REVIEW

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Use of proteomic-based MALDI-TOF mass spectra for identification of bacterial pathogens in aquaculture: a review

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Abstract

The fisheries and aquaculture sectors are growing rapidly, reflecting their importance in meeting the ever-increasing human population's demands for animal protein. Production progress in this sector, however, is challenging as a result of increased deaths from epidemics caused by bacterial infectious diseases in aquaculturally important species. In order to minimize the impact of such diseases, quick and reliable diagnosis of pathogens, timely intervention, and control of the disease are essential to ensure the health of aquaculture and fisheries stocks. Thus, high-throughput proteomics-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used as a rapid, accurate, and species-specific tool for the identification of bacterial fish pathogens and antibiotic resistance. The aim of this article is to review and evaluate the results of nearly three hundred papers published from 2010 to 2023 on the application of MALDI-TOF MS in aquaculture, showing that this method has been increasingly used over the years for the species-level identification and antibiotic resistance of fifty different gram-positive and negative bacterial aquatic pathogens encountered in wild or cultured fish, shellfish, invertebrates, and crustaceans. In addition, the history and general principles of the MALDI-TOF MS method are also mentioned in the article so that fish disease researchers interested in the use of this technology can see all aspects of the topic.

Keywords MALDI-TOF MS · Bacterial fish pathogens

Introduction

Aquaculture, a rapidly growing global industry, suffers tremendous financial losses each year as a result of bacterial, viral, fungal, and parasitic disease outbreaks (Woo and Bruno 2011; Fazio 2019). There are a number of bacterial pathogens that have been detected in

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wild or farmed fish, shellfish, and other aquatic organisms (Alderman 1996; Austin and Austin 2016). It is predicted that bacterial pathogens are responsible for about 35% of the diseases which damage the tissues of juvenile and adult fish, making them more vulnerable to infection (Dar 2022). Infection is particularly prevalent in fish under stress. Stressors include high stock densities, poor nutrition, the accumulation of hazardous toxic substances, contaminated water, sudden temperature changes, and low oxygenation (Stickney 2009; Natnan et al. 2021). Since bacterial infections are a major contributor to fish mortality in aquaculture, mitigating their impact is of great importance to the aquacultural and fisheries industry.

Bacterial pathogens commonly seen in inland and marine fish, invertebrates, and crustaceans are as follows: *Aeromonas* spp. (*A. hydrophila*, *A. salmonicida*, *A. veronii*, *A. caviae*, *A. sobria*), *Edwardsiella* spp. (*E. ictaluri*, *E. tarda*, *E. piscicida*), *Flavobacterium psychrophilum*, *F. columnare*, *Lactococcus garvieae*, *Mycobacterium* spp. (*M. fortuitum*, *M. marinum*), *Pseudomonas* spp. (*P. anguilliseptica*, *P. fluorescens*, *P. plecoglossicida*), *Photobacterium damselae*, *Renibacterium salmoninarum*, *Streptococcus* spp. (*S. iniae*, *S. agalactiae*), *Staphylococcus warneri*, *Vagacoccus salmoninarum*, *Vibrio* spp. (*V. anguillarum*, *V. splendidus*, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. coralliilyticus*), *Yersinia ruckeri*, and *Tenacibaculum maritinum* (Toranzo 2004; Noga 2010; Austin and Austin 2016; Vanamala 2022). It is crucial to monitor aquatic animal diseases through surveillance and rapid bacterial detection so that the appropriate interventions can be undertaken so that the diseases are diagnosed and managed before they present a serious threat (Adams and Thompson 2011; Crumlish 2017; Ashfaq et al. 2022) to both animal welfare and national economies that rely on marine and inland aquaculture. Accurate and rapid diagnostic techniques are, therefore, crucial.

Traditionally, bacterial fish diseases and aquatic pathogens have been identified using conventional microbiological, immunological, and molecular biological approaches. Microbiological methods are comparatively slow and require bacterial isolation and culturing and subsequent morphological observation and biochemical characterization of potential pathogens (Buller 2014; Smith 2019; Austin 2019; Duman et al. 2022). Bacterial isolates can be differentiated and classified either manually or using a number of kits and systems for biotyping based on the biochemical properties of bacteria, including the API (Biomerieux, USA), Vitek (Biomerieux, France), Biolog (Biolog, USA), and BD BBL (Becton Dickinson Microbiology Systems, USA) (Santos et al. 1993; Taylor et al. 1995; Popović et al. 2007). Serological and histopathological assays are also utilized in the diagnosis of bacterial fish diseases (Popovic et al. 2007; Timur et al. 2009; Austin 2019). Enzyme-linked immunosorbent assays (ELISA) have also been prominent in diagnosing bacterial fish diseases. ELISA detects specific antibodies or antigens associated with pathogens in fish samples, allowing for early disease identification and continuous monitoring of infection levels within fish populations (Tanrikul 2007; González and Santos 2009; Duman et al. 2022). In the late 1990s and early 2000s, molecular biological methods began to be used to identify bacterial fish diseases in aquaculture (Zlotkin et al. 1998a; Hassan et al. 2001; Altinok and Kurt 2003; González et al. 2004; Ruiz-Zarzuela et al. 2005; Altinok et al. 2008; Jung et al. 2010; Kayış et al. 2015). The most common and frequently used basic molecular techniques are standard polymerase chain reaction (PCR), PCR-based genotyping, and Sanger sequencing (SS). The basis of these techniques is the sensitive amplification of bacterial DNA, the identification of target genetic markers and their sequences as a tool for the identification of aquatic bacterial pathogens (del Cerro et al. 2002; Cunningham 2002; Rhodes et al. 2004; Beaz-Hidalgo et al. 2008; Onuk et al. 2010; Buller 2014; Kim et al. 2017; Çağatay 2022). The use of next-generation sequencing (NGS)

technique based on targeted amplicon or whole-genome sequencing (Hambuch and May-field 2014; Lefterova et al. 2015) has started a new era in aquaculture disease diagnosis since this basic PCR-based simple sequencing technique is not sensitive enough to distinguish some closely related aquatic bacterial species (Kumar and Kocour 2017; De Bruijn et al. 2018; Natnan et al. 2021; Jaies et al. 2024; Bohara et al. 2024). Although it is very beneficial to perform species-specific sequencing using an NSG device, the high cost and the time requirement are disadvantages for the users. In this regard, one of the alternative high throughput methods, the fast, highly sensitive, specific, and less demanding matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) method has become the new focus.

MALDI-TOF MS involves analyzing the protein profiles of bacterial strains, enabling rapid and definitive identification (Seng et al. 2009; Stevenson et al. 2017). This new and powerful proteomic approach can be used as a complement and validator of other techniques for microbial identification and allowing early recognition of bacterial populations in water and effective animal health management. Beyond microbial identification, it is also highly useful for characterizing pathogen life cycles and virulence components and investigating antibiotic resistance (Lauková et al. 2018; Moreira et al. 2021; Duman et al. 2022).

In this review article, unlike the articles in the literature on the application of the MALDI-TOF MS method in the diagnosis of bacterial diseases in aquaculture and fisheries, the findings of approximately three hundred studies from 2010 to 2023 are examined in detail. In addition, the general application areas and history of the method are briefly mentioned for the readers to better understand the topic. The results of incremental studies on the species identification of fifty different gram-negative and gram-positive aquatic bacterial pathogens using MALDI-TOF MS methods are presented and evaluated. This review also aims to assess the advantages, disadvantages, and potential future directions of the method in aquaculture.

History and applications of MALDI-TOF MS

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) was initially developed by Karas et al. (1985; 1987). Around the same period, Tanaka et al. (1988) reported a "soft desorption ionization" technique that allowed for the mass spectrometric study of biological macromolecules by integrating mass analyzer time of flight (TOF) with MALDI. MALDI was named after Karas and Hillenkamp's (1988) research on soft desorption ionization utilizing an organic compound matrix. Koichi Tanaka won the Chemistry Nobel Prize in 2002, and since then, MALDI-TOF MS has advanced significantly in proteomics and metabolomics research, allowing analysis of many different metabolites in biomedical research (Tanaka 2003). The American Food and Drug Administration (FDA) has approved several MALDI systems for commercial use in clinical microbiology laboratories globally, including the Clin-TOF (Bioyong Technology, China), the MassARRAY System (Agena Bioscience, USA), the VitekMS (bioMérieux Clinical Diagnostics, France), and the MALDI Biotyper (Bruker, Germany). These systems offer special combinations of databases, software, and mass spectrometers (Li et al. 2022). In addition, inclusive mass spectrum libraries are available for MALDI-TOF MS analysis (Table 1), which offer public access to mass spectra of numerous microorganisms (Böhme et al. 2012; Wieser et al. 2012; Torres-Sangiao et al. 2021; Zuffa et al. 2023), and users can select these libraries

Table 1 MALDI-TOF MS databases and libraries globally

No	Mass database/libraries	Description	Web address of database
1	Bruker-BioTyper®RUO/GP	4274 species of 704 microorganism genera	https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/
2	• RUO-Fungi library	Mass spectrum for 222 spp.	https://masst.gnps2.org/microbemasst/
	• Mycobacteria	Mass spectrum for 201 spp.	
	MicrobeMASST, USA	3262 Mass spectra of Bacteria, Archaea and Eukaryota	
3	SpectraBank, Spain, EU	Spectra and peak mass lists 200 bacterial spp.	http://www.spectrabank.org
4	SPECLUST, Sweden, EU	Generate clusters of mass spectra from similar proteins	http://bioinfo.thep.lu.se/speclust.html
5	Saramis™, Germany	Spectral Archive and Microbial Identification System	https://www.mass-spec-capital.com/product/saramis-database-maldi-tof-biomerieux-group-merieux-biological-2001-20066.html
6	VITEK® MS PLUS, UK	Bacteria, fungi, mycobacteria, *Nocardia*	https://www.biomerieux.co.uk/product/vitekr-ms
7	UniProt, UK	Proteins of 42 genera/1276 microbial spp.	http://www.uniprot.org/
8	Github, USA	83,452 protein data	https://github.com/dipcarbon/BacteriaMSLF
9	FoodBIMS, Italy	Mass spectra of bacteria from 11 genera	http://bioinformatica.isa.cnr.it/Descr_Bact_Dbase.htm
10	URMS Database, France	Mass spectra 90 microbial spp.	https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/urms-data-base/
11	EMbaRC Spectra, EU	Mass spectra of 204 microbial spp.	http://www.embarc.eu/
12	Folkhälsomyndigheten, Sweden	Mass spectra of 296 microorganisms	https://www.folkhalsomyndigheten.se/the-public-health-agency-of-sweden/microbiology-and-diagnostics/

according to their available current system. Studies have shown that one or more libraries can be used depending on the type of microorganism being investigated.

Among the many benefits of MALDI-TOF MS, which include ease of use, high throughput, and resistance to various contaminants, the equipment is extensively utilized in regular analysis and research in clinical microbiology laboratories throughout the world, making it a versatile investment. Uses of MALDI-TOF (Fig. 1) include microbial pathogen identification (Seng et al. 2010; Croxatto et al. 2012; De Marco and Burnham 2014; Cañas et al. 2015; Erler et al. 2015; Welker et al. 2019; Anwer et al. 2022), toxic substances and allergen detection, the diagnosis of human bloodstream infection, cancer diagnosis (Royo-Cebrecos et al. 2017), determination of antibiotic resistance (Wieser et al. 2012; Singhal et al. 2015; Patel 2015; Anwer et al. 2022; Özcan 2023), genetic disorders screening (Huang et al. 2016; Li et al. 2022), pharmaceutical analysis, and drug discovery (Koh et al. 2003; Kafka et al. 2011).

Although primarily developed for clinical purposes, MALDI-TOF MS technology has been applied to many different fields to identify a broad range of microorganisms (including viruses, bacteria, fungus, and yeast), parasites, and protozoa. It has also proven to be an effective tool for rapidly and precisely identifying aerobic, anaerobic, and fastidious (low growth rate or non-cultivatable) microorganisms (Seng et al. 2009; Biswas and Rolain 2013; Singhal et al. 2015; Patel 2015; Alcala et al. 2021; Popović et al. 2022). In fact, the Google Scholar search engine retrieved 18,500 research papers published between 2003 and 2023, based on the question "Other than clinical use, what are the applications of
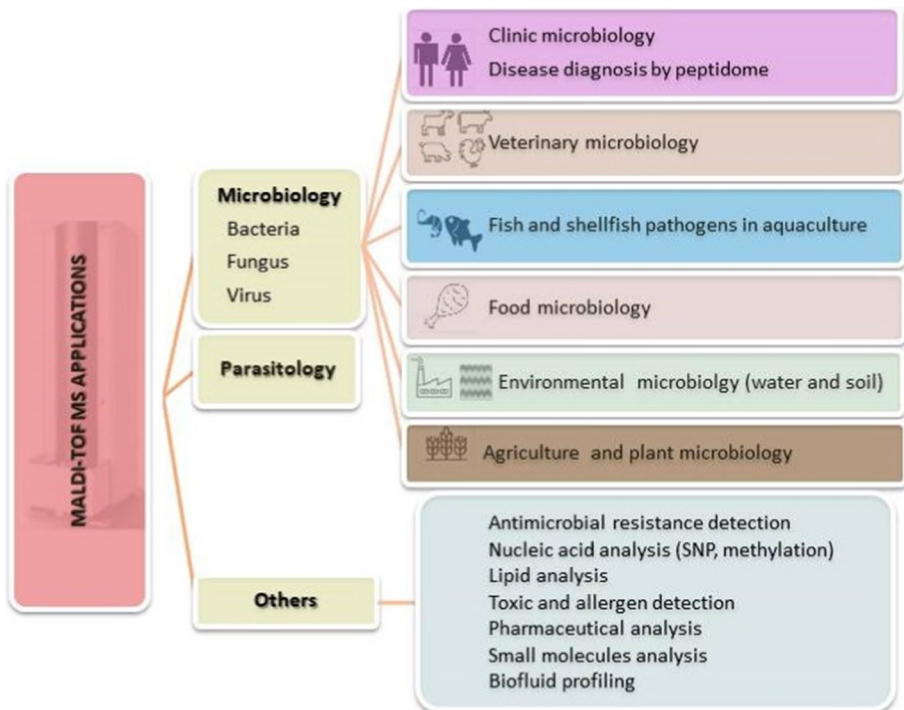

Fig. 1 Illustration of MALDI-TOF MS applications in different fields

MALDI-TOF mass spectrometry?" Applications of MALDI-TOF MS included the following: environmental microbiology for the identification of soil and waterborne pathogens and aquatic microorganisms (Singhal et al. 2015; Popović et al. 2017; Ashfaq et al. 2022; Freitas et al. 2022), veterinary microbiology to identify zoonotic pathogens (Thompson 2022; Popović et al. 2021; Arafa et al. 2023), food microbiology for the identification of foodborne pathogens (Böhme et al. 2013; Akimowicz et al. 2020; Brauge et al. 2021; Nissa et al. 2021; Haider et al. 2023), agricultural microbiology (Gandhi et al. 2013; Drissner et al. 2017; Tamaru 2023; Surányi et al. 2023), identification of plant pathogens (Wang et al. 2012; Ahmad et al. 2012; Chalupová et al. 2014; Chun et al. 2022; Sivanesan et al. 2023), and most recently in aquatic microbiology for identification of fish pathogens (Singhal et al. 2015; Assis et al. 2017; López-Cortés et al. 2017; Jansson et al. 2020; Piamsomboon et al. 2020; Moreira et al. 2021; Duman et al. 2022; Saticioglu et al. 2023).

General principles and methodology of MALDI-TOF MS

MALDI-TOF MS works by ionizing and measuring the mass-to-charge ratios (m/z) of ribosomal proteins in a microbial sample resulting in a mass spectrum that is a unique fingerprint (Singhal et al. 2015; Piamsomboon et al. 2020; Popović et al. 2022) which can be used to identify the microorganism by matching to existing bio-markers (López-Cortés et al. 2017; Duman et al. 2022). The microbial peptide mass fingerprints that are obtained are compared to the mass spectral library database of pre-existing reference samples (Brauge et al. 2021).

The MALDI-TOF MS process for microbial identification from different sample types can be summarized in three steps (Fig. 2). The first step is sample preparation from either a direct sample or culture, the second step is the analysis of the samples in the MALDI-TOF instrument, and the third step is the identification process (Patel 2015; Singhal et al. 2015; Sandalakis et al. 2017; Kazazić et al. 2019a; Kazazić et al. 2019b; Mishra et al. 2020; Dare 2006). In step one, a single colony from a cultured sample is spread onto the target plate, which can be either "direct sample spotting," "on-target extraction," or "full extraction" (Popović et al. 2021). One of these three methods is chosen according to the strains of microorganism, the cell wall structure, spore form, and biosafety requirements. It has been shown that the full extraction method has a higher efficacy rate in the identification of difficult bacteria, while in gram-negative bacteria, it is suitable for direct sample spotting and on-target extraction methods (Popović et al. 2021). The sample target plate is then coated with a matrix solution (such as 1 μL of 70% formic acid solution in 50% acetonitrile or 2.5% trifluoroacetic acid, or cyano 4-hydroxycinnamic acid-CHCA) and allowed to dry at room temperature. As the matrix dries, it crystallizes together with the sample, which is then analyzed in the MALDI-TOF instrument (Kazazić et al. 2019a; Kazazić et al. 2019b). The matrix plate is inserted into the instrument and exposed to a laser beam operating at a frequency of 60 Hz in auto-shot, which causes desorption and ionization. The matrix absorbs most of the energy and the microbial peptides are converted into protonated ions which are accelerated by an electrical field towards a detector with the time required to move under vacuum across the flight tube and measured according to the mass to charge ratio (m/z). Smaller analytes arrive at the detector first, followed by increasingly larger analytes. Information on TOF peaks is recorded by the instrument and used to generate a characteristic spectrum, called "a peptide mass fingerprint," allowing for the identification of the molecules present in the samples based on the unique mass fingerprints. The
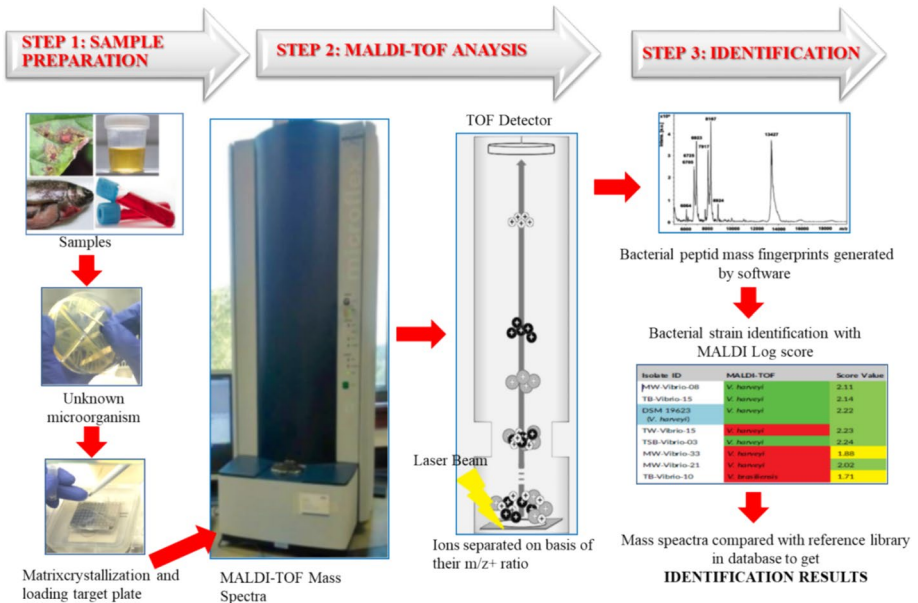
Fig. 2 Schematic steps for MALDI-TOF MS bacterial identification, STEP 1. Sample preparation, STEP 2. MALDI-TOF MS analysis in instrument, STEP 3. Identification of bacteria using the database

distinctive calibrated spectrum acquired for each species/strain is recorded and compared with the reference spectrum database. The mass spectra of the recorded proteins, peptides, and ribosomal peptides of the sample can range from 2000 to 21,000 Da depending on the type of microorganism. The generated peak lists are used directly for matches to the reference library using the software's integrated pattern-matching algorithm. The results of the pattern-matching process are expressed in scores ranging from 0 to 3.00 on a logarithmic scale as recommended by the manufacturer. Scores below 1.70 are considered unreliable identification, while a score ≥ 1.70 is considered a genus identification, and a score ≥ 2.00 indicates species identification (De Marco and Burnham 2014; Puk et al. 2018; Surányi et al. 2023).

Identification of bacterial fish pathogens with MALDI-TOF MS

In terms of aquatic health and fisheries management, MALDI-TOF MS serves as a state-of-the-art diagnostic tool for rapid and accurate identification of pathogens affecting aquatic organisms. The proteomics-based MALDI-TOF MS method described in the "General principles and methodology of MALDI-TOF MS" section has two different applications in the diagnosis of fish pathogens. One is the direct identification of the whole-cell bacterial pathogen (Jansson et al. 2020; Piamsomboon et al. 2020; Popović et al. 2022, and the other is the identification of antibiotic resistance biomarkers (for example to beta-lactam, carbapenem, methicillin, and vancomycin) in bacterial pathogens, enabling the instant detection of resistant isolates in real time during the standard routine identification process (Zhu et al. 2002; Fernández-Álvarez et al. 2017; Sandalakis et al. 2017; Popović et al. 2017;

Cordovana et al. 2019; Florio et al. 2020). This contributes to early disease detection and helps to develop timely and effective intervention strategies, promoting sustainable practices in aquaculture and healthier fish populations.

Studies have demonstrated that the MALDI-TOF MS method can identify a single bacterial disease agent or co-infectors which are of significance in marine and inland aquaculture fish species (Table 2) (Nissa et al. 2021; Natnan et al. 2021; Moreira et al. 2021; Parker-Graham et al. 2023). Many of the fish species listed in Table 2 are susceptible to disease, and some have high mortality rates resulting in substantial economic losses (Noga 2010; Austin and Austin 2016; Dar et al. 2022). Several bacterial species in the families of Aeromonadaceae and Pseudomonadaceae (Anagnostopoulos et al. 2022), Mycobacteriaceae (Ziarati et al. 2022), Streptococcaceae (Cañas et al. 2015; Kalimuddin et al. 2017), Vibrionaceae (Burbick et al. 2018), and Enterobacteriaceae (Boylan 2011; Zakrzewski et al. 2022) not only affect aquaculture production but they are also zoonoses transmitted between animal species to humans and are therefore also human public health concerns. The following sections review research publications from 2010 to 2023 retrieved using "MALDI-TOF MS" and "bacterial agents" as keywords or phrases in the Google Scholar search engine (Fig. 3). The bar graph in Fig. 3 shows the number of research studies that utilized MALDI-TOF MS for the identification of bacterial agents of aquatic diseases during this period has been gradually increasing. Table 2 summarizes the applications of MALDI-TOF MS reported in the academic literature to identify 50 major aquatic pathogens (37 g-negative and 13 g-positive) that cause common bacterial diseases in egg, larvae, juvenile, adult fish, shellfish, shrimp, and other aquatic organisms. In these publications, MALDI-TOF was either used alone or in combination with other methodologies in the identification of aquaculture pathogens. The following section summarizes the use of MALDI-TOF MS for the use of identification of both gram-negative and gram-positive fish pathogens.

Identification of gram-negative fish pathogens with MALDI-TOF MS

Aeromonadaceae

Aeromonas spp. are gram-negative, facultatively anaerobic, rod-shaped, non-spore-forming bacteria that morphologically resemble members of the family Aeromonadaceae. They are major infectious agents that cause significant financial losses and high mortality rates in many marine and freshwater fish, especially salmonids under stress (Doukas et al. 1998; Janda and Abbott 2010; Buller 2014; Austin 2017; Pekala-Safińska 2018). Well-known non-motile *A. salmonicida* causes furunculosis characterized by ulcers on fish skin (Balcazar et al. 2007; Beaz-Hidalgo et al. 2008), while motile aeromonads such as *A. hydrophila*, *A. bestiarum*, *A. veronii*, *A. media*, *A. caviae*, and *A. sobria* are the causative agents of motile aeromonas septicaemia (MAS) which causes abdominal edema, dermatitis, deep ulcers on skin and fins, exophthalmos, and septicemia in fish (Abreu et al. 2018; Beaz-Hidalgo et al. 2013). A number of studies have reported the identification of specific *Aeromonas* spp. from *Salmo trutta*, *S. gairdnerii* (Lauková et al. 2018; Jung-Schroers et al. 2019), *Oncorhynchus mykiss* (Popović et al. 2022; Tütmez et al. 2023), *Acipenser baerii* (Vázquez-Fernández et al. 2023), ornamental fish (Cardoso et al. 2021), *Danio rerio*, *Carassius auratus* (Walczak et al. 2017), and as a water-borne pathogens (Donohue et al. 2006) (Table 2). However, Pérez-Sancho et al. (2018) have demonstrated that the accuracy

Table 2 A list of fish pathogen bacteria identified with MALDI-TOF MS and their hosts and findings

Bacterial agents and diseases	Hosts/sources		Findings from MALDI-TOF MS	References
			Analysis/peaks (Da)/scores	
Gram-negative pathogens				
Aeromonadaceae				
Aeromonas spp. (motile aeromonas, hemorrhagic septicemia)	Salmo trutta (Brown trout), S. gairdnerii (Redband trout)	Antibiotic resistance profiles		Lauková et al. (2018)
	Oncorhynchus mykiss (Rainbow trout)	High score match		Tütmez et al. (2023)
	Waterborne pathogen	6301 to 13,450 Da		Donohue et al. (2006)
A. hydrophila	Ornamental fish	Score 1.700 to 2.000		Cardoso et al. (2021)
	Rapana venosa (Rapa whelk)	Antibiotic resistance profiles		Ozbey et al. (2023)
	Fresh water fish	Scores 2.018 to 2.333		Popović e. al. (2022)
	Oreochromis niloticus (Nile tilapia)	Scores 2.978 to 2.115		Piamsomboon et al. (2020)
	Danio rerio (Zebrafish), Carassius auratus (Goldfish)	Scores 1.700 to 1.900		Walczak et al. (2017)
A. veronii	O. mykiss	Antibiotic resistance profiles		Özcan (2022)
	Lates calcarifer (Asian seabass)	Scores 1.906 to 2.511		Piamsomboon et al. (2020)
A. media	O. mykiss	Antibiotic resistance profiles		Özcan (2023)
A. bestiarum	O. mykiss, S. trutta, Salvelinus alpinus (Arctic char)	Scores 1.917 to 2.214		Jansson et al. (2020)
A. salmonicida (furunculosis)	Acipenser baerii (Siberian sturgeon)	Scores > 2.000		Vázquez-Fernández et al. (2023)
	O. mykiss, S. trutta, S. alpinus	Scores 2.145 to 2.302		Jansson et al. (2020)
	S. trutta	Scores 1.750 to 2.950		Lauková et al. (2018)
	Salmonids	Scores 2.150 to 2.380 Antibiotic resistance profiles		Jung-Schroers et al. (2019) Jung-Schroers et al. (2019)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS — Analysis/peaks (Da)/scores	References
Gram-negative pathogens			
Flavobacteriaceae			
Flavobacterium spp.	*Perca fluviatilis* (Perch)	Identification with high score	Rupp et al. (2019)
F. columnare (columnaris disease)	*O. mykiss*	Scores 1.980 to 2.060	Fernández-Álvarez et al. (2018)
	O. mykiss	Scores 2.327 to 2.347	LaFrentz et al. (2022)
	Ictalurus punctatus (Channel catfish)	First protein expression analysis	Dumpala et al. (2010)
	C. carpio koi	Scores 2.30 to 2.37	Neidorf and Morozova (2021)
	Ctenopharyngodon idella (Grass carp)	Chondroitin lyases	Li et al. (2015)
	S. trutta, Cyprinus carpio (Common carp)	Scores 2.130 to 2.410	Jansson et al. (2020)
F. psychrophilum (bacterial cold water disease, fry syndrome)	*O. mykiss*	Scores 1.990 to 2.070	Fernández-Álvarez et al. (2018)
	O. mykiss, O. kisutch (Coho salmon)	Scores 2.270 to 2.500	Jansson et al. (2020)
	A. baerii	Score 2.030	Chinchilla et al. (2023)
	O. mykiss	Scores 2.494 to 2.644	Pérez-Sancho et al. (2017b)
F. bernardetii sp. nov	*O. mykiss*	Antimicrobial Resistance Genes	Saticioglu et al. (2021a)
F. kayseriense and *F. turcicum*	*O. mykiss*	Fatty acid composition	Saticioglu et al. (2021b)
F. collinsii	*Plecoglossus altivelis* (Ayu)	Proteins	Kondo et al. (2023)
Chryseobacterium spp. (opportunistic gills, kidney disease)	*O. mykiss*	Scores 2.800 to 2.940	Pérez-Sancho et al. (2017a)
	Fresh water fish	Scores 1.760 to 2.058	Popović et al. (2022)
Tenacibaculum spp. (tenacibaculosis)	Marine fish	2273 to 13,660 Da	Fernández-Álvarez et al. (2017)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS Analysis/peaks (Da)/scores	References
Gram-negative pathogens			
T. maritimum	Marine fish	6600 to 13,200 Da Scores 2.048 to 2.258	Bridel et al. (2020) Fernández-Álvarez et al. (2017)
Vibrionaceae			
Vibrio spp. (vibriosis)	Molluscs, human, environmental	4200 to 6500 Da	Dieckmann et al. (2010)
	Environmental samples	Scores 2.542 to 2.644	Erler et al. (2015)
	Sea water, shellfish, sediment	Scores 1.720 to 2.410	Cho et al. (2017)
	Hippocampus abdominalis, Seriola lalandi, Atractoscion nobilis, Pterapogon kauderni, Paralichthys californicus	Scores 1.700 to 3.010	Burbick et al. (2018)
	Litopenaeus vannamei	Scores 1.600 to 2.440	Bauer et al. (2018)
	Seawater, plankton, marine animals and plants	4276 to 9466 Da	Wu et al. (2020)
	Marine sources (corals, sponge, fish and seawater)	2590 to 9457 Da	Vidal et al. (2020)
	Clinical and environmental samples	Scores 1.700 to 2.590	Liu et al. (2022)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS Analysis/peaks (Da)/scores	References
Gram-negative pathogens			
V. vulnificus	Fish	Scores 2.218 to 2.418	Jansson et al. (2020)
	Anguilliformes spp.	Score 2.000	Boonstra et al. (2023)
	Moribund fish	Scores 1.755 to 2.233	Janampa-Sarmiento et al. (2024)
	Freshwater and ornamental fish	Score 1.921	Pastuszka et al. (2024)
	Sea water	Score 1.750	Haider et al. (2023)
V. splendidus (luminescent vibriosis)	Marine fish	Scores 1.780 to 2.030	Jansson et al. (2020)
V. anguillarum	Marine fish	Scores 2.123 to 2.318	Jansson et al. (2020)
	Dicentrarchus labrax	Score 2.500	Mougin et al. (2021)
	D. labrax, S. aurata	Score 2.232	Kazazić et al. (2019b)
V. alginolyticus	*Epinephelus fuscoguttatus*	910 to 2000 Da	Low et al. (2014)
	Perna Perna mussel	Score 2.000	Bronzato et al. (2018)
	Sea water	Score 2.040	Ashfaq et al. (2019)
	Coastal waters	Scores 2.000 to 2.400	Wang et al. (2024)
V. harveyi (luminescent vibriosis)	*D. labrax*	Score 2.480	Mougin et al. (2021)
	Shrimp	Scores 1.780 to 2.100	Culot et al. (2021)
	Sarpa salpa	Score 2.248	Yavuzcan et al. (2022)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS Analysis/peaks (Da)/scores	References
Gram-negative pathogens			
V. parahaemolyticus	shellfish, sea water and sediments	3000 to 11,000 Da	Malainine et al. (2013)
	D. labrax, Haliotis tuberculata, Crassostrea gigas	Scores 2.290 to 2.400	Mougin et al. (2020)
	Surface and bottom waters from ports	Scores 1.912 to 2.211	Khandeparker et al. (2023)
	Seafood samples	Score ≥ 2.200	Fasulkova et al. (2023)
V. tapetis (brown ring disease)	*Ruditapes philippinarum*	2137 to 7133 Da	Rahmani et al. (2021)
Photobacterium damselae (photobacteriosis)	Marine mollusks	Score 1.700	Moussa et al. (2021)
	S. aurata, O. mykiss	Scores 2.114 to 2.309	Perez-Sancho et al. (2016)
	D. labrax, S. aurata×M. saxatilis	Scores 1.900 to 2.080	Kazazić et al. (2019a)
	Sarpa salpa (Salema)	Scores 2.009 to 2.250	Yavuzcan et al. (2022)
	Colossoma macropomum (Tambaqui)	Scores 2.100 to 2.600	Reis et al. (2023)
Piscirickettsiaceae			
Piscirickettsia salmonis (piscirickettsiosis)	Salmon	5300 to 13,427 Da	Olate et al. (2016)
	S. salar, O. kisutch	5380 to 8012 Da	López-Cortés et al. (2017)
	S. salar	Enabled design probe	Zrnčić et al. (2021)
Gram-positive pathogens			
Renibacterium salmoninarum (bacterial kidney disease)	*S. alpinus, O. tshawytsch* (Chinook salmon), *O. mykiss*	Scores 2.036 to 2.400	Jansson et al. (2020)
	S. salar	Not correctly identified	Jia et al. (2023)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS Analysis/peaks (Da)/scores	References
Gram-negative pathogens			
Streptococcaceae			
Lactococcus garvieae (Lactococcosis)	*O. niloticus*	Scores 2.080 to 2.218	Assis et al. (2017)
	O. mykiss	3939 to 10,374 Da; scores 2.190 to 2.260	Torres-Corral and Santos (2021;2022)
	O. mykiss	Scores not given	Radosavljevic et al. (2020)
	O. mykiss	Scores 2.350	Sequeiros et al. (2015)
	O. mykiss	3500 to 10,457 Da	Saticioglu et al. (2023)
L. petauri	*O. mykiss*	2700 to 10,861 Da	Saticioglu et al. (2023)
Streptococcus iniae (Streptococcosis)	*O. niloticus*	Scores 1.480 to 1.850	Assis et al. (2017)
	Scophthalmus maximus (Turbot)	3419 to 10,887 Da	Torres-Corral et al. (2019)
	Paralichthys olivaceus (Olive flounder)	3137 to 10,139 Da	Kim et al. (2017)
	Acipenser transmontanus (White sturgeon)	Scores 1.720 to 1.970	Pierezan et al. (2020)
S. agalactiae	*O. niloticus, O. aureus* (Blue tilapia), *O. mossambicus* (Mozambique tilapia)	Scores 2.545 to 2.019	Piamsomboon et al. (2020)
S. parauberis	*O. mykiss*	2310 to 13,236 Da	Torres-Corral et al. (2019)
	P. olivaceus	2098 to 7935 Da	Kim et al. (2015)
S. dysgalactiae	*Leiarius marmoratus x Pseudoplatystoma corruscans* (Amazon catfish)	Scores not given	Tavares et al. (2018)

Table 2 (continued)

Bacterial agents and diseases	Hosts/sources	Findings from MALDI-TOF MS	References
		Analysis/peaks (Da)/scores	
Gram-negative pathogens			
Enterococcaceae			
Vagococcus salmoninarum (vagococcosis)	*O. mykiss*	4521 to 10,579 Da	Torres-Corral and Santos (2019)
Mycobacteriaceae			
Mycobacterium marinum (mycobacteriosis)	Marine fish	Scores 1.820 to 2.300	Kurokawa et al. (2013)
	Trichogaster lalius (Dwarf Gourami)	Score 1.737	Puk et al. (2018)
M. avium	Marine fish	Score 1.992	Kurokawa et al. (2013)
M. peregrinum	*Labidochromis caeruleus* (Yellow Lab Cichlid)	Score 2.096	Puk et al. (2018)
M. absces	*Mikrogeophagus ramirezi* (Ram cichlid)	Score 2.072	Puk et al. (2018)
M. fortuit	*Paracheirodon innesi* (Neon tetra)	Score 2.105	Puk et al. (2018)
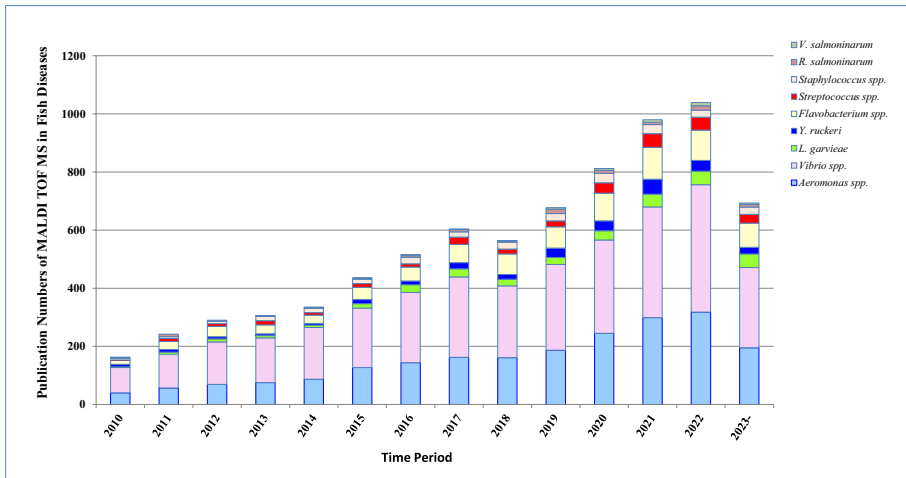
Fig. 3 The number of publications over 13 years found using the keywords, MALDI-TOF MS, bacterial fish diseases, and aquatic pathogens. The bar indicates the number of Web of Science searches associated with major bacterial pathogens in aquaculture

of the genus-level identification of *Aeromonas* using MALDI-TOF MS is inconsistent when it comes to correctly identifying some of the most prevalent species linked to fish infections. In addition to bacterial identification studies, antibiotic resistance profiles of *A. salmonicida* (Jung-Schroers et al. 2019), *A. hydrophila* (Ozbey et al. 2023), *A. media* (Özcan 2023), *A. bestiarum* (Jansson et al. 2020), and *A. veronii* (Özcan 2022) also been determined with MALDI-TOF MS (Table 2).

Flavobacteriaceae

Numerous bacterial species (*Flavobacterium* spp., *Tenacibaculum* spp., and *Chryseobacterium* spp.) belonging to the Flavobacteriaceae family cause flavobacterial diseases in fish, which have devastating impacts on fish stocks in both the wild and farms around the world. Species from flavobacterium cause worldwide economic losses in the aquaculture and fisheries due to infections with high mortality in young fish and morbidity in adult fish (Austin 2017; Crumlish 2017; Wahli and Madsen 2018). The yellow-pigmented, gram-negative *F. columnare* is the causative agent of one of the oldest known fish diseases, Columnaris, and has been identified by MALDI-TOF MS along with *F. psychrophilum* the causative agent of bacterial cold water disease (BCWD) and rainbow trout fry syndrome (RTFS) (Dumpala et al. 2010; Li et al. 2015; Pérez-Sancho et al. 2017b; Fernández-Álvarez et al. 2018; Jansson et al. 2020; Neidorf and Morozova 2021; LaFrentz et al. 2022; Chinchilla et al. 2023) (Table 2). In addition, the antimicrobial resistance profiles in *F. bernardetii* sp. nov. (Saticioglu et al. 2021a), fatty acid composition profiles of *F. kayseriense* and *F. turcium* (Saticioglu et al. 2021b), and bacterial protein profiles of *F. collinsii* (Kondo et al. 2023) have been identified by using the MALDI-TOF MS method (Table 2). In the studies summarized in Table 2, it was evident that the different isolates could be accurately identified by examining the mass score of bacterial protein or mass spectral profiles of other biomarkers from MALDI-TOF MS and confirmed to be *Flavobacterium* spp. by distinguishing them from other species.

The second genus of bacteria in the family Flavobacteriaceae identified with the MALDI-TOF MS is *Tenacibaculum maritimum* (Bridel et al. 2020; Fernández-Álvarez et al. 2017). This is a strictly aerobic, gram-negative, rod, and filamentous bacteria with pale yellow flat, thin colonies. It has been identified as the etiological agent of Tenacibaculosis in farmed and wild marine fish species (Toranzo et al. 2005; Avendaño-Herrera et al. 2006; Piñeiro-Vidal et al. 2007). The pathology of tenacibaculosis has been associated with characteristic gross lesions on the body surface of fish such as ulcers, necrosis, eroded mouth, frayed fins, tail rot, and sometimes necrosis on the gills and eyes (Austin and Austin 2016; Le Breton 2020; Mabrok et al. 2023). Bridel et al. (2020) evaluated the variability among *T. maritimum* isolates based on genome sequence data along with the mass spectral peak characteristics and values of several ribosomal proteins detected by MALDI-TOF MS. In another study by Fernández-Álvarez et al. (2017), the protein mass profiles of 53 *Tenacibaculum* strains were compared using mass-up software, and the genus-specific peak mass (*m/z*) values were 2273.50, 2433.48, 2661.82, 4789.03, 5048.15, 5314.34, 10,507.39, and 13,148.28 and were shown to be 100% specific to *T. maritimum* with score values between 2.048 and 2.258.

Chryseobacterium species belonging to the order flavobacteriales are chemoorganotrophic, rod-shaped, gram-negative fish pathogens forming characteristic yellow-orange colored colonies. This genus contains more than 100 described species from a variety of habitats, including freshwater sources, soil, humans, and marine fish (Loch and Faisal 2015; Austin and Austin 2016). *Chryseobacterium* species are generally isolated from external lesions, gills, kidneys, and fins of diseased fish. Pérez-Sancho et al. (2017a) reported on the first analysis of *Chryseobacterium* species and distinguished between the species such as *C. balustinum* (2.866), *C. oncorhynchi* (2.897), *C. piscicola* (2.881), *C. shigense* (2.790), *C. tructae* (2.802), *C. viscerum* (2.941), *C. chaponense* (2.832), and *C. piscium* (2.852) using the MALDI-TOF MS technique. Popović et al. (2022) compared the results of MALDI-TOF MS and API 20E identification for gram-negative strains isolated from freshwater, sediment, and fish tissues. Among 190 isolates, two isolates were identified as *C. indogenes* with a mass score between 1.768 and 2.058, and the other two isolates were identified as *C. scophtalmum* with a mass score between 1.870 and 2.058.

Vibrionaceae

The pathogenic Vibrionaceae family, which has multiple members, consists of gram-negative, mostly halophilic, flagellated, and facultative anaerobic bacteria (Austin and Austin 2016). Some species of this family, such as *Vibrio vulnificus*, *V. parahaemolyticus*, *V. mimicus*, and *V. cholerae*, cause enteric pathogenicity such as diarrheal disease, septicemia, and serious wound infections in humans (Sanjuán et al. 2007; Malainine et al. 2013; Burbick et al. 2018; Boonstra et al. 2023). *V. anguillarum*, *V. alginolyticus*, *V. vulnificus*, *V. parahaemolyticus*, *V. ordalii*, *V. splendidus, V. tubiashii*, *V. campbellii*, *V. harveyi*, *V. owensii*, *V. rotiferianus*, *V. azureus*, and *V. jasicida* are also known to be the causative agent of Vibriosis that causes symptoms of hemorrhagic septicemia in various marine fish and freshwater fish (salmon, rainbow trout) (Silva-Rubio et al. 2008; Mougin et al. 2020; Sanches-Fernandes et al. 2022) as well as shellfish, crustaceans, and bivalves (Tanrikul 2007; Mougin et al. 2020; Liu et al. 2004). Lastly, the bacterium *V. tapetis* causes brown ring disease (BRD) in Manila clams (Paillard et al. 2006).

Many studies (Table 2) have demonstrated that *Vibrio* species isolated from a wide range of aquatic organisms such as molluscs, seahorse, shrimp, oyster, and clam (Dieckmann

et al. 2010; Bauer et al. 2018; Burbick et al. 2018) and cod, char, halibut, eel, Japanese flounder, grouper, seabass, seabream, turbot, ayu, and dreamfish (Low et al. 2014; Ashfaq et al. 2022; Kazazić et al. 2019b; Jansson et al. 2020; Yavuzcan et al. 2022; Boonstra et al. 2023; Haider et al. 2023) can be accurately identified either by evaluating mass score values or peptide mass spectrum peaks. Dieckmann et al. (2010) compared the MALDI-TOF MS-based method with the RNA polymerase beta subunit gene (*rpoB*) sequence and showed that a total of 83 *Vibrio* strains were accurately identified by MALDI-TOF MS and species-specific score mass data were between 4200 and 6500 Da. In the study by Cho et al. (2017), four different MALDI-TOF preparation procedures were tested on fifty *Vibrio* isolates and five reference strains for the identification of *Vibrio* species. Formic acid and trifluoroacetic acid (TFA) extraction methods applied in the pretreatment of bacterial colonies plated on MALDI-TOF MS plates were considered to be the most suitable methods for the identification of *Vibrio* species. In a study conducted by Burbick et al. (2018), the MALDI-TOF MS method was compared with *rpoB* and *rpoD* gene sequence analysis to identify *Vibrio* species in isolates obtained from different aquatic organisms such as *Paralichthys californicus* (California halibut), *Hippocampus abdominalis* (big-bellied seahorse), *Seriola lalandi* (yellowtail kingfish), *Atractoscion nobilis* (white perch), and *Pterapogon kauderni* (Banggai cardinalfish). It was reported that 29 out of 35 *Vibrio* species (83%) were correctly identified at the species level using MALDI-TOF MS. Bauer et al. (2018) used MALDI-TOF MS analysis with score values between 1.700 and 2.410 and protein spectrum results to identify eleven pathogenic *Vibrio* species from *Litopenaeus vannamei*. These results were further compared with the 16S rRNA sequence and the sequence of the *pyrH* gene. Erler et al. (2015) carried out the identification of *Vibrios* in 997 environmental samples by comparing the MALDI-TOF MS and *rpoB* gene. The mass score value of *V. alginolyticus* was reported as 2.542, *V. cholerae* as 2.628, *V. parahaemolyticus* as 2.609, and *V. vulnificus* as 2.644. In order to identify *Vibrio* isolates, Vidal et al. (2020) compared the results of 16S rRNA, *pyrH* gene sequences, and MALDI-TOF MS analyses. Wu et al. (2020) identified 28 of 74 isolates obtained from seawater, plankton, marine plants, and animals, and Liu et al. (2022) identified isolates from clinical and environmental samples by the MALDI-TOF MS method. *V. vulnificus*, *V. fluvalis*, and *V. splendidus* were identified by analyzing peptide mass score values between 1.750 and 2.410 obtained from seawater (Haider et al. 2023) and *Anguilliformes* species (eels) (Boonstra et al. 2023), freshwater, ornamental fish, and moribund fish (Joansson et al. 2020; Janampa-Sarmiento et al. 2024; Pastuszka et al. 2024), respectively. *V. anguillarum*, another important bacterium causing vibriosis, was identified by MALDI-TOF MS from *Dicentrarchus labrax* and *Sparus aurata* with an average mass score of 2.120 to 2.500 (Kazazić et al. 2019b; Jansson et al. 2020; Mougin et al. 2021). *V. alginolyticus*, one of the most important causative bacteria causing vibriosis, was identified by Low et al. (2014) in *Epinephelus fuscoguttatus* (brown marble grouper) and by Ashfaq et al. (2019) in seawater by analyzing peptide mass values obtained from MALDI-TOF method. Bronzato et al. (2018) identified *V. alginolyticus* in 49 of 209 isolates obtained from *Perna perna* mussels using MALDI-TOF and *rpoA/pyrH* sequencing methods together. Wang et al. (2024) identified 35 of a total of 55 different bacterial isolates as *V. alginolyticus* by MALDI-TOF MS and confirmed the same isolates with a new method, rapid visual nucleic acid detection (RPA-CRISPR/Cas13a). Another bacterial species identified by the MALDI-TOF method was *V. harveyi*, which was also identified from *Sarpa salpa* (dreamfish) by Yavuzcan et al. (2022) and Culot et al. (2021) from shrimp. *V. parahaemolyticus*, the other causative agent of vibriosis, has been reported to be identified from *D. labrax*, *Haliotis tuberculata* (green ormer) (Malainine et al. 2013),

Crassostrea gigas (Pacific oyster) (Mougin et al. 2020), and seafood samples (Fasulkova et al. 2023) and seawater (Khandeparker et al. 2023) using the MALDI-TOF method. In addition, Rahmani et al. (2021) concluded that *V. tapetis* isolated from Manila clams was pathogenic based on protein profiles indicating the presence of a virulence gene. Moussa et al. (2021) reported a study on marine molluscs to identify *V. tapetis*.

Although many studies listed above have reported the identification of *Vibrio* species by MALDI-TOF MS, a study by Sanches-Fernandes et al. (2022) showed that isolates belonging to *V. tubiashii/V. europaeus* and *V. owensii/ V. jasicida/V. campbellii* species could not be correctly differentiated using some commonly available databases for MALDI-TOF MS-based classification. Similarly, Mougin et al. (2020) successfully identified several strains belonging to *V. campbellii*, *V. owensii*, *V. azureus*, *V. jasicida*, *V. sinaloensis*, and *V. rotiferianus* using an in-house database called Luvibase, which contains the master spectral profiles (MSPs) of 23 strains in combination with Bruker v.9.0.0.0 database, since Bruker v.9.0.0.0 could not distinguish some *Vibrio* species.

Photobacterium damsale is a gram-negative, halophilic, rod-shaped bacteria belonging to the family Vibrionaceae. This pathogen is an emerging threat in marine aquaculture and also occasionally causes disease in poikilotherms and mammals, including humans. *P. damselae* subsp. *piscicida* (formerly known as *Pasteurella piscicida*) is responsible for photobacteriosis (fish pasteurellosis) causing outbreaks characterized by multifocal necrosis in the spleen, liver, and kidneys leading to high mortality rates in larvae and juveniles fish (Austin and Austin 2016), and *P. damselae* subsp. *damselae* (formerly known as *Vibrio damsela*) which causes hemorrhagic ulcerative skin lesions in fish has also been isolated from humans and has been recognized as a zoonotic pathogen (Andreoni and Magnani 2014). Table 2 shows that both subspecies were identified and confirmed by the spp. names by Kazazić et al. (2019a) by trying longer incubation times and three MALDI-TOF methodologies such as direct bacterial sample spotting, on-target extraction, and full extraction. Pérez-Sancho et al. (2016) also showed that all tested isolates from *D. labrax*, *S. aurata*, and *M. saxatilis* were identified as *P. damselae* subsp. *damselae* with an average score value of 2.256 (2.114–2.353) and P. *damselae* subsp. *piscicida* with an average of 2.286 (2.250–2.331) by MALDI-Biotyper. Yavuzcan et al. (2022) identified *Photobacterium damselae* with MALDI-TOF with high identification score values (Table 2).

Pseudomonadaceae

Pseudomonas is a genus of gram-negative, rod-shaped, and non-spore-forming bacteria belonging to the family Pseudomonadaceae in the class Gammaproteobacteria. Due to their extensive metabolic diversity, the 313 members of the genus (Parte et al. 2020) can live in a wide range of habitats, including water, soil, plants, insects, fish, aquatic creatures, and animals (Khan et al. 2007; Duman et al. 2021). *P. putida*, *P. aeruginosa*, *P. lundensis*, *P. fluorescens*, and *P. baetica* are opportunistic human pathogens (Sadikot et al. 2005; De Bentzmann and Plesiat 2011; Tohya et al. 2022). A large number of *Pseudomonas* spp. (*P. aeruginosa*, *P. anguilliseptica*, *P. proteolytica*, *P. baetica*, *P. chlororaphis*, *P. fluorescens*, *P. koreensis*, *P. luteola*, *P. plecoglossicida*, *P. pseudoalcaligenes*, *P. lundensis*, *P. tructae*, *P. extremaustralis*, and *P. putida)* have been reported as the most common bacterial causative agent of stress-related infections in rainbow trout and tench (strawberry disease), sea bream, sea bass, ayu, and ornamental fish. Although the symptoms of the disease vary according to the fish, they are generally described as the

distended abdomen and hemorrhaging on the body surface, fin or tail rot, hemorrhagic septicemia, hemorrhagic ascites, exophthalmitis with extensive skin lesions, and external ulceration and Sekiten byo (red spot) (Altinok et al. 2006, 2007; von Siebenthal et al. 2009; Nishimori et al. 2000; López et al. 2012; Austin and Austin 2016; Walczak et al. 2017; Oh et al. 2019; Aydin et al. 2023). The MALDI-TOF MS method has been used to detect previously identified *Pseudomonas* spp. (Kačániová et al. 2019; Klūga et al. 2019; Popović et al. 2022) as well as the novel species *P. Sivasensis*, *P. anatoliensis* sp. nov., and *P. iridis* sp. nov. (Duman et al. 2020; 2021), *P. haemolytica* (Saticioglu et al. 2022).

Enterobacterales

Yersinia ruckeri is a gram-negative, non-spore-forming, round-tipped, rod-shaped, and facultative anaerobe bacteria belonging to the Yersiniaceae family of the Enterobacterales order. This bacterium is the causative agent of yersiniosis or enteric red mouth disease (ERM) in salmonids, especially in *O. mykiss* (Tobback et al. 2007; Huang et al. 2013; Austin and Austin 2016). Recent literature shows the successful identification of *Y. ruckeri* from *O. mykiss* (Jansson et al. 2020), *S. salar* (Ojasanya et al. 2022), and *Cyprinus carpio* (Kazarnikova et al. 2019) using the MALDI-TOF MS method (Table 2).

Edwardsiella tarda, a gram-negative, facultative anaerobic, small, motile, flagellated, and straight rod bacterium belonging to the order Enterobacterales and the family Hafniaceae, is a serious fish pathogen causing Edwardsiella septicemia (ES), Edwardsiellosis, fish gangrene, and red disease in both farmed and wild fish species (Plumb 1993; Thune et al. 1993; Park et al. 2012; Austin and Austin 2016). *E. tarta* is zoonotic and can infect fish as well as amphibians, reptiles, birds, and mammals (Xu and Zhang 2014; Verma et al. 2022). Table 2 shows two studies on the use of MALDI-TOF MS for the identification of *E. tarda*. The first study by Piamsomboon et al. (2020) showed that isolates from *O. niloticus*, a hybrid catfish, were *E. tarda*. Reis et al. (2023) also used MALDI-TOF MS for the identification of *E. tarda* isolated from *Colossoma macropomum*, as well as *dnaJ* gene sequencing to confirm the accuracy of the species identification.

Piscirickettsiaceae

Piscirickettsia salmonis is a gram-negative, recalcitrant, and psychotropic bacterium that is generally non-motile, aerobic, not encapsulated, highly fastidious, and very slow-growing, coccoid, and found in pairs or ring-shaped structures (Fryer et al. 1992; Rozas and Enríquez 2014; Makrinos and Bowden 2017). It is reported to be the causative agent of Piscirickettsiosis, also known as Salmonid Rickettsial Septicaemia (SRS) and Salmon Rickettsia Syndrome (Branson and Nieto Diaz-Munoz 1991; Lannan and Fryer 1993). It is an intracellular fish pathogen that can infect and reproduce in monocyte and macrophage cell lines. It is an economically important disease that greatly affects the production of cold-water salmonids and non-salmonids including sea bass grouper and Nile tilapia in many countries from the United States, North America, Canada, and Europe to Asia (Austin and Austin 2016). *P. salmonis* is very difficult to isolate, and culture from the fish cell, MALDI-TOF MS, and molecular identification have been used in combination as a powerful tool for accurate and early detection to facilitate disease control in the salmon farming industry (López-Cortés et al. 2017; Olate et al. 2016; Zrnčić et al. 2021).

Identification of gram-positive fish pathogens with MALDI-TOF MS

Renibacterium salmoninarum

R. salmoninarum, a member of the Micrococcaceae family, is a small, gram-positive, rod-shaped, fastidious, slow-growing, extracellular/intracellular pathogen causing bacterial kidney disease (BKD), especially in farmed and wild salmonid fish, reported from many countries around the world including Scotland, England, Germany, Spain, Turkey, Italy, France, Iceland, Balkan Peninsula, Canada, United States of America, Chile, and Japan (Fryer and Lannan 1993; Daly et al. 2001; Vardić et al. 2007). These bacteria can be transmitted vertically and horizontally via eggs (Evelyn et al. 1986) and primarily accumulate in the kidney and other internal organs, causing edema, granuloma lesions, and resulting in morbidity and mortality (Gutenberger et al. 1997). Clinical signs of BKD include loss of appetite, lethargy, dark colouration, exophthalmia, bloody ascites, and hemorrhages around the cloaca and fins (Rhodes et al. 2004; Austin and Austin 2016; Elliott 2017). Jansson et al. (2020) successfully identified bacterial isolates of *Renibacterium* at the species level from rainbow trout, Atlantic salmon, chinook salmon, coho salmon, and arctic char (Table 2). Conversely, Jia et al. (2023) compared different swab transport methods, along with molecular detection (qPCR) and MALDI-TOF methods for the identification of *R. salmoninarum* from bacterial colonies on selective kidney disease medium (SDKM) agar plates, but when samples from contaminated colonies on SDKM agar plates were tested with MALDI-TOF MS, the results obtained could not determine whether this isolate was *R. salmoninarum* because it was not taken from a single colony.

Streptococcaceae

Lactococcus garvieae is a gram-positive, facultative anaerobic, non-motile, non-spore-forming, ovoid cocci, pair, and short-chain bacterium (Ravelo et al. 2001; Vendrell et al. 2006). *L. garvieae* is the bacterial agent of lactococcosis, an important zoonotic disease affecting fish, other animals, and humans. It causes peritonitis, urinary tract infections, and infective endocarditis in humans (Ferrario et al. 2013; Cañas et al. 2015; Gibello 2016). In fish, it has been reported to cause lesions in the vascular endothelium, causing hemorrhages and petechiae on the surface of internal organs in rainbow trout, yellowtail, cobia, and mullet and causing large-scale deaths (Zlotkin et al. 1998b; Eldar and Ghittino 1999; Diler et al. 2002; Meyburgh et al. 2017). *L. garvieae* was successfully identified at the species level from salmonids by MALDI-TOF MS (Assis et al. 2017; Radosavljević et al. 2020; Saticioglu et al. 2023) and has a strong score at *m/z* 2.35 that corresponded to the nisin leader peptide mass peak (Sequeiros et al. 2015) (Table 2). Torres-Corral and Santos (2021) also showed that the two mass peaks belonging to the small (30S) subunit of the species-specific DNA-binding ribosomal protein were *m/z* 4717.04 and 9431.98 Da. They also found that a high intraspecies similarity (83% similarity) was observed among *L. garvieae* strains, regardless of the host species or geographical region from which the strains were isolated. Torres-Corral and Santos (2022) also showed that the identification of *L. garvieae* by determining the mass peaks associated with the antibiotic resistance pattern is a simple and rapid technique using protein mass patterns or biomarkers for oxytetracycline and florfenicol resistance that were associated with the expression of a peptide directly or indirectly related to phenotypic resistance.

Another bacterial species belonging to the Streptococcaceae family, which can be identified by MALDI-TOF MS, is a novel pathogen, gram-positive *L. petauri*, which is known as the second causative agent of lactococcosis in fish (Goodman 2017; Kotzamanidis et al. 2020; Altinok et al. 2022; Egger et al. 2023; Littman et al. 2023; Saticioglu et al. 2023).

MALDI-TOF MS can also be used to identify the gram-positive, cocci *Streptococcus iniae*, *S. agalactiae*, *S. dysgalactiae*, and *S. parauberis* belonging to the family Streptococcaceae (Mata et al. 2004; Buller 2014; Austin and Austin 2016). The disease known as warm water streptococcosis (mortalities at temperatures above 15 °C) caused by these species has signs of septicemia, suppurative exophthalmia (pop-eye), and meningoencephalitis in several aquatic species in marine and freshwater fish including rainbow trout (*O. mykiss*) (Lahav et al. 2004), olive flounder (*Paralichthys olivaceus*) (Nho et al. 2009; Kim et al. 2015), turbot (*Scophthalmus maximus*), flatfish (Domeénech et al. 1996), barramundi (*Lates calcalifera*), tilapia (*Oreochromis* spp.), and sturgeon (*Acipenser* spp.) (Deng et al. 2017). These *Streptococcus* species have been also recognized as zoonotic pathogens (Facklam et al. 2005; Gauthier 2015; Kim et al. 2017). Tavares et al. (2018) showed fifteen *Streptococcus* spp. isolates from Amazon catfish (*Leiarius marmoratus × Pseudoplatystoma corruscans*) were identified as with other diagnostic methods but also confirmed by MALDI-TOF MS. Table 2 summarizes the successful identification of *S. agalactiae* were from *O. niloticus* (Assis et al. 2017), *S. iniae*, and *S. parauberis* isolated from *P. olivaceus* (Kim et al. 2015; 2017) and from *S.maximus* and *O. mykiss* (Torres-Corral et al. 2019), *S. agalactiae* from *O. niloticus*, *O. aureus* and *O. mossambicus* (Piamsomboon et al. 2020), and *S. iniae* from *Acipenser transmontanus* (Pierezan et al. 2020).

Enterococcaceae

The gram-positive and rod-shaped *Vagococcus salmoninarum* (Wallbanks 1990; Schmidtke and Carson 1994) has been reported to be the causative agent of vagococcosis or "Cold Water Streptococcosis" with peritonitis in Atlantic salmon, rainbow trout, and brown trout (Michel et al. 1997; Ruiz-Zarzuela et al. 2005; Salogni et al. 2007). Although there are many articles in the literature showing the identification of *V. salmoninarum* by classical methods, the application of the MALDI-TOF method has been limited due to a lack of reference data (Buller and Hair 2016). Nevertheless, Torres-Corral and Santos (2019) recently reported that *V. salmoninarum* strains can be successfully identified by MALDI-TOF MS methods.

Mycobacteriaceae

Four *Mycobacterium* species from fish identified by the MALDI-TOF MS method are known as pleomorphic, acid-fast, gram-positive, aerobic, non-motile, and rods form (Gauthier and Rhodes 2009). These species are known to be the causative agents of mycobacteriosis, also known as fish tuberculosis, which develops chronically and rapidly in many fish species (Kaattari et al. 2005; Whipps et al. 2007; Zanoni et al. 2008; Jacob et al. 2009; Mugetti et al. 2021) and progresses with external symptoms such as emaciation, skin swelling, exophthalmos, open lesions, and ulceration symptoms in several organs (Austin and Austin 2016). Some *Mycobacterium* species are also known to be zoonotic (Bhatty et al. 2000; Ucko and Colorni 2005). The slow-growing *M. marinum* was first identified by Aronson in (1926), and it has been observed and identified from many fish worldwide (Lansdell et al. 1993; Puk et. al. 2018; Elgendy et al. 2023). Kurokawa et al. (2013)

demonstrated that *M. marinum* and *M. avium* could be reliably identified with the MALDI-TOF MS, and Puk et al. (2018) showed accurate detection of *M. marinum*, *M. peregrinum*, *M. fortuitum*, and *M. abscessus* in aquarium fish with MALDI-TOF MS in combination with two other techniques (16S rRNA and *Hsp65*) to maximize the identification results.

Conclusion

In conclusion, the proteomics-based MALDI-TOF MS method appears to be an effective and reliable microbial identification approach for rapid, accurate, and specific identification and classification of many bacteria at the species level with unique fingerprints (spectral protein peaks) and mass score values generated by cellular ribosomal proteins or antimicrobial resistance markers present in bacteria compared to reference mass spectral databases. In this review, it is shown that the number of published studies that utilized MALDI-TOF MS between 2010 and 2023 has increased rapidly, reflecting the effectiveness and efficiency of the technique.

Although the use of this method in aquatic studies has been validated with numerous outstanding achievements, its use for the identification of pathogens has a number of advantages. Firstly, in addition to speeding up the detection process of diseases that damage aquatic organisms, it can efficiently screen a large number of field samples at one time due to its high throughput and low sample preparation needs. It is also a beneficial approach as it eliminates the need for expensive reagents, labor-intensive steps, and highly trained operators that are part of traditional techniques.

There are a few limitations to consider, nevertheless. The main disadvantage that may discourage users from using this technique is the high initial cost of acquiring a MALDI-TOF MS instrument, which has a low unit sample cost, although not as expensive as the NGS platforms that perform the next-generation sequencing technique. Another limitation of current devices is that bacteria cannot be sampled and identified directly from the diseased tissue sample on the instrument. Although it can accurately identify most bacterial species, the inability to detect some species not found in global proteomics databases may be one of the limitations of this method. Therefore, the potential for in-house databases such as the EnviBase library and Luvibase database, which have been developed to identify *Vibrio* species, is needed to identify other aquatic pathogens.

Overall, MALDI TOF MS technology has ongoing innovations and system refinement for future user convenience. Efforts to optimize sample preparation protocols and streamline data analyses can make MALDI-TOF MS more accessible and user-friendly for aquaculture practitioners and hold great promise for advancing disease diagnosis and promoting sustainable aquaculture practices in the future. Nevertheless, more fish pathogens' score values and peak data should be uploaded to global protein databases for accurate discrimination and identification of fish pathogens. The use of this method in the aquaculture disease sector promotes sustainable practices and healthier fish populations in aquaculture by contributing to the early detection of disease and the development of timely and effective intervention strategies. Therefore, more research is crucial to facilitating the routine use of MALDI-TOF MS for the identification of pathogens of importance to the aquaculture and fisheries sectors.

Declarations

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