus*) at subgroups level based on specific biomarkers [60–62]. The *Campylobacter* community is not an exception to the rule, and several reports bring biomarkers links with allelic profiles to light [39,40,42,43]. In the case of *C. jejuni*, it was already described in the literature that it was possible to discriminate some STs with 14 specific biomarkers while using ethanol/acetonitrile extraction and direct smear deposit based on PCA-dendrograms [39,43]. One biomarker retained in this study was commonly described previously by Zautner and colleagues (e.g., 10,276.02 Da). They ambiguously identified the previous biomarker as a 30S ribosomal protein S18 while it was uncharacterizable in our study. In the same process, the latter authors tentatively tried to identify relevant biomarkers based on the calculated masses ORFs from WGS data. While most of their biomarkers were ribosomal proteins, several current identified proteins are not related to known functions or involved in metabolic pathways encoded by housekeeping genes. However, it is worth to notice that the comparison of biomarkers in both studies is tricky due to the choice of the internal calibration point: recombinant human insulin peak (5808.29 m/z) and the shared BTS peak (4365.00 m/z), in the two studies. Interestingly, the DT algorithm used the shift between the 4159.99 Da and 4174.19 Da peaks to distinguish ST-2254 and ST-6175. It is worth highlighting that isolates from ST-2254 and ST-6175 have different phenotypic behaviour, in term of adhesion and biofilm formation (Nennig et al., manuscript in preparation). The only known protein matching this molecular weight was the flagellin subunit protein FlaA. Combination of MLST with the major outer membrane protein gene (*porA*) and/or with flagellin A gene (*flaA*), called extended MLST, has been widely described in the literature, for the typing of *C. jejuni*, underlining one more time the close similarity between genomics and proteomics methods [9,63–65]. Nevertheless, *flaA* is an accessory gene, giving more flexibility to bacteria for environmental adaptation. In addition, *flaA* shows common and highly variable domains, suggesting that this protein alone, could not be considered as a stable biomarker to assess *C. jejuni* genetic diversity [66,67]. As well, no biomarkers retained in this study had a direct link with housekeeping genes classically used for MLST typing. This assessment is not aberrant, as most of these genes produced proteins with a molecular weight exceeding the 2–20 kDa windows (e.g., AspA: 51,765 Da, GlnA: 53,945 Da) used in the study. To go further, a real peptides sequencing should be performed to assess the actual identity and function of each biomarker.

Several reports highlighted the ability of MALDI-TOF MS to classify different *Staphylococcus aureus* clonal lineages with the help of different machine learning models (e.g., supervised neural network, support vector machine (SVM) and genetic algorithm (GA)). Camoez et al. (2016) reported a sensitivity and specificity of 100.0% and 99.1% for the classification of four *S. aureus* CCs, i.e., which is a group compiling close STs and therefore more general than the ST level. While Zhang et al. (2015) described for the assessment of *S. aureus* ST-239, ST-5, ST-59 and ST-45 by using GA, a sensitivity between 81% and 100% and a specificity between 92% and 100%, we observed an overall sensitivity and specificity for four STs of ranging from 98.1% to 100.0%, respectively. Wang et al. (2018) also described close results on same STs by using a SVM model with an accuracy of 86.4%. All previous cited studies support the idea that machine learning and MALDI-TOF MS

present obvious advantages for MRSA typing, such as rapidity, accuracy and cost-efficiency in comparison with MLST and it can be carried out at the same time that routine identification of isolates. In addition, such an approach may remove the need of retrospective epidemiological analysis. While cgMLST is the reference method for the surveillance of *Campylobacter* spp., the combination of mass spectrometry and artificial intelligence may be a suitable tool to make a pre-selection of what need to be sequenced. Indeed, it has been shown recently that genetic lineages were frequently identified in human infection over-time in Luxembourg [19]. Using the same lineage isolates of Nennig et al. (2021), we pushed the study at the clone level, confirmed by three cgMLST schemes and by comparing the pangenome (wgMLST). Eleven specific MALDI-TOF profiles have been linked to specific lineages identified over-time in Luxembourg. In addition, several close-related MALDI-TOF profiles where only related to these three clones. Thus, *C. jejuni* protein mass spectra may be enough to make an early detection of these recurring lineages. Machine learning is for supporting decision making process and giving suggestions on possible outcomes that lead research in a specific direction. Machine learning prediction based on MALDI-TOF mass spectra may be a frontline tool to make a preliminary screening of these recurring genotypes and identify related MALDI-TOF profiles. Nevertheless, WGS may still follow to further elucidate molecular details in case of an outbreak as it has been recently described in Denmark [5].

Along the same line, a recent preprint introduced a pipeline using a surveillance system recording routine results from clinical laboratories, among them MALDI-TOF mass spectra identification [68]. The system detected an abnormal increase of *Streptococcus pneumoniae* identification in a short interval of time. More identification than planned were recorded by the system. Spectra responsible of the alert and the other records from previous months were retained for clustering analyses, resulting in two subtrees which may be associated to two epidemiological events. Authors highlighted that such an investigation technique is not for subtyping but helps in detecting a possible suspicion of bacterial species spread and to prevent or slow down possible outbreaks. In summary, combination of MALDI-TOF MS, machine learning and WGS could be valuable tools for accurate epidemiological surveillance of *Campylobacter* and potentially other relevant clinical or foodborne pathogens.

Nevertheless, the present study presents several limitations. Thus, so far, only four different STs were used to build the current model. Therefore, if the latter is used to identify another STs, then it will be misclassified. Additionally, to build the current model only strains from the Luxembourg monitoring program have been used, hence model's adaptation will be needed, with the implementation of STs depending on the area of utilization. In the case of *Campylobacter* spp., where the question of the possibility of cross-border genotype existing, it may be critical for long-term monitoring, while using MALDI-TOF MS and machine learning only. However, further analysis must be done to include additional STs, to avoid misclassification, for an accurate and robust screening tool. Additionally, all MALDI-TOF MS analyses were carried out by a unique operator the whole study. Therefore, the operator variation has not been established and may affect conclusions [69]. Finally, spectra were investigated after using the standard off-plate protein extraction using ethanol, formic acid and acetonitrile, used to obtain high-resolution spectra. However, such extraction is not straightforward in a routine context. For that reason, further analyses must be carried out to evaluate the ability of MALDI-TOF MS to subtype *C. jejuni* with spectra obtained with on-plate extraction or direct deposit.

5. Conclusions

In the present study our results provide evidence that MALDI-TOF MS could be a valuable tool to swiftly subtype *C. jejuni*. Such applications may be suitable as a cost-efficient alternative to NGS technologies, with several advantages such as rapidness or congruency with genomics methods up to the CC, ST and CT level. For a "universal", accurate, and early surveillance and integration of routine laboratories, a single mass spectrum analysis

could combine several tests into one examination, i.e., species identification, antimicrobial susceptibility screening and the assessment of genetic diversity. However, WGS may still be needed in addition to MALDI-TOF MS to further assess the relatedness between isolates (e.g., source attribution) in case of an outbreak.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/diagnostics11111949/s1. Supplementary File S1—list and details of isolates used for MALDI-TOF mass spectrometry and machine learning analysis. Supplementary File S2—similarity cluster size plot. Supplementary File S3—Python algorithm for machine learning analysis. Supplementary File S4—contingency table for MALDI, ST and CT profiles comparison. Supplementary File S5—mass spectra peak matching table used for machine learning analysis.

Author Contributions: M.F. carried out MALDI-TOF MS work, data analysis and drafted the manuscript with M.N., C.R., H.-M.C., M.N. and C.R. isolated and performed the molecular and phenotypic characterisation of the *Campylobacter* collection and bring their expertise on the *Campylobacter* surveillance problematic. S.L. gave access his lab for all mass spectrometry analysis. C.P. wrote the project proposal, obtained funding, and aided to interpret mass spectrometry results. H.-M.C. supervised the project. D.M. and S.L.B. reviewed the work and provided a critical analysis of the work. All authors equally contributed to the formal analysis, writing, review, and editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The MALDI-TOF mass spectra peak matching table is available in Supplementary File S5. Raw MALDI-TOF spectra are available upon request.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Butzler, J.-P. *Campylobacter*, from obscurity to celebrity. *Clin. Microbiol. Infect.* **2004**, *10*, 868–876. [CrossRef] [PubMed]
2. EFSA. ECDC the European Union One Health 2019 Zoonoses Report. *EFSA J.* **2021**, *19*, e06406.
3. Devleesschauwer, B.; Bouwknegt, M.; Mangen, M.-J.J.; Havelaar, A.H. Health and economic burden of *Campylobacter*. In *Campylobacter: Features, Detection, and Prevention of Foodborne Disease*; Klein, G., Ed.; Elsevier BV: Amsterdam, The Netherlands, 2017; pp. 27–40.
4. Murray, C.J.L.; Vos, T.; Lozano, R.; Naghavi, M.; Flaxman, A.D.; Michaud, C.; Ezzati, M.; Shibuya, K.; Salomon, J.A.; Abdalla, S.; et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990–2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **2013**, *380*, 2197–2223. [CrossRef]
5. Joensen, K.G.; Schjørring, S.; Gantzhorn, M.R.; Vester, C.T.; Nielsen, H.L.; Engberg, J.H.; Holt, H.M.; Ethelberg, S.; Müller, L.; Sandø, G.; et al. Whole genome sequencing data used for surveillance of *Campylobacter* infections: Detection of a large continuous outbreak, Denmark, 2019. *Eurosurveillance* **2021**, *26*, 2001396. [CrossRef] [PubMed]
6. Llarena, A.-K.; Taboada, E.; Rossi, M. Whole-Genome Sequencing in Epidemiology of *Campylobacter jejuni* Infections. *J. Clin. Microbiol.* **2017**, *55*, 1269–1275. [CrossRef]
7. ISO 23418:2018(E): Microbiology of the Food Chain—Whole Genome Sequencing for Typing and Genomic Characterization of Foodborne Bacteria—General Requirements and Guidance. Available online: https://www.iso.org/standard/75509.html (accessed on 2 August 2021).
8. European Food Safety Authority (EFSA). ECDC The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018 European. *EFSA J.* **2020**, *18*, e06007.
9. Ragimbeau, C.; Schneider, F.; Losch, S.; Even, J.; Mossong, J. Multilocus Sequence Typing, Pulsed-Field Gel Electrophoresis, and fla Short Variable Region Typing of Clonal Complexes of *Campylobacter jejuni* Strains of Human, Bovine, and Poultry Origins in Luxembourg. *Appl. Environ. Microbiol.* **2008**, *74*, 7715–7722. [CrossRef]

10. Ragimbeau, C.; Colin, S.; Devaux, A.; Decruyenaere, F.; Cauchie, H.-M.; Losch, S.; Penny, C.; Mossong, J. Investigating the host specificity of *Campylobacter jejuni* and *Campylobacter coli* by sequencing gyrase subunit A. *BMC Microbiol.* **2014**, *14*, 205. [CrossRef]
11. Mossong, J.; Mughini-Gras, L.; Penny, C.; Devaux, A.; Olinger, C.; Losch, S.; Cauchie, H.-M.; Van Pelt, W.; Ragimbeau, C. Human Campylobacteriosis in Luxembourg, 2010–2013: A Case-Control Study Combined with Multilocus Sequence Typing for Source Attribution and Risk Factor Analysis. *Sci. Rep.* **2016**, *6*, 20939. [CrossRef]
12. Sheppard, S.K.; Jolley, K.A.; Maiden, M.C.J. A Gene-By-Gene Approach to Bacterial Population Genomics: Whole Genome MLST of *Campylobacter*. *Genes* **2012**, *3*, 261–277. [CrossRef]
13. Dingle, K.E.; Colles, F.M.; Wareing, D.R.A.; Ure, R.; Fox, A.J.; Bolton, F.E.; Bootsma, H.J.; Willems, R.J.L.; Urwin, R.; Maiden, M.C.J. Multilocus Sequence Typing System for *Campylobacter jejuni*. *J. Clin. Microbiol.* **2001**, *39*, 14–23. [CrossRef] [PubMed]
14. Dekker, J.P.; Frank, K.M. Commentary: Next-Generation Epidemiology: Using Real-Time Core Genome Multilocus Sequence Typing To Support Infection Control Policy. *J. Clin. Microbiol.* **2016**, *54*, 2850–2853. [CrossRef] [PubMed]
15. Gona, F.; Comandatore, F.; Battaglia, S.; Piazza, A.; Trovato, A.; Lorenzin, G.; Cichero, P.; Biancardi, A.; Nizzero, P.; Moro, M.; et al. Comparison of core-genome MLST, coreSNP and PFGE methods for *Klebsiella pneumoniae* cluster analysis. *Microb. Genom.* **2020**, *6*, 1–12. [CrossRef]
16. Cody, A.J.; Bray, J.E.; Jolley, K.A.; McCarthy, N.D.; Maiden, M.C.J. Core Genome Multilocus Sequence Typing Scheme for Stable, Comparative Analyses of *Campylobacter jejuni* and *C. coli* Human Disease Isolates. *J. Clin. Microbiol.* **2017**, *55*, 2086–2097. [CrossRef] [PubMed]
17. Rossi, M.; Silva, M.; Gonçalves, B.; Silva, D.N.; Machado, M.P.; Oleastro, M.; Borges, V.; Isidro, J.; Viera, L.; Halkilahti, J.; et al. Innuendo Whole Genome and Core Genome Mlst Schemas and Datasets for Salmonella enterica. Available online: https://zenodo.org/record/1322564 (accessed on 11 August 2021).
18. Llarena, A.; Ribeiro-Gonçalves, B.F.; Nuno Silva, D.; Halkilahti, J.; Machado, M.P.; Da Silva, M.S.; Jaakkonen, A.; Isidro, J.; Hämäläinen, C.; Joenperä, J.; et al. INNUENDO: A cross-sectoral platform for the integration of genomics in the surveillance of food-borne pathogens. *EFSA Support. Publ.* **2018**, *15*, 1498E. [CrossRef]
19. Nennig, M.; Llarena, A.-K.; Herold, M.; Mossong, J.; Penny, C.; Losch, S.; Tresse, O.; Ragimbeau, C. Investigating Major Recurring *Campylobacter jejuni* Lineages in Luxembourg Using Four Core or Whole Genome Sequencing Typing Schemes. *Front. Cell. Infect. Microbiol.* **2021**, *10*, 8020. [CrossRef]
20. Magana, M.; Chatzipanagiotou, S.; Burriel, A.R.; Ioannidis, A. Inquiring into the Gaps of *Campylobacter* Surveillance Methods. *Vet. Sci.* **2017**, *4*, 36. [CrossRef]
21. Angeletti, S.; Ciccozzi, M. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry in clinical microbiology: An updating review. *Infect. Genet. Evol.* **2019**, *76*, 104063. [CrossRef]
22. Robert, M.-G.; Cornet, M.; Hennebique, A.; Rasamoelina, T.; Caspar, Y.; Pondérand, L.; Bidart, M.; Durand, H.; Jacquet, M.; Garnaud, C.; et al. MALDI-TOF MS in a Medical Mycology Laboratory: On Stage and Backstage. *Microorganisms* **2021**, *9*, 1283. [CrossRef]
23. Alcalá, L.; Marín, M.; Ruiz, A.; Quiroga, L.; Zamora-Cintas, M.; Fernández-Chico, M.A.; Muñoz, P.; Rodríguez-Sánchez, B. Identifying Anaerobic Bacteria Using MALDI-TOF Mass Spectrometry: A Four-Year Experience. *Front. Cell. Infect. Microbiol.* **2021**, *11*, 300. [CrossRef] [PubMed]
24. Sy, I.; Margardt, L.; Ngbede, E.O.; Adah, M.I.; Yusuf, S.T.; Keiser, J.; Rehner, J.; Utzinger, J.; Poppert, S.; Becker, S.L. Identification of Adult *Fasciola* spp. Using Matrix-Assisted Laser/Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry. *Microorganisms* **2021**, *9*, 82. [CrossRef]
25. Bredtmann, C.M.; Krücken, J.; Murugaiyan, J.; Balard, A.; Hofer, H.; Kuzmina, T.A.; Von Samson-Himmelstjerna, G.; Samson-Himmelstjerna, G. Concurrent Proteomic Fingerprinting and Molecular Analysis of Cyathostomins. *Proteomics* **2019**, *19*, e1800290. [CrossRef]
26. Marzano, V.; Pane, S.; Foglietta, G.; Mortera, S.L.; Vernocchi, P.; Muda, A.O.; Putignani, L. Mass Spectrometry Based-Proteomic Analysis of *Anisakis* spp.: A Preliminary Study towards a New Diagnostic Tool. *Genes* **2020**, *11*, 693. [CrossRef] [PubMed]
27. Rybicka, M.; Miłosz, E.; Bielawski, K.P. Superiority of MALDI-TOF Mass Spectrometry over Real-Time PCR for SARS-CoV-2 RNA Detection. *Viruses* **2021**, *13*, 730. [CrossRef]
28. Iles, R.K.; Zmuidinaite, R.; Iles, J.K.; Carnell, G.; Sampson, A.; Heeney, J.L. A clinical MALDI-ToF Mass spectrometry assay for SARS-CoV-2: Rational design and multi-disciplinary team work. *Diagnostics* **2020**, *10*, 746. [CrossRef]
29. Hernandez, M.M.; Banu, R.; Shrestha, P.; Patel, A.; Chen, F.; Cao, L.; Fabre, S.; Tan, J.; Lopez, H.; Chiu, N.; et al. RT-PCR/MALDI-TOF mass spectrometry-based detection of SARS-CoV-2 in saliva specimens. *J. Med. Virol.* **2021**, *93*, 5481–5486. [CrossRef]
30. Yan, L.; Yi, J.; Huang, C.; Zhang, J.; Fu, S.; Li, Z.; Lyu, Q.; Xu, Y.; Wang, K.; Yang, H.; et al. Rapid Detection of COVID-19 Using MALDI-TOF-Based Serum Peptidome Profiling. *Anal. Chem.* **2021**, *93*, 4782–4787. [CrossRef] [PubMed]
31. Axelsson, C.; Rehnstam-Holm, A.-S.; Nilson, B. Rapid detection of antibiotic resistance in positive blood cultures by MALDI-TOF MS and an automated and optimized MBT-ASTRA protocol for *Escherichia coli* and *Klebsiella pneumoniae*. *Infect. Dis.* **2020**, *52*, 45–53. [CrossRef]
32. Sparbier, K.; Schubert, S.; Kostrzewa, M. MBT-ASTRA: A suitable tool for fast antibiotic susceptibility testing? *Methods* **2016**, *104*, 48–54. [CrossRef] [PubMed]
33. Oviaño, M.; Bou, G. Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond. *Clin. Microbiol. Rev.* **2018**, *32*, e00037-18. [CrossRef]

34. Wolters, M.; Rohde, H.; Maier, T.; Belmar-Campos, C.; Franke, G.; Scherpe, S.; Aepfelbacher, M.; Christner, M. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. *Int. J. Med. Microbiol.* **2011**, *301*, 64–68. [CrossRef] [PubMed]
35. Wang, H.-Y.; Lee, T.-Y.; Tseng, Y.-J.; Liu, T.-P.; Huang, K.-Y.; Chang, Y.-T.; Chen, C.-H.; Lu, J.-J. A new scheme for strain typing of methicillin-resistant *Staphylococcus aureus* on the basis of matrix-assisted laser desorption ionization time-of-flight mass spectrometry by using machine learning approach. *PLoS ONE* **2018**, *13*, e0194289. [CrossRef] [PubMed]
36. Egli, A.; Tschudin-Sutter, S.; Oberle, M.; Goldenberger, D.; Frei, R.; Widmer, A.F. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass-Spectrometry (MALDI-TOF MS) Based Typing of Extended-Spectrum β-Lactamase Producing *E. coli*—A Novel Tool for Real-Time Outbreak Investigation. *PLoS ONE* **2015**, *10*, e0120624. [CrossRef]
37. Meng, X.; Yang, J.; Duan, J.; Liu, S.; Huang, X.; Wen, X.; Huang, X.; Fu, C.; Li, J.; Dou, Q.; et al. Assessing Molecular Epidemiology of Carbapenem-resistant *Klebsiella pneumoniae* (CR-KP) with MLST and MALDI-TOF in Central China. *Sci. Rep.* **2019**, *9*, 2271. [CrossRef]
38. Giacometti, F.; Piva, S.; Vranckx, K.; De Bruyne, K.; Drigo, I.; Lucchi, A.; Manfreda, G.; Serraino, A. Application of MALDI-TOF MS for the subtyping of *Arcobacter butzleri strains* and comparison with their MLST and PFGE types. *Int. J. Food Microbiol.* **2018**, *277*, 50–57. [CrossRef] [PubMed]
39. Zautner, A.E.; Masanta, W.O.; Tareen, A.M.; Weig, M.; Lugert, R.; Groß, U.; Bader, O. Discrimination of multilocus sequence typing-based *Campylobacter jejuni* subgroups by MALDI-TOF mass spectrometry. *BMC Microbiol.* **2013**, *13*, 247. [CrossRef]
40. Emele, M.F.; Karg, M.; Hotzel, H.; Bloois, L.G.-V.; Groß, U.; Bader, O.; Zautner, A.E. Differentiation of *Campylobacter fetus* subspecies by proteotyping. *Eur. J. Microbiol. Immunol.* **2019**, *9*, 62–71. [CrossRef]
41. Zautner, A.E.; Masanta, W.O.; Weig, M.; Groß, U.; Bader, O. Mass Spectrometry-based PhyloProteomics (MSPP): A novel microbial typing Method. *Sci. Rep.* **2015**, *5*, 13431. [CrossRef]
42. Emele, M.F.; Možina, S.S.; Lugert, R.; Bohne, W.; Masanta, W.O.; Riedel, T.; Groß, U.; Bader, O.; Zautner, A.E. Proteotyping as alternate typing method to differentiate *Campylobacter coli* clades. *Sci. Rep.* **2019**, *9*, 4244. [CrossRef] [PubMed]
43. Zautner, A.E.; Lugert, R.; Masanta, W.O.; Weig, M.; Groß, U.; Bader, O. Subtyping of *Campylobacter jejuni* ssp. doylei Isolates Using Mass Spectrometry-based PhyloProteomics (MSPP). *J. Vis. Exp.* **2016**, e54165. [CrossRef] [PubMed]
44. Weis, C.; Jutzeler, C.; Borgwardt, K.M. Machine learning for microbial identification and antimicrobial susceptibility testing on MALDI-TOF mass spectra: A systematic review. *Clin. Microbiol. Infect.* **2020**, *26*, 1310–1317. [CrossRef]
45. Weis, C.; Cuénod, A.; Rieck, B.; Llinares-López, F.; Dubuis, O.; Graf, S.; Lang, C.; Oberle, M.; Soegaard, K.K.; Osthoff, M.; et al. Direct Antimicrobial Resistance Prediction from MALDI-TOF mass spectra profile in clinical isolates through Machine Learning. *bioRxiv* **2020**, *1*, 1–35.
46. Delavy, M.; Cerutti, L.; Croxatto, A.; Prod'Hom, G.; Sanglard, D.; Greub, G.; Coste, A.T. Machine Learning Approach for *Candida albicans* Fluconazole Resistance Detection Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. *Front. Microbiol.* **2020**, *10*, 3000. [CrossRef] [PubMed]
47. Ling, J.; Wang, H.; Li, G.; Feng, Z.; Song, Y.; Wang, P.; Shao, H.; Zhou, H.; Chen, G. A novel short-term high-lactose culture approach combined with a matrix-assisted laser desorption ionization-time of flight mass spectrometry assay for differentiating *Escherichia coli* and Shigella species using artificial neural networks. *PLoS ONE* **2019**, *14*, e0222636. [CrossRef] [PubMed]
48. Nakano, S.; Matsumura, Y.; Ito, Y.; Fujisawa, T.; Chang, B.; Suga, S.; Kato, K.; Yunoki, T.; Hotta, G.; Noguchi, T.; et al. Development and evaluation of MALDI-TOF MS-based serotyping for *Streptococcus pneumoniae*. *Eur. J. Clin. Microbiol. Infect. Dis.* **2015**, *34*, 2191–2198. [CrossRef]
49. Camoez, M.; Sierra, J.M.; Dominguez, M.; Ferrer-Navarro, M.; Vila, J.; Roca, I. Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin. Microbiol. Infect.* **2016**, *22*, 161.e1–161.e7. [CrossRef] [PubMed]
50. Zhang, T.; Ding, J.; Rao, X.; Yu, J.; Chu, M.; Ren, W.; Wang, L.; Xue, W. Analysis of methicillin-resistant *Staphylococcus aureus* major clonal lineages by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI–TOF MS). *J. Microbiol. Methods* **2015**, *117*, 122–127. [CrossRef]
51. Severiano, A.; Pinto, F.; Ramirez, M.; Carriço, J.A. Adjusted Wallace Coefficient as a Measure of Congruence between Typing Methods. *J. Clin. Microbiol.* **2011**, *49*, 3997–4000. [CrossRef]
52. Hunter, P.R.; Gaston, M.A. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *J. Clin. Microbiol.* **1988**, *26*, 2465–2466. [CrossRef]
53. Grandini, M.; Bagli, E.; Visani, G. Metrics for Multi-Class Classification: An Overview. *arXiv* **2020**, arXiv:2008.05756.
54. Uelze, L.; Grützke, J.; Borowiak, M.; Hammerl, J.A.; Juraschek, K.; Deneke, C.; Tausch, S.H.; Malorny, B. Typing methods based on whole genome sequencing data. *One Health Outlook* **2020**, *2*, 3. [CrossRef] [PubMed]
55. McCarthy, N.D.; Colles, F.M.; Dingle, K.; Bagnall, M.C.; Manning, G.; Maiden, M.C.J.; Falush, D. Host-associated Genetic Import in *Campylobacter jejuni*. *Emerg. Infect. Dis.* **2007**, *13*, 267–272. [CrossRef]
56. Denis, M.; Rose, V.; Nagard, B.; Serghine, J.; Meunier, M.; Benoit, F.; Rince, A.; Cauvin, E.; Gourmelon, M. Comparison of *Campylobacter jejuni* using MALDI-TOF to identify relationships between different origins: Poultry, bovine, sheep, shorebird, river and shellfish. In Proceedings of the Conference: MICROBE, 15th Congress of the French Society of Microbiology (SFM), Paris, France, 30 September–2 October 2019.

57. Lawton, S.J.; Weis, A.M.; Byrne, B.A.; Fritz, H.; Taff, C.C.; Townsend, A.K.; Weimer, B.; Mete, A.; Wheeler, S.; Boyce, W.M. Comparative analysis of *Campylobacter* isolates from wild birds and chickens using MALDI-TOF MS, biochemical testing, and DNA sequencing. *J. Vet. Diagn. Investig.* **2018**, *30*, 354–361. [CrossRef] [PubMed]
58. Jadhav, S.; Gulati, V.; Fox, E.M.; Karpe, A.; Beale, D.J.; Sevior, D.; Bhave, M.; Palombo, E.A. Rapid identification and source-tracking of *Listeria monocytogenes* using MALDI-TOF mass spectrometry. *Int. J. Food Microbiol.* **2015**, *202*, 1–9. [CrossRef]
59. Cuénod, A.; Egli, A. Advanced Applications of MALDI-TOF MS—Typing and Beyond. In *Application and Integration of Omics-powered Diagnostics in Clinical and Public Health Microbiology*; Moran-Gilad, J., Yagel, Y., Eds.; Springer: Heidelebrg/Berlin, Germany, 2021; pp. 153–173.
60. Josten, M.; Reif, M.; Szekat, C.; Al-Sabti, N.; Roemer, T.; Sparbier, K.; Kostrzewa, M.; Rohde, H.; Sahl, H.-G.; Bierbaum, G. Analysis of the Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrum of *Staphylococcus aureus* Identifies Mutations That Allow Differentiation of the Main Clonal Lineages. *J. Clin. Microbiol.* **2013**, *51*, 1809–1817. [CrossRef]
61. Kuhns, M.; Zautner, A.E.; Rabsch, W.; Zimmermann, O.; Weig, M.; Bader, O.; Groß, U. Rapid Discrimination of *Salmonella enterica* Serovar Typhi from Other Serovars by MALDI-TOF Mass Spectrometry. *PLoS ONE* **2012**, *7*, e40004. [CrossRef]
62. Reil, M.; Erhard, M.; Kuijper, E.; Kist, M.; Zaiss, H.; Witte, W.; Gruber, H.; Borgmann, S. Recognition of *Clostridium difficile* PCR-ribotypes 001, 027 and 126/078 using an extended MALDI-TOF MS system. *Eur. J. Clin. Microbiol. Infect. Dis.* **2011**, *30*, 1431–1436. [CrossRef] [PubMed]
63. Djordjevic, S.P.; Unicomb, L.E.; Adamson, P.J.; Mickan, L.; Rios, R.; Adamson, P.; Cheung, K.; Combs, B.; Dalton, C.; Doyle, R.; et al. Clonal Complexes of *Campylobacter jejuni* Identified by Multilocus Sequence Typing Are Reliably Predicted by Restriction Fragment Length Polymorphism Analyses of the flaA Gene. *J. Clin. Microbiol.* **2007**, *45*, 102–108. [CrossRef]
64. Mohan, V.; Habib, I. Multilocus sequence typing (MLST), porA and flaA typing of *Campylobacter jejuni* isolated from cats attending a veterinary clinic. *BMC Res. Notes* **2019**, *12*, 76. [CrossRef]
65. Dingle, K.E.; McCarthy, N.D.; Cody, A.J.; Peto, T.E.A.; Maiden, M.C.J. Extended Sequence Typing of *Campylobacter* spp. *Emerg. Infect. Dis.* **2008**, *14*, 1620–1622. [CrossRef]
66. Harrington, C.S.; Thomson-Carter, F.M.; Carter, P.E. Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: Implications for the flagellin gene typing scheme. *J. Clin. Microbiol.* **1997**, *35*, 2386–2392. [CrossRef] [PubMed]
67. Fischer, S.H.; Nachamkin, I. Common and variable domains of the flagellin gene, flaA, in *Campylobacter jejuni*. *Mol. Microbiol.* **1991**, *5*, 1151–1158. [CrossRef]
68. Giraud-gatineau, A. Using MALDI-TOF spectra in epidemiological surveillance for the detection of bacterial subgroups with a possible epidemic potential. *Res. Sq.* **2021**, *1*, 1–16.
69. Oberle, M.; Wohlwend, N.; Jonas, D.; Maurer, F.; Jost, G.; Tschudin-Sutter, S.; Vranckx, K.; Egli, A. The Technical and Biological Reproducibility of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) Based Typing: Employment of Bioinformatics in a Multicenter Study. *PLoS ONE* **2016**, *11*, e0164260. [CrossRef] [PubMed]

Part III

Discussion and Conclusions

CHAPTER 8

General discussion and perspectives

Contents

1. A look back on the research project

For decades, AMR has been considered as a global long-lasting challenge. If no action is taken, AMR-related diseases could give a rise up to 10 million deaths each year by 2050 and 24 million people might end into extreme poverty, mainly in low-income countries (O'Neill, 2016; IACG, 2019). In this first section of the discussion, societal challenges as well as means used in the presented research work will be put back in context.

1.1 The urge to use a One-Health approach

Two extensive drivers of AMR could be highlighted. The first one is the use of antibiotics exerting a pressure of selection on bacterial population, enabling the emergence or the persistence of resistant microorganisms (ECDC, 2014). The second one is the spread and cross-transmission of drug-resistant organisms between human, animal and environment (ECDC, 2014). For example, top ranking zoonotic pathogens including *Campylobacter* and *Salmonella* are present all along the farm-

to-fork chain (Wielinga and Schlundt, 2014). Because of the overuse of antibiotics in food-producing animals for therapeutic and non-therapeutic (e.g. prophylaxis or growth promotion (banished in 2006 in the EU)) purposes, they could carry multidrug resistant pathogens that could be transmitted from animals to humans via food sources or from direct animal contact (EU, 2005; ECDC, 2014). A significant positive association between consumption of fluroquinolones by food-producing animals and occurrences of resistance in *C. jejuni* and *C. coli* from these animals were reported between 2013 and 2015 (ECDC et al., 2017). Similar observation was made between the occurrence of resistance to fluoroquinolones in *C. jejuni* and *C. coli* from animals and the occurrence of resistance in *C. jejuni* and *C. coli* from human infections (ECDC et al., 2017). Therefore, over the last years, international policies makers, e.g. WHO, ECDC, CDC, OIE and FAO, strongly encouraged a One-Health approach to solve this global burden, recognizing that the human health is connected to animals and environments (FAO et al.; CDC, 2015; WHO, 2019; ECDC, 2021; European Commission, 2021).

1.2 Rapid diagnostics tests to tackle AMR

Nowadays, rapid diagnostics tests are considered as a key tool in diagnostics for the swift identification of infectious organisms as well as the fight against AMR. While such tests were reported to reduce mortality, decreased healthcare costs and lessen hospital stays, they were proved to reduce antibiotics use (Kaprou et al., 2021). Holmes et al. (2018) evaluate the use of point-of-care C-Reactive protein testing in routine primary care with patient with acute respiratory tract infections. They evaluated a decreased of 74% and 89% in antibiotic and unnecessary prescribing, respectively (Holmes et al., 2018). By detecting earlier AMR, adapted antibiotherapy might be administrated promptly shifting from empirical to evidence-based practices, conserving effectiveness of certain antimicrobials (Holmes et al., 2018; Vasala et al., 2020). While the development of such diagnostics tests is actively strengthened by policies makers, competitions (e.g. Longitude prize, https://longitudeprize.org/), are also calling for innovative ideas to tackle AMR. In 2020, Visby Medical Inc. received the 19 million USD prize of the AMR Diagnostic Challenge, for the development of a rapid test able to identify organisms that cause gonorrhoea and its related susceptibility profile under 30 min (NIH, 2020).

The already implemented cost- and time-efficient MALDI-TOF MS in routine laboratories for the identification of microorganisms based on expressed protein profiles, was successfully applied for bacterial typing and detection of specific AMR peak in a research context (Feucherolles et al., 2019). Over the last five years, the breakthrough combination of MALDI-TOF MS with ML was investigated for both AMR prediction and subtyping (Weis et al., 2020b). While, conventional mass spectra analysis relies on few features, such as peak height or area under the peak, ML algorithms

are able to extract and analyse useful information which are embedded in mass spectra, that conventional approaches cannot detect (Weis et al., 2020b). In the line of developing rapid tests for diagnostics, MALDI-TOF MS seemed to be an ideal candidate for a powerful and promising "One fit-all" diagnostics tool.

1.3 Play your part

As a reminder, the presented research work aimed to investigate two principal axes. The first one was to determine if mass spectra profiles generated by MALDI-TOF MS, mainly based on the expression of mainly highly conserved housekeeping proteins comprised in the 2-20 kDa range (e.g. ribosomal proteins L35 at 7159 m/z), commonly used for microorganism's identification could reflect the AMR background of *Campylobacter* spp. (Chapter 6) (Ryzhov and Fenselau, 2001). If such observation turned out to be accurate, so putative protein biomarkers were explored to make a possible link between the protein expression and known or unknown biological mechanisms. Additionally, spectra generated for previous purposes were analysed to determine if they could give a vision on the population structure and diversity of *Campylobacter* spp (Chapter 7). For this, a *Campylobacter* collection, based on *C. jejuni* and *C. coli* isolates from humans, livestocks, including poultry, ruminants, ovine, pigs, and environment sources, such as surface water and wildlife was established. Before MS analysis isolates were phenotypically and genotypically characterized at the AMR and subtype level by conventional methods, i.e. disk-diffusion antibiogram and WGS.

2. Key information embedded in protein profiles

In this second section, results obtained in the results part will be discussed with a One-Health perspective regarding the technical and scientific questions.

2.1 MALDI-TOF MS as an important tool for tackling AMR

One of the objectives of this research work was to determine if the analysis of the proteins expressed in the 2-20 kDa MALDI-TOF mass spectrometer's range could allow the profiling of AMR in *Campylobacter* spp. Through the Chapter 6, protein mass spectra of 340 *Campylobacter* isolates, tested phenotypically for seven antibiotics, were explored by using a ML prediction approach as an AMR screening tool. The key findings of this part were the (1) high performance of three Machine Learning (ML) classifiers, i.e. susceptibility, ciprofloxacin and tetracycline, (2) regardless or not of the microbial identification and (3) the type of protein extractions performed. While a specific expression of proteins might be linked to the fluoroquinolones and tetracyclines resistance profile, lower prediction performances for other antimicrobial classes, i.e. aminoglycosides, β-lactams and

macrolides were obtained. Such approach for the rapid screening of fluoroquinolones and tetracyclines in both veterinary and human medicine could be interesting.

On the one hand, 61% of *C. jejuni* and *C. coli* isolated in 2019 in humans were resistant to ciprofloxacin, while association of macrolides and fluoroquinolones are still drugs of choice for the treatment of enteric illness (Sproston et al., 2018; EFSA and ECDC, 2021). Therefore, fluoroquinolones resistant *Campylobacter* spp. are responsible of treatment failure and symptomatic relapsed (Yang et al., 2019). Already in the late 1980's, 20% of patients with *Campylobacter* infections were relapsing because of resistant isolates (Piddock, 1999). Additionally, the co-resistance to fluoroquinolones and macrolides (which is generally low for *C. jejuni* (1%) and moderate for *C. coli* (10%)) is concerning in Europe from human clinical isolates (EFSA and ECDC, 2021). On the other hand, 74% and 87% of respectively *C. jejuni* and *C. coli* isolated in broilers were resistant to ciprofloxacin in Europe in 2018-2019. As well, 7% and 17% of *C. coli* isolated in broilers and turkey respectively were resistant to erythromycin (EFSA and ECDC, 2021). While it is known that occurrence of fluoroquinolones in food animal producing and human is strongly associated, there are still common antibiotic classes used in both human and animal medicine for therapeutic or prophylactic purposes (ECDC et al., 2017). For example, erythromycin is used in poultry for the control of respiratory diseases caused by *Mycoplasma* but also for the treatment of complicated *Campylobacter* infections in humans (Trott et al., 2021). Likewise for human diagnosis, the European commission encouraged the used of rapid diagnostic tests to reduce and improve antibiotics use in food-producing animals (European Commission, 2015; Buller et al., 2020; Chan et al., 2020). Nevertheless, over the last past years significant advances were made for rapid diagnostics in humans in comparison to the veterinary field (Chan et al., 2020).

In the presented study, the fluoroquinolone prediction model was able to predict positively in 75% of the cases *Campylobacter* ciprofloxacin resistance based on MALDI-TOF protein profile. Few isolates were erythromycin resistant (n = 33/340 isolates). Therefore, little isolates were available to build a reliable and precise prediction model. For example, when a spectrum was returned as macrolides resistant, there was 43% of probability that the actual spectrum was resistant to macrolides. In order to ensure that erythromycin resistance could not be detected using protein spectra, additional resistant strains should be analysed to balance the current dataset. Furthermore, the size of the training set was underlined as critically important for classifier's predictive power and pattern recognition (Figueroa et al., 2012; Vabalas et al., 2019). While in this context it could be asked how much the size of the initial training set should had be to validate the current research work, it is a complex question, and it is unknowable in advance (e.g. complexity of the problem or learning algorithm). At the time of the experimental design, it was not known that

ML based methods exist to estimate the training sample size required to a specific accuracy target (Brownlee, 2017). Figueroa et al. (2012) designed a sample size prediction algorithm that can help to determine sample size for supervised machine learning. Also by investigating the learning curve, i.e. visual representation of the training and validation scores with an increasing number of training sample, it might be able to identify how much or little data you actually need (Brownlee, 2017). According to the learning curve of the erythromycin dataset, while up to 200 Main Spectra profiles are used as a training set, the accuracy remains unchanged between the training and test set. However, beyond 200 spectra it exists a gap between both sets. Therefore, it might be wiser to use this ratio for the training of the prediction model.

Due to the weaknesses of the *Campylobacter* European surveillance and the poor information regarding macrolides resistance predictions using MALDI-TOF protein mass spectra, there is a niche to develop rapid tests for the detection of such resistance. Thus, it will be worth to further explore the ability of prediction of erythromycin resistance based on proteins profile, by implementing additional isolates (Weis et al., 2020a; Yoon and Jeong, 2021). By developing such prediction approach for the screening of fluoroquinolones and macrolides resistances in clinical or veterinary diagnostic practice, it might rationalize antimicrobials use by adapting earlier the definitive antibiotic treatment. Therefore, it could limit the empirical treatment strategy and optimize treatment success. However, as mentioned in Chapter 6 discussion, little is known on the impact of combination of ML and MALDI-TOF MS on health management. Reports suggested that applications of such technique could provide a treatment guidance from 12 to 72 hours earlier than classical approaches (Weis et al., 2020a). On the veterinarian side, the implementation of rapid tests might be also useful to tackle AMR. Likewise for human diagnostics, rapid AMR tests could help reduce the emergence of AMR, by enabling the use of the most appropriate antimicrobial when therapy is required (OIE, 2015). Nevertheless, Chan et al. (2020) reported that in the United Kingdom 70% of farm vets rather rely on their skills and experiences, instead of diagnostic tests for prescribing antibiotics treatments (Chan et al., 2020). Therefore, an additional awareness-raising work concerning the need for accurate testing might be performed upstream with farmers and veterinarians.

The proof of concept to use MALDI-TOF and ML to screen AMR was performed for *C. jejuni* and *C. coli*. Other enteric pathogens, such as *E. coli*, *Yersinia enterolitica*, *Salmonella* spp. and *Shigella* spp., tested for four different antibiotics were also submitted for MALDIT-TOF MS analysis (**Figure 8.1**). Protein mass spectra were grouped according their similarity. While distinct groups could be observed for *E. coli*, *Y. enterolitica* and *S. flexneri*, no clear distinction was obtained for *Salmonella* spp. and *S. sonnei*. Thus, proteins expressed in the 2-20 kDa range might be relevant to rapidly

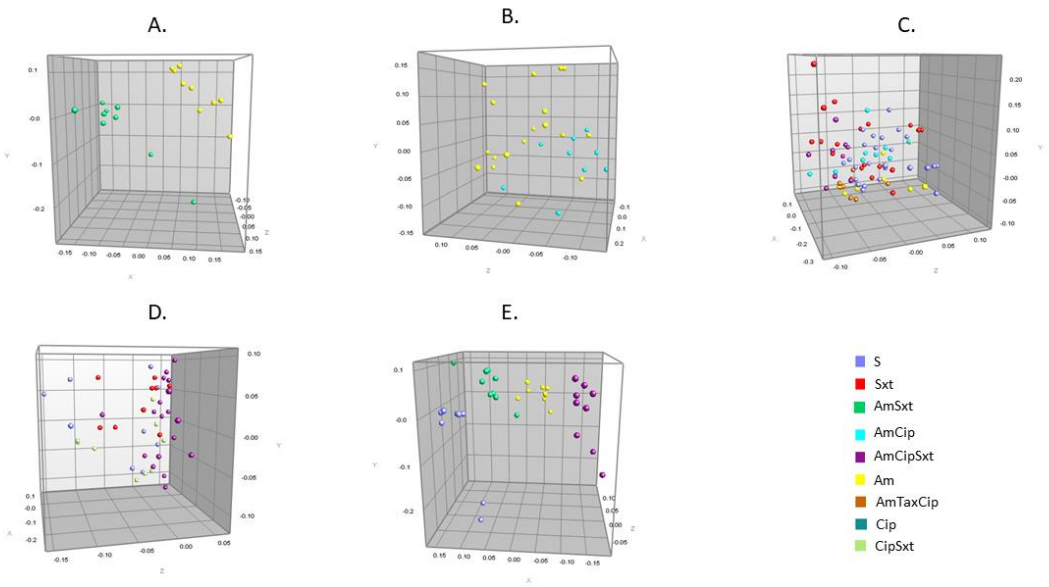

Figure 8.1. *Multidimensional Dimensional Scaling (MDS) of different enteric pathogens based on their MALDI-TOF MS protein profiles grouped by their phenotypic AMR. A:* Escherichia coli *(n = 2). B:* Yersinia enterolitica *(n = 3). C:* Salmonella *spp. (n = 10).* D: Shigella sonnei *(n = 5). E:* Shigella flexneri *(n = 4). Abbreviations: S, susceptible to the four tested antibiotics; Am, ampicillin; Cip, ciprofloxacin; Sxt, trimethoprim/sulfamethoxazole; Tax, cefotaxime.*

screen resistance for those species. However, due to the small sample size of each species it should be cautiously interpreted. While, specific MALDI-TOF MS biomarker related to AMR were reported in the literature for *E. coli* and other enterococci, few data is available for *Shigella* or *Yersinia* (Sabença et al., 2020; Sousa et al., 2020). Additionally, MALDI-TOF MS is mainly investigated as an alternative to serotyping methods for the identification of *Salmonella* serovars (e.g. Enteritidis, Typhimurium and Thompson)(Yang et al., 2021). However, there is no documentation on its application for AMR screening. Recently more and more research works are focusing on the use of WGS data and ML for the *in silico* assessment of AMR (Nguyen et al., 2019; Steinkey et al., 2020; Barros, 2021). For example, Nguyen et al. (2019) trained an algorithm on numerous non-typhoidal *S. enterica* WGS data to predict the minimal inhibition concentration for 15 antibiotics. Their model obtained an overall 95% of accuracy (Nguyen et al., 2019). In line with the previous example, it is worth noting that WGS has a higher resolution and discriminatory power than MALDI-TOF MS, and is on its way to become the reference method for AMR detection in routine diagnostics (Rossen et al., 2018). Additionally, from 2025 AMR monitoring of food-producing isolates, such as ESBL/AmpC/carbapenemase-producing *E. coli*, will be done by WGS (Aerts et al., 2019). Therefore, a combination of MALDI-TOF MS, ML and WGS could be an

interesting monitoring tool with a relevant impact on the control of the emergence of AMR in the European Union.

To wrap up the AMR part of the discussion, work findings suggested that proteins involved in the acquisition of MALDI-TOF mass spectra could reflect the AMR profile of certain resistances, such as ciprofloxacin and tetracycline in *Campylobacter* spp. Regarding other resistances, further analysis with additional isolates are needed to confirm current observation. For further reasoning, while performant prediction signals were obtained while investigating specifically AMR, the latter were obtained for resistance due to a mutation in the gene, i.e. fluoroquinolones, and the acquisition of a specific resistance gene, i.e. tetracyclines. Therefore, it could be interesting to explore the possibility that beyond the species identification, it is the resistance mechanisms themselves (e.g. MOMP or CmeABC efflux pump) that could be highlighting through protein spectra and potentially transferable to other foodborne pathogens. As ciprofloxacin is a drug of interest in both clinical and veterinary practice, implementation of MALDI-TOF MS and ML for rapid species identification and susceptibility screening could be an interesting tool to tackle AMR by optimizing antibiotic prescription.

2.2 MALDI-TOF MS as a powerful subtyping method

The second objective of this study was to determine if the same protein profile used for AMR screening could also give an appreciation on the genetic diversity of *Campylobacter* spp. and hence its population structure. Through Chapter 7, protein mass spectra generated by MALDI-TOF MS of 126 *C. jejuni* isolates featuring different CC, ST and CT determined by the SeqSphere+ cgMLST were subjected to congruence and ML analysis. The main findings of this part were that (1) protein profiles allowed to predict *C. jejuni* CCs, STs, and CTs at 100%, 93% and 85% respectively, and (2) ML based on protein fingerprints enables to efficiently predict STs with a sensitivity and specificity of 98.1% and 100%, respectively.

Currently WGS remains the reference method for the typing and hence surveillance and outbreak investigation of *Campylobacter* spp. (Uelze et al., 2020). On the one hand, as already mentioned, and reviewed in the discussion of the Chapter 7, due to extensive number of isolates to sequence, high-throughput and cost-efficient MALDI-TOF MS could pre-screen relevant isolates that warrant further sequencing. Therefore, by combining WGS and MS it could increase *Campylobacter* typeability through a rapid, accurate and early surveillance by routine laboratories and national reference centres. On the other hand, a gene-by-gene approach like MLST reflects *Campylobacter* spp. population genetics (i.e. variation in the genes found within group of individuals and changes in allele frequency) and STs are often assimilated to specific or multi hosts (Dingle et al., 2001; McCarthy et al., 2007; Sheppard et al., 2011). While isolates belonging to ST-

257 and ST-61 are strongly associated to chickens and ruminants, respectively, most common disease strains (e.g. ST-21, ST-45 and ST-828) causing human infections could be isolates from various animal species and are burdensome to attribute to a specific source by using MLST (Sheppard et al., 2011; Dearlove et al., 2016). Gripp et al. (2011) highlighted extensive genomic and phenotypic micro-diversity within the ST-21 group, which could be caused by the recombination and acquisition of phage related genes. Therefore such strains carry the stigmata, i.e. high genetic flexibility, of a generalist variant (Gripp et al., 2011). Due to its clinical importance, source tracking *Campylobacter* spp. infections is important to identify source of infection and take preventive and control measures to eliminate the contamination.

The development of efficient bacterial typing methods for source-attribution and source tracking studies is essential to investigate and determine the source and origin of a specific strains causing human infection (Dieckmann et al., 2016). On the one hand, genotyping methods such as MLST are the reference method for *Campylobacter* source-attribution (Mossong et al., 2016; Cody et al., 2019). To date there is few studies reporting the use of WGS data for such purpose (Thépault et al., 2017; Mughini-Gras et al., 2021). Nevertheless, such techniques are still time-demanding and could be considered as expensive. On the other hand, our present work suggested that MALDI-TOF MS could be a suitable tool for the typing of *Campylobacter* at the ST level. Bacterial typing by MALDI-TOF MS was suggested to determine the origin of specific strains by grouping isolate's protein profile by sources, so-called bacterial source tracking (Santos et al., 2016). Two ground-breaking studies evaluated the use of MALDI-TOF MS to identify the source of bacteria in recreational and surface water (Siegrist et al., 2007; Giebel et al., 2008). Giebel et al. (2008) analysed protein mass spectra of *Enterococcus* isolates from sources, including human, dog, duck, cow, goose, gull and chicken. Siegrist et al. (2007) used mass spectra to characterize closely related *E. coli* and classify them according their respective sources. Both studies recognized MALDI-TOF MS as a potential promising and rapid tool to address the problem of fecal contamination of water. Jadhav et al. (2015) explored MALDI-TOF MS as a single identification and source-tracking tool for *Listeria monocytogenes*. They compared source-tracking discriminatory index and congruence between MALDI-TOF MS and PFGE methods (Jadhav et al., 2015). Chiefly, they underlined MALDI-TOF MS as a rapid and cost-effective source-tracking technique for *L. monocytogenes*. Concerning MALDI-TOF MS based source-tracking for *Campylobacter* spp., only a conference poster and presentation recounted its potential application. Denis et al. (2019) compared *C. jejuni* by protein profiles to identify the relationship between different origins such as poultry, bovine, sheep, shorebird, river and shellfish (Denis et al., 2019). Their preliminary study suggested that rivers' contamination might be attributed to shorebirds, bovines and sheep, which is congruous with

results obtained by MLST (Mughini-Gras et al., 2016). Along the same line, Py et al. (2021) explored the ability of MALDI-TOF MS to identify the origin of a *Campylobacter* contamination. Several *C. jejuni* and *C. coli* isolates from various origins were subjected to MALDI-TOF MS (Py et al., 2021). On the one hand, the distinction between *C. jejuni* isolates sampled from sheep, bovine and poultry was hazy. Also, *C. jejuni* birds and rivers isolates were close-related. On the other hand, *C. coli* isolates sampled from avian, i.e. birds and poultry, were close related to environmental samples, i.e. shellfish and rivers, by sharing a similar mass peak at 6126 Da, identified by a gel view approach. Nevertheless, while in the case of *C. jejuni* it might be complex to assess the source of the contamination, further investigations (e.g. classification with MLST profiles) are required to conclude on *C. coli* (Py et al., 2021). Also, authors used conventional approaches, i.e. heatmap or pseudo gel view, to identify specific biomarkers. Nevertheless, such approaches could be limiting considering the high genetic diversity of *Campylobacter*, resulting in a potential shift within the species or origins group. Indeed, by looking to a unique biomarker for source attribution without knowing MLST or AMR profiles might be a laborious task.

In the current research work, a ML approach was used to identify biomarkers related to the genetic diversity of *C. jejuni*. Nevertheless, the source attribution side of the MALDI-TOF MS was not explored. Integration of ML for the identification of specific or signatures of protein peaks might be relevant for source tracking. Actually, reports are growingly using ML to make bacterial source attribution (Lupolova et al., 2019; Mathai et al., 2020; Munck et al., 2020; Wu et al., 2021). Munck et al. (2020) considered ML to predict the animal source of strains isolated from human salmonellosis based on WGS data. The Logit boost algorithm was able to predict the origin of 81% of sporadic human salmonellosis (Munck et al., 2020). Recently, a similar experiment was performed for campylobacteriosis, including cgMLST and WGS data (Arning et al., 2021). Authors reported an overall accuracy improvement of 33% over existing methods that use a subset of genes. Therefore such approach could be used to understand the global epidemiology of *Campylobacter* and enable a continuous disease surveillance (Arning et al., 2021). For instance, in 2019, contamination of drinking water caused a large campylobacteriosis waterborne outbreak in Norway (Hyllestad et al., 2020; Paruch et al., 2020). After investigation, it was highlighted that the *Campylobacter* contamination was due to cracks in the back of the holding water drinking pool. Paruch et al. (2020) determined the origin and source of the *Campylobacter* water contamination by using DNA-based methods, such as quantitative PCR, as well as quantitative microbial source tracking using genetic markers. They highlighted a 100% zoogenic contamination, where the test displayed 69% coming from horses, 6% from ruminants, and 25% from other animals (e.g wildlife). Two transport routes through the defective water tank were suggested. The first one was that

horses defecated on the hill over the pool cave and their faeces were washed down directly to the crack. The second one could be the scattering of horses faeces by other animals directly into the pool as openings between the wall and the ground were identified (Paruch et al., 2020). The whole process was composed of several steps. Considering a MALDI-TOF single mass spectrum approach to identify the microbial contaminant at the species level, determine the genetic diversity and potentially track the source of the contamination, could have been faster giving the possibility to undertaken pre-actions with local regulatory agencies (e.g. expansion or creation of groundwater protection zone). While in theory, the faster the information is known, the faster solutions and actions could be carried out to contain the contamination, in reality decision making is a long process including several stakeholders. In the end, while protein mass spectra could be investigated for source tracking, it will mostly be used as a complementary information for surveillance, whereas WGS will remain the reference method for decision making. Therefore, such approach might be worth exploring to assess MALDI-TOF MS as an utmost tool for a fast and complete screening of isolates. At the time of writing this dissertation, no reports investigated the use of ML and on MALDI-TOF protein profile for bacterial source attribution.

To conclude on the subtyping part, work findings underlined that protein released during protein extraction and detected in the 2-20 kDa range could displayed *Campylobacter* genetic diversity in addition to ciprofloxacin and tetracycline resistance. Additionally, protein fingerprints generated by MALDI-TOF MS combined to a prediction approach might be relevant to source track bacterial contamination. However, further work needs to be addressed in the case of *Campylobacter* spp. Overall, a single spectrum based on bacterial expressed protein could be used for species identification, AMR screening and potentially as a complete pre-screening for daily surveillance, including genetic diversity and source attribution after further analysis.

3 A fresh look on rapid mass spectra-based diagnostics tests: outlook and challenges

In this final section, MALDI-TOF protein mass spectra as a key tool for the establishment of a "One-fit all" screening method in human and veterinary *Campylobacter*'s diagnostics will be discussed in relation to the current diagnostic market and its emerging technologies, including a look at the challenges and prerequisites.

3.1 The emphasis of biomarkers: characterization and validation

3.1.1 Characterization

All along the project, a crosscut objective was to determine if identified biomarkers, based on protein expression, were linkable to known biological mechanisms related to AMR or genetic

diversity of *Campylobacter*. As a reminder, a biomarker could be defined in our case as a MALDI-TOF MS protein peak which could be an indicator of normal biological processes or pathogenic processes (Hunter et al., 2010). By using the online Uniprot database, putative biomarkers linked to (1) AMR mechanisms and (2) population structure, i.e. variation in the genes found within group of individuals and changes in allele frequency, were identified for *Campylobacter.* On the one hand, biomarkers putatively associated to fluoroquinolones, aminoglycosides and tetracyclines resistances were identified. The one for aminoglycosides at 3257.41 Da, i.e. GCN5-related N-acetyltransferase fragment, and tetracyclines at 4365.25 Da, i.e. 50S ribosomal protein L36 may potentially be linked to biological known mechanisms of *Campylobacter*. Moreover, the first biomarker identified for fluoroquinolones (6436.22 Da) was assimilated at a predicted (i.e. in Uniprot protein predicted refers to entries without evidence at protein, transcript or homology level) DNA methyltransferase in *H. pylori* (former *C. pylori*). While DNA methylation was suggested to be capable to induce fluoroquinolones resistance, such mechanisms was never suggested for *Campylobacter* spp. (Chapter 3) (Yugendran and Harish, 2016). On the other hand, the single biomarker represented as a shift between the 4159.99 Da and 4174.19 Da peaks, suggested as the *flaA* gene, were able to distinguish ST-2254 and ST-6175. Nevertheless, interpretation on the biological role of these features may be cautiously interpreted as no peptide sequencing was carried out.

Griffin et al. (2012) identified a specific protein peak at 5092 Da after examining a set of vancomycin-resistant *Enterococcus faecium* showing a sensitivity of 92.4% and specificity of 85.2% after internal validation (Griffin et al., 2012). However, a recent study investigated the suitability of this approach (Brackmann et al., 2020). After identifying the same peak than Griffin and colleagues, authors determined the protein sequence using a tandem mass spectrometry. The peak was identified as a hiracin protein (HirJM79), a sec-dependent bacteriocin. Then they investigated the usability of this protein as a biomarker for VanB-type vancomycin-resistant *E. faecium* by analysing available vanB- and hirJM79 encoding genome. Interestingly both genes co-occurred in several strains, yielding a predictive power of 36%. They concluded that the hiracin gene could not be directly linked to the presence of a VanB-type *E. faecium* and hence cannot be used routinely in diagnostics (Brackmann et al., 2020). Such study underlines the need to properly characterize MALDI-TOF MS biomarkers to completely understand information hidden behind the protein fingerprint. Additionally, further exploration has been conducted concerning the previous identified *flaA* biomarker. Two extra isolates, identified in 2006 and 2010, classified as belonging to the CC-257 and ST-257 harbouring the *flaA16* allele were analysed by MALDI-TOF MS. Interestingly a peak at 4174 Da was also identified, like for isolates of the ST-2254. While the peak shift was

thought to be associated with ST-2254 (CC-257), it might actually be possibly linked either to the flagellin itself or to the CC-257. As mentioned by Brackmann et al. (2020), deeper proteomic analysis, notably peptide sequencing, may be performed to ensure the usability of a biomarker.

3.1.2 Validation

There are important requirements to further explore pre-analytical steps and to develop standardized MALDI-TOF MS protocols. Even more important will be the transfer of identified and characterized predictive biomarkers to diagnostics companies to turn them into in vitro diagnostics tests and potentially "companion diagnostics" (CDx) for a personalized medicine. CDx is defined by the European medicines agency (EMA) as "an IVD test that supports the safe and effective use of a specific medicinal product, by identifying patients that are suitable or unsuitable for treatment". They are mainly developed in oncology but its development in infectious disease treatment is still rare (Zhou et al., 2018; Dailey et al., 2020). During the presented research work, putative predictive biomarkers related to *Campylobacter* spp. AMR and population genetics were identified based on a machine learning approach. AMR biomarkers could predict if a specific antimicrobial will be suitable for the treatment of patients. Currently there are numerous microbial biomarkers which have been described in the literature (Zautner et al., 2013; Camoez et al., 2016; Emele et al., 2019; Feucherolles et al., 2019). Nevertheless, only few of them are now available under commercial solutions for IVD or research use only purposes (Bruker, 2019b). This could be explained by the fact that the path between the biomarker discovery and validation is full of pitfalls. Firstly, biomarker development is a long process divided in five steps (**Figure 8.2**):

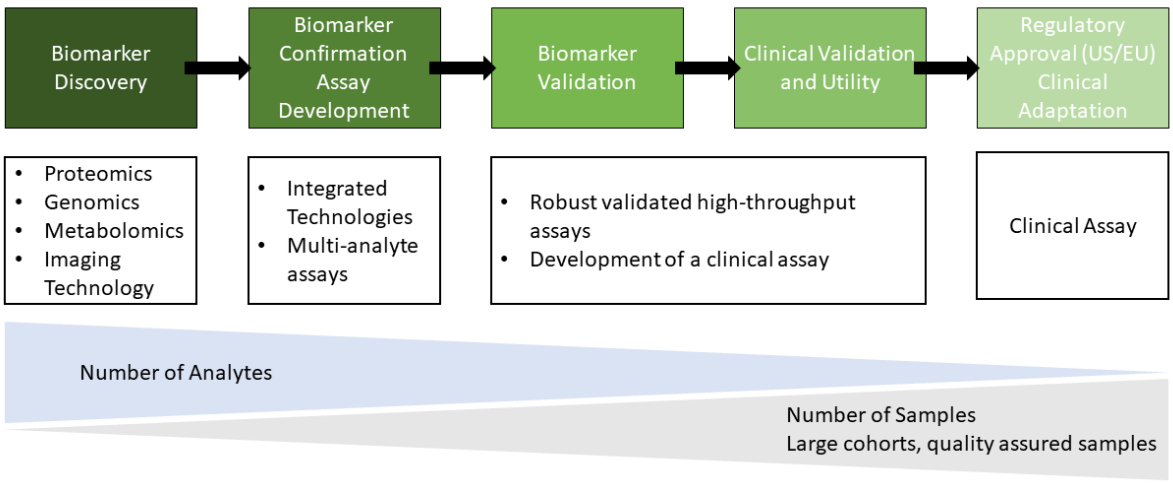

Figure 8.2. *Typical biomarker discovery and validation workflow (adapted from Marshall (2020))*

the discovery, qualification, verification, validation and the marketing (Lee, 2009; Research Advocacy Network, 2013; Karlsson et al., 2020).

The discovery part is the unbiased process of identifying specific protein or peptides in e.g. bacterial cultures, employing binary comparison between susceptible and resistant isolates (Rifai et al., 2006). Highlighted protein consistency, i.e. the biomarker must be found to distinguish resistant to susceptible isolate using different methods. In the verification and validation stage, the analysis is carried out on a larger number of isolates from various sources to evaluate genetic or biological variation in the population tested. This allows computing the sensitivity and specificity of candidates. Biomarkers performing well during the previous steps may be selected for a potential commercialisation for clinical use and hence go through regulatory pathway to have the clearance of the US Food and drug administration (FDA) or EMA (Rifai et al., 2006; Research Advocacy Network, 2013; Ritzhaupt et al., 2020). Similarly to our research work, the majority of studies stopped at the discovery stage and in-house solution were developed (Zautner et al., 2016). For example, Zautner et *al*. (2016) published in *The Journal of Visualized Experiments* a step-by-step and in-house protocol to proteo-type *C. jejuni* ssp. *doylei* based on allelic isoforms biomarkers identified during a previous study (Zautner et al., 2013, 2016). Secondly, while in some cases protein biomarker candidates may be not as performant as expected, development of diagnostic biomarkers requires tremendous investments of time. As well, the establishment of benefits and drawbacks in the context of a clinical use, i.e. clinical utility, and compliance regarding regulatory administration (e.g. FDA and EMA) remain fastidious, uncertain and costly (Rifai et al., 2006). The fondness for straightforward and high-throughput omics technologies, including proteomics, combined to artificial intelligence to address the current global microbial public health issues, such as septicaemia or AMR, will hopefully reverse the trend in the coming years.

3.2 The emergence of new applicable technologies in diagnostics

3.2.1 Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) is a non-destructive biophysical method conventionally used in chemistry to characterize molecules of different samples (Novais and Peixe, 2021). Such characterization is possible by observing changes in vibrational mode of chemicals bonds in a sample after the absorption of the IR (Novais and Peixe, 2021). In a case of a microbiological sample, IR interactions with cells component such as proteins, lipids, nucleic acid and carbohydrates, result in a spectrum expressing abundance of the different group at different wavenumbers (**Figure 8.3**) (Novais and Peixe, 2021).

FT-IR was firstly attempted for analysing microorganisms in the 1950s (Randall et al., 1951). However, It is only in the 1990s that FT-IR started to be mainly used in microbiology for bacterial discrimination at the genus, species and even strain level (Wenning and Scherer, 2013; Lasch and Naumann, 2015). In the case of *Campylobacter* spp. Mouwen et al. (2006) successfully used supervised machine learning algorithms to analyse *Campylobacter* infrared spectral data (Mouwen et al., 2006). Similar to MALDI-TOF MS, cluster analysis based on biochemical *Campylobacter* fingerprints showed a differentiation between different species, which was in agreement with 16S rRNA based phylogenetic tree. (Muhamadali et al., 2016). Josefsen et al. (2012) compared the discriminatory power of *fla* Short Variable Region sequencing-based method (SVR) to FT-IR on *C. jejuni* strains. Both techniques showed a high degree of congruence, by assigning isolates to similar cluster

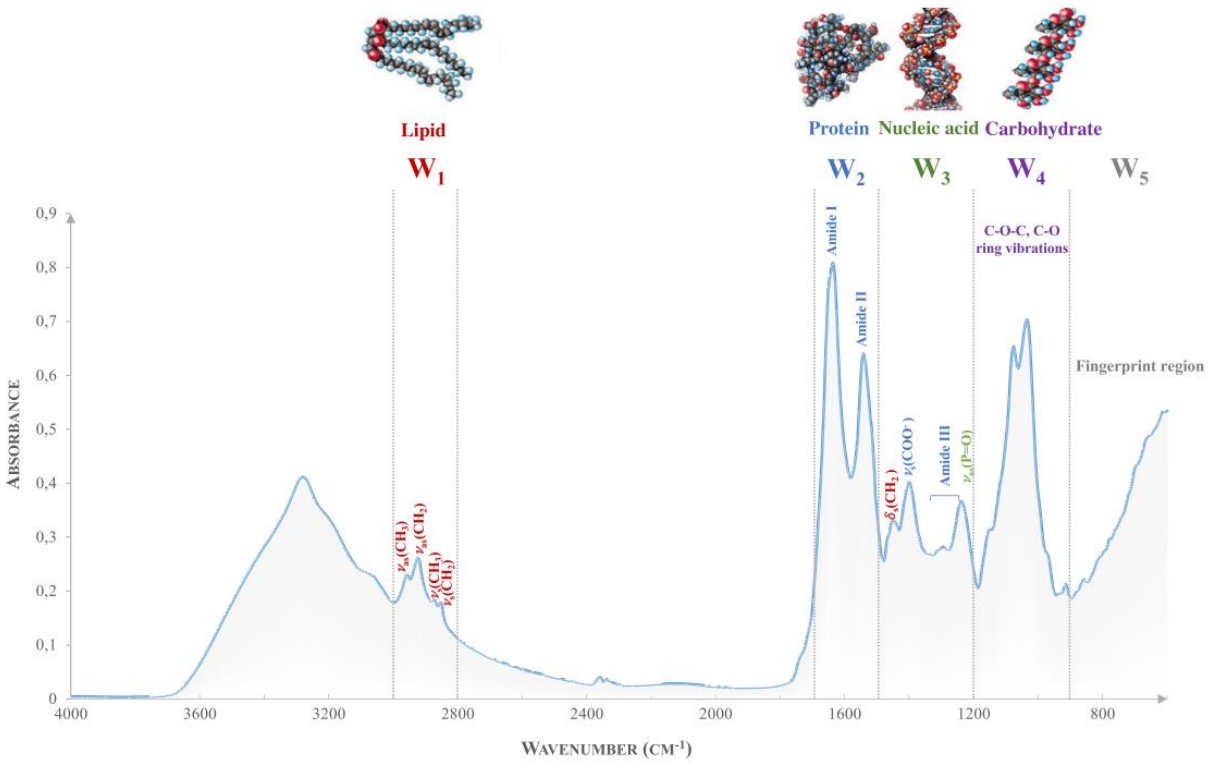

Figure 8.3. Klebsiella pneumoniae *Fourier Transform infrared spectrum (FT-IR) (Adapted from Novais et al. (2019))*

structures (Josefsen et al., 2012). Therefore, FT-IR might be suitable for the bacterial typing for large numbers of strains in the same manner as MALDI-TOF MS was suggested in the present research work. Nonetheless, due to inaccuracy of bacterial classification systems at that time (e.g. scarcity of performant typing methods), lack of standardised protocols and databases, as well as the development of DNA-based methods in the 1990s, FT-IR was dropped out. Additionally, at this time such methods were complicated and time-consuming (Novais and Peixe, 2021). Nevertheless, the

culmination of WGS resulted in bacterial taxonomy improvement, bringing FT-IR to the forefront of microbial diagnosis (Novais and Peixe, 2021).

The renewal of interest of FT-IR over the last five years could be seen on the current analytical market. In 2017, Bruker Daltonics introduced the bench-top IR Biotyper® (IRBT). It is an automated system for microorganisms typing based on FT-IR technology. During the 31st European Congress of Clinical Microbiology and Infectious Disease, the company launched the IRBT®3.1 software, including artificial neural network (ANN) machine learning algorithm, for strain typing for hospital hygiene management (Bruker, 2021a). Therefore, manufacturers such as Bruker are signing up machine learning as a concrete approach for microbial routine diagnostics. IRBT was used for the microbial typing of typhoid and paratyphoid fever-associated *Salmonella* isolates using an automated classifier using a ANN model (Cordovana et al., 2021). Classifier accuracy ranges from 87.0% to 99.9% for the different strains. Authors underline FT-IR as a fast, cost-effective and reliable technique as a suitable alternative to conventional approaches for surveillance and diagnostic purposes.

While IRBT is suggested as comparable to reference DNA-based molecular methods (e.g. MLST, PFGE and WGS) often considered as time consuming and demanding in resources, such method could also provide solutions to some MALDI-TOF MS limitations (Hu et al., 2021). As mentioned in the Chapter 2 of the introduction part, it is the incapacity of the protein-based MALDI-TOF MS to distinguish specific species within bacterial complex (e.g. *Mycobacterium tuberculosis* complex). In the case of *Enterobacter cloacae* complex, the distinction between *E. cloacae* and other closely related species and subspecies are challenging while using MALDI-TOF MS as they are genotypically close and the taxonomy of the genus is still under debate (e.g. *Enterobacter aerogenes* suggested to be reclassified as *Klebsiella*)(Davin-Regli et al., 2019). Two studies explored IRBT systems for the discrimination of species within the *E. cloacae* complex (Vogt et al., 2019; Candela et al., 2021). Vogt et al. (2019) demonstrated that with an ANN trained on FT-IR spectra, it improved the recognition of close isolates. Furthermore, authors investigated strains from an *E. cloacae* complex outbreak and obtained fast typing results confirmed by WGS. Candela et al. (2021) reported in a pre-print the rapid and accurate discrimination of species within the *Enterobacter cloacae* complex using MALDI-TOF MS and FT-IR coupled with ML tools (Candela et al., 2021). However, while FT-IR enabled differentiation between *E. hormaechei* from non-*E. hormaechei*, the distinction within the non-*E. hormaechei* was difficult (62.7% of correct identification) (Candela et al., 2021).

LikeMALDI-TOF MS, FT-IR is an attractive technology due to its high-throughput, time- and cost-efficient aspect for bacterial typing. Therefore, development of FT-IR in diagnostics seems to be promising for real-time surveillance and outbreak analysis.

3.2.2 Mass spectrometry based Lipidomics

Lipids are highly abundant in microbial cells making them an interesting molecular component to explore by using MALDI-TOF MS. Over the last decade, reports underlined the successful application of MALDI-TOF MS based lipidomics for bacterial identification based on lipids fingerprints (Voorhees et al., 2013; Larrouy-Maumus and Puzo, 2015; Leung et al., 2017; Jia Khor et al., 2021). Leung et al. (2017) reported the possible identification of ESKAPE pathogens (E*nterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) based on a library of glycolipid mass spectra, which might provide an alternative to current diagnostics methods. However, it is only over the past few years that its applicability in diagnostics was put in the spotlight.

On the one hand, the current study investigated protein profiles generated by a benchtop MALDI-TOF MS in a linear positive ion mode. On the other hand, to study lipids a negative ion mode is required. In 2019 Bruker Daltonics introduced the MALDI Biotyper Sirius benchtop system at the American Society for Microbiology conference (Bruker, 2019a). The apparatus combines a negative and positive ion mode enabling rapid microbial identification as well as analysis of lipids for research purposes. Research and development of such technology was based on pioneering studies (Larrouy-Maumus et al., 2016; Dortet et al., 2018a). Initially Larrout-Maumus and al. (2016) reported a method using 2, 5-dihydroxybenzoic acid matrix for the direct detection of lipid A from intact Gram-negative bacteria by investigating spectrum between 1000 and 2200 m/z. From it, Dortet et al. (2018) developed a MALDI-TOF MS based method, so-called MALDIxin test, enabling the detection of polymyxin resistance linked to modification of the lipid A in *E. coli*. The test was developed on the fact that polymyxin resistance is associated with addition of phosphoethanolamine (pETN) on the phosphate group at position 4’ or 1 of lipid A, resulting in an increase of 123 Da (**Figure 8.4**).

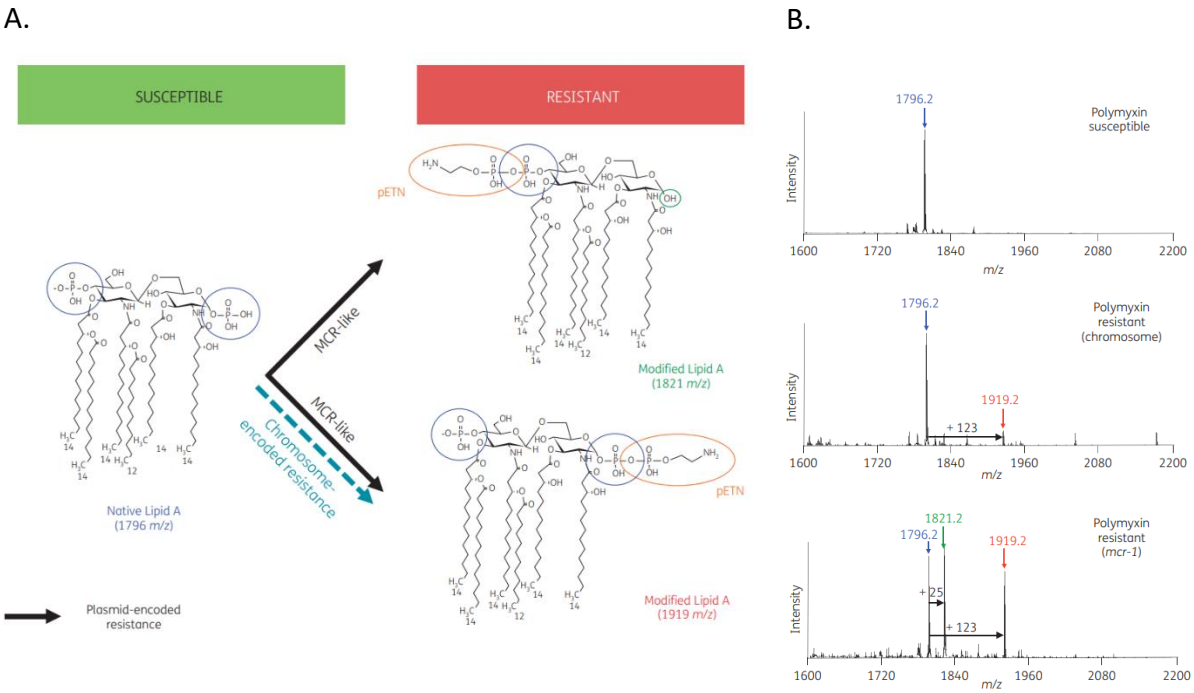

***Figure 8.4**. A. Lipid A modifications caused either by chromosome encoded determinant or enzymes. B. Results of the MALDIxin test on polymyxin-susceptible and -resistant* E. coli *(Adapted from Dortet et al. (2018a))*

Negative mass spectra were scanned between 1600 and 2200 m/z. The major peak at 1796.2 was known to be the native lipid A and the 1919.2 m/z one corresponded at the addition of the pETN on native lipid A. Additionally, a third peak at 1821.2 m/z appeared to be a specific marker of MCR-like enzymes (Dortet et al., 2018a). Therefore, by the identification of these specific peaks, MALDIxin test managed to reliably and rapidly identify plasmid-encoded pETN transferase-producing strains. Since several original articles were published assessing the MALDI-TOF MS technology as an utmost tool for rapid diagnosis of AMR spectra (Dortet et al., 2018b, 2020; Furniss et al., 2019). To make such workflow closer to its potential future integration in diagnostics settings, a new RUO Bruker kit of lipid extraction, MBT Lipid Xtract™ kit was developed and launched at the occasion of the 31st European Congress of Clinical Microbiology and Infectious Disease (Bruker, 2021a). This kit allows the sample preparation of the Lipid A molecule and related modification for colistin resistance detection within less than 15 min (Bruker, 2021b). Accompanying this new fast kit, the MBT LipidART software module was developed to identify mass differences related or not to the resistance induced by the Lipid A modification (Bruker, 2021b).

Likewise, FT-IR, MS-based lipidomics might overcome limitations encountered with MS-based proteomics. Indeed, protein profiling demonstrated limitations for the closely related species and subspecies (Kostrzewa et al., 2019). As previously mentioned, MALDI-TOF MS is unable to distinguish the *Mycobacterium tuberculosis* complex into specific species (Saleeb et al., 2011;

Neuschlova et al., 2017; Akyar et al., 2018; Body et al., 2018). It is known that specific lipids such as sulphoglycolipids or polyacyltrehaloses are specific to the *Mycobacterium tuberculosis* complex, while C-mycoside glycopeptidolipids are only found in non-tuberculous mycobacteria (Gonzalo et al., 2021). Therefore, a deeper investigation of mycobacterial lipids might be relevant for the rapid discrimination of *Mycobacterium tuberculosis* complex species. Larrouy-Maumus and Puzo (2015) analysed mycobacterial envelope lipid fingerprints from direct MALDI-TOF MS analysis of intact cells. Interestingly different lipid profiles were obtained for *M. tuberculosis* lineages (Larrouy-Maumus and Puzo, 2015). Another study reported high performances for the identification of isolates belonging to the *M. tuberculosis complex* (96.7%) and non-tuberculous mycobacteria (91.7%) based on lipid fingerprint generated by MALDI-TOF MS (Gonzalo et al., 2021). Along the same line, species-specific lipid profiles were explored for the rapidly discriminate mycobacteria within the *Mycobacterium abscessus* complex (Jia Khor et al., 2021). Overall, the development of simple workflow to identify *Mycobacterium* spp. and screen AMR using time- and cost-efficient MS-based lipidomics could be a significant advantage for clinical microbiology laboratories for decision making improving patient outcomes.

To date, no study reported the use of lipidomics based MALDI-TOF MS for *Campylobacter*. Therefore, it opens the field of possibilities to create new research projects. Lipooligosaccharide (LOS), i.e. glycopeptides including core oligosaccharide and lipid A, is considered to be implied in Guillain-Barré syndrome induced by *Campylobacter* (Nachamkin et al., 2002; Moran, 2010). Mimicry between LOS and gangliosides presented on human peripheral nerve drives immune response, resulting in immune-mediated nerve damages (Yuki et al., 2004). However, not all *Campylobacter* synthetize ganglioside mimics. Currently identification of high-risk strains for Guillain-Barré syndrome is carried out by the genomic analysis of the LOS biosynthesis locus. Several LOS locus classes exist from A to W, where LOS A, B and C types are highly dominant in Guillain-Barré-induced *C. jejuni* population (Hameed et al., 2020; Zang et al., 2021). Interestingly, several studies reported concordance between ST and LOS classes (Islam et al., 2009; Ellström et al., 2013). Islam et al. (2009) reported concordance between LOS B class and ST-403 complex. To echo this present study, where it was suggested that protein based MALDI-TOF mass spectra were concordant to genomic typing tools, it might be worth investigating lipid-based mass spectra to swiftly determine LOS classes and hence highlighted high-risk strains for Guillain-Barré syndrome in routine diagnostics.

While, the microbial diagnostics market seems attentive to the future of this technology, reports already acknowledge Bruker MBT Sirius as the next generation clinical microbiology system.

3.2.3 Shotgun proteomics

Currently, there are remaining questions concerning the use of proteomic top-down approach in microbiology such as whole-cell MALDI-TOF MS (Grenga et al., 2019). To quote some of them: the discriminative power for strain epidemiology, addressed in this this study for *C. jejuni*, the possibility to identify mixtures of organisms, or the possibility to directly identify microorganisms from clinical samples (e.g. stools) (Grenga et al., 2019). MALDI-TOF MS applied in routine laboratories is based on a culture step of pure bacterial colonies, even if research reports highlighted the potential to identify bacteria from complex polymicrobial mixtures (see Chapter 2) (Mahé et al., 2014; Yang et al., 2018; Mörtelmaier et al., 2019).

However, by using a proteomic independent culture approach, such as bottom-up proteomics also referred as shotgun proteomics, such issue might be overcome. Briefly, shotgun proteomics consists in a first place of the proteolysis of proteins isolated from biological samples, i.e. clinical samples (e.g. stools) (Gouveia et al., 2020). Then most abundant generated peptides are analysed and sequenced by a high-resolution tandem mass spectrometer (MS/MS) coupled to a chromatography system (**Figure 8.5**). Then the numerous MS/MS spectra obtained could be assigned to peptide sequences using genome-derived protein sequences databases (e.g. NCBInr) (Pible and Armengaud, 2015). In comparison to targeted proteomics such as MALDI-TOF MS, shotgun proteomics enables to obtain a general cellular metabolic view on samples without an *a priori* identification of peptides (Armengaud, 2020). It enables the identification and the quantification of unique or polymicrobial mixtures (Hayoun et al., 2020; Pible et al., 2020). Pible et al. (2020) developed a new method, so-called phylopeptidomics, which evaluates the biomass contribution based on the signature of peptide sequences shared with all other organisms. They evaluated their new tool to estimate the abundance used in artificial mixture of closely related pathogens, i.e. *Salmonella bongori* and *Shigella flexneri*, and complex microbiota models. Concerning *Salmonella* and *Shigella*

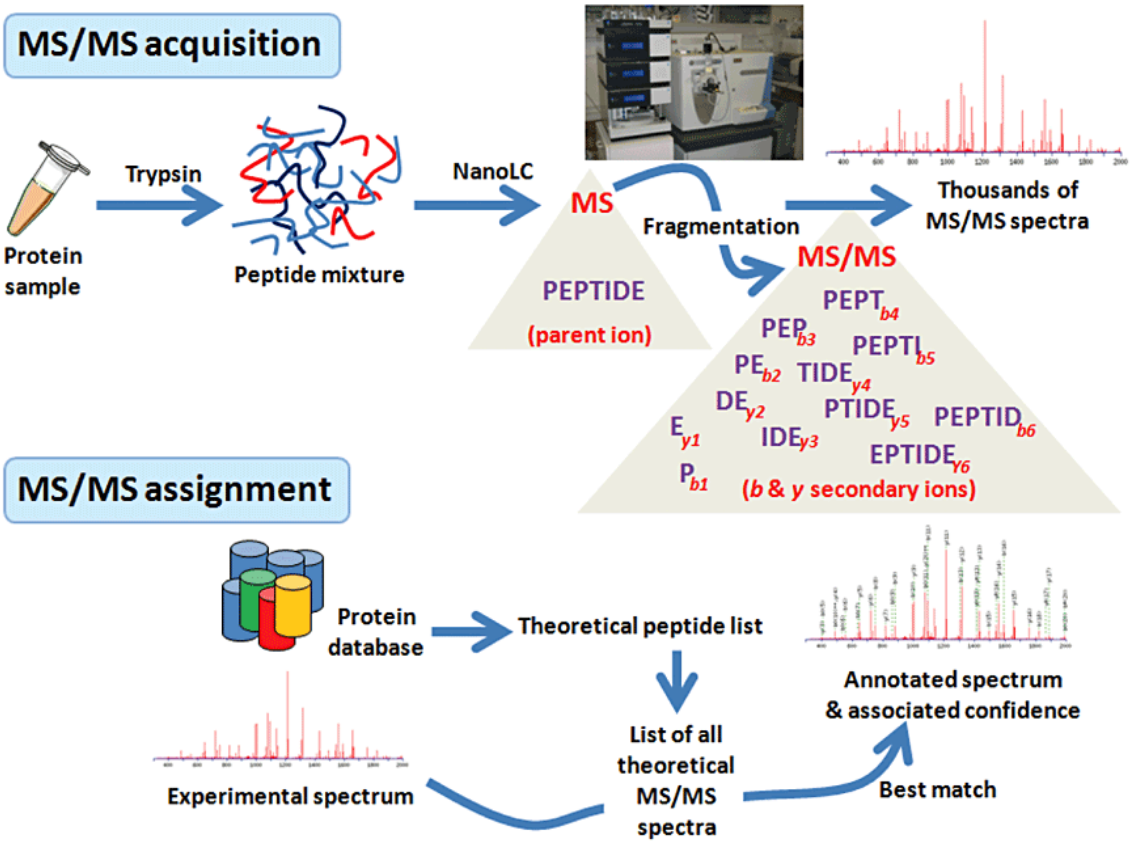

Figure 8.5. *Shotgun proteomics and tandem mass spectrometry workflow (Adapted from Armengaud (2013)*

mixtures, two distinct phylopeptidomics signatures were obtained as well as a linear response with a performant estimation of the bacterial ratio (Pible et al., 2020). Finally, the same observation was made for two data sets of 10 and 22 microorganisms, including Gram-positive and -negative bacteria, fungi, archaea and phage viruses. Therefore, such methods could be relevant to rapidly and directly screen clinical polymicrobial mixtures in diagnostics (Kondori et al., 2021). Recently, Chen et al. (2020) explored shotgun liquid chromatography-MS/MS to screen AMR determinants in *C. jejuni* isolates using the Comprehensive Antibiotic Resistance Database (CARD) (Chen et al., 2020). Interestingly, three isolates harboured the $bla_{OXA\text{-}61}$ gene (occurring β-lactams resistance), but only one isolate presented a higher OXA-61 protein abundance. This was consistent with an elevated ampicillin MIC. As detailed in Chapter 3, the single nucleotide G-T transversion in the $bla_{OXA\text{-}61}$-like promoter area is associated with high levels of ampicillin resistance, due to the overproduction of OXA-61 proteins (Ocejo et al., 2021). After genomic investigation, Chen et al. (2020) observed this specific mutation in the related isolate. They also underlined the fact that neither bioinformatics tools like ResFinder or databases such as CARD give such information, which could lead to wrong AMR predictions (Chen et al., 2020). In summary, their proof-of-concept suggested that despite the fact that phenotypic antibiograms are reference methods and WGS is

faster, whole-proteome sequencing could be relevant for AMR predictions and provide additional information to *in silico* genomics. Likewise, during the current study, WGS of the AMR isolates panels were also submitted to the ResFinder online platform to compare phenotypic and molecular antibiograms (data not shown). Over the 340 isolates analysed, 148 were phenotypically resistant to ampicillin. However, Resfinder detected bla_{OXA} genes in 279 isolates resulting in *in silico* predicted β-lactams resistance phenotype. It might be possible by investigating the relative protein abundance of our isolates to obtain the same scheme than Chen et al. (2020).

Overall shotgun proteomics could obtain the same information than MALDI-TOF MS based-protein or -lipids, and FT-IR all together combined, with a theoretically greater discriminatory power (Grenga et al., 2019). However, it presents some obstacles for its implementation in laboratories routine. The major constrains are that such methods are time-consuming, could be laborious and require expertise for sample preparation, analysis and data processing (Grenga et al., 2019). Beside the fact that high-resolution MS/MS apparats could be expensive, there is an significant need of accurate and high quality genomic sequence databases with a stable taxonomy (Pible and Armengaud, 2015). The presence of errors in DNA sequences leads to erroneous protein sequences, which results in false peptides identification and quantification (Pible and Armengaud, 2015). In a study, over 486 polypeptides identified, 64 were originally wrongly annotated (Christie-Oleza et al., 2012). Errors in sample handling (e.g. initial culture contamination) and taxonomic characterization (e.g. 16S RNA sequence anomalies) might be as well a source of confusion in meta-omics analysis (Ashelford et al., 2005; Shrestha et al., 2013; Pible and Armengaud, 2015). Finally, while bioinformatics tools (e.g. DeconSeq) exist to identify and remove sequence contaminations from genomic and metagenomics datasets, cross-contamination among genome sequences is still a problem (Schmieder and Edwards, 2011; Pible and Armengaud, 2015). For example, *Wolbochia* genomes were identified in several *Drosophilia* genomesn (Salzberg et al., 2005). More recently, Pible and Amengaud (2015) investigated the cucumber genome because it was systematically appearing in metaproteomic analysis comprising *Enterobacter* spp., initially present in plants roots for nitrogen fixation. After investigation, they concluded that these cross-contaminations probably came from the preparation of DNA extraction *priori* genome sequencing.

Despite the previous introduced limitations, shotgun proteomics seems to be a promising high-throughput tool to rapidly identify at the species level, quantify, subtype, AMR screening polymicrobial mixtures from the three life tree branches from complex matrices (e.g. blood, urine or stool).

3.3 Ongoing digitalization of microbiology laboratories

Over the last decades, introduction of (meta)omics technologies in life sciences transcended routine laboratories, resulting into an unavoidable digitalization of these latter. Digitalization of laboratories could be defined as the changes associated with the use of digital technologies, such as DNA sequencing, in laboratories. AI and digital technologies kindle high interest for healthcare, giving birth to a digital microbiology (Egli et al., 2020). Egli et al. (2020) defined digital microbiology as "the usage of big data, ML based algorithms, and other digital technologies in the diagnostic process of clinical microbiology". Development and utilization of (meta)omics, bioinformatics tool as well as ML based approaches in microbiology enhance to bring clinical microbiology to a new level (Krüger et al., 2020).

In the context of the global AMR crisis, identification of multidrug resistant pathogens or design of rapid diagnostics tests for personalised medicine might be at hand in this digital era. While reference methods for AMR identification in routine diagnostics is phenotypic antibiograms, i.e. disk diffusion or microdilution antibiogram, new meta(omics) tools are on their way to be implemented for additional insights of AMR diagnostic. Currently, the development of online platforms to investigate genomic and metagenomic datasets is flourishing. For example, De Nies et al. (2021) developed the freely available PathoFact pipeline (https://pathofact.lcsb.uni.lu) for the prediction of virulence factors, bacterial toxins and AMR genes from metagenomics datasets with high accuracy (de Nies et al., 2021). As already mentioned in the manuscript, the EFSA will request the use of WGS for the harmonization of the monitoring of antimicrobial resistances in food-producing animals and derived meat by 2026 (EFSA and ECDC, 2020). As a support to analyses, this genomics data could be either investigated by online open access platform such as ResFinder 4.0, for identification of known AMR genes, or the implementation of ML workflow, for the estimation of e.g. minimum inhibitory concentrations (Nguyen et al., 2019; Bortolaia et al., 2020). Additionally, the wide range of possibilities offered by the recent fusion of ML and protein expression-based method, such as MALDI-TOF MS, in clinical microbiology will play a key role in the fast screening of AMR (Egli, 2020; Egli et al., 2020). As highlighted by Egli (2020), such advances in data-driven technologies in medicine already changed and will considerably change analytical workflow in diagnostics laboratories (Egli, 2020).

Nevertheless, there is still a stony way before their full implementation as reference methods in routine settings. Indeed, as alluded to in the introduction part, the application of such powered-omics methods in clinical laboratory requires sufficient data storage infrastructure, bioinformatical expertise to support microbiologists and infectious diseases specialists, and standardized data format (Egli, 2020). Indeed, the ever-exponential accumulation of these daily

gigabytes of data, including MALDI-TOF mass spectra, sequencing data, real-time PCR or serological results, need to be stored for quality controls, legal reasons and researches (Egli et al., 2020). Egli et al. (2020) summarized the need of data collected through healthcare data warehouse, which is centralized repository for electronic health records and clinical data, as well as the need of large structured, interoperable and interconnected datasets. Along the same line, they underlined the need of further standardization and annotation of clinical data.

In parallel to data storage, data management and security, private issues are also important to consider (Galetsi et al., 2019). Due to the rapid evolution of form, transfer, sharing of data, this topic is the centre of attention. A study reported 1042 data safety breaches involving sensitive demographic and financial information enabling the theft of identity (Jiang and Bai, 2020). Enforced in May 2018, the General Data Protection Regulation (GDPR) aims to provide an ensemble of data protection laws in the EU. Therefore, the GDPR has a direct impact on medical data science (Rumbold and Pierscionek, 2017). The GDPR defined medical data as "personal data related to the physical or mental health of a natural person, including the provision of health care services, which reveal information about his or her health status." Currently, pseudonymized data is considered as personal data and will require consent or authorization (Rumbold and Pierscionek, 2017). However, ML algorithms are "data-hungry" and require large datasets to learn. In some cases, AI in medicine encounters dataset limitation due to the lack of standardization of data ensuring patient privacy (Kaissis et al., 2020). Therefore, for long-term development of accurate ML, there is an important need to assure patient privacy while promoting scientific research in order to improve patient management and outcomes (Kaissis et al., 2020).

Finally, while ML decision-making algorithms would probably improve diagnosis and "physician workflow", there are still several uncertainties regarding its transparency. Indeed, understandability of model prediction from both patient and clinician is important for a long term success of these methods for moral, scientific and legal reasons (Watson et al., 2019). However, despite the will of tech companies to make ML algorithms more explainable, ML opacity, often referred to as "black box", is well known for top performing algorithms such as deep neural network (Watson et al., 2019; Grote and Berens, 2020). It is feared that superficial understanding of black box predictions could prohibit decision makers, here clinicians, to build knowledge on phenomena or disease, which will consequently make them lose their ability to make decisions (Newell and Marabelli, 2015; Galetsi et al., 2019). Another interesting fact is the ethical aspect of such decision-making algorithms. Grote and Berens (2020) extensively described this pitfall in their report entitled "On the ethics of algorithmics decision-making in health care". Briefly, clinicians are normally being held accountable for their decisions. However, in the case when the decision is made by the

algorithm, how much blame should the clinician receive? As well, what if there is a disagreement between the machine and the clinician on the results outcome? Authors summed up that ML algorithms might drive to a mechanisms of “defensive medicine” among clinicians (Grote and Berens, 2020). Toker et al. (2004) defined defensive medicine as “physician’s deviation from what is considered to be good practice to prevent complaints from patients or their families” (Toker et al., 2004).

Therefore, by gaining a better understanding of microbial cells and a better explainable and transparency of ML algorithms, it will bring microbiology to an all-new level. Therefore, combination of omics technologies, bioinformatics and artificial intelligence might be the new black to develop fast, accurate, personalised and complete screening strategies for tailor-made treatment in routine diagnostics.

CHAPTER 9

Conclusions

AMR is considered as a global long-lasting challenge. If no action is taken, AMR-related diseases could give a rise to up to 10 million deaths each year by 2050 and 24 million people might end into extreme poverty, mainly in low-income countries (O'Neill, 2016; IACG, 2019). One of the numerous solutions to curb AMR is to develop rapid diagnostics tests. Indeed, by detecting earlier AMR, an adapted antibiotherapy might be administrated promptly, shifting from empirical to evidence-based practices, conserving effectiveness of certain antimicrobials. Since 2005, *Campylobacter* is considered as the major cause of foodborne gastrointestinal diseases worldwide. As well, ever-growing *Campylobacter*'s resistances to critically important antibiotics, such as quinolones or macrolides, both used for human and veterinary therapeutic purposes, are particularly of concern. Indeed, around 61% and up to 86.7% for both *C. coli* and *C. jejuni* isolates from human and poultry respectively, were resistant to fluroquinolones (EFSA and ECDC, 2021b). Along the same line, it is known that an occurrence of resistance to fluoroquinolones exists in *C. jejuni* and *C. coli* from animals, as well as the occurrence of resistance in *C. jejuni* and *C. coli* from human infections (ECDC et al., 2017). Therefore, the increase of fluroquinolones resistance among *Campylobacter* in food-producing animals had limited treatment options for human patients (CDC, 2019). While protein based MALDI-TOF MS was partially investigated for the typing of several *Campylobacter* species, identification of resistances by MALDI-TOF MS within foodborne pathogens is poorly documented.

During the presented research work, different key findings were highlighted. The first one was that MALDI-TOF MS protein profiles combined to ML displayed promising results for the prediction of the susceptibility and the ciprofloxacin and tetracycline *Campylobacter*'s resistances. Additionally, MALDI-TOF MS *C. jejuni* protein clusters were highly concordant to conventional DNA-based typing methods, such as MLST and cgMLST, when a similarity cut-off of 94% was applied. As well, a similar discriminatory power between 2-20 kDa expressed protein and cgMLST profiles was underlined. Finally, putative biomarkers either linked to known or unknown AMR mechanisms, or genetic structural population of *Campylobacter* were identified.

Through this work, the following questions were investigated: could conserved ribosomal and surface protein profiles reflect the AMR profile of *Campylobacter* spp.? Could the same protein profiles be used to have an insight on the genetic diversity and population structure of *Campylobacter* spp.? If it was the case for both parameters, how could it be explained? According to the previous paragraph, our work findings suggested that proteins involved in the acquisition of

MALDI-TOF mass spectra could reflect the AMR profile of certain *Campylobacter*'s resistances, such as ciprofloxacin and tetracycline in *Campylobacter* spp. Likewise, results underlined that protein detected in the 2-20 kDa range could display *Campylobacter* genetic diversity. It seems that according to *Campylobacter* characteristics, i.e. AMR or cgMLST profiles, the presence of certain peaks could be linked to specific known biological mechanisms (e.g. protein synthesis inhibition)

Such suggestions can be put into perspective with current diagnostics needs. On the one hand, the combination of MALDI-TOF MS protein spectra and ML approach could be a useful diagnostics tool for a fast and precise AMR screening of relevant foodborne pathogens, such as *C. coli* and *C. jejuni*. While campylobacteriosis is mainly self-limiting and does not require specific antibiotherapy, such a combination strategy may aid to swiftly prescribe a definitive antimicrobial therapy and therefore limit an empirical broad-spectrum strategy for other pathogens. This work could serve as a proof-of-concept, and future research should include other important foodborne pathogens such as *Salmonella* spp. On the other hand, high-throughput and cost-efficient MALDI-TOF MS could be an efficient pre-screening tool to relevant isolates that warrants further sequencing. In fact, due to its status as the first bacterial human zoonosis, there are an important number of isolates to sequence, which is the main drawback of *Campylobacter* surveillance. By coupling WGS with MS supported by ML, it could increase typing ability and therefore, elucidate genotypes circulating in human infections, animal production and environment. In the end, MALDI-TOF MS protein-based method coupled with ML turned to be the perfect candidate for a "universal", accurate, and early surveillance and integration of routine laboratories. Through a single protein mass spectrum analysis of several tests, i.e. species identification, antimicrobial susceptibility screening and the assessment of genetic diversity, could be summed up into one examination. Therefore, MALDI-TOF MS protein-based seems to be a promising and utmost "One-fit all" diagnostics tool.

Bibliography

Abdallah, C., Dumas-Gaudot, E., Renaut, J., and Sergeant, K. (2012). Gel-based and gel-free quantitative proteomics approaches at a glance. *Int. J. Plant Genomics* 2012. 2012 ,1-17. doi:10.1155/2012/494572.

Aerts, M., Battisti, A., Hendriksen, R., Kempf, I., Teale, C., Tenhagen, B. A., et al. (2019). Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. *EFSA J.* 17, 5709. doi:10.2903/j.efsa.2019.5709.

Aeschbacher, M., and Piffaretti, J. C. (1989). Population genetics of human and animal enteric *Campylobacter* strains. *Infect. Immun.* 57, 1432–1437. doi:10.1128/iai.57.5.1432-1437.1989.

Agnew, E., Dolecek, C., Hasan, R., Lahra, M., Merk, H., Perovic, O., et al. (2021). Global antimicrobial resistance and use surveillance system (GLASS) report. Available at: https://www.who.int/initiatives/glass [Accessed August 10, 2021]

Agunos, A., Waddell, L., Léger, D., and Taboada, E. (2014). A systematic review characterizing on-farm sources of *Campylobacter* spp. for broiler chickens. *PLoS One* 9, e104905. doi:10.1371/journal.pone.0104905.

Agyare, C., Etsiapa Boamah, V., Ngofi Zumbi, C., and Boateng Osei, F. (2019). "Antibiotic Use in Poultry Production and Its Effects on Bacterial Resistance," in *Antimicrobial Resistance - A Global Threat*, IntechOpen. doi:10.5772/intechopen.79371.

Ahuja, A. S. (2019). The impact of artificial intelligence in medicine on the future role of the physician. *PeerJ* 7:e7702. doi:10.7717/peerj.7702.

Akyar, I., Çavuşoğlu, C., Ayaş, M., Sürücüoğlu, S., İlki, A., Kaya, D. E., et al. (2018). Evaluation of the performance of maldi-tof ms and dna sequence analysis in the identification of mycobacteria species. *Turkish J. Med. Sci.* 48, 1351–1357. doi:10.3906/sag-1801-198.

Alcalá, L., Marín, M., Ruiz, A., Quiroga, L., Zamora-Cintas, M., Fernández-Chico, M. A., et al. (2021). Identifying Anaerobic Bacteria Using MALDI-TOF Mass Spectrometry: A Four-Year Experience. *Front. Cell. Infect. Microbiol.* 11, 1-9. doi:10.3389/fcimb.2021.521014.

Allcock, S., Young, E. H., Holmes, M., Gurdasani, D., Dougan, G., Sandhu, M. S., et al. (2017). Antimicrobial resistance in human populations: Challenges and opportunities. *Glob. Heal. Epidemiol. Genomics* 2, 1–7. doi:10.1017/gheg.2017.4.

Alliance save our antibiotics (2020). New European Union rules on farm antibiotic use. Available at: https://ec.europa.eu/eurostat/statistics- [Accessed October 15, 2021].

Ames, G. F. L., and Nikaido, K. (1975). Two-Dimensional Gel Electrophoresis of Membrane Proteins.

Biochemistry 15, 616–623. doi:10.1021/bi00648a026.

Amisha, Malik, P., Pathania, M., and Rathaur, V. (2019). Overview of artificial intelligence in medicine. *J. Fam. Med. Prim. Care* 8, 2328-2331. doi:10.4103/jfmpc.jfmpc_440_19.

Angel, T. E., and Aryal, U. K. (2020). "Impact of Gut Microbiota on Host by Exploring Proteomics," in *Gut Microbiome and Its Impact on Health and Diseases* (Springer, Cham), 229–250. doi:10.1007/978-3-030-47384-6_11.

Anhalt, J. P., and Fenselau., C. (1975). Identification of bacteria using mass spectrometry techniques. *Anal. Chem.* 47, 219–225. doi:10.1016/j.ijms.2013.04.016.

Anjum, M. F., Marco-Jimenez, F., Duncan, D., Marín, C., Smith, R. P., and Evans, S. J. (2019). Livestock-Associated Methicillin-Resistant *Staphylococcus aureus* From Animals and Animal Products in the UK. *Front. Microbiol.* 10, 2136. doi:10.3389/fmicb.2019.02136.

Ann Luna, R. (2016). "Molecular Typing Instruments and Methods," in *Manual of Commercial Methods in Clinical Microbiology*, John Wiley & Sons, Ltd, 336–345. doi:10.1002/9781119021872.ch18.

Appala, K., Bimpeh, K., Freeman, C., and Hines, K. M. (2020). Recent applications of mass spectrometry in bacterial lipidomics. *Anal. Bioanal. Chem.* 412, 5935–5943. doi:10.1007/s00216-020-02541-8.

Araujo, R., and Sampaio-Maia, B. (2018). "Fungal Genomes and Genotyping," in *Advances in Applied Microbiology*, Academic Press Inc., 37–81. doi:10.1016/bs.aambs.2017.10.003.

Arena, F., Giani, T., Pollini, S., Viaggi, B., Pecile, P., and Rossolini, G. M. (2017). Molecular antibiogram in diagnostic clinical microbiology: Advantages and challenges. *Future Microbiol.* 12, 361–364. doi:10.2217/fmb-2017-0019.

Armengaud, J. (2013). Microbiology and proteomics, getting the best of both worlds! *Environ. Microbiol.* 15, 12–23. doi:10.1111/j.1462-2920.2012.02811.x.

Armengaud, J. (2020). The proteomics contribution to the counter-bioterrorism toolbox in the post-COVID-19 era. *Expert Rev. Proteomics* 17, 507–511. doi:10.1080/14789450.2020.1822745.

Arning, N., Sheppard, S. K., Bayliss, S., Clifton, D. A., and Wilson, D. J. (2021). Machine learning to predict the source of campylobacteriosis using whole genome data. *PLOS Genet.* 17, e1009436. doi:10.1371/journal.pgen.1009436.

Asakura, K., Azechi, T., Sasano, H., Matsui, H., Hanaki, H., Miyazaki, M., et al. (2018). Rapid and easy detection of low-level resistance to vancomycin in methicillin-resistant *Staphylococcus aureus* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *PLoS One* 13, 1-10. doi:10.1371/journal.pone.0194212.

Ashelford, K. E., Chuzhanova, N. A., Fry, J. C., Jones, A. J., and Weightman, A. J. (2005). At least 1 in

20 16S rRNA sequence records currently held in public repositories is estimated to contain substantial anomalies. *Appl. Environ. Microbiol.* 71, 7724–7736. doi:10.1128/AEM.71.12.7724-7736.2005.

Awofisayo-Okuyelu, A., Hall, I., Adak, G., Hawker, J. I., Abbott, S., and McCarthy, N. (2017). A systematic review and meta-analysis on the incubation period of Campylobacteriosis. *Epidemiol. Infect.* 145, 2241–2253. doi:10.1017/S0950268817001303.

Backert, S. (2021). *Fighting Campylobacter infections - Towards a One-Health approach*., ed. S. Backert Springer. doi:10.3109/08820137709055812.

Banerji, S., Simon, S., Tille, A., Fruth, A., and Flieger, A. (2020). Genome-based *Salmonella* serotyping as the new gold standard. *Sci. Rep.* 10, 1–10. doi:10.1038/s41598-020-61254-1.

Barlam, T. F., Cosgrove, S. E., Abbo, L. M., Macdougall, C., Schuetz, A. N., Septimus, E. J., et al. (2016). Implementing an antibiotic stewardship program: Guidelines by the Infectious Diseases Society of America and the Society for Healthcare Epidemiology of America. *Clin. Infect. Dis.* 62, e51–e77. doi:10.1093/cid/ciw118.

Barros, C. C. (2021). Neural network-based predictions of antimicrobial resistance in *Salmonella* spp. using k-mers counting from whole-genome sequences. *bioRxiv*, 2021.08.10.455825. doi:10.1101/2021.08.10.455825.

Benagli, C., Rossi, V., Dolina, M., Tonolla, M., and Petrini, O. (2011). Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. *PLoS One* 6, e16424. doi:10.1371/journal.pone.0016424.

Benkova, M., Soukup, O., and Marek, J. (2020). Antimicrobial susceptibility testing: currently used methods and devices and the near future in clinical practice. *J. Appl. Microbiol.* 129, 806–822. doi:10.1111/jam.14704.

Berg, R. L., Jutila, J. W., and Firehammer, B. D. (1971). A revised classification of *Vibrio fetus*. *Am. J. Vet. Res.* 32, 11–22.

Bergeron, M. G., and Ouellette, M. (1998). Preventing antibiotic resistance through rapid genotypic identification of bacteria and of their antibiotic resistance genes in the clinical microbiology laboratory. *J. Clin. Microbiol.* 36, 2169–2172. doi:10.1128/jcm.36.8.2169-2172.1998.

Bertelli, C., and Greub, G. (2013). Rapid bacterial genome sequencing: Methods and applications in clinical microbiology. *Clin. Microbiol. Infect.* 19, 803–813. doi:10.1111/1469-0691.12217.

Biemann, K., Gapp, F., and Seibl, J. (1959). Application of Mass Spectrometry to Structure Problems. I. Amino Acid Sequence in Peptides. *J. Am. Chem. Soc.* 81, 2274–2275. doi:10.1021/ja01518a069.

BioMérieux. VITEK® MS Mycobacterium/Nocardia Kit. Available at:

https://biomerieuxdirect.com/clinical/Bacteriology/ID-AST-Auto/MASS-SPECTROMETRY/Mass-Spectrometry-Reagents-%26-Consumables/Mass-Spec-Reagents-%26-Consumables/VITEK®-MS-Mycobacterium-Nocardia-Kit-/p/415659 [Accessed October 21, 2021].

BioMérieux (2021). bioMérieux announces CE-marking of the new MALDI-TOF mass spectrometry identification system : VITEK ® MS PRIME. Available at: https://www.biomerieux.com/en/biomerieux-announces-ce-marking-new-maldi-tof-mass-spectrometry-identification-system-vitekr-ms [Accessed October 21, 2021]

Bizzini, A., Durussel, C., Bille, J., Greub, G., and Prod'hom, G. (2010). Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a Clinical Microbiology Laboratory. *J. Clin. Microbiol.* 48, 1549–1554. doi:10.1128/JCM.01794-09.

Body, B. A., Beard, M. A., Slechta, E. S., Hanson, K. E., Barker, A. P., Babady, N. E., et al. (2018). Evaluation of the vitek MS v3.0 matrix-Assisted laser desorption ionization–Time of flight mass spectrometry system for identification of *Mycobacterium* and *Nocardia* species. *J. Clin. Microbiol.* 56, e00237-18. doi:10.1128/JCM.00237-18.

Bolinger, H., and Kathariou, S. (2017). The Current State of Macrolide Resistance in *Campylobacter* spp.: Trends and Impacts of Resistance Mechanisms. *Appl. Environ. Microbiol.* 83, e00416-17. doi:10.1128/aem.00416-17.

Bookstaver, P. B., Nimmich, E. B., Smith, T. J., Justo, J. A., Kohn, J., Hammer, K. L., et al. (2017). Cumulative effect of an antimicrobial stewardship and rapid diagnostic testing bundle on early streamlining of antimicrobial therapy in Gram-negative bloodstream infections. *Antimicrob. Agents Chemother.* 61, 1–10. doi:10.1128/AAC.00189-17.

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., et al. (2020). ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother.* 75, 3491–3500. doi:10.1093/jac/dkaa345.

Boucheikhchoukh, M., Laroche, M., Aouadi, A., Dib, L., Benakhla, A., Raoult, D., et al. (2018). MALDI-TOF MS identification of ticks of domestic and wild animals in Algeria and molecular detection of associated microorganisms. *Comp. Immunol. Microbiol. Infect. Dis.* 57, 39–49. doi:10.1016/j.cimid.2018.05.002.

Bouton, C. E., Shaikhouni, A., Annetta, N. V., Bockbrader, M. A., Friedenberg, D. A., Nielson, D. M., et al. (2016). Restoring cortical control of functional movement in a human with quadriplegia. *Nature* 533, 247–250. doi:10.1038/nature17435.

Bowman, J. P. (2014). "Advantages and applications of gel-free proteomic approaches in the study

of prokaryotes," in *Comprehensive Analytical Chemistry*, 157–200. doi:10.1016/B978-0-444-62650-9.00007-5.

Brackmann, M., Leib, S. L., Tonolla, M., Schürch, N., and Wittwer, M. (2020). Antimicrobial resistance classification using MALDI-TOF-MS is not that easy: lessons from vancomycin-resistant *Enterococcus faecium*. *Clin. Microbiol. Infect.* 26, 391–393. doi:10.1016/j.cmi.2019.10.027.

Bredtmann, C. M., Krücken, J., Murugaiyan, J., Kuzmina, T., and von Samson-Himmelstjerna, G. (2017). Nematode Species Identification—Current Status, Challenges and Future Perspectives for Cyathostomins. *Front. Cell. Infect. Microbiol.* 7, 1–8. doi:10.3389/fcimb.2017.00283.

Bruker (2018). MALDI Biotyper® Changing Microbiology. Available at: https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification.html [Accessed July 8, 2019]

Bruker (2019a). Bruker Launches MALDI Biotyper Sirius at ASM Microbe Conference. Available at: www.asm.org [Accessed July 8, 2019].

Bruker (2019b). Innovation with Integrity Changing Microbiology MALDI Biotyper ® Subtyping Module. Available at: https://www.bruker.com/fileadmin/user_upload/8-PDF-Docs/Separations_MassSpectrometry/Literature/Brochures/1862837_MBT_Subtyping_brochure_01-2019_ebook.pdf [Accessed June 18, 2019].

Bruker (2020). Notes de publication. MBT IVD Library, Révision K.

Bruker (2021a). Bruker Announces New Microbiology Products and Workflows for Microbial Identification and Infection Control at ECCMID 2021 | Bruker. Available at: https://www.bruker.com/en/news-and-events/news/2021/bruker_announces_new_microbiology_products_and_workflows_for_microbial_identification.html [Accessed November 9, 2021].

Bruker (2021b). MBT Filamentous Fungi Library. Available at: https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/mbt-filamentous-fungi-library.html [Accessed October 21, 2021].

Bruker (2021c). MBT Lipid Xtract ™ Kit. Available at: https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/mbt-lipid-xtract-kit.html [Accessed October 21, 2021].

Bruker (2021d). MBT *Mycobacteria* IVD Module. Available at: https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification-for-clinical-laboratories-ivd-ce/mbt-mycobacteria-ivd-

module.html [Accessed October 21, 2021].

Bruker (2021e). MBT *Mycobacteria* RUO Module. Available at: https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/mbt-mycobacteria-ruo.html [Accessed October 21, 2021].

Buckwalter, S. P., Olson, S. L., Connelly, B. J., Lucas, B. C., Rodning, A. A., Walchak, R. C., et al. (2016). Evaluation of matrix-Assisted laser desorption ionization-Time of flight mass spectrometry for identification of *Mycobacterium* species, *Nocardia* species, and other aerobic Actinomycetes. *J. Clin. Microbiol.* 54, 376–384. doi:10.1128/JCM.02128-15.

Buller, H., Adam, K., Bard, A., Bruce, A., Chan, K. W., Hinchliffe, S., et al. (2020). Veterinary Diagnostic Practice and the Use of Rapid Tests in Antimicrobial Stewardship on UK Livestock Farms. *Front. Vet. Sci.* 7, 765. doi:10.3389/fvets.2020.569545.

Bush, K., and Bradford, P. A. (2016). β-lactams and β-lactamase inhibitors: An overview. *Cold Spring Harb. Perspect. Med.* 6, 1-22. doi:10.1101/cshperspect.a025247.

Butzler, J. P., Dekeyser, P., Detrain, M., and Dehaen, F. (1973). Related vibrio in stools. *J. Pediatr.* 82, 493–495. doi:10.1016/S0022-3476(73)80131-3.

Caballé, N. C., Castillo-Sequera, J. L., Gómez-Pulido, J. A., Gómez-Pulido, J. M., and Polo-Luque, M. L. (2020). Machine learning applied to diagnosis of human diseases: A systematic review. *Appl. Sci.* 10, 1–27. doi:10.3390/app10155135.

Calderaro, A., Arcangeletti, M. C., Rodighiero, I., Buttrini, M., Gorrini, C., Motta, F., et al. (2014). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification. *Sci. Rep.* 4, 1–10. doi:10.1038/srep06803.

Camoez, M., Sierra, J. M., Dominguez, M. A., Ferrer-Navarro, M., Vila, J., and Roca, I. (2016). Automated categorization of methicillin-resistant *Staphylococcus aureus* clinical isolates into different clonal complexes by MALDI-TOF mass spectrometry. *Clin. Microbiol. Infect.* 22, 161.e1-161.e7. doi:10.1016/j.cmi.2015.10.009.

Candela, A., Arroyo, M. J., Mancera, L., Microbiology, C., General, H., Software, C. B., et al. (2021a). Rapid and reproducible MALDI-TOF-based method for detection Vancomycin- resistant *Enterococcus faecium* using classifying algorithms. *bioRxiv*, 1–24. doi:10.1101/2021.06.23.449689.

Candela, A., Mateos, M., Gómez-Asenjo, A., Arroyo, M. J., Hernandez-García, M., Campo, R. del, et al. (2021b). Discrimination of species within the *Enterobacter cloacae* complex using MALDI-TOF Mass Spectrometry and Fourier-Transform Infrared Spectroscopy coupled with Machine Learning tools. *bioRxiv*, 2021.11.02.467040. doi:10.1101/2021.11.02.467040.

Carbonnelle, E., Grohs, P., Jacquier, H., Day, N., Tenza, S., Dewailly, A., et al. (2012). Robustness of two MALDI-TOF mass spectrometry systems for bacterial identification. *J. Microbiol. Methods* 89, 133–136. doi:10.1016/J.MIMET.2012.03.003.

Carbonnelle, É., and Nassif, X. (2011). Utilisation en routine du MALDI-TOF-MS pour l'identification des pathogènes en microbiologie médicale. *Medecine/Sciences* 27, 882–888. doi:10.1051/medsci/20112710017.

Cardoso, M. J., Ferreira, V., Truninger, M., Maia, R., and Teixeira, P. (2021). Cross-contamination events of *Campylobacter* spp. in domestic kitchens associated with consumer handling practices of raw poultry. *Int. J. Food Microbiol.* 338, 1–10. doi:10.1016/j.ijfoodmicro.2020.108984.

CASFM (2020). Comité de l'antibiogramme de la Société Française de Microbiologie Recommandations 2020. Available at: https://www.sfm-microbiologie.org/2020/10/02/casfm-eucast-v1-2-octobre-2020/ [Accessed December 10, 2020]

Cassini, A., Högberg, L. D., Plachouras, D., Quattrocchi, A., Hoxha, A., Simonsen, G. S., et al. (2019). Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect. Dis.* 19, 56–66. doi:10.1016/S1473-3099(18)30605-4.

Castro-Escarpulli, G., Alonso-Aguilar, N. M., Sánchez, G. R., Bocanegra-Garcia, V., Guo, X., Juárez-Enríquez, S. R., et al. (2015). Identification and Typing Methods for the Study of Bacterial Infections: a Brief Review and Mycobacterial as Case of Study. *iMedPub Journals* 7, 1–10.

Catherman, A. D., Skinner, O. S., and Kelleher, N. L. (2014). Top Down proteomics: Facts and perspectives. *Biochem. Biophys. Res. Commun.* 445, 683–693. doi:10.1016/j.bbrc.2014.02.041.

CDC (2015). The AMR Challenge. Available at: https://www.cdc.gov/drugresistance/intl-activities/amr-challenge.html [Accessed November 3, 2021].

CDC (2019). Antibiotic resistance threats in the United States. Available at: https://stacks.cdc.gov/view/cdc/82532 [Accessed November 3, 2021].

Chabriere, E., Bassène, H., Drancourt, M., and Sokhna, C. (2018). MALDI-TOF-MS and Point of Care are disruptive diagnostic tools in Africa. *New Microbes New Infect. S83-S88*. doi:10.1016/j.nmni.2018.08.020.

Chan, K. W., Bard, A. M., Adam, K. E., Rees, G. M., Morgans, L., Cresswell, L., et al. (2020). Diagnostics and the challenge of antimicrobial resistance: A survey of UK livestock veterinarians' perceptions and practices. *Vet. Rec.* 187, E125. doi:10.1136/vr.105822.

Chen, C. Y., Clark, C. G., Langner, S., Boyd, D. A., Bharat, A., McCorrister, S. J., et al. (2020). Detection of Antimicrobial Resistance Using Proteomics and the Comprehensive Antibiotic Resistance Database: A Case Study. *Proteomics - Clin. Appl.* 14, 1800182. doi:10.1002/prca.201800182.

Chen, J. C., Tagg, K. A., Joung, Y. J., Bennett, C., Watkins, L. F., Eikmeier, D., et al. (2018). Report of *erm(B) Campylobacter jejuni* in the United States. *Antimicrob. Agents Chemother.* 62. doi:10.1128/AAC.02615-17.

Chen, Y., Wu, T., Lu, W., Yuan, W., Pan, M., Lee, Y., et al. (2021). Predicting the Role of the Human Gut Microbiome in Constipation Using Machine-Learning Methods : A Meta-Analysis. *Microorganisms* 9, 1–15.

Chlebicz, A., and Śliżewska, K. (2018). Campylobacteriosis, Salmonellosis, Yersiniosis, and Listeriosis as Zoonotic Foodborne Diseases: A Review. *Int. J. Environ. Res. Public Health* 15, 1–29. doi:10.3390/ijerph15050863.

Christie-Oleza, J. A., Miotello, G., and Armengaud, J. (2012). High-throughput proteogenomics of Ruegeria pomeroyi: Seeding a better genomic annotation for the whole marine *Roseobacter* clade. *BMC Genomics* 13, 1-13. doi:10.1186/1471-2164-13-73.

Chun, K., Syndergaard, C., Damas, C., Trubey, R., Mukindaraj, A., Qian, S., et al. (2015). Sepsis Pathogen Identification. *J. Lab. Autom.* 20, 539–561. doi:10.1177/2211068214567345.

Clark, A. E., Kaleta, E. J., Arora, A., and Wolk, D. M. (2013). Matrix-Assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clin. Microbiol. Rev.* 26, 547–603. doi:10.1128/CMR.00072-12.

Claydon, M. A., Davey, S. N., Edwards-Jones, V., and Gordon, D. B. (1996). The Rapid Identification of Intact Microorganisms Using Mass Spectrometry. *Nat. Biotechnol.* 14, 1584–1586. doi:10.1038/nbt1196-1584.

Cody, A. J., Maiden, M. C., Strachan, N. J., and McCarthy, N. D. (2019). A systematic review of source attribution of human campylobacteriosis using multilocus sequence typing. *Eurosurveillance* 24. doi:10.2807/1560-7917.ES.2019.24.43.1800696.

Cody, A. J., Mccarthy, N. D., Bray, J. E., Wimalarathna, H. M. L., Colles, F. M., Jansen van Rensburg, M. J., et al. (2015). Wild bird-associated *Campylobacter jejuni* isolates are a consistent source of human disease, in Oxfordshire, United Kingdom. *Environ. Microbiol. Rep.* 7, 782–788. doi:10.1111/1758-2229.12314.

Cody, A. J., McCarthy, N. D., Van Rensburg, M. J., Isinkaye, T., Bentley, S. D., Parkhill, J., et al. (2013). Real-time genomic epidemiological evaluation of human *Campylobacter* isolates by use of whole-genome multilocus sequence typing. *J. Clin. Microbiol.* 51, 2526–2534. doi:10.1128/JCM.00066-13.

Cohen, S., Rokach, L., Motro, Y., Moran-Gilad, J., and Veksler-Lublinsky, I. (2021). minMLST: machine learning for optimization of bacterial strain typing. *Bioinformatics* 37, 303–311. doi:10.1093/bioinformatics/btaa724.

Cordovana, M., Kostrzewa, M., Glandorf, J., Bienia, M., Ambretti, S., and Pranada, A. B. (2018). A full MALDI-based approach to detect plasmid-encoded KPC-producing *Klebsiella pneumoniae*. *Front. Microbiol.* 9, 2854, 1-8. doi:10.3389/fmicb.2018.02854.

Cordovana, M., Mauder, N., Kostrzewa, M., Wille, A., Rojak, S., Hagen, R. M., et al. (2021). Classification of *Salmonella enterica* of the (Para-)Typhoid Fever Group by Fourier-Transform Infrared (FTIR) Spectroscopy. *Microorganisms* 9, 853, 1-13. doi:10.3390/microorganisms9040853.

Costa, D., and Iraola, G. (2019). Pathogenomics of emerging *Campylobacter* species. *Clin. Microbiol. Rev.* 32, 1-24. doi:10.1128/CMR.00072-18.

Cottrell, J. S. (1994). Protein identification by peptide mass fingerprinting. *Pept. Res.* 7, 115–124. doi:10.1533/9781908818058.185.

Couto, N., and Rossen, J. W. (2021). "Overview of Microbial NGS for Clinical and Public Health Microbiology," in *Application and Integration of Omics-powered Diagnostics in Clinical and Public Health Microbiology*, Springer, Cham, 9–24. doi:10.1007/978-3-030-62155-1_2.

Crespo-Piazuelo, D., and Lawlor, P. G. (2021). Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) prevalence in humans in close contact with animals and measures to reduce on-farm colonisation. *Ir. Vet. J.* 74, 1–12. doi:10.1186/s13620-021-00200-7.

Croxatto, A., Prod'hom, G., and Greub, G. (2012). Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* 36, 380–407. doi:10.1111/j.1574-6976.2011.00298.x.

Cuénod, A., Foucault, F., Pflüger, V., and Egli, A. (2021). Factors Associated With MALDI-TOF Mass Spectral Quality of Species Identification in Clinical Routine Diagnostics. *Front. Cell. Infect. Microbiol.* 11, 1-15. doi:10.3389/fcimb.2021.646648.

Culebras, D. E. (2018). "Application of MALDI-TOF MS in Bacterial Strain Typing and Taxonomy," in *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*, Academic Press, 213–233. doi:10.1016/B978-0-12-814451-0.00015-0.

Cunningham, S. A., Mainella, J. M., and Patel, R. (2014). Misidentification of *Neisseria polysaccharea* as *Neisseria meningitides* with the use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 52, 2270–2271. doi:10.1128/JCM.00664-14.

Dailey, P. J., Elbeik, T., and Holodniy, M. (2020). Companion and complementary diagnostics for

infectious diseases. *Expert Rev. Mol. Diagn.* 20, 619–636. doi:10.1080/14737159.2020.1724784.

Davenport, T., and Kalakota, R. (2019). The potential for artificial intelligence in healthcare. *Futur. Healthc. J.* 6, 94–98. doi:10.7861/futurehosp.6-2-94.

Davin-Regli, A., Lavigne, J. P., and Pagès, J. M. (2019). Enterobacter spp.: update on taxonomy, clinical aspects, and emerging antimicrobial resistance. *Clin. Microbiol. Rev.* 32. doi:10.1128/CMR.00002-19.

De Bruycker, K., Welle, A., Hirth, S., Blanksby, S. J., and Barner-Kowollik, C. (2020). Mass spectrometry as a tool to advance polymer science. *Nat. Rev. Chem.* 4, 257–268. doi:10.1038/s41570-020-0168-1.

De Nies, L., Lopes, S., Busi, S. B., Galata, V., Heintz-Buschart, A., Laczny, C. C., et al. (2021). PathoFact: a pipeline for the prediction of virulence factors and antimicrobial resistance genes in metagenomic data. *Microbiome* 9, 1–14. doi:10.1186/s40168-020-00993-9.

Deak, E., Green, N., and Humphries, R. M. (2014). Microbiology test reliability in differentiation of *Neisseria meningitidis* and *Neisseria polysaccharea*. *J. Clin. Microbiol.* 52, 3496. doi:10.1128/JCM.01703-14.

Dearlove, B. L., Cody, A. J., Pascoe, B., Méric, G., Wilson, D. J., and Sheppard, S. K. (2016). Rapid host switching in generalist *Campylobacter* strains erodes the signal for tracing human infections. *ISME J.* 10, 721–729. doi:10.1038/ismej.2015.149.

Del Chierico, F., Di Cave, D., Accardi, C., Santoro, M., Masotti, A., D'Alfonso, R., et al. (2016). Identification and typing of free-living *Acanthamoeba* spp. by MALDI-TOF MS Biotyper. *Exp. Parasitol.* 170, 82–89. doi:10.1016/j.exppara.2016.09.007.

Demirev, P. A., Ho, Y. P., Ryzhov, V., and Fenselau, C. (1999). Microorganism identification by mass spectrometry and protein database searches. *Anal. Chem.* 71, 2732–2738. doi:10.1021/ac990165u.

Deng, J., Fu, L., Wang, R., Yu, N., Ding, X., Jiang, L., et al. (2014). Comparison of MALDI-TOF MS, gene sequencing and the Vitek 2 for identification of seventy-three clinical isolates of enteropathogens. *J. Thorac. Dis.* 6, 539–544. doi:10.3978/j.issn.2072-1439.2014.02.20.

Denis, M., Rose, V., Nagard, B., Serghine, J., Meunier, M., Benoit, F., et al. (2019). Comparison of *Campylobacter jejuni* using MALDI-TOF to identify relationships between different origins: poultry, bovine, sheep, shorebird, river and shellfish. in *Conference: MICROBE, 15eme congress of the French Society of Microbiology (SFM)* Available at: https://www.researchgate.net/publication/336140746_Comparison_of_Campylobacter_jejuni_using_MALDI-

TOF_to_identify_relationships_between_different_origins_poultry_bovine_sheep_shorebird_river_and_shellfish [Accessed April 3, 2020].

Deurenberg, R. H., Bathoorn, E., Chlebowicz, M. A., Couto, N., Ferdous, M., García-Cobos, S., et al. (2017). Application of next generation sequencing in clinical microbiology and infection prevention. *J. Biotechnol.* 243, 16–24. doi:10.1016/j.jbiotec.2016.12.022.

Devi, A. (2019). Prevalence of *Campylobacter* spp. in human clinical samples. Available at: https://researchoutput.csu.edu.au/ws/portalfiles/portal/30761296/Devi_A_PhDThesis_2019.pdf [Accessed July 8, 2019].

Devleesschauwer, B., Bouwknegt, M., Mangen, M. J. J., and Havelaar, A. H. (2016). "Health and economic burden of *Campylobacter*," in *Campylobacter: Features, Detection, and Prevention of Foodborne Disease*, ed. G. Klein, Amsterdam, Netherlands: Elsevier Inc., 27–40. doi:10.1016/B978-0-12-803623-5.00002-2.

Dieckmann, R., Hammerl, J. A., Hahmann, H., Wicke, A., Kleta, S., Dabrowski, P. W., et al. (2016). Rapid characterisation of: *Klebsiella oxytoca* isolates from contaminated liquid hand soap using mass spectrometry, FTIR and Raman spectroscopy. *Faraday Discuss.* 187, 353–375. doi:10.1039/c5fd00165j.

Ding, S., Bale, N. J., Hopmans, E. C., Villanueva, L., Arts, M. G. I., Schouten, S., et al. (2021). Lipidomics of Environmental Microbial Communities. II: Characterization Using Molecular Networking and Information Theory. *Front. Microbiol.* 12, 1-15. doi:10.3389/fmicb.2021.659315.

Dingle, K. E., Colles, F. M., Wareing, D. R. A., Ure, R., Fox, A. J., Bolton, F. E., et al. (2001). Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* 39, 14–23. doi:10.1128/JCM.39.1.14-23.2001.

Dingle, K. E., McCarthy, N. D., Cody, A. J., Peto, T. E. A., and Maiden, M. C. J. (2008). Extended Sequence Typing of *Campylobacter* spp., United Kingdom. *Emerg. Infect. Dis.* 14, 1620–1622. doi:10.3201/EID1410.071109.

Dortet, L., Bonnin, R. A., Le Hello, S., Fabre, L., Bonnet, R., Kostrzewa, M., et al. (2020). Detection of Colistin Resistance in *Salmonella enterica* Using MALDIxin Test on the Routine MALDI Biotyper Sirius Mass Spectrometer. *Front. Microbiol.* 11, 1–6. doi:10.3389/fmicb.2020.01141.

Dortet, L., Bonnin, R. A., Pennisi, I., Gauthier, L., Jousset, A. B., Dabos, L., et al. (2018a). Rapid detection and discrimination of chromosome-And MCR-plasmid-mediated resistance to polymyxins by MALDI-TOF MS in *Escherichia coli*: The MALDIxin test. *J. Antimicrob. Chemother.* 73, 3359–3367. doi:10.1093/jac/dky330.

Dortet, L., Potron, A., Bonnin, R. A., Plesiat, P., Naas, T., Filloux, A., et al. (2018b). Rapid detection of colistin resistance in *Acinetobacter baumannii* using MALDI-TOF-based lipidomics on intact

bacteria. *Sci. Rep.* 8, 1–5. doi:10.1038/s41598-018-35041-y.

Draker, K. A., and Wright, G. D. (2004). Molecular Mechanism of the Enterococcal Aminoglycoside 6' -N-Acetyltransferase': Role of GNAT-Conserved Residues in the Chemistry of Antibiotic Inactivation. *Biochemistry* 43, 446–454. doi:10.1021/bi035667n.

Du, J., Luo, J., Huang, J., Wang, C., Li, M., Wang, B., et al. (2019). Emergence of genetic diversity and multi-drug resistant *Campylobacter jejuni* from wild birds in Beijing, china. *Front. Microbiol.* 10, 2433, 1-14. doi:10.3389/fmicb.2019.02433.

Dupree, E. J., Jayathirtha, M., Yorkey, H., Mihasan, M., Petre, B. A., and Darie, C. C. (2020). A critical review of bottom-up proteomics: The good, the bad, and the future of this field. *Proteomes* 8, 1–26. doi:10.3390/proteomes8030014.

Eberle, K. N., and Kiess, A. S. (2012). Phenotypic and genotypic methods for typing *Campylobacter jejuni* and *Campylobacter coli* in poultry. *Poult. Sci.* 91, 255–264. doi:10.3382/ps.2011-01414.

ECDC (2014). Factsheet for experts - Antimicrobial resistance. *Fact sheet Expert. - Antimicrob. Resist.* Available at: https://www.ecdc.europa.eu/en/antimicrobial-resistance/facts/factsheets/experts [Accessed November 3, 2021].

ECDC (2020). Antimicrobial consumption in the EU/EEA – Annual Epidemiological Report 2019. Available at: https://www.ecdc.europa.eu/en/publications-data/surveillance-antimicrobial-consumption-europe-2019 [Accessed November 3, 2021].

ECDC (2021). Assessment tool for joint ' One-Health ' country visits in relation to antimicrobial resistance. Available at: https://www.ecdc.europa.eu/en/publications-data/assessment-tool-joint-one-health-country-visits-relation-antimicrobial-resistance [Accessed November 3, 2021].

ECDC, EFSA, and EMA (2017). ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals: Joint Interagency Antimicrobial Consumption and Resistan. *EFSA J.* 15, 4872. doi:10.2903/j.efsa.2017.4872.

Edwards-Jones, V., Claydon, M. A., Evason, D. J., Walker , J., Fox ,A. J., and Gordon ,D. B. (2000). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* by intact cell mass spectrometry. *J. Med. Microbiol.* 49, 295–300. doi:10.1099/0022-1317-49-3-295.

EFSA (2010). Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. *EFSA J.* 8, 1–89. doi:10.2903/j.efsa.2010.1437.

EFSA (2014). EFSA explains zoonotic diseases: Food-borne zoonotic diseases. Available at: https://www.efsa.europa.eu/fr/corporate/pub/factsheetfoodbornezoonoses2014#:~:text=E

FSA%20explains%20zoonotic%20diseases%3A%20Food%2Dborne%20zoonotic%20diseases,-Published%3A&text=Zoonoses%20are%20infections%20or%20diseases,through%20contact%20with%20infected%20animals.[Accessed November 15, 2021]

EFSA, and ECDC (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA J.* 15, 5077. doi:10.2903/j.efsa.2017.5077.

EFSA, and ECDC (2020). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018 European. *EFSA J.* 18, 1–166. doi:10.2903/j.efsa.2020.6007.

EFSA, and ECDC (2021a). The European Union One Health 2019 Zoonoses Report. *EFSA J.* 19, 1–286. doi:10.2903/j.efsa.2021.6406.

EFSA, and ECDC (2021b). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA J.* 19, 1–179. doi:10.2903/j.efsa.2021.6490.

Egli, A. (2020). Digitalization, clinical microbiology and infectious diseases. *Clin. Microbiol. Infect.* 26, 1289–1290. doi:10.1016/j.cmi.2020.06.031.

Egli, A., Schrenzel, J., and Greub, G. (2020). Digital microbiology. *Clin. Microbiol. Infect.* 26, 1324–1331. doi:10.1016/j.cmi.2020.06.023.

El-Bouri, K., Johnston, S., Rees, E., Thomas, I., Bome-Mannathoko, N., Jones, C., et al. (2012). Comparison of bacterial identification by MALDI-TOF mass spectrometry and conventional diagnostic microbiology methods: Agreement, speed and cost implications. *Br. J. Biomed. Sci.* 69, 47–55. doi:10.1080/09674845.2012.12002436.

Emami, K., Askari, V., Ullrich, M., Mohinudeen, K., Anil, A. C., Khandeparker, L., et al. (2012). Characterization of bacteria in Ballast water using MALDI-TOF mass spectrometry. *PLoS One* 7, e38515. doi:10.1371/journal.pone.0038515.

Emele, M. F., Karg, M., Hotzel, H., Graaf-van Bloois, L., Groß, U., Bader, O., et al. (2019a). Differentiation of *Campylobacter fetus* subspecies by proteotyping. *Eur. J. Microbiol. Immunol.* 9, 62–71. doi:10.1556/1886.2019.00006.

Emele, M. F., Možina, S. S., Lugert, R., Bohne, W., Masanta, W. O., Riedel, T., et al. (2019b). Proteotyping as alternate typing method to differentiate *Campylobacter coli* clades. *Sci. Rep.* 9, 1–11. doi:10.1038/s41598-019-40842-w.

Epping, L., Walther, B., Piro, R. M., Knüver, M. T., Huber, C., Thürmer, A., et al. (2021). Genome-wide insights into population structure and host specificity of *Campylobacter jejuni*. *Sci. Rep.* 11, 1–15. doi:10.1038/s41598-021-89683-6.

Erickson, B. J., Korfiatis, P., Akkus, Z., and Kline, T. L. (2017). Machine learning for medical imaging. *Radiographics* 37, 505–515. doi:10.1148/rg.2017160130.

European Commission (2015). Guidelines for the prudent use of antimicrobials in veterinary medicine. *Off. J. Eur. Union*, C 299/7.

European Commission (2017). EU Guidelines for the prudent use of antimicrobials in human health. *Off. J. Eur. Union*, C 212/1.

European Commission (2021). A European One Health Action Plan against Antimicrobial Resistance (AMR). Available at: http://www.who.int/entity/drugresistance/documents/surveillancereport/en/index.html [Accessed November 3, 2021].

Fabre, A., Oleastro, M., Nunes, A., Santos, A., Sifré, E., Ducournau, A., et al. (2018). Whole-Genome Sequence Analysis of Multidrug-Resistant *Campylobacter* Isolates: a Focus on Aminoglycoside Resistance Determinants. *J. Clin. Microbiol.* 56, 1–12. doi:10.1128/jcm.00390-18.

Facciolà, A., Riso, R., Avventuroso, E., Visalli, G., Delia, S. A., and Laganà, P. (2017). *Campylobacter*: From microbiology to prevention. *J. Prev. Med. Hyg.* 58, E79–E92. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28900347 [Accessed July 21, 2018].

Falagas, M. E., Grammatikos, A. P., and Michalopoulos, A. (2008). Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Rev. Anti. Infect. Ther.* 6, 593–600. doi:10.1586/14787210.6.5.593.

Fall, B., Lo, C. I., Samb-Ba, B., Perrot, N., Diawara, S., Gueye, M. W., et al. (2015). The ongoing revolution of MALDI-TOF mass spectrometry for microbiology reaches tropical Africa. *Am. J. Trop. Med. Hyg.* 92, 641–647. doi:10.4269/ajtmh.14-0406.

FAO, WHO, and OIE. WHO, FAO, and OIE unite in the fight against Antimicrobial Resistance. Available at: https://www.oie.int/en/document/fao_oie_who_amrfactsheet/ [Accessed November 15, 2021].

Ferone, M., Gowen, A., Fanning, S., and Scannell, A. G. M. (2020). Microbial detection and identification methods: Bench top assays to omics approaches. *Compr. Rev. Food Sci. Food Saf.* 19, 3106–3129. doi:10.1111/1541-4337.12618.

Feucherolles, M., Cauchie, H., and Penny, C. (2019a). MALDI-TOF Mass Spectrometry and Specific Biomarkers : Potential New Key for Swift Identification of Antimicrobial Resistance in Foodborne Pathogens . *Microorganisms* 7, 1–16. doi:10.3390/microorganisms7120593.

Feucherolles, M., Nennig, M., Becker, S. L., Martiny, D., Losch, S., Penny, C., et al. (2021). Investigation of MALDI - TOF Mass Spectrometry for Assessing the Molecular Diversity of *Campylobacter jejuni* and Comparison with MLST and cgMLST : A Luxembourg One - Health

Study. *Diagnostics* 11, 1–17. doi:10.3390/diagnostics11111949.

Feucherolles, M., Poppert, S., Utzinger, J., and Becker, S. L. (2019b). MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: a systematic review. *Parasit. Vectors* 12, 1–13. doi:10.1186/s13071-019-3493-9.

Fitzgerald, C. (2015). *Campylobacter*. *Clin. Lab. Med.* 35, 289–298. doi:10.1016/j.cll.2015.03.001.

Flores-Treviño, S., Garza-González, E., Mendoza-Olazarán, S., Morfín-Otero, R., Camacho-Ortiz, A., Rodríguez-Noriega, E., et al. (2019). Screening of biomarkers of drug resistance or virulence in ESCAPE pathogens by MALDI-TOF mass spectrometry. *Sci. Rep.* 9, 1-10. doi:10.1038/s41598-019-55430-1.

Florez-Cuadrado, D., Ugarte-Ruiz, M., Quesada, A., Palomo, G., Domínguez, L., and Porrero, M. C. (2016). Description of an *erm(B)*-carrying *Campylobacter coli* isolate in Europe. *J. Antimicrob. Chemother.* 71, 841–843. doi:10.1093/jac/dkv383.

Florio, W., Tavanti, A., Ghelardi, E., and Lupetti, A. (2018). MALDI-TOF MS Applications to the Detection of Antifungal Resistance: State of the Art and Future Perspectives. *Front. Microbiol.* 9, 1–7. doi:10.3389/fmicb.2018.02577.

Foudraine, D. E., Strepis, N., Stingl, C., ten Kate, M. T., Verbon, A., Klaassen, C. H. W., et al. (2021). Exploring antimicrobial resistance to beta-lactams, aminoglycosides and fluoroquinolones in *E. coli* and *K. pneumoniae* using proteogenomics. *Sci. Rep.* 11, 1–18. doi:10.1038/s41598-021-91905-w.

Furniss, R. C. D., Dortet, L., Bolland, W., Drews, O., Sparbier, K., Bonnin, R. A., et al. (2019). Detection of colistin resistance in *Escherichia coli* by use of the MALDI biotyper sirius mass spectrometry system. *J. Clin. Microbiol.* 57, 1–7. doi:10.1128/JCM.01427-19.

Galetsi, P., Katsaliaki, K., and Kumar, S. (2019). Values, challenges and future directions of big data analytics in healthcare: A systematic review. *Soc. Sci. Med.* 241, 112533. doi:10.1016/j.socscimed.2019.112533.

Ge, M. C., Kuo, A. J., Liu, K. L., Wen, Y. H., Chia, J. H., Chang, P. Y., et al. (2017). Routine identification of microorganisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: Success rate, economic analysis, and clinical outcome. *J. Microbiol. Immunol. Infect.* 50, 662–668. doi:10.1016/j.jmii.2016.06.002.

Gibbs, E. P. J. (2014). The evolution of one health: A decade of progress and challenges for the future. *Vet. Rec.* 174, 85–91. doi:10.1136/vr.g143.

Gibson, D., and Costello, C. E. (2000). “Mass spectrometry of biomolecules,” in *Separation Science and Technology*, 299–327. doi:10.1016/S0149-6395(00)80053-7.

Giebel, R. A., Fredenberg, W., and Sandrin, T. R. (2008). Characterization of environmental isolates

of *Enterococcus* spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Water Res.* 42, 931–940. doi:10.1016/j.watres.2007.09.005.

Girolamo, F. Di, Lante, I., Muraca, M., and Putignani, L. (2013). The Role of Mass Spectrometry in the "Omics" Era. *Curr. Org. Chem.* 17, 2891–2905. doi:10.4172/jpb.s2-e001.

Gölz, G., Rosner, B., Hofreuter, D., Josenhans, C., Kreienbrock, L., Löwenstein, A., et al. (2014). Relevance of *Campylobacter* to public health-The need for a One Health approach. *Int. J. Med. Microbiol.* 304, 817–823. doi:10.1016/j.ijmm.2014.08.015.

Gonzalo, X., Broda, A., Drobniewski, F., and Larrouy-Maumus, G. (2021). Performance of lipid fingerprint-based MALDI-ToF for the diagnosis of mycobacterial infections. *Clin. Microbiol. Infect.* 27, 912.e1-912.e5. doi:10.1016/j.cmi.2020.08.027.

Goodswen, S. J., Barratt, J. L. N., Kennedy, P. J., Kaufer, A., Calarco, L., and Ellis, J. T. (2021). Machine learning and applications in microbiology. *FEMS Microbiol. Rev.* 015, 1–19. doi:10.1093/femsre/fuab015.

Gorman, R., Bloomfield, S., and Adley, C. C. (2002). A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland. *Int. J. Food Microbiol.* 76, 143–150. doi:10.1016/S0168-1605(02)00028-4.

Gouveia, D., Grenga, L., Pible, O., and Armengaud, J. (2020). Quick microbial molecular phenotyping by differential shotgun proteomics. *Environ. Microbiol.* 22, 2996–3004. doi:10.1111/1462-2920.14975.

Grandini, M., Bagli, E., and Visani, G. (2020). Metrics for Multi-Class Classification: an Overview. *A white Pap.*, 1–17. Available at: http://arxiv.org/abs/2008.05756 [Accessed June 18, 2021].

Graves, P. R., and Haystead, T. A. J. (2002). Molecular Biologist's Guide to Proteomics. *Microbiol. Mol. Biol. Rev.* 66, 39–63. doi:10.1128/mmbr.66.1.39-63.2002.

Greaves, J., and Roboz, J. (2014). *Mass spectrometry for the novice, First edition*. CRC Press: Boca Rato, FL, USA.

Grenga, L., Pible, O., and Armengaud, J. (2019). Pathogen proteotyping: A rapidly developing application of mass spectrometry to address clinical concerns. *Clin. Mass Spectrom.* 14, 9–17. doi:10.1016/j.clinms.2019.04.004.

Griffin, P. M., Price, G. R., Schooneveldt, J. M., Schlebusch, S., Tilse, M. H., Urbanski, T., et al. (2012). Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify vancomycin-resistant enterococci and investigate the epidemiology of an outbreak. *J. Clin. Microbiol.* 50, 2918–2931. doi:10.1128/JCM.01000-12.

Griggs, D. J., Peake, L., Johnson, M. M., Ghori, S., Mott, A., and Piddock, L. J. V. (2009). β-lactamase-mediated β-lactam resistance in *Campylobacter* species: Prevalence of Cj0299 (blaOXA-61)

and evidence for a novel β-lactamase in *C. jejuni*. *Antimicrob. Agents Chemother.* 53, 3357–3364. doi:10.1128/AAC.01655-08.

Gripp, E., Hlahla, D., Didelot, X., Kops, F., Maurischat, S., Tedin, K., et al. (2011). Closely related *Campylobacter jejuni* strains from different sources reveal a generalist rather than a specialist lifestyle. *BMC Genomics* 12, 584. doi:10.1186/1471-2164-12-584.

Grote, T., and Berens, P. (2020). On the ethics of algorithmic decision-making in healthcare. *J. Med. Ethics* 46, 205–211. doi:10.1136/medethics-2019-105586.

Guerry, P. (2007). *Campylobacter* flagella: not just for motility. *Trends Microbiol.* 15, 456–461. doi:10.1016/j.tim.2007.09.006.

Hald, B., Skov, M. N., Nielsen, E. M., Rahbek, C., Madsen, J. J., Wainø, M., et al. (2016). *Campylobacter jejuni* and *Campylobacter coli* in wild birds on Danish livestock farms. *Acta Vet. Scand.* 58, 11. doi:10.1186/s13028-016-0192-9.

Hald, B., Skovgård, H., Pedersen, K., and Bunkenborg, H. (2008). Influxed insects as vectors for *Campylobacter jejuni* and *Campylobacter coli* in Danish broiler houses. *Poult. Sci.* 87, 1428–1434. doi:10.3382/ps.2007-00301.

Hambali, M. A., and Jimoh, M. K. (2014). Rule-Based Expert System for Disease Diagnosis. Proceedings of the International Conference on Science, Technology, Education, Arts, Management and Social Sciences (iSTEAMS Research Nexus).

Hardy, K. J., Hawkey, P. M., Gao, F., and Oppenheim, B. A. (2004). Methicillin resistant *Staphylococcus aureus* in the critically ill. *Br. J. Anaesth.* 92, 121–130. doi:10.1093/bja/aeh008.

Harrison, L., Mukherjee, S., Hsu, C. H., Young, S., Strain, E., Zhang, Q., et al. (2021). Core Genome MLST for Source Attribution of *Campylobacter coli*. *Front. Microbiol.* 12, 1936. doi:10.3389/fmicb.2021.703890.

Hayoun, K., Pible, O., Petit, P., Allain, F., Jouffret, V., Culotta, K., et al. (2020). Proteotyping environmental microorganisms by phylopeptidomics: Case study screening water from a radioactive material storage pool. *Microorganisms* 8, 1–14. doi:10.3390/microorganisms8101525.

He, Y., Li, H., Lu, X., Stratton, C. W., and Tang, Y. W. (2010). Mass spectrometry biotyper system identifies enteric bacterial pathogens directly from colonies grown on selective stool culture media. *J. Clin. Microbiol.* 48, 3888–3892. doi:10.1128/JCM.01290-10.

Health First Europe (2017). White Paper on Rapid Diagnostic Technologies to Tackle Antimicrobial Resistance. Available at: https://healthfirsteurope.eu/wp-content/uploads/2015/05/White-Paper-on-rapid-diagnostic-technologies-to-tackle-AMR.pdf. [Accessed on October 15, 2021].

Heather, J. M., and Chain, B. (2016). The sequence of sequencers: The history of sequencing DNA.

Genomics 107, 1–8. doi:10.1016/j.ygeno.2015.11.003.

Hedberg, C. W., Smith, K. E., Besser, J. M., Boxrud, D. J., Hennessy, T. W., Bender, J. B., et al. (2001). Limitations of pulsed-field gel eiectrophoresis for the routine surveillance of *Campylobacter* infections (multiple letters). *J. Infect. Dis.* 184, 242–244. doi:10.1086/322005.

Hicks, N. D., Yang, J., Zhang, X., Zhao, B., Grad, Y. H., Liu, L., et al. (2018). Clinically prevalent mutations in *Mycobacterium tuberculosis* alter propionate metabolism and mediate multidrug tolerance. *Nat. Microbiol.* 3, 1032–1042. doi:10.1038/s41564-018-0218-3.

Higgins, P., Fluit, A., and Schmitz, F.- (2005). Fluoroquinolones: Structure and Target Sites. *Curr. Drug Targets* 4, 181–190. doi:10.2174/1389450033346920.

Holmes, E. A. F., Harris, S. D., Hughes, A., Craine, N., and Hughes, D. A. (2018). Cost-effectiveness analysis of the use of point-of-care C-reactive protein testing to reduce antibiotic prescribing in primary care. *Antibiotics* 7, 1-29. doi:10.3390/antibiotics7040106.

Holzmann, T., Frangoulidis, D., Simon, M., Noll, P., Schmoldt, S., Hanczaruk, M., et al. (2012). Fatal anthrax infection in a heroin user from Southern Germany, June 2012. *Eurosurveillance* 17, 38–42. doi:10.2807/ese.17.26.20204-en.

Hong, E., Bakhalek, Y., and Taha, M. K. (2019). Identification of *Neisseria meningitidis* by MALDI-TOF MS may not be reliable. *Clin. Microbiol. Infect.* 25, 717–722. doi:10.1016/j.cmi.2018.09.015.

Hormeño, L., Ugarte-Ruiz, M., Palomo, G., Borge, C., Florez-Cuadrado, D., Vadillo, S., et al. (2018). Ant(6)-I genes encoding aminoglycoside O-nucleotidyltransferases are widely spread Among streptomycin resistant strains of *Campylobacter jejuni* and *Campylobacter coli*. *Front. Microbiol.* 9, 2515. doi:10.3389/fmicb.2018.02515.

Hou, T. Y., Chiang-Ni, C., and Teng, S. H. (2019). Current status of MALDI-TOF mass spectrometry in clinical microbiology. *J. Food Drug Anal.*, 27, 1–11. doi:10.1016/j.jfda.2019.01.001.

Hrabák, J., Chudáčková, E., and Walková, R. (2013). Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry for detection of antibiotic resistance mechanisms: From research to routine diagnosis. *Clin. Microbiol. Rev.* 26, 103–114. doi:10.1128/CMR.00058-12.

Hsieh, S. Y., Tseng, C. L., Lee, Y. S., Kuo, A. J., Sun, C. F., Lin, Y. H., et al. (2008). Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. *Mol. Cell. Proteomics* 7, 448–456. doi:10.1074/mcp.M700339-MCP200.

Hsu, C. H., Harrison, L., Mukherjee, S., Strain, E., McDermott, P., Zhang, Q., et al. (2020). Core genome multilocus sequence typing for food animal source attribution of human *Campylobacter jejuni* infections. *Pathogens* 9, 1–12. doi:10.3390/pathogens9070532.

Hu, Y., Zhou, H., Lu, J., Sun, Q., Liu, C., Zeng, Y., et al. (2021). Evaluation of the IR Biotyper for *Klebsiella pneumoniae* typing and its potentials in hospital hygiene management. *Microb. Biotechnol.* 14, 1343–1352. doi:10.1111/1751-7915.13709.

Hugo, A., Baxter, D. J., Cannon, W. R., Kalyanaraman, A., Kulkarni, G., and Callister, S. J. (2012). Proteotyping of microbial communities by optimization of tandem mass spectrometry data interpretation. in *Pacific Symposium on Biocomputing*, 225–234. doi:10.1142/9789814366496_0022.

Hunter, D. J., Losina, E., Guermazi, A., Burstein, D., Lassere, M. N., and Kraus, V. (2010). A Pathway and Approach to Biomarker Validation and Qualification for Osteoarthritis Clinical Trials. *Curr. Drug Targets* 11, 536. doi: 10.2174/138945010791011947

Hunter, P. R. (1990). Reproducibility and indices of discriminatory power of microbial typing methods. *J. Clin. Microbiol.* 28, 1903–1905. doi:10.1128/jcm.28.9.1903-1905.1990.

Hutchings, M., Truman, A., and Wilkinson, B. (2019). Antibiotics: past, present and future. *Curr. Opin. Microbiol.* 51, 72–80. doi:10.1016/j.mib.2019.10.008.

IACG (2019). No time to wait: Securing the future from drug-resistant infections. Available at: https://www.who.int/publications/i/item/no-time-to-wait-securing-the-future-from-drug-resistant-infections. [Accessed November 5, 2021]

Igwaran, A., and Okoh, A. I. (2019). Human campylobacteriosis: A public health concern of global importance. *Heliyon* 5, e02814. doi:10.1016/j.heliyon.2019.e02814.

Iles, R. K., Zmuidinaite, R., Iles, J. K., Carnell, G., Sampson, A., and Heeney, J. L. (2020). A clinical MALDI-ToF Mass spectrometry assay for SARS-CoV-2: Rational design and multi-disciplinary team work. *Diagnostics* 10, 1–15. doi:10.1101/2020.08.22.20176669.

Ingresa-Capaccioni, S., Jiménez-Trigos, E., Marco-Jiménez, F., Catalá, P., Vega, S., and Marin, C. (2016). *Campylobacter* epidemiology from breeders to their progeny in Eastern Spain. *Poult. Sci.* 95, 676–683. doi:10.3382/ps/pev338.

Iovine, N. M. (2013). Resistance mechanisms in *Campylobacter jejuni*. *Virulence* 4, 230–240. doi:10.4161/viru.23753.

ISO (2020). 23418:2018(E): Microbiology of the food chain — Whole genome sequencing for typing and genomic characterization of foodborne bacteria — General requirements and guidance. Available at: https://www.iso.org/standard/75509.html [Accessed August 3, 2021].

Jadhav, S., Gulati, V., Fox, E. M., Karpe, A., Beale, D. J., Sevior, D., et al. (2015). Rapid identification and source-tracking of *Listeria monocytogenes* using MALDI-TOF mass spectrometry. *Int. J. Food Microbiol.* 202, 1–9. doi:10.1016/j.ijfoodmicro.2015.01.023.

Jamin, C., de Koster, S., van Koeveringe, S., de Coninck, D., Mensaert, K., de Bruyne, K., et al. (2021).

Harmonization of whole-genome sequencing for outbreak surveillance of enterobacteriaceae and enterococci. *Microb. Genomics* 7, 000567. doi:10.1099/mgen.0.000567.

Jarman, K. H., Cebula, S. T., Saenz, A. J., Petersen, C. E., Valentine, N. B., Kingsley, M. T., et al. (2000). An algorithm for automated bacterial identification using matrix- assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* 72, 1217–1223. doi:10.1021/ac990832j.

Jevons, M. P. (1961). "Celbenin"-resistant Staphylococci. *Br. Med. J.* 1, 124–125. doi:10.1136/bmj.1.5219.124-a.

Jia Khor, M., Broda, A., Kostrzewa, M., Drobniewski, F., and Larrouy-Maumus, G. (2021). An Improved Method for Rapid Detection of *Mycobacterium abscessus* Complex Based on Species-Specific Lipid Fingerprint by Routine MALDI-TOF. *Front. Chem.* 9, 545. doi:10.3389/FCHEM.2021.715890/BIBTEX.

Jiang, F., Jiang, Y., Zhi, H., Dong, Y., Li, H., Ma, S., et al. (2017). Artificial intelligence in healthcare: Past, present and future. *Stroke Vasc. Neurol.* 2, 230–243. doi:10.1136/svn-2017-000101.

Jiang, J., and Bai, G. (2020). Types of information compromised in breaches of protected health information. *Ann. Intern. Med.* 172, 159–160. doi:10.7326/M19-1759.

Johnson, T. J., Shank, J. M., and Johnson, J. G. (2017). Current and potential treatments for reducing *Campylobacter* colonization in animal hosts and disease in humans. *Front. Microbiol.* 8, 487. doi:10.3389/fmicb.2017.00487.

Josefsen, M. H., Bonnichsen, L., Larsson, J. T., Nielsen, E. M., Fricker, M., Ehling-Schulz, M., et al. (2012). Evaluation of flaA Short Variable Region Sequencing, Multilocus Sequence Typing, and Fourier Transform Infrared Spectroscopy for Discrimination between *Campylobacter jejuni* Strains. *Food Anal. Methods* 5, 980–987. doi:10.1007/s12161-011-9333-y.

Josten, M., Dischinger, J., Szekat, C., Reif, M., Al-Sabti, N., Sahl, H. G., et al. (2014). Identification of agr-positive methicillin-resistant *Staphylococcus aureus* harbouring the class A mec complex by MALDI-TOF mass spectrometry. *Int. J. Med. Microbiol.* 304, 1018–1023. doi:10.1016/J.IJMM.2014.07.005.

Kaakoush, N. O., Castaño-Rodríguez, N., Mitchell, H. M., and Man, S. M. (2015). Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* 28, 687–720. doi:10.1128/CMR.00006-15.

Kaissis, G. A., Makowski, M. R., Rückert, D., and Braren, R. F. (2020). Secure, privacy-preserving and federated machine learning in medical imaging. *Nat. Mach. Intell.* 2, 305–311. doi:10.1038/s42256-020-0186-1.

Kang, S. M., Kim, D. H., Jin, C., and Lee, B. J. (2018). A systematic overview of type II and III toxin-antitoxin systems with a focus on druggability. *Toxins.* 10, 1–21. doi:10.3390/toxins10120515.

Kapperud, G., Espeland, G., Wahl, E., Walde, A., Herikstad, H., Gustavsen, S., et al. (2003). Factors associated with increased and decreased risk of *Campylobacter* infection: A prospective case-control study in Norway. *Am. J. Epidemiol.* 158, 234–242. doi:10.1093/aje/kwg139.

Kaprou, G. D., Bergšpica, I., Alexa, E. A., Alvarez-Ordóñez, A., and Prieto, M. (2021). Rapid methods for antimicrobial resistance diagnostics. *Antibiotics* 10, 1–30. doi:10.3390/antibiotics10020209.

Karas, M., Bachmann, D., Bahr, U., and Hillenkamp, F. (1987). Matrix-assisted ultraviolet laser desorption of non-volatile compounds. *Int. J. Mass Spectrom. Ion Process.* 78, 53–68. doi:10.1016/0168-1176(87)87041-6.

Karlsson, R., Gonzales-Siles, L., Boulund, F., Svensson-Stadler, L., Skovbjerg, S., Karlsson, A., et al. (2015). Proteotyping: Proteomic characterization, classification and identification of microorganisms - A prospectus. *Syst. Appl. Microbiol.* 38, 246–257. doi:10.1016/j.syapm.2015.03.006.

Karlsson, R., Thorsell, A., Gomila, M., Salvà-Serra, F., Jakobsson, H. E., Gonzales-Siles, L., et al. (2020). Discovery of species-unique peptide biomarkers of bacterial pathogens by tandem mass spectrometry-based proteotyping. *Mol. Cell. Proteomics* 19, 518–528. doi:10.1074/mcp.RA119.001667.

Kathera, C. (2018). "Microbial proteomics and their importance in medical microbiology," in *Recent Developments in Applied Microbiology and Biochemistry*, Elsevier, 21–31. doi:10.1016/B978-0-12-816328-3.00003-9.

Kaul, V., Enslin, S., and Gross, S. A. (2020). History of artificial intelligence in medicine. *Gastrointest. Endosc.* 92, 807–812. doi:10.1016/j.gie.2020.06.040.

Kayin, M., Mert, B., Aydemir, S., and Özenci, V. (2019). Comparison of rapid BACpro® II, Sepsityper® kit and in-house preparation methods for direct identification of bacteria from blood cultures by MALDI-TOF MS with and without Sepsityper® module analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 2133–2143. doi:10.1007/s10096-019-03654-4.

Keim, P. (2005). "Bacterial Pathogens," in *Microbial Forensics*, Academic Press, 99–107. doi:10.1016/B978-012088483-4/50008-1.

Khot, P. D., and Fisher, M. A. (2013). Novel Approach for Differentiating *Shigella* Species and *Escherichia coli* by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *J. Clin. Microbiol* 51, 3711-3716. doi:10.1128/JCM.01526-13.

Kittler, S., Shakeri, G., Peh, E., and Plötz, M. (2021). A One Health Perspective on a Multi-hurdle Approach to Combat *Campylobacter* spp. in Broiler Meat. *Curr. Clin. Microbiol. Reports* 8, 49–61. doi:10.1007/s40588-021-00167-7.

Köfeler, H. C., Fauland, A., Rechberger, G. N., and Trötzmüller, M. (2012). Mass spectrometry based lipidomics: An overview of technological platforms. *Metabolites* 2, 19–38. doi:10.3390/metabo2010019.

Kondori, N., Kurtovic, A., Piñeiro-Iglesias, B., Salvà-Serra, F., Jaén-Luchoro, D., Andersson, B., et al. (2021). Mass Spectrometry Proteotyping-Based Detection and Identification of *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* in Blood. *Front. Cell. Infect. Microbiol.* 11, 1-15. doi:10.3389/fcimb.2021.634215.

Kostrzewa, M., Nagy, E., Schröttner, P., and Pranada, A. B. (2019). How MALDI-TOF mass spectrometry can aid the diagnosis of hard-to-identify pathogenic bacteria–the rare and the unknown. *Expert Rev. Mol. Diagn.* 19, 667–682. doi:10.1080/14737159.2019.1643238.

Krishnamurthy, T., and Ross, P. L. (1996). Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun. Mass Spectrom.* 10, 1992–1996. doi:10.1002/(SICI)1097-0231(199612)10:15<1992::AID-RCM789>3.0.CO;2-V.

Krüger, A., Schäfers, C., Busch, P., and Antranikian, G. (2020). Digitalization in microbiology – Paving the path to sustainable circular bioeconomy. *N. Biotechnol.* 59, 88–96. doi:10.1016/j.nbt.2020.06.004.

Kuhns, M., Zautner, A. E., Rabsch, W., Zimmermann, O., Weig, M., Bader, O., et al. (2012). Rapid discrimination of *Salmonella enterica* serovar typhi from other serovars by MALDI-TOF mass spectrometry. *PLoS One* 7, e40004. doi:10.1371/journal.pone.0040004.

Kumar, S., Singh, S., Kumar, V., Datta, S., Dhanjal, D. S., Sharma, P., et al. (2020). "Pathogenesis and Antibiotic Resistance of *Staphylococcus aureus*," in *Model Organisms for Microbial Pathogenesis, Biofilm Formation and Antimicrobial Drug Discovery*, Springer Singapore, 99–115. doi:10.1007/978-981-15-1695-5_7.

Kwong, J. C., Mccallum, N., Sintchenko, V., and Howden, B. P. (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology* 47, 199–210. doi:10.1097/PAT.0000000000000235.

Kyle, J. E. (2021). How lipidomics can transform our understanding of virus infections. *Expert Rev. Proteomics* 18, 329–332. doi:10.1080/14789450.2021.1929177.

Pinault L., Charbière E., Raoult D., and Fenollar F. (2019). Direct Identification of Pathogens in Urine by Use of a Specific Matrix-Assisted Laser Desorption Ionization-Time of Flight Spectrum Database. *J. Clin. Microbiol.* 57, 1-9. doi:10.1128/JCM.01678-18.

Lagarde, M., Géloën, A., Record, M., Vance, D., and Spener, F. (2003). Lipidomics is emerging. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 1634, 61. doi:10.1016/j.bbalip.2003.11.002.

Lambert, P. A. (2005). Bacterial resistance to antibiotics: Modified target sites. *Adv. Drug Deliv. Rev.* 57, 1471–1485. doi:10.1016/j.addr.2005.04.003.

Lane, T. (2018). A short history of robotic surgery. *Ann. R. Coll. Surg. Engl.* 100, 5–7. doi:10.1308/rcsann.supp1.5.

Larrouy-Maumus, G., Clements, A., Filloux, A., McCarthy, R. R., and Mostowy, S. (2016). Direct detection of lipid A on intact Gram-negative bacteria by MALDI-TOF mass spectrometry. *J. Microbiol. Methods* 120, 68–71. doi:10.1016/j.mimet.2015.12.004.

Larrouy-Maumus, G., and Puzo, G. (2015). Mycobacterial envelope lipids fingerprint from direct MALDI-TOF MS analysis of intact bacilli. *Tuberculosis* 95, 75–85. doi:10.1016/j.tube.2014.11.001.

Lasch, P., and Naumann, D. (2015). "Infrared Spectroscopy in Microbiology," in *Encyclopedia of Analytical Chemistry*, American Cancer Society, 1–32. doi:10.1002/9780470027318.a0117.pub2.

Lastovica, A. J., On, S. L. W., and Zhang, L. (2014). "The Family *Campylobacteraceae*," in *The Prokaryotes: Deltaproteobacteria and Epsilonproteobacteria*, Springer, Berlin, Heidelberg, 1–413. doi:10.1007/978-3-642-39044-9.

Lau, A. F., Wang, H., Weingarten, R. A., Drake, S. K., Suffredini, A. F., Garfield, M. K., et al. (2014). A rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry-based method for single-plasmid tracking in an outbreak of carbapenem-resistant *Enterobacteriaceae*. *J. Clin. Microbiol.* 52, 2804–2812. doi:10.1128/JCM.00694-14.

Lavezzo, E., Barzon, L., Toppo, S., and Palù, G. (2016). Third generation sequencing technologies applied to diagnostic microbiology: benefits and challenges in applications and data analysis. *Expert Rev. Mol. Diagn.* 16, 1011–1023. doi:10.1080/14737159.2016.1217158.

Layre, E., Al-Mubarak, R., Belisle, J. T., and Branch Moody, D. (2014). Mycobacterial Lipidomics. *Microbiol. Spectr.* 2. doi:10.1128/microbiolspec.mgm2-0033-2013.

Leaper, S., and Owen, R. J. (1981). Identification of catalase-producing *Campylobacter* species based on biochemical characteristics and on cellular fatty acid composition. *Curr. Microbiol.* 6, 31–35. doi:10.1007/BF01566723.

Lee, H. S., Shin, J. H., Choi, M. J., Won, E. J., Kee, S. J., Kim, S. H., et al. (2017a). Comparison of the Bruker biotyper and VITEK MS matrix- assisted laser desorption/ionization time-of-flight mass spectrometry systems using a formic acid extraction method to identify common and uncommon yeast isolates. *Ann. Lab. Med.* 37, 223–230. doi:10.3343/alm.2017.37.3.223.

Lee, J. W. (2009). Method validation and application of protein biomarkers: Basic similarities and differences from biotherapeutics. *Bioanalysis* 1, 1461–1474. doi:10.4155/BIO.09.130.

Lee, Y., Sung, J. Y., Kim, H., Yong, D., and Lee, K. (2017b). Comparison of a new matrix-assisted laser desorption/ionization time-of-flight mass spectrometry platform, ASTA MicroIDSys, with Bruker Biotyper for species identification. *Ann. Lab. Med.* 37, 531–535. doi:10.3343/alm.2017.37.6.531.

Leo, S., Cherkaoui, A., Renzi, G., and Schrenzel, J. (2020). Mini Review: Clinical Routine Microbiology in the Era of Automation and Digital Health. *Front. Cell. Infect. Microbiol.* 10, 1-8. doi:10.3389/fcimb.2020.582028.

Leung, L. M., Fondrie, W. E., Doi, Y., Johnson, J. K., Strickland, D. K., Ernst, R. K., et al. (2017). Identification of the ESKAPE pathogens by mass spectrometric analysis of microbial membrane glycolipids. *Sci. Rep.* 7, 1-10. doi:10.1038/s41598-017-04793-4.

Lévesque, S., Dufresne, P. J., Soualhine, H., Domingo, M. C., Bekal, S., Lefebvre, B., et al. (2015). A Side by Side Comparison of Bruker Biotyper and VITEK MS: Utility of MALDI-TOF MS Technology for Microorganism Identification in a Public Health Reference Laboratory. *PLoS One* 10, e0144878. doi:10.1371/journal.pone.0144878.

Li, L. G., Yin, X., and Zhang, T. (2018). Tracking antibiotic resistance gene pollution from different sources using machine-learning classification. *Microbiome* 6, 1-12. doi:10.1186/s40168-018-0480-x.

Li, W., Atkinson, G. C., Thakor, N. S., Allas, U., Lu, C. C., Yan Chan, K., et al. (2013). Mechanism of tetracycline resistance by ribosomal protection protein Tet(O). *Nat. Commun.* 4, 1–8. doi:10.1038/ncomms2470.

Liébana-Martos, C. (2018). "Indications, Interpretation of Results, Advantages, Disadvantages, and Limitations of MALDI-TOF," in *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*, Academic Press, 75–86. doi:10.1016/B978-0-12-814451-0.00005-8.

Lin, J., Overbye Michel, L., and Zhang, Q. (2002). CmeABC functions as a multidrug efflux system in *Campylobacter jejuni*. *Antimicrob. Agents Chemother.* 46, 2124–2131. doi:10.1128/AAC.46.7.2124-2131.2002.

Lior, H. (1984). New, extended biotyping scheme for *Campylobacter jejuni, Campylobacter coli*, and "*Campylobacter laridis*." *J. Clin. Microbiol.* 20, 636–640. doi:10.1128/jcm.20.4.636-640.1984.

Lior, H., Woodward, D. L., Edgar, J. A., Laroche, L. J., and Gill, P. (1982). Serotyping of *Campylobacter jejuni* by slide agglutination based on heat-labile antigenic factors. *J. Clin. Microbiol.* 15, 761–768. doi:10.1128/JCM.15.5.761-768.1982.

Liu, D., Liu, W., Li, X., Yao, H., Shen, Z., Wang, Y., et al. (2020). Presence and Antimicrobial Susceptibility of RE-cmeABC-Positive *Campylobacter* Isolated from Food-Producing Animals, 2014–2016. *Engineering* 6, 34–39. doi:10.1016/j.eng.2019.10.013.

Llarena, A. K., Taboada, E., and Rossi, M. (2017). Whole-genome sequencing in epidemiology of *Campylobacter jejuni* infections. *J. Clin. Microbiol.* 55, 1269–1275. doi:10.1128/JCM.00017-17.

Logan, S. M., and Trust, T. J. (1982). Outer membrane characteristic of *Campylobacter jejuni*. *Infect. Immun.* 38, 898–906. doi:10.1128/iai.38.3.898-906.1982.

Longo, A., Petrin, S., Mastrorilli, E., Tiengo, A., Lettini, A. A., Barco, L., et al. (2019). Characterizing *Salmonella enterica* Serovar Choleraesuis, var. Kunzendorf: A Comparative Case Study. *Front. Vet. Sci.* 6, 1-5. doi:10.3389/fvets.2019.00316.

Lowy, F. D. (2003). Antimicrobial resistance: The example of *Staphylococcus aureus*. *J. Clin. Invest.* 111, 1265–1273. doi:10.1172/JCI18535.

Luangtongkum, T., Han, J., Zhang, Q., Logue, C. M., Plummer, P., Jeon, B., et al. (2009). Antibiotic resistance in *Campylobacter* : emergence, transmission and persistence . *Future Microbiol.* 4, 189–200. doi:10.2217/17460913.4.2.189.

Luber, P., Brynestad, S., Topsch, D., Scherer, K., and Bartelt, E. (2006). Quantification of *Campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. *Appl. Environ. Microbiol.* 72, 66–70. doi:10.1128/AEM.72.1.66-70.2006.

Luepke, K. H., and Mohr, J. F. (2017). The antibiotic pipeline: reviving research and development and speeding drugs to market. *Expert Rev. Anti. Infect. Ther.* 15, 425–433. doi:10.1080/14787210.2017.1308251.

Lupolova, N., Lycett, S. J., and Gally, D. L. (2019). A guide to machine learning for bacterial host attribution using genome sequence data. *Microb. Genomics* 5, 1-14. doi:10.1099/MGEN.0.000317.

MacDonald, E., White, R., Mexia, R., Bruun, T., Kapperud, G., Lange, H., et al. (2015). Risk factors for sporadic domestically acquired *Campylobacter* infections in Norway 2010-2011: A national prospective case-control study. *PLoS One* 10, e0139636. doi:10.1371/journal.pone.0139636.

Macklin, A., Khan, S., and Kislinger, T. (2020). Recent advances in mass spectrometry based clinical proteomics: Applications to cancer research. *Clin. Proteomics* 17, 1–25. doi:10.1186/s12014-020-09283-w.

Magana, M., Chatzipanagiotou, S., Burriel, A. R., and Ioannidis, A. (2017). Inquiring into the gaps of *Campylobacter* surveillance methods. *Vet. Sci.* 4, 1–14. doi:10.3390/vetsci4030036.

Mahé, P., Arsac, M., Chatellier, S., Monnin, V., Perrot, N., Mailler, S., et al. (2014). Automatic identification of mixed bacterial species fingerprints in a MALDI-TOF mass-spectrum. *Bioinformatics* 30, 1280–1286. doi:10.1093/bioinformatics/btu022.

Maiden, M. C. J., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., et al. (1998). Multilocus sequence typing: A portable approach to the identification of clones within populations of

pathogenic microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* 95, 3140–3145. doi:10.1073/pnas.95.6.3140.

Maiden, M. C. J., and Harrison, O. B. (2016). Population and functional genomics of *Neisseria* revealed with gene-by-gene approaches. *J. Clin. Microbiol.* 54, 1949–1955. doi:10.1128/JCM.00301-16.

Mangioni, D., Viaggi, B., Giani, T., Arena, F., D'Arienzo, S., Forni, S., et al. (2019). Diagnostic stewardship for sepsis: the need for risk stratification to triage patients for fast microbiology workflows. *Future Microbiol.* 14, 169–174. doi:10.2217/fmb-2018-0329.

Marcus, K., Lelong, C., and Rabilloud, T. (2020). What room for two-dimensional gel-based proteomics in a shotgun proteomics world? *Proteomes* 8, 1-26. doi:10.3390/PROTEOMES8030017.

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: Impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi:10.1128/CMR.00002-11.

Marshall, G. (2020). Designing a biomarker strategy. Available at: https://md.catapult.org.uk/designing-a-biomarker-strategy/ [Accessed October 14, 2021].

Martins, D., McKay, G., Sampathkumar, G., Khakimova, M., English, A. M., and Nguyen, D. (2018). Superoxide dismutase activity confers (p)ppGppmediated antibiotic tolerance to stationary-phase *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U. S. A.* 115, 9797–9802. doi:10.1073/pnas.1804525115.

Mathai, P. P., Staley, C., and Sadowsky, M. J. (2020). Sequence-enabled community-based microbial source tracking in surface waters using machine learning classification: A review. *J. Microbiol. Methods* 177, 1-6. doi:10.1016/J.MIMET.2020.106050.

Mather, C. A., Werth, B. J., Sivagnanam, S., SenGupta, D. J., and Butler-Wu, S. M. (2016). Rapid detection of vancomycin-intermediate *Staphylococcus aureus* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 54, 883–890. doi:10.1128/JCM.02428-15.

Mattar, C., Edwards, S., Baraldi, E., and Hood, J. (2020). An overview of the global antimicrobial resistance research and development hub and the current landscape. *Curr. Opin. Microbiol.* 57, 56–61. doi:10.1016/j.mib.2020.06.009.

McArthur, A. G., and Wright, G. D. (2015). Bioinformatics of antimicrobial resistance in the age of molecular epidemiology. *Curr. Opin. Microbiol.* 27, 45–50. doi:10.1016/j.mib.2015.07.004.

McCarthy, N. D., Colles, F. M., Dingle, K. E., Bagnall, M. C., Manning, G., Maiden, M. C. J., et al. (2007). Host-associated genetic import in *Campylobacter jejuni*. *Emerg. Infect. Dis.* 13, 267–272. doi:10.3201/eid1302.060620.

McElvania Tekippe, E., and Burnham, C.-A. A. D. (2014). Evaluation of the Bruker Biotyper and VITEK MS MALDI-TOF MS systems for the identification of unusual and/or difficult-to-identify microorganisms isolated from clinical specimens. *Eur. J. Clin. Microbiol. Infect. Dis.* 33, 2163–2171. doi:10.1007/s10096-014-2183-y.

Mehndiratta, P. L., and Bhalla, P. (2014). Use of antibiotics in animal agriculture & emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) clones: Need to assess the impact on public health. *Indian J. Med. Res.* 140, 339–344.

Michael, C. A., Dominey-Howes, D., and Labbate, M. (2014). The antimicrobial resistance crisis: Causes, consequences, and management. *Front. Public Heal.* 2, 1-8. doi:10.3389/fpubh.2014.00145.

Miller, W. G., On, S. L. W., Wang, G., Fontanoz, S., Lastovica, A. J., and Mandrell, R. E. (2005). Extended multilocus sequence typing system for *Campylobacter coli, C. lari, C. upsaliensis*, and *C. helveticus*. *J. Clin. Microbiol.* 43, 2315–2329. doi:10.1128/JCM.43.5.2315-2329.2005.

Mitchell, S. L., and Alby, K. (2017). Performance of microbial identification by MALDI-TOF MS and susceptibility testing by VITEK 2 from positive blood cultures after minimal incubation on solid media. *Eur. J. Clin. Microbiol. Infect. Dis.* 36, 2201–2206. doi:10.1007/s10096-017-3046-0.

Morales, M. P. F., Lim, C. K., Shephard, L., and Weldhagen, G. F. (2018). Mycobacterial inactivation protein extraction protocol for matrix-assisted laser desorption ionization time-of-flight characterization of clinical isolates. *Int. J. Mycobacteriology* 7, 217–221. doi:10.4103/ijmy.ijmy_104_18.

Morgenthaler, N. G., and Kostrzewa, M. (2015). Rapid identification of pathogens in positive blood culture of patients with sepsis: Review and meta-analysis of the performance of the Sepsityper kit. *Int. J. Microbiol.* 2015, 1-10. doi:10.1155/2015/827416.

Mörtelmaier, C., Panda, S., Robertson, I., Krell, M., Christodoulou, M., Reichardt, N., et al. (2019). Identification performance of MALDI-TOF-MS upon mono- and bi-microbial cultures is cell number and culture proportion dependent. *Anal. Bioanal. Chem.* 411, 7027–7038. doi:10.1007/s00216-019-02080-x.

Mossong, J., Mughini-Gras, L., Penny, C., Devaux, A., Olinger, C., Losch, S., et al. (2016). Human Campylobacteriosis in Luxembourg, 2010-2013: A Case-Control Study Combined with Multilocus Sequence Typing for Source Attribution and Risk Factor Analysis. *Sci. Rep.* 6, 1–12. doi:10.1038/srep20939.

Mouwen, D. J. M., Capita, R., Alonso-Calleja, C., Prieto-Gómez, J., and Prieto, M. (2006). Artificial neural network based identification of *Campylobacter* species by Fourier transform infrared spectroscopy. *J. Microbiol. Methods* 67, 131–140. doi:10.1016/j.mimet.2006.03.012.

Mughini-Gras, L., Penny, C., Ragimbeau, C., Schets, F. M., Blaak, H., Duim, B., et al. (2016). Quantifying potential sources of surface water contamination with *Campylobacter jejuni* and *Campylobacter coli*. *Water Res.* 101, 36–45. doi:10.1016/j.watres.2016.05.069.

Mughini-Gras, L., Pijnacker, R., Coipan, C., Mulder, A. C., Fernandes Veludo, A., de Rijk, S., et al. (2021). Sources and transmission routes of campylobacteriosis: A combined analysis of genome and exposure data. *J. Infect.* 82, 216–226. doi:10.1016/j.jinf.2020.09.039.

Mughini Gras, L., Smid, J. H., Wagenaar, J. A., de Boer, A. G., Havelaar, A. H., Friesema, I. H. M., et al. (2012). Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: A combined case-control and source attribution analysis. *PLoS One* 7, 1-13. doi:10.1371/journal.pone.0042599.

Muhamadali, H., Weaver, D., Subaihi, A., Almasoud, N., Trivedi, D. K., Ellis, D. I., et al. (2016). Chicken, beams, and Campylobacter: Rapid differentiation of foodborne bacteria via vibrational spectroscopy and MALDI-mass spectrometry. *Analyst* 141, 111–122. doi:10.1039/c5an01945a.

Munck, N., Njage, P. M. K., Leekitcharoenphon, P., Litrup, E., and Hald, T. (2020). Application of Whole-Genome Sequences and Machine Learning in Source Attribution of *Salmonella Typhimurium*. *Risk Anal.* 40, 1693–1705. doi:10.1111/risa.13510.

Murff, H. J., FitzHenry, F., Matheny, M. E., Gentry, N., Kotter, K. L., Crimin, K., et al. (2011). Automated identification of postoperative complications within an electronic medical record using natural language processing. *J. Am. Med. Assoc.* 306, 848–855. doi:10.1001/jama.2011.1204.

Murray, C. J. L., Vos, T., Lozano, R., Naghavi, M., Flaxman, A. D., Michaud, C., et al. (2012). Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: A systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 380, 2197–2223. doi:10.1016/S0140-6736(12)61689-4.

Murray, P. R. (2012). What Is new in clinical microbiologymicrobial identification by MALDI-TOF mass spectrometry: A paper from the 2011 William Beaumont Hospital symposium on molecular pathology. *J. Mol. Diagnostics* 14, 419–423. doi:10.1016/j.jmoldx.2012.03.007.

Nadon, C., Van Walle, I., Gerner-Smidt, P., Campos, J., Chinen, I., Concepcion-Acevedo, J., et al. (2017). Pulsenet international: Vision for the implementation of whole genome sequencing (WGS) for global foodborne disease surveillance. *Eurosurveillance* 22, 1-12. doi:10.2807/1560-7917.ES.2017.22.23.30544.

Nami, Y., Imeni, N., and Panahi, B. (2021). Application of Machine Learning in bacteriophage research. *BMC Microbiol.* 21, 1–8. doi:10.1186/s12866-021-02256-5.

Nennig, M., Llarena, A.-K., Herold, M., Mossong, J., Penny, C., Losch, S., et al. (2021). Investigating Major Recurring *Campylobacter jejuni* Lineages in Luxembourg Using Four Core or Whole Genome Sequencing Typing Schemes. *Front. Cell. Infect. Microbiol.* 10, 1–16. doi:10.3389/fcimb.2020.608020.

Neuschlova, M., Vladarova, M., Kompanikova, J., Sadlonova, V., and Novakova, E. (2017). "Identification of *Mycobacterium* species by MALDI-TOF mass spectrometry," in *Advances in Experimental Medicine and Biology*, Springer, Cham, 37–42. doi:10.1007/5584_2017_26.

Newell, S., and Marabelli, M. (2015). Strategic opportunities (and challenges) of algorithmic decision-making: A call for action on the long-term societal effects of "datification." *J. Strateg. Inf. Syst.* 24, 3–14. doi:10.1016/j.jsis.2015.02.001.

Nguyen, A. P., and Downard, K. M. (2013). Proteotyping of the parainfluenza virus with high-resolution mass spectrometry. *Anal. Chem.* 85, 1097–1105. doi:10.1021/ac302962u.

Nguyen, M., Long, S. W., Mcdermott, P. F., Olsen, R. J., Olson, R., Stevens, R. L., et al. (2019). Using Machine Learning To Predict Antimicrobial MICs and Associated Genomic Features for Nontyphoidal *Salmonella*. *J. Clin. Microbiol.* 57, 1-15 doi:10.1128/JCM.01260-18.

Nichols, J. A., Herbert Chan, H. W., and Baker, M. A. B. (2019). Machine learning: applications of artificial intelligence to imaging and diagnosis. *Biophys. Rev.* 11, 111–118. doi:10.1007/s12551-018-0449-9.

NIH (2020). Rapid diagnostic for *Gonorrhea* wins $19 million federal prize competition to combat antibiotic resistance. Available at: https://www.nih.gov/news-events/news-releases/rapid-diagnostic-gonorrhea-wins-19-million-federal-prize-competition-combat-antibiotic-resistance [Accessed November 3, 2021].

Nilsson, C. L. (1999). Fingerprinting of Helicobacter pylori strains by matrix-assisted laser desorption/ionization mass spectrometric analysis. *Rapid Commun. Mass Spectrom.* 13, 1067–1071. doi:10.1002/(SICI)1097-0231(19990615)13:11<1067::AID-RCM612>3.0.CO;2-N.

Ning, F., Wu, X., and Wang, W. (2016). Exploiting the potential of 2DE in proteomics analyses. *Expert Rev. Proteomics* 13, 901–903. doi:10.1080/14789450.2016.1230498.

Nomura, F., Tsuchida, S., Murata, S., Satoh, M., and Matsushita, K. (2020). Mass spectrometry-based microbiological testing for blood stream infection. *Clin. Proteomics* 17, 1–11. doi:10.1186/s12014-020-09278-7.

Noreen, Z., Siddiqui, F., Javed, S., Wren, B. W., and Bokhari, H. (2019). Transmission of Multidrug Resistant *Campylobacter jejuni* to Children from Different Sources in Pakistan. *J. Glob. Antimicrob. Resist.* 20, 219-224. doi:10.1016/j.jgar.2019.07.018.

Novais, Â., Freitas, A. R., Rodrigues, C., and Peixe, L. (2019). Fourier transform infrared

spectroscopy: unlocking fundamentals and prospects for bacterial strain typing. *Eur. J. Clin. Microbiol. Infect. Dis.* 38, 427–448. doi:10.1007/s10096-018-3431-3.

Novais, Â., and Peixe, L. (2021). "Fourier Transform Infrared Spectroscopy (FT-IR) for Food and Water Microbiology," in *Application and Integration of Omics-powered Diagnostics in Clinical and Public Health Microbiology*, Springer, Cham, 191–217. doi:10.1007/978-3-030-62155-1_11.

Nyvang Hartmeyer, G., Kvistholm Jensen, A., Böcher, S., Damkjaer Bartels, M., Pedersen, M., Engell Clausen, M., et al. (2010). Mass spectrometry: *Pneumococcal meningitis* verified and *Brucella* species identified in less than half an hour. *Scand. J. Infect. Dis.* 42, 716–718. doi:10.3109/00365541003754493.

O'Neill, J. (2016). Tackling drug-resistant infections globally: final report and recommendations. Available at: https://amr-review.org/Publications.html [Accessed September 12, 2021].

Ocejo, M., Oporto, B., Lavín, J. L., and Hurtado, A. (2021). Whole genome-based characterisation of antimicrobial resistance and genetic diversity in *Campylobacter jejuni* and *Campylobacter coli* from ruminants. *Sci. Rep.* 11, 1–11. doi:10.1038/s41598-021-88318-0.

Olkkola, S., Juntunen, P., Heiska, H., Hyytiäinen, H., and Hänninen, M. L. (2010). Mutations in the rpsL gene are involved in streptomycin resistance in *Campylobacter coli*. *Microb. Drug Resist.* 16, 105–110. doi:10.1089/mdr.2009.0128.

Omondi Asimba, B. (2019). Machine Learning basics. Available at: https://brianasimba.github.io/MachineLearningblog//Introduction-post/ [Accessed September 10, 2021].

On, S. L. W. (2001). Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter* and related bacteria: current status, future prospects and immediate concerns. *J. Appl. Microbiol.* 90, 1S-15S. doi:10.1046/j.1365-2672.2001.01349.x.

On, S. L. W., Zhang, J., Cornelius, A. J., and Anderson, T. P. (2021). Markers for discriminating *Campylobacter concisus* genomospecies using MALDI-TOF analysis. *Curr. Res. Microb. Sci.* 2, 1–5. doi:10.1016/j.crmicr.2020.100019.

Opota, O., Prod'hom, G., and Greub, G. (2016). "Applications of MALDI-TOF Mass Spectrometry in Clinical Diagnostic Microbiology," in *MALDI-TOF and Tandem MS for Clinical Microbiology*, John Wiley & Sons, Ltd, 55–92. doi:10.1002/9781118960226.ch3.

Ota, Y., Furuhashi, K., Hirai, N., Ishikawa, J., Nagura, O., Yamanaka, K., et al. (2021). Evaluation of MBT STAR-Cepha and MBT STAR-Carba kits for the detection of extended-spectrum β-lactamases and carbapenemase producing microorganisms using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Microbiol. Methods* 183, 1-9.

doi:10.1016/j.mimet.2021.106166.

Oviaño, M., and Bou, G. (2019). Matrix-assisted laser desorption ionization–time of flight mass spectrometry for the rapid detection of antimicrobial resistance mechanisms and beyond. *Clin. Microbiol. Rev.* 32, 1–29. doi:10.1128/CMR.00037-18.

Oviaño, M., Ingebretsen, A., Steffensen, A. K., Croxatto, A., Prod'hom, G., Quiroga, L., et al. (2021). Evaluation of the rapidBACpro® II kit for the rapid identification of microorganisms directly from blood cultures using MALDI-TOF MS. *bioRxiv*, 2021.01.25.428200. doi:10.1101/2021.01.25.428200.

Parisi, A., Chiara, M., Caffara, M., Mion, D., Miller, W. G., Caruso, M., et al. (2021). *Campylobacter vulpis* sp. nov. isolated from wild red foxes. *Syst. Appl. Microbiol.* 44, 1-11. doi:10.1016/j.syapm.2021.126204.

Parker, M. T., and Hewitt, J. H. (1970). Methicillin resistance in *Staphylococcus aureus*. *Lancet* 295, 800–804. doi:10.1016/S0140-6736(70)92408-6.

Parkhill, J., Wren, B. W., Mungall, K., Ketley, J. M., Churcher, C., Basham, D., et al. (2000). The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665–668. doi:10.1038/35001088.

Pataki, B. Á., Matamoros, S., van der Putten, B. C. L., Remondini, D., Giampieri, E., Aytan-Aktug, D., et al. (2020). Understanding and predicting ciprofloxacin minimum inhibitory concentration in *Escherichia coli* with machine learning. *Sci. Rep.* 10, 1–9. doi:10.1038/s41598-020-71693-5.

Patton, C. M., Barrett, T. J., and Morris, G. K. (1985). Comparison of the Penner and Lior methods for serotyping *Campylobacter* spp. *J. Clin. Microbiol.* 22, 558–565. doi:10.1128/JCM.22.4.558-565.1985.

Payot, S., Bolla, J. M., Corcoran, D., Fanning, S., Mégraud, F., and Zhang, Q. (2006). Mechanisms of fluoroquinolone and macrolide resistance in *Campylobacter* spp. *Microbes Infect.* 8, 1967–1971. doi:10.1016/j.micinf.2005.12.032.

Pearson, B. M., Gaskin, D. J. H., Segers, R. P. A. M., Wells, J. M., Nuijten, P. J. M., and Van Vliet, A. H. M. (2007). The complete genome sequence of *Campylobacter jejuni* strain 81116 (NCTC11828). *J. Bacteriol.* 189, 8402–8403. doi:10.1128/JB.01404-07.

Peiffer-Smadja, N., Dellière, S., Rodriguez, C., Birgand, G., Lescure, F. X., Fourati, S., et al. (2020). Machine learning in the clinical microbiology laboratory: has the time come for routine practice? *Clin. Microbiol. Infect.* 26, 1300–1309. doi:10.1016/j.cmi.2020.02.006.

Penner, J. L., and Hennessy, J. N. (1980). Passive hemagglutination technique for serotyping *Campylobacter fetus* subsp. *jejuni* on the basis of soluble heat-stable antigens. *J. Clin. Microbiol.* 12, 732–737. doi:10.1128/jcm.12.6.732-737.1980.

Penny, C., Grothendick, B., Zhang, L., Borror, C. M., Barbano, D., Cornelius, A. J., et al. (2016). A Designed Experiments Approach to Optimizing MALDI-TOF MS Spectrum Processing Parameters Enhances Detection of Antibiotic Resistance in *Campylobacter jejuni*. *Front. Microbiol.* 7, 1–9. doi:10.3389/fmicb.2016.00818.

Pereira, R., Oliveira, J., and Sousa, M. (2020). Bioinformatics and computational tools for next-generation sequencing analysis in clinical genetics. *J. Clin. Med.* 9, 1-30. doi:10.3390/jcm9010132.

Pérez-Llarena, F. J., and Bou, G. (2016). Proteomics as a tool for studying bacterial virulence and antimicrobial resistance. *Front. Microbiol.* 7, 1–21. doi:10.3389/fmicb.2016.00410.

Pible, O., Allain, F., Jouffret, V., Culotta, K., Miotello, G., and Armengaud, J. (2020). Estimating relative biomasses of organisms in microbiota using “phylopeptidomics.” *Microbiome* 8, 1-13. doi:10.1186/s40168-020-00797-x.

Pible, O., and Armengaud, J. (2015). Improving the quality of genome, protein sequence, and taxonomy databases: A prerequisite for microbiome meta-omics 2.0. *Proteomics* 15, 3418–3423. doi:10.1002/pmic.201500104.

Piddock, L. J. V. (1999). Quinolone resistance and *Campylobacter*. *Clin. Microbiol. Infect.* 5, 239–243. doi:10.1111/j.1469-0691.1999.tb00135.x.

Pinar-Méndez, A., Fernández, S., Baquero, D., Vilaró, C., Galofré, B., González, S., et al. (2021). Rapid and improved identification of drinking water bacteria using the Drinking Water Library, a dedicated MALDI-TOF MS database. *Water Res.* 203, 1-10. doi:10.1016/j.watres.2021.117543.

Py, J.-S., Nagard, B., Rose, V., Couturier, L., Mathieu, C., Gassilloud, B., et al. (2021). Capacité du MALDI-TOF à identifier l’origine d’une contamination par *Campylobacter*. in *Réunion d'information et d'échanges sur Campylobacter*. Attended the 12.10.2021.

Qin, S., Wang, Y., Zhang, Q., Chen, X., Shen, Z., Deng, F., et al. (2012). Identification of a novel genomic island conferring resistance to multiple aminoglycoside antibiotics in *Campylobacter coli*. *Antimicrob. Agents Chemother.* 56, 5332–5339. doi:10.1128/AAC.00809-12.

Qin, S., Wang, Y., Zhang, Q., Zhang, M., Deng, F., Shen, Z., et al. (2014). Report of ribosomal RNA methylase gene *erm(B)* in multidrug-resistant *Campylobacter coli*. *J. Antimicrob. Chemother.* 69, 964–968. doi:10.1093/jac/dkt492.

Qu, K., Guo, F., Liu, X., Lin, Y., and Zou, Q. (2019). Application of machine learning in microbiology. *Front. Microbiol.* 10, 1-10. doi:10.3389/fmicb.2019.00827.

Ragimbeau, C., Colin, S., Devaux, A., Decruyenaere, F., Cauchie, H. M., Losch, S., et al. (2014). Investigating the host specificity of *Campylobacter jejuni* and *Campylobacter coli* by sequencing gyrase subunit A. *BMC Microbiol.* 14, 1–12. doi:10.1186/s12866-014-0205-7.

Rahi, P., Prakash, O., and Shouche, Y. S. (2016). Matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry (MALDI-TOF MS) based microbial identifications: Challenges and scopes for microbial ecologists. *Front. Microbiol.* 7, 1-12. doi:10.3389/fmicb.2016.01359.

Randall, H. M., Smith, D. W., Colm, A. C., and Nungester, W. J. (1951). Correlation of biologic properties of strains of *Mycobacterium* with infra-red spectrums. *Am. Rev. Tuberc.* 63, 372–380. doi:10.1164/art.1951.63.4.372.

Rani, P., Kotwal, S., Manhas, J., Sharma, V., and Sharma, S. (2021). Machine Learning and Deep Learning Based Computational Approaches in Automatic Microorganisms Image Recognition: Methodologies, Challenges, and Developments. *Arch. Comput. Methods Eng.* 1, 1-37. doi:10.1007/s11831-021-09639-x.

Ren, Z., Wang, H., Cui, G., Lu, H., Wang, L., Luo, H., et al. (2021). Alterations in the human oral and gut microbiomes and lipidomics in COVID-19. *Gut* 70, 1253–1265. doi:10.1136/gutjnl-2020-323826.

Research Advocacy Network (2013). Biomarker Validation. Available at: www.researchadvocacy.org [Accessed March 10, 2021].

Ribot, E. M., Freeman, M., Hise, K. B., and Gerner-Smidt, P. (2019). PulseNet: Entering the Age of Next-Generation Sequencing. *Foodborne Pathog. Dis.* 16, 451–456. doi:10.1089/fpd.2019.2634.

Riekeles, M., Schirmack, J., and Schulze-Makuch, D. (2021). Machine learning algorithms applied to identify microbial species by their motility. *Life* 11, 1–13. doi:10.3390/life11010044.

Rifai, N., Gillette, M. A., and Carr, S. A. (2006). Protein biomarker discovery and validation: The long and uncertain path to clinical utility. *Nat. Biotechnol.* 24, 971–983. doi:10.1038/nbt1235.

Ritzhaupt, A., Hayes, I., and Ehmann, F. (2020). Implementing the EU in vitro diagnostic regulation–a European regulatory perspective on companion diagnostics. *Expert Rev. Mol. Diagn.* 20, 565–567. doi:10.1080/14737159.2020.1720653.

Robert, M. G., Cornet, M., Hennebique, A., Rasamoelina, T., Caspar, Y., Pondérand, L., et al. (2021). MALDI-TOF MS in a medical mycology laboratory: On stage and backstage. *Microorganisms* 9, 1–16. doi:10.3390/microorganisms9061283.

Roberts, M. C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett.* 245, 195–203. doi:10.1016/j.femsle.2005.02.034.

Rodriguez-Granger, J., Mendiola, J. C., and Martínez, L. A. (2018). "Identification of Mycobacteria by Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry," in *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*, ed. F. Cobo, Academic Press, 181–195.

Rodríguez-Sánchez, B., Alcalá, L., Marín, M., Ruiz, A., Alonso, E., and Bouza, E. (2016). Evaluation of MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time-of-Flight Mass Spectrometry) for routine identification of anaerobic bacteria. *Anaerobe* 42, 101–107. doi:10.1016/j.anaerobe.2016.09.009.

Rodríguez-Sánchez, B., Cercenado, E., Coste, A. T., and Greub, G. (2019). Review of the impact of MALDI-TOF MS in public health and hospital hygiene, 2018. *Eurosurveillance* 24, 1-12. doi:10.2807/1560-7917.ES.2019.24.4.1800193.

Rojo-Martín, M. D. (2018). "Detecting Bacterial Resistance, Biomarkers, and Virulence Factors by MALDI-TOF Mass Spectrometry," in *The Use of Mass Spectrometry Technology (MALDI-TOF) in Clinical Microbiology*, Academic Press, 113–126. doi:10.1016/B978-0-12-814451-0.00008-3.

Rolim, A. E. H., Henrique-Araújo, R., Ferraz, E. G., de Araújo Alves Dultra, F. K., and Fernandez, L. G. (2015). Lipidomics in the study of lipid metabolism: Current perspectives in the omic sciences. *Gene* 554, 131–139. doi:10.1016/j.gene.2014.10.039.

Rossen, J. W. A., Friedrich, A. W., and Moran-Gilad, J. (2018). Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. *Clin. Microbiol. Infect.* 24, 355–360. doi:10.1016/j.cmi.2017.11.001.

Rotcheewaphan, S., Lemon, J. K., Desai, U. U., Henderson, C. M., and Zelazny, A. M. (2019). Rapid one-step protein extraction method for the identification of mycobacteria using MALDI-TOF MS. *Diagn. Microbiol. Infect. Dis.* 94, 355–360. doi:10.1016/j.diagmicrobio.2019.03.004.

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., and Domig, K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in *Escherichia coli*: A global overview. *Poult. Sci.* 98, 1791–1804. doi:10.3382/ps/pey539.

Roth, S., Berger, F. K., Link, A., Nimmesgern, A., Lepper, P. M., Murawski, N., et al. (2021). Application and clinical impact of the RESIST-4 O.K.N.V. rapid diagnostic test for carbapenemase detection in blood cultures and clinical samples. *Eur. J. Clin. Microbiol. Infect. Dis.* 40, 423–428. doi:10.1007/s10096-020-04021-4.

Rudd, K. E., Johnson, S. C., Agesa, K. M., Shackelford, K. A., Tsoi, D., Kievlan, D. R., et al. (2020). Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. *Lancet* 395, 200–211. doi:10.1016/S0140-6736(19)32989-7.

Rudrik, J. T., Soehnlen, M. K., Perry, M. J., Sullivan, M. M., Reiter-Kintz, W., Lee, P. A., et al. (2017). Safety and accuracy of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of highly pathogenic organisms. *J. Clin. Microbiol.* 55, 3513–3529. doi:10.1128/JCM.01023-17.

Rumbold, J. M. M., and Pierscionek, B. (2017). The Effect of the General Data Protection Regulation on Medical Research. *J Med Internet Res* 19, 1-5. doi:10.2196/JMIR.7108.

Rybicka, M., Miłosz, E., and Bielawski, K. P. (2021). Superiority of MALDI-TOF Mass Spectrometry over Real-Time PCR for SARS-CoV-2 RNA Detection. *Viruses* 13, 1–11. doi:10.3390/v13050730.

Rychert, J. (2019). Benefits and Limitations of MALDI-TOF Mass Spectrometry for the Identification of Microorganisms. *J. Infect.* 2, 1–5. doi:10.29245/2689-9981/2019/4.1142.

Ryzhov, V., and Fenselau, C. (2001). Characterization of the protein subset desorbed by MALDI from whole bacterial cells. *Anal. Chem.* 73, 746–750. doi:10.1021/ac0008791.

Sabença, C., de Sousa, T., Oliveira, S., Viala, D., Théron, L., Chambon, C., et al. (2020). Next-generation sequencing and maldi mass spectrometry in the study of multiresistant processed meat vancomycin-resistant enterococci (VRE). *Biology* 9, 1–17. doi:10.3390/biology9050089.

Saito, T., and Rehmsmeier, M. (2015). The precision-recall plot is more informative than the ROC plot when evaluating binary classifiers on imbalanced datasets. *PLoS One* 10, 1–21. doi:10.1371/journal.pone.0118432.

Sajedi, H., Mohammadipanah, F., and Pashaei, A. (2020). Image-processing based taxonomy analysis of bacterial macromorphology using machine-learning models. *Multimed. Tools Appl.* 79, 32711–32730. doi:10.1007/s11042-020-09284-9.

Saleeb, P. G., Drake, S. K., Murray, P. R., and Zelazny, A. M. (2011). Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 49, 1790–1794. doi:10.1128/JCM.02135-10.

Salzberg, S. L., Dunning Hotopp, J. C., Delcher, A. L., Pop, M., Smith, D. R., Eisen, M. B., et al. (2005). Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. *Genome Biol.* 6, 1–8. doi:10.1186/gb-2005-6-3-r23.

Sandle, T. (2016). "Microbial identification," in *Pharmaceutical Microbiology*, 103–113. doi:10.1016/b978-0-08-100022-9.00009-8.

Santos, I. C., Hildenbrand, Z. L., and Schug, K. A. (2016). Applications of MALDI-TOF MS in environmental microbiology. *Analyst* 141, 2827–2837. doi:10.1039/c6an00131a.

Sauget, M., Valot, B., Bertrand, X., and Hocquet, D. (2017). Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria? *Trends Microbiol.* 25, 447–455. doi:10.1016/j.tim.2016.12.006.

Schmieder, R., and Edwards, R. (2011). Fast identification and removal of sequence contamination from genomic and metagenomic datasets. *PLoS One* 6, 1-11. doi:10.1371/journal.pone.0017288.

Schwartz, D. C., and Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* 37, 67–75. doi:10.1016/0092-8674(84)90301-5.

Segata, N., and Huttenhower, C. (2011). Toward an efficient method of identifying core genes for evolutionary and functional microbial phylogenies. *PLoS One* 6, 1-10. doi:10.1371/journal.pone.0024704.

Seng, P., Drancourt, M., Gouriet, F., Scola, B. La, Fournier, P. E., Rolain, J. M., et al. (2009). Ongoing revolution in bacteriology: Routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clin. Infect. Dis.* 49, 543–551. doi:10.1086/600885.

Seuylemezian, A., Aronson, H. S., Tan, J., Lin, M., Schubert, W., and Vaishampayan, P. (2018). Development of a custom MALDI-TOF MS database for species-level identification of bacterial isolates collected from spacecraft and associated surfaces. *Front. Microbiol.* 9, 1-8. doi:10.3389/fmicb.2018.00780.

Shaheen, A., Tariq, A., Shehzad, A., Iqbal, M., Mirza, O., Maslov, D. A., et al. (2020). Transcriptional regulation of drug resistance mechanisms in *Salmonella*: where we stand and what we need to know. *World J. Microbiol. Biotechnol.* 36, 1–8. doi:10.1007/s11274-020-02862-x.

Shao, W., Zhang, M., Lam, H., and Lau, S. C. K. (2015). A peptide identification-free, genome sequence-independent shotgun proteomics workflow for strain-level bacterial differentiation. *Sci. Rep.* 5, 1–12. doi:10.1038/srep14337.

Shen, Z., Wang, Y., Zhang, Q., and Shen, J. (2018). Antimicrobial Resistance in *Campylobacter* spp. *Microbiol. Spectr.* 6, 1-13. doi:10.1128/microbiolspec.arba-0013-2017.

Sheppard, S. K., Cheng, L., Méric, G., De Haan, C. P. A., Llarena, A. K., Marttinen, P., et al. (2014). Cryptic ecology among host generalist *Campylobacter jejuni* in domestic animals. *Mol. Ecol.* 23, 2442–2451. doi:10.1111/mec.12742.

Sheppard, S. K., Colles, F. M., McCarthy, N. D., Strachan, N. J. C., Ogden, I. D., Forbes, K. J., et al. (2011). Niche segregation and genetic structure of *Campylobacter jejuni* populations from wild and agricultural host species. *Mol. Ecol.* 20, 3484–3490. doi:10.1111/j.1365-294X.2011.05179.x.

Sheppard, S. K., Colles, F., Richardson, J., Cody, A. J., Elson, R., Lawson, A., et al. (2010). Host association of *Campylobacter* genotypes transcends geographic variations. *Appl. Environ. Microbiol.* 76, 5269–5277. doi:10.1128/AEM.00124-10.

Sheppard, S. K., Jolley, K. A., and Maiden, M. C. J. (2012). A gene-by-gene approach to bacterial population genomics: Whole genome MLST of *Campylobacter*. *Genes* 3, 261–277. doi:10.3390/genes3020261.

Shrestha, P. M., Nevin, K. P., Shrestha, M., and Lovley, D. R. (2013). When is a microbial culture "Pure"? Persistent cryptic contaminant escapes detection even with deep genome

sequencing. *MBio* 4, 1-4. doi:10.1128/mBio.00591-12.

Siegrist, T. J., Anderson, P. D., Huen, W. H., Kleinheinz, G. T., McDermott, C. M., and Sandrin, T. R. (2007). Discrimination and characterization of environmental strains of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). *J. Microbiol. Methods* 68, 554–562. doi:10.1016/j.mimet.2006.10.012.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P. A., and Teixeira, P. (2011). *Campylobacter* spp. As a foodborne pathogen: A review. *Front. Microbiol.* 2, 1-12. doi:10.3389/fmicb.2011.00200.

Silvan, J. M., and Martinez-Rodriguez, A. J. (2018). "Food by-products as natural source of bioactive compounds against *Campylobacter*," in *Encyclopedia of Food Security and Sustainability*, Elsevier, 336–350. doi:10.1016/B978-0-08-100596-5.22139-2.

Singer, A. C., Kirchhelle, C., and Roberts, A. P. (2019). Reinventing the antimicrobial pipeline in response to the global crisis of antimicrobial-resistant infections. *F1000Research* 8, 1-6. doi:10.12688/f1000research.18302.1.

Singh, A., Mahto, K. K., and Prasad, R. (2013). Lipidomics and in Vitro Azole Resistance in *Candida albicans*. *Omi. A J. Integr. Biol.* 17, 84–93. doi:10.1089/omi.2012.0075.

Singhal, N., Kumar, M., Kanaujia, P. K., and Virdi, J. S. (2015). MALDI-TOF mass spectrometry: An emerging technology for microbial identification and diagnosis. *Front. Microbiol.* 6, 1–16. doi:10.3389/fmicb.2015.00791.

Singhal, N., Kumar, M., and Virdi, J. S. (2016). MALDI-TOF MS in clinical parasitology: Applications, constraints and prospects. *Parasitology* 143, 1491–1500. doi:10.1017/S0031182016001189.

Skirrow, M. B. (1977). *Campylobacter enteritis*: A "new" disease. *Br. Med. J.* 2, 9–11. doi:10.1136/bmj.2.6078.9.

Skirrow, M. B. (2006). John McFadyean and the Centenary of the First Isolation of *Campylobacter* Species. *Clin. Infect. Dis.* 43, 1213–1217. doi:10.1086/508201.

Soeny, K., Pandey, G., Gupta, U., Trivedi, A., Gupta, M., and Agarwal, G. (2021). Attended robotic process automation of prescriptions' digitization. *Smart Heal.* 20, 1-20. doi:10.1016/j.smhl.2021.100189.

Sogawa, K., Watanabe, M., Ishige, T., Segawa, S., Miyabe, A., Murata, S., et al. (2017). Rapid discrimination between methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* using MALDI-TOF mass spectrometry. *Biocontrol Sci.* 22, 163–169. doi:10.4265/bio.22.163.

Solntceva, V., Kostrzewa, M., and Larrouy-Maumus, G. (2021). Detection of Species-Specific Lipids by Routine MALDI TOF Mass Spectrometry to Unlock the Challenges of Microbial Identification and Antimicrobial Susceptibility Testing. *Front. Cell. Infect. Microbiol.* 10, 1–12. doi:10.3389/fcimb.2020.621452.

Sousa, T. De, Viala, D., Th, L., and Chambon, C. (2020). Putative Protein Biomarkers of *Escherichia coli* Antibiotic Multiresistance Identified by MALDI Mass spectrometry. *Biology* 9, 1–14. doi: 10.3390/biology9030056

Spener, F., Lagarde, M., Géloën, A., and Record, M. (2003). What is lipidomics? *Eur. J. Lipid Sci. Technol.* 105, 481-482. doi:10.1002/ejlt.200390101.

Sproston, E. L., Wimalarathna, H. M. L., Sheppard, S. K., L Wimalarathna, H. M., and Sheppard, S. K. (2018). Trends in fluoroquinolone resistance in *Campylobacter*. *Microb. Genomics* 4, 1-8. doi:10.1099/mgen.0.000198.

Steinkey, R., Moat, J., Gannon, V., Zovoilis, A., and Laing, C. (2020). Application of artificial intelligence to the in silico assessment of antimicrobial resistance and risks to human and animal health presented by priority enteric bacterial pathogens. *Canada Commun. Dis. Rep.*, 180–185. doi:10.14745/CCDR.V46I06A05.

Su, W., Kumar, V., Ding, Y., Ero, R., Serra, A., Lee, B. S. T., et al. (2018). Ribosome protection by antibiotic resistance ATP-binding cassette protein. *Proc. Natl. Acad. Sci. U. S. A.* 115, 5157–5162. doi:10.1073/pnas.1803313115.

Sy, I., Margardt, L., Ngbede, E. O., Adah, M. I., Yusuf, S. T., Keiser, J., et al. (2021). Identification of adult *Fasciola* spp. Using matrix-assisted laser/desorption ionization time-of-flight (maldi-tof) mass spectrometry. *Microorganisms* 9, 1–11. doi:10.3390/microorganisms9010082.

Sykes, E. (2018). Tackling antimicrobial resistance through rapid diagnostics. *Biochem. (Lond).* 40, 42–43. doi:10.1042/bio04001042.

Taboada, E. N., Clark, C. G., Sproston, E. L., and Carrillo, C. D. (2013). Current methods for molecular typing of *Campylobacter* species. *J. Microbiol. Methods* 95, 24–31. doi:10.1016/j.mimet.2013.07.007.

Tahir, D., Almeras, L., Varloud, M., Raoult, D., Davoust, B., and Parola, P. (2017). Assessment of MALDI-TOF mass spectrometry for filariae detection in *Aedes aegypti* mosquitoes. *PLoS Negl. Trop. Dis.* 11, 1–18. doi:10.1371/journal.pntd.0006093.

Tan, C. C. S., Acman, M., van Dorp, L., and Balloux, F. (2021). Metagenomic evidence for a polymicrobial signature of sepsis. *Microb. Genomics* 7, 1-13. doi:10.1099/mgen.0.000642.

Tan, K. E., Ellis, B. C., Lee, R., Stamper, P. D., Zhang, S. X., and Carroll, K. C. (2012). Prospective evaluation of a matrix-assisted laser desorption ionization-time of flight mass spectrometry system in a hospital clinical microbiology laboratory for identification of bacteria and yeasts: A bench-by-bench study for assessing the impact on ti. *J. Clin. Microbiol.* 50, 3301–3308. doi:10.1128/JCM.01405-12.

Tanaka, K., Waki, H., Ido, Y., Akita, S., Yoshida, Y., and Yoshida, T. (1988). Protein and Polymer

Analyses up to mlz 100 000 by Laser Ionization Time-of-flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* 2, 151–153.

Tandina, F., Laroche, M., Davoust, B., K Doumbo, O., and Parola, P. (2018). Blood meal identification in the cryptic species *Anopheles gambiae* and *Anopheles coluzzii* using MALDI-TOF MS. *Parasite* 25, 1–7. doi:10.1051/parasite/2018041.

Tang, W., Ranganathan, N., Shahrezaei, V., and Larrouy-Maumus, G. (2019). MALDI-TOF mass spectrometry on intact bacteria combined with a refined analysis framework allows accurate classification of MSSA and MRSA. *PLoS One* 14, 1–16. doi:10.1371/journal.pone.0218951.

Tang, Y., Dai, L., Sahin, O., Wu, Z., Liu, M., and Zhang, Q. (2017a). Emergence of a plasmid-borne multidrug resistance gene *cfr(C)* in foodborne pathogen *Campylobacter*. *J. Antimicrob. Chemother.* 72, 1581–1588. doi:10.1093/jac/dkx023.

Tang, Y., Fang, L., Xu, C., and Zhang, Q. (2017b). Antibiotic resistance trends and mechanisms in the foodborne pathogen, *Campylobacter*. *Anim. Heal. Res. Rev.* 18, 87–98. doi:10.1017/S1466252317000135.

Thépault, A., Méric, G., Rivoal, K., Pascoe, B., Mageiros, L., Touzain, F., et al. (2017). Genome-wide identification of host-segregating epidemiological markers for source attribution in *Campylobacter jejuni*. *Appl. Environ. Microbiol.* 83, 1-13. doi:10.1128/AEM.03085-16.

Toker, A., Shvarts, S., Perry, Z. H., Doron, Y., and Reuveni, H. (2004). Clinical guidelines, defensive medicine, and the physician between the two. *Am. J. Otolaryngol. - Head Neck Med. Surg.* 25, 245–250. doi:10.1016/j.amjoto.2004.02.002.

Tolar, B., Joseph, L. A., Schroeder, M. N., Stroika, S., Ribot, E. M., Hise, K. B., et al. (2019). An Overview of PulseNet USA Databases. *Foodborne Pathog. Dis.* 16, 457–462. doi:10.1089/fpd.2019.2637.

Tong, S., Ma, L., Ronholm, J., Hsiao, W., and Lu, X. (2021). Whole genome sequencing of *Campylobacter* in agri-food surveillance. *Curr. Opin. Food Sci.* 39, 130–139. doi:10.1016/j.cofs.2020.12.020.

Torres-Sangiao, E., Leal Rodriguez, C., and García-riestra, C. (2021). Application and perspectives of MALDI-TOF mass spectrometry in clinical microbiology laboratories. *Microorganisms* 9, 1–19. doi:10.3390/microorganisms9071539.

Tran, A., Alby, K., Kerr, A., Jones, M., and Gilligan, P. H. (2015). Cost savings realized by implementation of routine microbiological identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J. Clin. Microbiol.* 53, 2473–2479. doi:10.1128/JCM.00833-15.

Trott, D. J., Turnidge, J., Kovac, J. H., Simjee, S., Wilson, D., and Watts, J. (2021). Comparative

macrolide use in humans and animals: Should macrolides be moved off the World Health Organisation's critically important antimicrobial list? *J. Antimicrob. Chemother.* 76, 1955–1961. doi:10.1093/jac/dkab120.

Uchil, R. R., Kohli, G. S., Katekhaye, V. M., and Swami, O. C. (2014). Strategies to combat antimicrobial resistance. *J. Clin. Diagnostic Res.* 8, 1-4. doi:10.7860/JCDR/2014/8925.4529.

Uelze, L., Grützke, J., Borowiak, M., Hammerl, J. A., Juraschek, K., Deneke, C., et al. (2020). Typing methods based on whole genome sequencing data. *One Heal. Outlook* 2, 1–19. doi:10.1186/s42522-020-0010-1.

Valiente, E., Cairns, M. D., and Wren, B. W. (2014). The *Clostridium difficile* PCR ribotype 027 lineage: A pathogen on the move. *Clin. Microbiol. Infect.* 20, 396–404. doi:10.1111/1469-0691.12619.

Van Belkum, A., Welker, M., Erhard, M., and Chatellier, S. (2012). Biomedical mass spectrometry in today's and tomorrow's clinical microbiology laboratories. *J. Clin. Microbiol.* 50, 1513–1517. doi:10.1128/JCM.00420-12.

Van Breemen, R. B., Hathout, Y., and Fabris, D. (2020). Catherine Fenselau: A distinguished career dedicated to biomedical mass spectrometry. *J. Mass Spectrom.* 55, 1-8. doi:10.1002/jms.4500.

Van Hoek, A. H. A. M., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P., and Aarts, H. J. M. (2011). Acquired antibiotic resistance genes: An overview. *Front. Microbiol.* 2, 1-27. doi:10.3389/fmicb.2011.00203.

Vasala, A., Hytönen, V. P., and Laitinen, O. H. (2020). Modern Tools for Rapid Diagnostics of Antimicrobial Resistance. *Front. Cell. Infect. Microbiol.* 10, 1-23. doi:10.3389/FCIMB.2020.00308.

Vega-Castaño, S., Ferreira, L., González-Ávila, M., Sánchez-Juanes, F., García-García, M. I., García-Sánchez, J. E., et al. (2012). Reliability of MALDI-TOF mass spectrometry in the identification of anaerobic bacteria. *Enferm. Infecc. Microbiol. Clin.* 30, 597–601. doi:10.1016/j.eimc.2012.03.002.

Vogt, S., Löffler, K., Dinkelacker, A. G., Bader, B., Autenrieth, I. B., Peter, S., et al. (2019). Fourier-Transform Infrared (FTIR) Spectroscopy for Typing of Clinical *Enterobacter cloacae* Complex Isolates. *Front. Microbiol.* 10, 1-11. doi:10.3389/fmicb.2019.02582.

Voorhees, K. J., Jensen, K. R., McAlpin, C. R., Rees, J. C., Cody, R., Ubukata, M., et al. (2013). Modified MALDI MS fatty acid profiling for bacterial identification. *J. Mass Spectrom.* 48, 850–855. doi:10.1002/jms.3215.

Wagenaar, J. A., Newell, D. G., Kalupahana, R. S., and Mughini-Gras, L. (2014). "*Campylobacter*: Animal reservoirs, human infections, and options for control," in *Zoonoses-Infections Affecting*

Humans and Animals: Focus on Public Health Aspects, Springer, Dordrecht, 159–177. doi:10.1007/978-94-017-9457-2_6.

Waldvogel, F. A. (2004). Infectious diseases in the 21st century: Old challenges and new opportunities. *Int. J. Infect. Dis.* 8, 5–12. doi:10.1016/j.ijid.2003.01.001.

Wang, H. Y., Chen, C. H., Lee, T. Y., Horng, J. T., Liu, T. P., Tseng, Y. J., et al. (2018). Rapid detection of heterogeneous vancomycin-intermediate *Staphylococcus aureus* based on matrix-assisted laser desorption ionization time-of-flight: Using a machine learning approach and unbiased validation. *Front. Microbiol.* 9, 1–10. doi:10.3389/fmicb.2018.02393.

Watkins, L. K. F., Laughlin, M. E., Joseph, L. A., Chen, J. C., Nichols, M., Basler, C., et al. (2021). Ongoing Outbreak of Extensively Drug-Resistant *Campylobacter jejuni* Infections Associated With US Pet Store Puppies, 2016-2020. *JAMA Netw. Open* 4, e2125203–e2125203. doi:10.1001/JAMANETWORKOPEN.2021.25203.

Watson, D. S., Krutzinna, J., Bruce, I. N., Griffiths, C. E. M., McInnes, I. B., Barnes, M. R., et al. (2019). Clinical applications of machine learning algorithms: Beyond the black box. *BMJ* 364, 1-4. doi:10.1136/bmj.l886.

Webber, M. A., and Piddock, L. J. V. (2003). The importance of efflux pumps in bacterial antibiotic resistance. *J. Antimicrob. Chemother.* 51, 9–11. doi:10.1093/jac/dkg050.

Weis, C., Cuénod, A., Rieck, B., Llinares-López, F., Dubuis, O., Graf, S., et al. (2020a). Direct Antimicrobial Resistance Prediction from MALDI-TOF mass spectra profile in clinical isolates through Machine Learning. *bioRxiv* 1, 1–35. doi:10.1101/2020.07.30.228411.

Weis, C. V., Jutzeler, C. R., and Borgwardt, K. (2020b). Machine learning for microbial identification and antimicrobial susceptibility testing on MALDI-TOF mass spectra: a systematic review. *Clin. Microbiol. Infect.* 26, 1310–1317. doi:10.1016/j.cmi.2020.03.014.

Welker, M., and Belkum, A. Van (2019). One system for all: Is mass spectrometry a future alternative for conventional antibiotic susceptibility testing? *Front. Microbiol.* 10, 1–12. doi:10.3389/fmicb.2019.02711.

Wendel, T., Feucherolles, M., Rehner, J., Keiser, J., Poppert, S., Utzinger, J., et al. (2021). Evaluating Different Storage Media for Identification of *Taenia saginata* proglottids using MALDI-TOF Mass Spectrometry. *Microorganisms*, 1–11. doi:10.3390/ microorganisms9102006.

Wenning, M., and Scherer, S. (2013). Identification of microorganisms by FTIR spectroscopy: Perspectives and limitations of the method. *Appl. Microbiol. Biotechnol.* 97, 7111–7120. doi:10.1007/s00253-013-5087-3.

Wensley, A., Padfield, S., and Hughes, G. J. (2020). An outbreak of campylobacteriosis at a hotel in England: The ongoing risk due to consumption of chicken liver dishes. *Epidemiol. Infect.* 148,

1-5. doi:10.1017/S095026882000028X.

WHO (2001). WHO Global Strategy for Containment of Antimicrobial Resistance. Available at: https://apps.who.int/iris/handle/10665/66860 [Accessed September 13, 2021].

WHO (2011). Regional Committee for Europe European strategic action plan on antibiotic resistance. 6, 12–15. Available at: http://www.euro.who.int/__data/assets/pdf_file/0008/147734/wd14E_AntibioticResistance_111380.pdf [Accessed September 13, 2021].

WHO (2019a). Averting the AMR crisis: What are the avenues for policy action for countries in Europe? *Eur. Obs. Policy Briefs*. Available at: http://www.euro.who.int/en/about-us/partners/ [Accessed November 3, 2021].

WHO (2019b). Critically Important Antimicrobials for Human Medicine 6th Re.vision 2018. Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. Available at: https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf?ua=1 [Accessed May 15, 2019].

WHO (2020). Antimicrobial resistance. Available at: https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance [Accessed 18 November, 2021]

Wieczorek, K., and Osek, J. (2013). Antimicrobial Resistance Mechanisms among *Campylobacter*. *Biomed Res. Int.* 2013, 1–12. doi:10.1155/2013/340605.

Wielinga, P. R., and Schlundt, J. (2014). "One health and food safety," in *Confronting Emerging Zoonoses: The One Health Paradigm*, Nature Publishing Group, 213–232. doi:10.1007/978-4-431-55120-1_10.

Wu, J., Song, C., Dubinsky, E. A., and Stewart, J. R. (2021). Tracking Major Sources of Water Contamination Using Machine Learning. *Front. Microbiol.* 11, 1-13. doi:10.3389/fmicb.2020.616692.

Yagel, Y., and Moran-Gilad, J. (2021). "Introduction to Advanced Diagnostics in Microbiology," in *Application and Integration of Omics-powered Diagnostics in Clinical and Public Health Microbiology*, Springer, Cham, 1–7. doi:10.1007/978-3-030-62155-1_1.

Yang, K., and Han, X. (2016). Lipidomics: Techniques, Applications, and Outcomes Related to Biomedical Sciences. *Trends Biochem. Sci.* 41, 954–969. doi:10.1016/j.tibs.2016.08.010.

Yang, S. M., Kim, E., Kim, D., Baek, J., Yoon, H., and Kim, H. Y. (2021). Rapid detection of *Salmonella enteritidis*, *typhimurium*, and *thompson* by specific peak analysis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Foods* 10, 1-16. doi:10.3390/foods10050933.

Yang, Y., Feye, K. M., Shi, Z., Pavlidis, H. O., Kogut, M., J. Ashworth, A., et al. (2019). A Historical Review on Antibiotic Resistance of Foodborne *Campylobacter*. *Front. Microbiol.* 10, 1-8. doi:10.3389/fmicb.2019.01509.

Yang, Y., Lin, Y., and Qiao, L. (2018). Direct MALDI-TOF MS Identification of Bacterial Mixtures. *Anal. Chem.*, 90, 1400-1408. doi:10.1021/acs.analchem.8b02258.

Yao, H., Liu, D., Wang, Y., Zhang, Q., and Shen, Z. (2017). High prevalence and predominance of the aph(2')-if gene conferring aminoglycoside resistance in *Campylobacter*. *Antimicrob. Agents Chemother.* 61, 1-9. doi:10.1128/AAC.00112-17.

Yao, H., Shen, Z., Wang, Y., Deng, F., Liu, D., Naren, G., et al. (2016). Emergence of a potent multidrug efflux pump variant that enhances *Campylobacter* resistance to multiple antibiotics. *MBio* 7, 1-11. doi:10.1128/mBio.01543-16.

Yao, H., Zhang, N., Zhang, R., Duan, M., Xie, T., Pan, J., et al. (2020). Severity Detection for the Coronavirus Disease 2019 (COVID-19) Patients Using a Machine Learning Model Based on the Blood and Urine Tests. *Front. Cell Dev. Biol.* 8, 1–10. doi:10.3389/fcell.2020.00683.

Yates III, J. R. (2011). A century of mass spectrometry: from atoms to proteomes. *Nat. Methods* 8, 633-637. Available at: http://www.imbb.forth.gr/ProFI/pdf/nmeth_1659.pdf%5Cnhttp://www.nature.com.gate2.inist.fr/nmeth/journal/v8/n8/pdf/nmeth.1659.pdf. [Accessed October 12, 2021].

Yi, Q., Xiao, M., Fan, X., Zhang, G., Yang, Y., Zhang, J.-J., et al. (2021). Evaluation of Autof MS 1000 and Vitek MS MALDI-TOF MS System in Identification of Closely-Related Yeasts Causing Invasive Fungal Diseases. *Front. Cell. Infect. Microbiol.* 11, 1-7. doi:10.3389/FCIMB.2021.628828.

Yoon, E., and Jeong, S. H. (2021). MALDI-TOF Mass Spectrometry Technology as a Tool for the Rapid Diagnosis of Antimicrobial Resistance in Bacteria. *Antibiotics* 10, 1–13. doi:10.3390/antibiotics10080982.

Yugendran, T., and Harish, B. N. (2016). Global DNA methylation level among ciprofloxacin-resistant clinical isolates of *Escherichia coli*. *J. Clin. Diagnostic Res.* 10, 27–30. doi:10.7860/JCDR/2016/19034.7830.

Zautner, A. E., Lugert, R., Masanta, W. O., Weig, M., Groß, U., and Bader, O. (2016). Subtyping of *Campylobacter jejuni* ssp. doylei Isolates Using Mass Spectrometry-based PhyloProteomics (MSPP). *J. Vis. Exp.*, 1–9. doi:10.3791/54165.

Zautner, A. E., Masanta, W. O., Tareen, A. M., Weig, M., Lugert, R., Groß, U., et al. (2013). Discrimination of multilocus sequence typing-based *Campylobacter jejuni* subgroups by MALDI-TOF mass spectrometry. *BMC Microbiol.* 13, 1–8. doi:10.1186/1471-2180-13-247.

Zautner, A. E., Masanta, W. O., Weig, M., Gross, U., and Bader, O. (2015). Mass Spectrometry-based PhyloProteomics (MSPP): A novel microbial typing Method. *Sci. Rep.* 5, 1–11. doi:10.1038/srep13431.

Zeng, X., Brown, S., Gillespie, B., and Lin, J. (2014). A single nucleotide in the promoter region modulates the expression of the β-lactamase OXA-61 in *Campylobacter jejuni*. *J. Antimicrob. Chemother.* 69, 1215–1223. doi:10.1093/jac/dkt515.

Zhang, S., Yin, Y., Jones, M. B., Zhang, Z., Kaiser, B. L. D., Dinsmore, B. A., et al. (2015). *Salmonella* serotype determination utilizing high-throughput genome sequencing data. *J. Clin. Microbiol.* 53, 1685–1692. doi:10.1128/JCM.00323-15.

Zhang, X.-D. (2017). "Machine Learning," in *A Matrix Algebra Approach to Artificial Intelligence*, 223–439. doi: 10.1007/978-981-15-2770-8

Zhao, S., Tyson, G. H., Chen, Y., Li, C., Mukherjee, S., Young, S., et al. (2016). Whole-Genome Sequencing Analysis Accurately Predicts Antimicrobial Resistance Phenotypes in *Campylobacter* spp. *Appl. Environ. Microbiol.* 82, 459–466. doi:10.1128/AEM.02873-15.

Zhou, Y. J., Wang, G., and Tang, Y. W. (2018). Companion diagnostics: New opportunities for safe and effective anti-infectious disease therapies news-and-views. *Emerg. Microbes Infect.* 7, 1-2. doi:10.1038/s41426-017-0014-9.

Appendices

Appendix 1

Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry

While pursuing the current research project, close collaboration with the former employer, i.e. the University of Saarland, on the previous MALDI-TOF MS and parasites project was maintained. In this context, an original research article was published on the evaluation of liquid chromatography (LC)-MS grade water, sodium chloride solution, ethanol, and formalin as storage media for *T. saginata* proglottids for MALDI-TOF MS analysis*.

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Evaluating Different Storage Media for Identification of *Taenia saginata* Proglottids Using MALDI-TOF Mass Spectrometry

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Abstract: *Taenia saginata* is a helminth that can cause taeniasis in humans and cysticercosis in cattle. A species-specific diagnosis and differentiation from related species (e.g., *Taenia solium*) is crucial for individual patient management and disease control programs. Diagnostic stool microscopy is limited by low sensitivity and does not allow discrimination between *T. saginata* and *T. solium*. Molecular diagnostic approaches are not routinely available outside research laboratories. Recently, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) was proposed as a potentially suitable technique for species-specific helminth diagnosis. However, standardized protocols and commercial databases for parasite identification are currently unavailable, and pre-analytical factors have not yet been assessed. The purpose of this study was to employ MALDI-TOF MS for the identification of *T. saginata* proglottids obtained from a human patient, and to assess the effects of different sample storage media on the technique's diagnostic accuracy. We generated *T. saginata*-specific main spectral profiles and added them to an in-house database for MALDI-TOF MS-based diagnosis of different helminths. Based on protein spectra, *T. saginata* proglottids could be successfully differentiated from other helminths, as well as bacteria and fungi. Additionally, we analyzed *T. saginata* proglottids stored in (i) LC–MS grade water; (ii) 0.45% sodium chloride; (iii) 70% ethanol; and (iv) 37% formalin after 2, 4, 6, 8, 12, and 24 weeks of storage. MALDI-TOF MS correctly identified 97.2–99.7% of samples stored in water, sodium chloride, and ethanol, with log-score values $\geq$2.5, thus indicating reliable species identification. In contrast, no protein spectra were obtained for samples stored in formalin. We conclude that MALDI-TOF-MS can be successfully employed for the identification of *T. saginata*, and that water, sodium chloride, and ethanol are equally effective storage solutions for prolonged periods of at least 24 weeks.

Keywords: cestodes; diagnosis; helminth infections; matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry; neglected tropical diseases; taeniasis

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1. Introduction

The beef tapeworm, *Taenia saginata*, is a zoonotic cestode that can cause taeniasis, an intestinal infection in humans, and cysticercosis in bovines [1]. It is the most common and most widely distributed *Taenia* species. While humans are the definitive host, cattle serve as intermediate hosts for *T. saginata*. *Taenia solium* and *Taenia asiatica* are less frequently occurring species, with *T. solium* being of particular clinical relevance, as it gives rise to intestinal disease and the potentially fatal human (neuro-)cysticercosis [2]. Humans acquire intestinal *Taenia* infection through the consumption of raw or undercooked meat of infected animals. Intestinal taeniasis mainly causes mild and unspecific symptoms, such as weight

loss and general malaise. More pronounced symptoms (e.g., diarrhea, abdominal pain, and nausea) are less frequent [3]. Severe complications, such as appendicitis or gall bladder perforation, have rarely been reported [4].

After the ingestion of infected bovine muscle tissue, a *Taenia* cysticercus develops within the human host's intestine into an adult worm during a prepatency period of approximately 2 months, and produces eggs and gravid proglottids, which are shed with the feces. In settings with poor sanitation, eggs can spread through water, wind, or simply attach to vegetation. Cattle become infected by ingesting contaminated plants [5].

Taeniasis is considered a neglected tropical disease (NTD) [6]. In recent years, several studies carried out by the European CystiNet network and others investigated the global occurrence of taeniasis. It was found that *Taenia* tapeworms occur worldwide, and that *T. saginata* is particularly frequent in East, Southeast, and South Asia [7]. In Europe, taeniasis cases are reported in 12 out of 18 surveyed countries, with an estimated prevalence ranging from 0.02 to 0.67% [1]. As taeniasis is associated with poor sanitation, low-income settings, and understaffed meat inspectorates, the disease is also frequently reported from parts of the Middle East, Africa [8], and Central and South America [9]. However, prevalence estimates lack accuracy, as taeniasis is a non-notifiable disease in most countries, and as for many NTDs, public health campaigns pay little attention to this disease [10]. In 2007, it was estimated that at least 60 million people were infected with *T. saginata* [11]. However, the global burden of taeniasis, as expressed in disability-adjusted life years (DALYs), has yet to be determined [12].

The diagnosis of human taeniasis mainly relies on the direct visualization of proglottids, or the microscopic detection of eggs in stool samples [4]. In research settings, other methods are also used, such as stool-based polymerase chain reaction (PCR) assays or copro-antigen enzyme-linked immunosorbent assay (ELISA) tests, which detect specific secretory antigens in fecal samples [3]. However, these techniques have several limitations. While the commonly employed microscopy can be rapidly performed and does not require well-equipped laboratories, its sensitivity is low [4], and a species differentiation between *T. saginata* and *T. solium* is only possible if proglottids are shed in the feces, because the eggs of both species are indistinguishable [13]. The copro-antigen ELISA is characterized by a relatively low specificity, as studies carried out on samples stemming from cattle reported relatively high rates of cross-reactivity with related species of veterinary importance, such as *Taenia hydatigena* and *Taenia multiceps* [14]. PCR-based assays allow highly sensitive species identification, but are costly, rarely available outside research laboratories, and require specific technical expertise. Hence, there is a need for simple-to-use, accurate diagnostic methods for taeniasis, as the correct identification of *Taenia* infections at the species level is an important requirement for clinical management and contact screening, particularly in case of *T. solium* infections that pose the risk of human neurocysticercosis [15].

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is an extensively validated diagnostic technique, which is nowadays routinely used in clinical microbiology laboratories for the species-specific diagnosis of bacteria and fungi in high-income countries [16]. Recently, several studies also reported MALDI-TOF MS, which analyzes pathogen-specific protein spectra to reach a specific diagnosis, as a suitable method for the identification of parasites [13], including helminths (e.g., *Fasciola* spp. [17], *Trichinella* spp. [18], and *Anisakis* spp. [19]). Besides high accuracy, the low cost of reagents needed for MALDI-TOF analysis in comparison to reagents required for PCR assays is a competitive advantage. However, there is uncertainty regarding the standardization of MALDI-TOF analytical protocols, and the effects of pre-analytical factors need to be elucidated. In this study, we utilized *T. saginata* proglottids to systematically assess whether the use of different sample storage media or the duration of storage affect the composition of the resulting protein spectra, and hence, the ability of MALDI-TOF MS to reach species-specific identification.

2. Materials and Methods

2.1. Ethics Statement

The *T. saginata* sample used in this study was obtained from an infected patient who sought routine diagnostic work-up for suspected parasite infection at the Swiss Tropical and Public Health Institute (Swiss TPH) in Basel, Switzerland. All procedures adhered to local laws and regulations.

2.2. Sample Collection

T. saginata proglottids were collected by an experienced medical laboratory technician from the stool sample of an infected patient at Swiss TPH in Basel. The specimen was stored in a freezer at −20 °C in 0.45% (*v*/*v*) sodium chloride solution. In October 2018, the sample was transferred to the Institute of Medical Microbiology and Hygiene in Homburg, Germany for further examination.

2.3. Study Design and Experimental Set-Up

Upon receipt at the Institute of Medical Microbiology and Hygiene in Homburg, the *Taenia* specimen was subjected to nucleic acid extraction, PCR, and partial sequencing for species-specific identification as *T. saginata*. Next, MALDI-TOF MS was carried out to generate protein spectral profiles, which were then transferred into an in-house database for MS-based identification of helminths. Subsequently, proglottids were put into different storage media and re-analyzed by MALDI-TOF MS after 2, 4, 6, 8, 12, and 24 weeks. At each time, the obtained spectra were compared to the initially measured spectra.

2.4. Molecular Diagnosis Using PCR and Partial Sequencing

For confirmatory molecular species identification, one proglottid of the *Taenia* specimen was thawed and subjected to DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen GmbH; Hilden, Germany). In brief, a sample measuring approximately 1 cm was pounded into small pieces. Next, 180 µL of ATL buffer was added, the sample was vortexed, and 20 µL of proteinase K was added. The mix was vortexed and incubated at 56 °C in a thermomixer (Eppendorf; Hamburg, Germany) for 1 h. After incubation, the mix was vortexed again, and both 200 µL of AL buffer and 200 µL of 100% (*v*/*v*) ethanol were added. Subsequently, the DNeasy Mini column system (Qiagen; Hilden, Germany) was used for nucleic acid extraction, adhering to the manufacturer's protocol.

For gene amplification, the partial mitochondrial cytochrome oxidase 1 gene (COX-1) was used to perform a PCR as previously described [20]. Specific forward (5′-CATCATATGTTTACGGTTGG-3′) and reverse (5′-GACCCTAATGACATAACATAAT-3′) primers were used to amplify a gene of around 350 base pairs (bp), utilizing a peqSTAR thermocycler (VWR; Radnor, PA, USA). In brief, the assay consists of 12.5 µL Hotstart Mix (Qiagen; Hilden, Germany), 0.5 µL of forward primer, 0.5 µL of reverse primer, 9.5 µL of water, and 2 µL of *Taenia* DNA. The cycling conditions comprised an initial denaturation step at 95 °C for 5 min, followed by 56 °C for 1 min, and 72 °C for 2 min. Then, 45 amplification cycles were performed, each consisting of a denaturation step at 95 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s. Afterwards, a final elongation step at 72 °C for 4 min was performed.

For sequencing of the generated amplicons, the Capillary Electophoretic GenomeLab genetic analysis system (Beckman Coulter; Brea, CA, USA) was used. Consensus sequences were created by editing and merging raw forward and reverse sequences, using the BioEdit© software version 7.2.5 (Tom Hall; Carlsbad, CA, USA). The consensus sequence was aligned with sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank database for final identification.

2.5. Differential Sample Storage Conditions

Taenia proglottids were removed from the original storage medium (sodium chloride 0.45% (*v*/*v*)) and placed on a Petri dish. Using a sterile scalpel, individual proglottids were

cut into small pieces of approximately 1 cm. Next, each specimen was placed into a 1.5 mL Eppendorf tube, and 1 mL of one of the following four different storage solutions was added: (i) sodium chloride 0.45% (*v*/*v*) (Merck KG; Darmstadt, Germany); (ii) ethanol 70% (*v*/*v*) (Merck KG); (iii) liquid chromatography (LC) MS grade water (Merck KG); and (iv) formalin 37% (*v*/*v*) (Merck KG). All samples were then stored at −20 °C in these media, before being consecutively subjected to MALDI-TOF MS after the aforementioned exposure periods. The experiment was carried out with 6 specimens for each storage medium, i.e., 24 proglottids in total.

2.6. MALDI-TOF Analysis

2.6.1. Protein Extraction

Prior to analysis, each proglottid sample was thawed and cut into three equal parts. Each part was then transferred to a new tube for subsequent MALDI-TOF MS measurements. For protein extraction, we employed a previously developed protocol [17].

2.6.2. MALDI-TOF Target Plate Preparation and Measurements

Using the protein extract, 1 µL of the supernatant was spotted onto the MALDI target plate. For each sample, eight specific spots on the target plate were used, as recommended by the manufacturer (MSP creation protocol V1.1; Bruker Daltonics; Bremen, Germany). After drying, 1 µL of α-cyano-4-hydroxycinamic acid (CHCA) matrix solution (Bruker Daltonics), composed of saturated CHCA, 50% (*v*/*v*) of acetonitrile, 2.5% (*v*/*v*) of trifluoracetic, and 47.5% (*v*/*v*) of LC–MS grade water, was added to each spot. A commercially available Bacterial Test Standard (BTS; i.e., *Escherichia coli* extract connected with two high molecular weight proteins) was used to calibrate the mass spectrometer. After drying at room temperature, the MALDI target plate was placed into the Microflex LT Mass Spectrometer (Bruker Daltonics; Bremen, Germany) for MALDI-TOF MS analysis. Each sample spot was measured four times to generate a total of 32 raw spectra (8 spots × 4). This procedure was carried out on two replicates on the same day (repeatability analysis), and on one additional replicate on a subsequent day (reproducibility analysis). Hence, a total of 96 raw spectra were acquired for each sample.

2.6.3. MALDI-TOF MS Parameters

All measurements were performed using the AutoXecute algorithm in the FlexControl® software version 3.4. (Bruker Daltonics; Bremen, Germany). For each spot, 240 laser shots (40 laser shots each using six random positions) were used to generate protein spectral profiles in linear positive ion mode. The laser frequency was 60 Hz, and a high voltage of 20 kV and pulsed ion extraction of 180 ns were employed. The mass charge ratio range (m/z) was measured between 2 and 20 k Da.

2.6.4. Spectral Analysis, MSP Creation, and Clustering Analysis

All raw spectra were analyzed with the FlexAnalysis® software version 3.4 (Bruker Daltonics; Bremen, Germany). To improve the spectral quality, raw spectra were edited by removing all flatlines and outlier peaks. The intensities were smoothened, and baseline subtraction was performed, as appropriate. Peak shifts within spectra were also edited when they exceeded 500 ppm. Following these steps, replicates containing at least 22 remaining spectra were maintained, and the measurement was repeated if these conditions were not reached.

The edited spectra of the initial *Taenia* sample were used to create a species-specific main spectral profile (MSP), utilizing the automated function of the MALDI Biotyper Compass Explorer® software version 4.1 (Bruker Daltonics; Bremen, Germany). The newly created *Taenia* MSP was added to a previously developed in-house database with several species, including cestodes (e.g., *Diphyllobothrium* spp.), nematodes (e.g., *Ascaris* spp.), and trematodes (e.g., *Fasciola* spp.), for helminth identification, and served as a reference spectrum for comparative analysis under different storage conditions. Subsequently, a

clustering analysis was performed on the edited spectra obtained after 2, 12, and 24 weeks using the BioNumerics® software version 7.6 (Applied Maths N.V.; Sint-Martens-Latem, Belgium). A dendrogram was generated using an unweighted pair group method with the arithmetic mean (UPGMA), and a curve-based similarity matrix was calculated using Pearson correlation. A principal components analysis (PCA) and a discriminant analysis were carried out using quantitative values.

2.6.5. MALDI-TOF Identification Parameters

All measured spectra were initially analyzed using the official Bruker Taxonomy Database designed for bacteria and fungi, containing 8936 MSPs, which is routinely used in clinical microbiology laboratories, to detect possible contamination with bacterial or fungal organisms. Next, protein spectra were analyzed by a combination of this official Bruker database (Bruker Taxonomy) and the previously developed in-house helminth database with around 98 MSPs, including the MSP of the initially analyzed *Taenia* proglottid. The reliability of identification was evaluated by log score values (LSVs), which were generated by MALDI-TOF MS. We followed the LSV thresholds used in routine microbiology for the identification of bacteria and fungi, i.e., LSVs $\leq$1.69, indicating an unreliable identification; LSVs ranging between 1.70 and 1.99, indicating an accurate genus and probable species identification; and LSVs $\geq$2.0, suggesting a reliable species identification.

3. Results

3.1. Molecular Identification of Taenia Proglottids

PCR and sequencing of the initial *Taenia* proglottid sample using primers of the COX1-gene confirmed the species diagnosis. An analysis using NCBI GenBank showed 100% sequence homologies with a previously described *T. saginata* sequence (reference accession number: MT074048.1). The sequence of our *Taenia* sample was deposited in the GenBank database (accession number: MZ720823).

3.2. Comparative MALDI-TOF MS Analysis after Different Storage Periods

3.2.1. Protein Spectra and LSV Analysis

A representative protein spectral profile for each storage medium is displayed in Figure 1. High peak intensities were observed and reached up to 1.0×10^4 arbitrary units (a.u.). With regard to the position and the intensity of the measured peaks, *Taenia* samples stored in LC–MS grade water, ethanol, and sodium chloride showed a similar profile to the original sample, with no significant changes over time. For samples stored in formalin, no protein spectra were found at any time point.

For all samples, the commercially available MALDI-TOF database for the identification of bacteria and fungi did not yield a reliable identification, with an LSV of 1.37 for the bacterium *Arthrobacter monumenti* being the highest score. When submitting the spectra to a combination of the commercially available and in-house helminth databases, a correct identification was achieved in 97.2%, 99.7%, and 99.0%, for samples stored in sodium chloride, ethanol, and LC–MS grade water, respectively, with LSVs ranging between 2.53 and 2.57. No identification was achieved for spectra of *Taenia* proglottids stored in formalin (Table 1).

When analyzing identification patterns over time, a high LSV ($\geq$2.3) was constantly observed at all measurements for each storage solution, except formalin. Small fluctuations of LSVs were found for all storage solutions, with slightly more fluctuation in the sodium chloride medium (Figure 2).

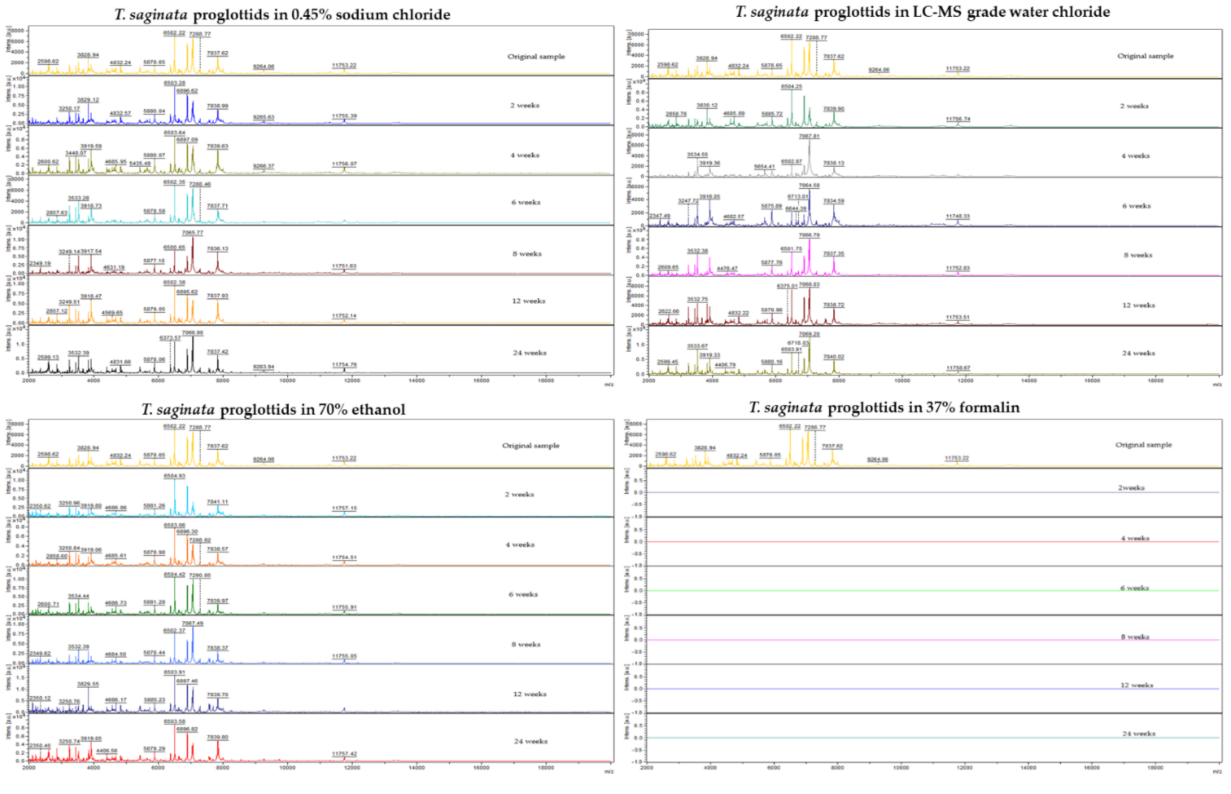

Figure 1. Protein spectral profiles of *Taenia saginata* proglottids. The peaks obtained when measuring the original sample, and protein profiles after prolonged storage in four different media, are displayed. *X*-axis, mass-to-charge ratio of (*m*/*z*); *Y*-axis, peak intensities of ionized molecules; a.u., arbitrary unit.

Table 1. Identification of *Taenia saginata* proglottids stored in different storage media (**A**) using Bruker Taxonomy, the commercially available database for bacteria and fungi, and (**B**) using a combination of Bruker Taxonomy and an in-house helminth database.

(A)					
Sample Preservation Medium	**Number of Samples**	**Number of Spectra**	**Bruker Taxonomy Database**		
			Correct Identification	**Average LSV**	**Most Frequently Suggested Result**
0.45% sodium chloride	6	560	0%	1.38	*Arthrobacter monumenti*
70% ethanol	6	574	0%	1.39	*Arthrobacter monumenti*
LC–MS grade water	6	570	0%	1.38	*Arthrobacter monumenti*
37% formalin	6	0	0%	0	None
(B)					
Sample Preservation Medium	**Number of Samples**	**Number of Spectra**	**Combination of Bruker Taxonomy and In-House Helminth Database**		
			Correct Identification	**Average LSV**	**Most Frequently Suggested Result**
0.45% sodium chloride	6	560	97.2% (560/576)	2.54	*T. saginata* proglottid
70% ethanol	6	574	99.7% (574/576)	2.53	*T. saginata* proglottid
LC–MS grade water	6	570	99.0% (570/576)	2.57	*T. saginata* proglottid
37% formalin	6	0	0%	0	-

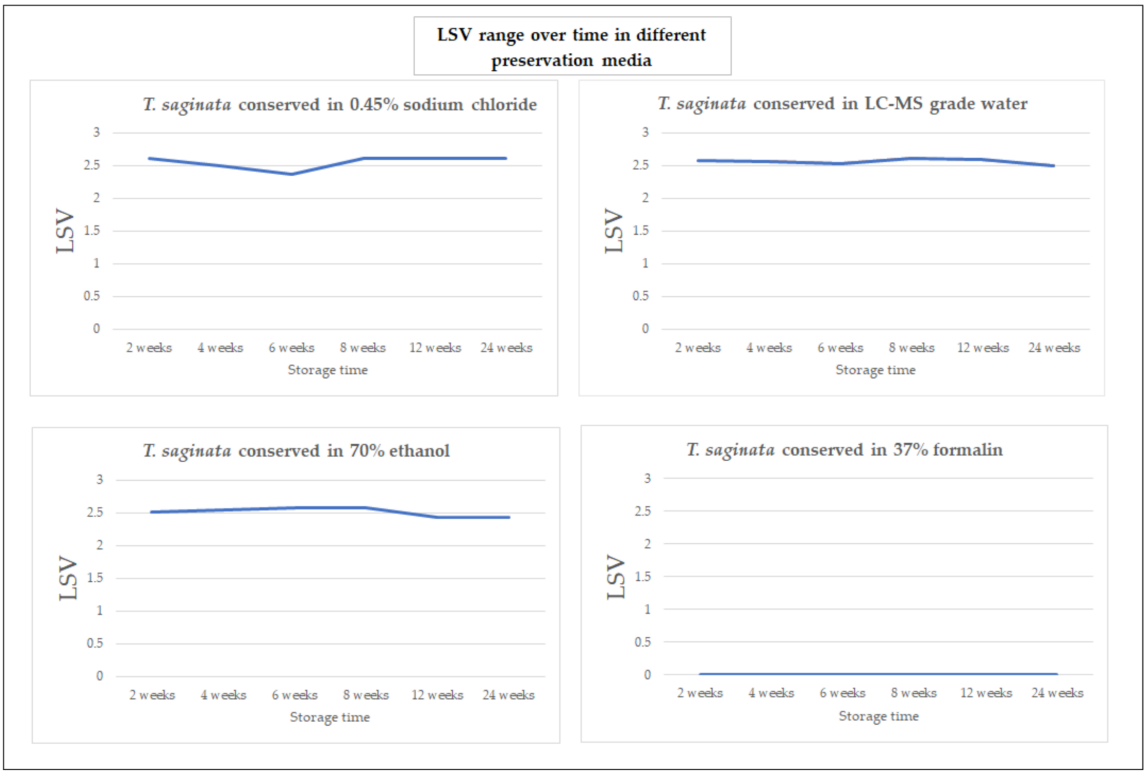

Figure 2. Average LSVs of protein spectra stemming from *Taenia* proglottids in different preservation media during a 24-week observation period. Spectra were identified using a combination of Bruker Taxonomy and an in-house helminth database.

3.2.2. Cluster Analysis

Cluster analysis to display the relatedness of the *Taenia* proglottids stored in sodium chloride, ethanol, and LC–MS grade water showed that all these proglottids clustered together and showed relatedness levels >85% (Figure 3). Subsequent statistical analyses (both PCA and discriminant analysis) performed on the summary spectra of *T. saginata* proglottids did not show specific differences pertaining to the different preservation media or the duration of storage (Figure 4), thus indicating an almost identical pattern of the protein spectra.

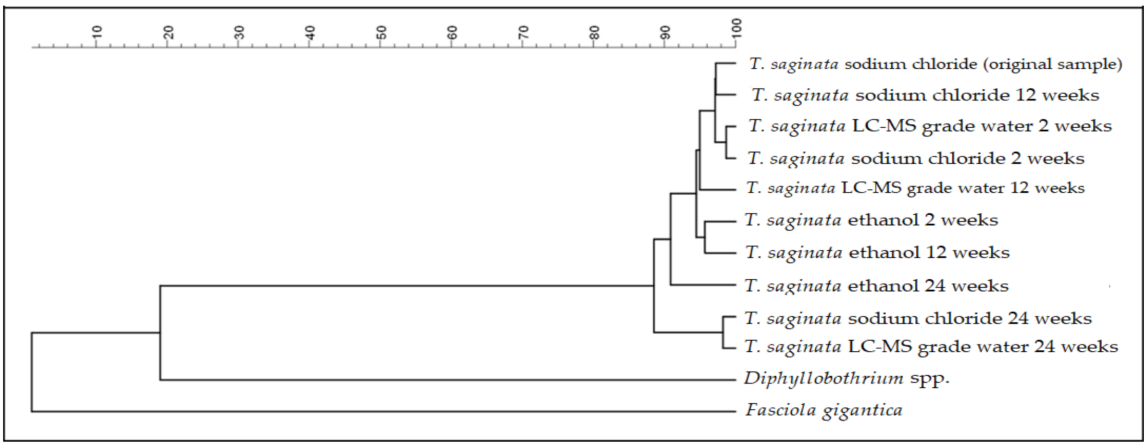

Figure 3. Dendrogram derived from a clustering analysis to assess and compare the different protein spectra of *Taenia saginata* proglottids stored in three storage media for different time periods. The cestode *Diphyllobothrium* spp. and the trematode *Fasciola gigantica* were added as outgroup samples.

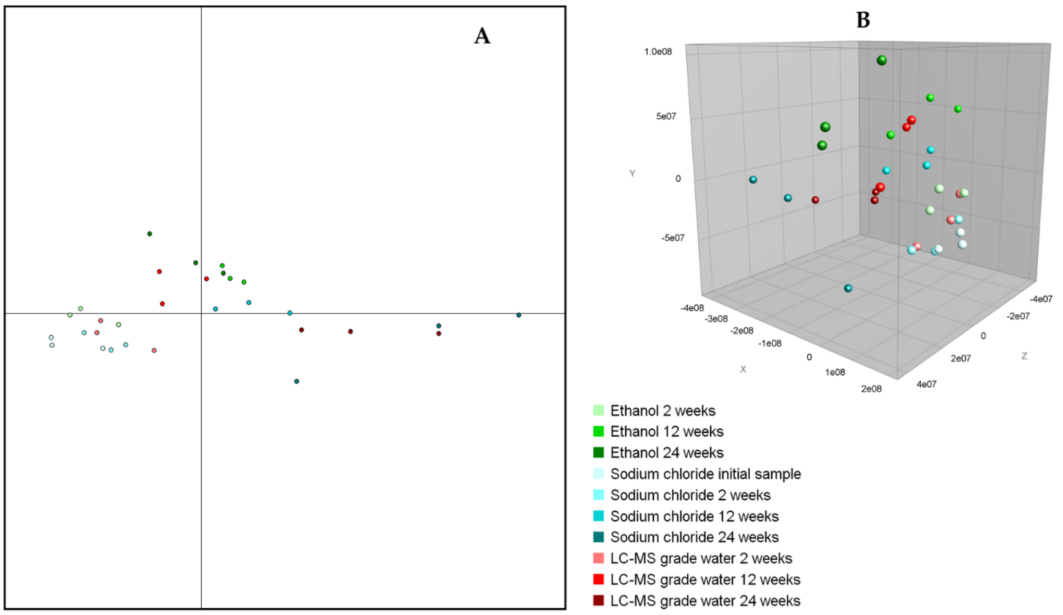

Figure 4. Discriminant analysis and principal components analysis (PCA) of *Taenia saginata* proglottids stored in different preservation media for different time periods. Each storage medium is depicted with a different color. Both statistical analyses indicate that the clusters are highly related and cannot be separated. (**A**) Two-dimensional view of the discriminant analysis. (**B**) Three-dimensional view of the PCA.

4. Discussion

The purpose of this study was to determine whether MALDI-TOF MS can be used as a diagnostic tool for the identification of *T. saginata* proglottids, and whether the use of different storage media may affect the technique's diagnostic accuracy. We found that *T. saginata* can be diagnosed by MALDI-TOF MS, and that its protein spectral analysis allows for reliable differentiation from other helminths, bacteria, and fungi. Indeed, *T. saginata* was consistently identified correctly in $\geq$97% of cases if LC–MS grade water, ethanol, or 0.45% sodium chloride was used as a storage solution, with no changes over time for storage periods of up to 24 weeks. Notably, preservation in 37% formalin did not allow for subsequent MALDI-TOF MS examinations.

Our findings might have important implications for future helminth diagnosis in epidemiologic studies. Indeed, MALDI-TOF MS is a widely used diagnostic tool in microbiologic routine diagnosis [21,22], which will also be increasingly available in laboratories of low- and middle-income countries [23]. Besides the identification of bacteria and fungi, this technique has also been successfully used for the differentiation of ticks and fleas [24], mosquitos [25], lice [26], and more recently, different helminths of medical and veterinary importance [13]. Hence, MALDI-TOF MS could also be employed for confirmatory testing of helminths in reference laboratories, for example, when no unambiguous identification is reached by conventional methods. However, prolonged transport periods of samples from peripheral healthcare centers to such reference laboratories are likely to be expected, and hence, information on the most appropriate sample storage media is key to ensure a reliable analysis by MALDI-TOF MS. In this context, it is important to note that different protocols were utilized in studies conducted thus far, as there is no consensus on the most suitable storage media. For the identification of *Fasciola* spp. [17], cyathostomins [27], and lice [26], 70% (v/v) ethanol was used as a storage solution, while studies on *Anisakis* spp. [19], *Dirofilaria* spp., and *Ascaris* spp. [28] employed a sodium chloride solution, which was sometimes even supplemented with antibiotics to prevent bacterial contamination. Nebbak et al. [24] analyzed the effects of different storage conditions on the identification of arthropods. The authors concluded that the immediate freezing of samples without the

addition of any fixative might be the best approach, closely followed by storage in 70% (*v*/*v*) ethanol at room temperature.

Only a few investigations have assessed the potential effects of different storage conditions on the subsequent MALDI-TOF MS-based identification of helminths. A study focusing on *Trichinella* spp. did not observe significant differences in identification rates when either freezing without any fixative or using 70% ethanol. Indeed, only minor alterations of measured peak intensities were reported, but no change in peak patterns or obtained LSVs [29]. In our study, LC–MS grade water, ethanol, and sodium chloride were equally effective in maintaining a high quality of protein spectra for up to 24 weeks, with correct identification rates ranging from 97.2% for sodium chloride to 99.7% for ethanol at −20 °C. In addition, a statistical analysis of the protein spectra did not reveal fixative-related clusters, thus confirming that all three media can be equally used as storage solutions for *T. saginata* proglottids until MALDI-TOF MS is carried out. Notably, preservation in formalin and subsequent protein extraction using formic acid and acetonitrile impeded any MALDI-TOF-based identification, and hence, should not be employed. This observation is not surprising, as formalin induces considerable molecular cross-linking that may change protein structures [30].

Several limitations restrict the generalizability of our findings. First, the proglottids used in this study were originally stored in sodium chloride for 12 months, before being assigned to the different storage media. Hence, future studies should employ fresh specimens. However, data from a study on suitable buffers for MALDI-based screening of biochemical targets suggest no concerns with regard to the use of sodium chloride [31]. The results obtained in this study may confirm this fact. Second, we only assessed potential effects on *T. saginata*; the in-house database is restricted as it does not contain other *Taenia* species, such as *T. solium*. While it is unlikely that other helminth species would react differently, a broader validation on similar cestodes—most importantly *T. solium*—as well as on nematodes and trematodes is desirable. Specifically, all developmental stages of helminths, including their eggs, should be subjected to MALDI-TOF-based examinations. Third, we compared the effects of different media stored at −20 °C, while future research should also assess the potential effects of storage at different temperatures.

5. Conclusions

We conclude that MALDI-TOF MS is a promising tool for the rapid and accurate identification of *T. saginata* proglottids. Samples can be reliably identified after prolonged storage in LC–MS grade water, sodium chloride solution, and ethanol, while formalin cannot be used as a fixative for later MALDI-TOF MS analysis.

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Data Availability Statement: The data presented in this study are available on reasonable request from the corresponding author.

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References

1. Laranjo-González, M.; Devleesschauwer, B.; Trevisan, C.; Allepuz, A.; Sotiraki, S.; Abraham, A.; Afonso, M.B.; Blocher, J.; Cardoso, L.; Correia da Costa, J.M.; et al. Epidemiology of taeniosis/cysticercosis in Europe, a systematic review: Western Europe. *Parasit. Vectors* **2017**, *10*, 349. [CrossRef] [PubMed]
2. Garcia, H.H.; Gonzalez, A.E.; Gilman, R.H. *Taenia solium* cysticercosis and its impact in neurological disease. *Clin. Microbiol. Rev.* **2020**, *33*, e00085-19. [CrossRef] [PubMed]
3. Krecek, R.C. *WHO/FAO/OIE Guidelines for the Surveillance, Prevention and Control of Taeniosis/Cysticercosis*; Murrell, K.D., Dorny, P., Flisser, A., Geerts, S., Kyvsgaard, N.C., McManus, D.P., Nash, T.E., Pawlowski, Z.S., Eds.; Book Review; WHO/FAO/OIE: Paris, France, 2006; Volume 77, pp. 1–45. [CrossRef]
4. Okello, A.L.; Thomas, L.F. Human taeniasis: Current insights into prevention and management strategies in endemic countries. *Risk Manag. Healthc. Policy* **2017**, *10*, 107–116. [CrossRef]
5. Symeonidou, I.; Arsenopoulos, K.; Tzilves, D.; Soba, B.; Gabriël, S.; Papadopoulos, E. Human taeniasis/cysticercosis: A potentially emerging parasitic disease in Europe. *Ann. Gastroenterol.* **2018**, *31*, 406–412. [CrossRef] [PubMed]
6. Utzinger, J.; Becker, S.L.; Knopp, S.; Blum, J.; Neumayr, A.L.; Keiser, J.; Hatz, C.F. Neglected tropical diseases: Diagnosis, clinical management, treatment and control. *Swiss Med. Wkly.* **2012**, *142*, w13727. [CrossRef] [PubMed]
7. Eichenberger, R.M.; Thomas, L.F.; Gabriël, S.; Bobić, B.; Devleesschauwer, B.; Robertson, L.J.; Saratsis, A.; Torgerson, P.R.; Braae, U.C.; Dermauw, V.; et al. Epidemiology of *Taenia saginata* taeniosis/cysticercosis: A systematic review of the distribution in East, Southeast and South Asia. *Parasit. Vectors* **2020**, *13*, 234. [CrossRef]
8. Saratsis, A.; Sotiraki, S.; Braae, U.C.; Devleesschauwer, B.; Dermauw, V.; Eichenberger, R.M.; Thomas, L.F.; Bobić, B.; Dorny, P.; Gabriël, S.; et al. Epidemiology of *Taenia saginata* taeniosis/cysticercosis: A systematic review of the distribution in the Middle East and North Africa. *Parasit. Vectors* **2019**, *12*, 113. [CrossRef]
9. Braae, U.C.; Thomas, L.F.; Robertson, L.J.; Dermauw, V.; Dorny, P.; Willingham, A.L.; Saratsis, A.; Devleesschauwer, B. Epidemiology of *Taenia saginata* taeniosis/cysticercosis: A systematic review of the distribution in the Americas. *Parasit. Vectors* **2018**, *11*, 518. [CrossRef]
10. Dermauw, V.; Dorny, P.; Braae, U.C.; Devleesschauwer, B.; Robertson, L.J.; Saratsis, A.; Thomas, L.F. Epidemiology of *Taenia saginata* taeniosis/cysticercosis: A systematic review of the distribution in Southern and Eastern Africa. *Parasit. Vectors* **2018**, *11*, 578. [CrossRef]
11. Craig, P.; Ito, A. Intestinal cestodes. *Curr. Opin. Infect. Dis.* **2007**, *30*, 524–532. [CrossRef] [PubMed]
12. GBD 2017 DALYs and HALE Collaborators. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **2018**, *392*, 1859–1922. [CrossRef]
13. Feucherolles, M.; Poppert, S.; Utzinger, J.; Becker, S.L. MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: A systematic review. *Parasit. Vectors* **2019**, *12*, 245. [CrossRef] [PubMed]
14. Nonaka, N.; Nakamura, S.; Inoue, T.; Oku, Y.; Katakura, K.; Matsumoto, J.; Mathis, A.; Chembesofu, M.; Phiri, I.G.K. Coprological survey of alimentary tract parasites in dogs from Zambia and evaluation of a coproantigen assay for canine echinococcosis. *Ann. Trop. Med. Parasitol.* **2011**, *105*, 521–530. [CrossRef] [PubMed]
15. González, L.M.; Montero, E.; Harrison, L.J.S.; Parkhouse, R.M.E.; Garate, T. Differential diagnosis of *Taenia saginata* and *Taenia solium* infection by PCR. *J. Clin. Microbiol.* **2000**, *38*, 737–744. [CrossRef]
16. Croxatto, A.; Prod'hom, G.; Greub, G. Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. *FEMS Microbiol. Rev.* **2012**, *36*, 380–407. [CrossRef]
17. Sy, I.; Margardt, L.; Ngbede, E.O.; Adah, M.I.; Yusuf, S.T.; Keiser, J.; Rehner, J.; Utzinger, J.; Poppert, S.; Becker, S.L. Identification of adult *Fasciola* spp. using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. *Microorganisms* **2021**, *9*, 82. [CrossRef]
18. Karadjian, G.; Bilska-Zając, E.; Bahn, P.; Py, J.-S.; Johne, A.; Gassilloud, B.; Różycki, M.; Cencek, T.; Mayer-Scholl, A.; Vallée, I. Species identification of *Trichinella* originated from various host and different geographical location by MALDI-TOF. *Exp. Parasitol.* **2020**, *213*, 107890. [CrossRef]
19. Marzano, V.; Pane, S.; Foglietta, G.; Mortera, S.L.; Vernocchi, P.; Muda, A.O.; Putignani, L. Mass spectrometry based-proteomic analysis of *Anisakis* spp.: A preliminary study towards a new diagnostic tool. *Genes* **2020**, *11*, 693. [CrossRef]
20. Ng-Nguyen, D.; Stevenson, M.A.; Dorny, P.; Gabriël, S.; Vo, T.V.; Nguyen, V.A.T.; Phan, T.V.; Hii, S.F.; Traub, R.J. Comparison of a new multiplex real-time PCR with the Kato Katz thick smear and copro-antigen ELISA for the detection and differentiation of *Taenia* spp. in human stools. *PLoS Negl. Trop. Dis.* **2017**, *11*, e0005743. [CrossRef]
21. Welker, M. Proteomics for routine identification of microorganisms. *Proteomics* **2011**, *11*, 3143–3153. [CrossRef]
22. Schubert, S.; Kostrzewa, M. MALDI-TOF MS in microbiology laboratory: Current trends. *Curr. Issues Mol. Biol.* **2017**, *23*, 17–20. [CrossRef]
23. Ngoy, S.; Diarra, A.Z.; Laudisoit, A.; Gembu, G.-C.; Verheyen, E.; Mubenga, O.; Mbalitini, S.G.; Baelo, P.; Laroche, M.; Parola, P. Using MALDI-TOF mass spectrometry to identify ticks collected on domestic and wild animals from the Democratic Republic of the Congo. *Exp. Appl. Acarol.* **2021**, *84*, 637–657. [CrossRef] [PubMed]

24. Nebbak, A.; El Hamzaoui, B.; Berenger, J.M.; Bitam, I.; Raoult, D.; Almeras, L.; Parola, P. Comparative analysis of storage conditions and homogenization methods for tick and flea species for identification by MALDI-TOF MS. *Med. Vet. Entomol.* **2017**, *31*, 438–448. [CrossRef]
25. Nebbak, A.; Almeras, L. Identification of *Aedes* mosquitoes by MALDI-TOF MS biotyping using protein signatures from larval and pupal exuviae. *Parasit. Vectors* **2020**, *13*, 161. [CrossRef] [PubMed]
26. Ouarti, B.; Laroche, M.; Righi, S.; Meguini, M.N.; Benakhla, A.; Raoult, D.; Parola, P. Development of MALDI-TOF mass spectrometry for the identification of lice isolated from farm animals. *Parasite* **2020**, *27*, 28. [CrossRef]
27. Bredtmann, C.M.; Krücken, J.; Murugaiyan, J.; Balard, A.; Hofer, H.; Kuzmina, T.A.; von Samson-Himmelstjerna, G. Concurrent proteomic fingerprinting and molecular analysis of cyathostomins. *Proteomics* **2019**, *19*, e1800290. [CrossRef]
28. Nagorny, S.A.; Aleshukina, A.V.; Aleshukina, I.S.; Ermakova, L.A.; Pshenichnaya, N.Y. The application of proteomic methods (MALDI-TOF MS) for studying protein profiles of some nematodes (*Dirofilaria* and *Ascaris*) for differentiating species. *Int. J. Infect. Dis.* **2019**, *82*, 61–65. [CrossRef]
29. Mayer-Scholl, A.; Murugaiyan, J.; Neumann, J.; Bahn, P.; Reckinger, S.; Nöckler, K. Rapid identification of the foodborne pathogen *Trichinella* spp. by matrix-assisted laser desorption/ionization mass spectrometry. *PLoS One* **2016**, *11*, e0152062. [CrossRef] [PubMed]
30. Lu, K.; Ye, W.; Zhou, L.; Collins, L.B.; Chen, X.; Gold, A.; Ball, L.M.; Swenberg, J.A. Structural characterization of formaldehyde-induced cross-links between amino acids and deoxynucleosides and their oligomers. *J. Am. Chem. Soc.* **2010**, *132*, 3388–3399. [CrossRef]
31. Chandler, J.; Haslam, C.; Hardy, N.; Leveridge, M.; Marshall, P. A systematic investigation of the best buffers for use in screening by MALDI-mass spectrometry. *SLAS Discov.* **2017**, *22*, 1262–1269. [CrossRef] [PubMed]

Appendix 2

Scientific outputs

1. Oral presentations

Feucherolles M, Poppert S, Endriss Y, Hanspeter M, Hermosilla C, Lundström-Stadelmann B, Utzinger J, Becker S (2019) MALDI-TOF mass spectrometry in diagnostic helminthology: a proof-of-concept study. **Conference on Tropical Medicine** – Munich (Germany).

Feucherolles M, Cauchie HM and Penny C. (2019) CampyTOF: MALDI-TOF Mass spectrometry enabled subtyping and antimicrobial resistance screening of the food and waterborne pathogens *Campylobacter jejuni* and *Campylobacter coli*. **3rd Luxembourg Microbiology Day** – Strassen (Luxembourg).

Feucherolles M (2020) Application of the MALDI-TOF Mass spectrometry for antimicrobial Resistance screening and typing of *Campylobacter* spp. : an irksome pathogen. **Research in Food Safety** – Online.

Feucherolles M, Nennig M, Martiny D, Becker S, Losch S, Ragimbeau C, Penny C (2021) Use of MALDI-TOF mass spectrometry and Machine Learning for swift detection of antimicrobial resistances: The *Campylobacter* example. Bruker M&D BeLux Virtual Users Meeting 2021 – Online.

Feucherolles M, Nennig M, Martiny D, Becker S, Losch S, Penny C, Ragimbeau C (2021) MALDI-TOF Mass Spectrometry and Machine Learning for detection of resistant and susceptible isolates, the *Campylobacter* example: a proof-of-concept study. **31st European Society of Clinical Microbiology and Infectious Diseases** – Online.

Feucherolles M (2021) MALDI-TOF MS as a One-fits all diagnostic tool for the characterization of *Campylobacter* spp. **ESGMD Webinar** – Online.

2. Posters

Feucherolles M, Cauchie HM and Penny C. (2019) **CampyTOF:** MALDI-TOF Mass spectrometry enabled subtyping and antimicrobial resistance screening of the food and waterborne pathogens *Campylobacter jejuni* and *Campylobacter coli*. **3rd Luxembourg Microbiology Day** – Strassen (Luxembourg).

Feucherolles M (2019) Antimicrobial resistance is the new black. **Luxembourg Institute of Science and Technology PhD Day** - Belvaux (Luxembourg).

Sy I, Wendel T, **Feucherolles M**, Nimmesgern A, Stuermann A, Endriss Y, Utzinger J, Poppert S, Becker S (2020) Application of MALDI-TOF MS for identification of helminths in clinical samples. **30th European Society of Clinical Microbiology and Infectious Diseases** – Paris (France).

Feucherolles M, Nennig M, Martiny D, Becker S, Losch S, Penny C, Ragimbeau C (2021) Investigation of MALDI-TOF mass spectrometry as a tool for assessing the genetic diversity of *Campylobacter jejuni*: A One-Health approach. **31st European Society of Clinical Microbiology and Infectious Diseases** – Online.

3. Published papers:

M Feucherolles, S Poppert, J Utzinger, SL Becker - *Parasites & vectors*, 2019. MALDI-TOF mass spectrometry as a diagnostic tool in human and veterinary helminthology: a systematic review.

M Feucherolles, HM Cauchie, C Penny - *Microorganisms*, 2019. MALDI-TOF mass spectrometry and specific biomarkers: potential new key for swift identification of antimicrobial resistance in foodborne pathogens.

M Feucherolles, M Nennig, SL Becker, D Martiny, S Losch, C Penny, HM Cauchie, C Ragimbeau – *Diagnostics*, 2021. Investigation of MALDI-TOF mass spectrometry for assessing the molecular diversity of *Campylobacter jejuni* and comparison with MLST and cgMLST: a Luxembourg One-Health study.

M Feucherolles, M Nennig, SL Becker, D Martiny, S Losch, C Penny, HM Cauchie, C Ragimbeau – *Frontiers in Microbiology*, 2022. Combination of MALDI-TOF mass spectrometry and Machine Learning for rapid antimicrobial resistances screening: the case of *Campylobacter* spp.

Appendix 3

Achievements

1. Awards

- Luxembourg Society for Microbiology Best PhD presentation at the 3rd Luxembourg Microbiology Day (2019).
- Luxembourg Society for Microbiology Student Conference achievement grant (2020).
- ESCMID 30 under 30 outstanding young scientist (2020).

2. Symposium

- Initiating and organizing the "**What's up with MALDI-TOF mass spectrometry in Microbiology?**" (2021).
- **Assistant guest editor** at the Conference Special Issue "What's up with MALDI-TOF mass spectrometry in Microbiology?" empowered by *Diagnostics* (MDPI) (2021).