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Virulence of *Enterococcus* Species Isolated from the Nile Tilapia and Human: Evaluation of MALDI-TOF-MS Diagnostic Techniques Accuracy in Comparison to PCR

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ABSTRACT

Enterococci are widely distributed bacteria incriminated in various clinical conditions in humans and economic losses in fish farms. The aim of this study was to recognize *Enterococcus* sp. and their virulence in the diseased Nile tilapia and humans, and assess the accuracy of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in recognizing *Enterococcus* species. Septicemic farmed *O. niloticus* (n=70) and human blood, urine, and pus samples (n=45) were collected from Sharkia Governorate and Suez Canal University Hospital, Ismailia, Egypt, respectively. Samples were bacteriologically and molecularly tested for the identification of *Enterococcus* sp.; moreover, carriage of virulence genes by the isolates was evaluated. Proteomic (MALDI-TOF MS) was also used to assess its ability to identify *Enterococcus* sp. in comparison to conventional PCR technique. Overall, *Enterococcus* sp. was identified in the Nile tilapia (32.9%) and human samples (28.9%). Two species of enterococci; *E. faecalis* and *E. faecium*, were identified. *E. faecalis* was the most prevalent species in both fish and human samples. High occurrence of four virulence genes (*gelE*) (86.1%), (*cylA*) (86.1%), (*asa1*) (77.8%), (*esp*) (72.2%) were detected in *Enterococcus* sp. isolates; however, (*hyl*) (19.4%) was recorded the lowest. Using MALDI-TOF MS, five of ten examined isolates were identified as *Enterococcus* sp.; however, all isolates were confirmed to be *Enterococcus* using conventional PCR. The study confirmed that PCR is the most accurate method for the identification of *Enterococcus* sp., while using MALDI-TOF MS as the only identification method is not advisable.

1. INTRODUCTION

Enterococci are Gram-positive bacteria distributed in soil, sewage, sediments, freshwater, and gastrointestinal tracts of both animals and humans **(Lebreton *et al.*,**

2014). These organisms are facultative anaerobic bacteria that are resistant to harsh environmental conditions, allowing its survival in unfavorable settings such as those existing in hospitals **(Ch'ng *et al.*, 2019)**.

The Nile tilapia (*Oreochromis niloticus*) is the second most common farmed fish species in the world, with estimated global production of around 5.5 million metric tons **(Arumugam *et al.*, 2017)**. *Enterococcus* species is a commensal bacterium, dominant in fish, shellfish and other aquatic animals **(Paganelli *et al.*, 2017)**; however, the virulence of *E. faecalis* in tilapia varied from high, moderate, to low **(Rahman *et al.*, 2017)**. Enterococcosis (also known as 'pop-eye 'disease) is an infectious disease in fish characterized by high mortality rate in the aquarium leading to economic losses **(Anshary *et al.*, 2014)**.

In human, enterococci are incriminated in nosocomial infections in hospitals such as endocarditis, meningitis, pneumonia, wounds, burns, skin and soft tissue, urinary tract infection (UTI), bone and joint infections **(Singh *et al.*, 2010)**. Furthermore, other non-health care associated diseases such as inflammation of the bowel and Crohn's disease may also result **(Kushkevych *et al.*, 2019)**. The transmission of enterococci to human is mainly foodborne through handling and consumption of contaminated food products that may have been cross-contaminated in the kitchen **(Bortolaia *et al.*, 2016)**.

Among enterococci, *E. faecalis* and *E. faecium* are the most prevailing species in humans **(Guzman Prieto *et al.*, 2016)**. Ninety percent of human infections produced by enterococci are due to *E. faecalis*, while the other 10% are caused by *E. faecium* **(Golob *et al.*, 2019)**. Moreover, isolation of the same strains of *E. faecalis* from human and animals suggest the probable zoonotic transmission of this bacterium **(Hammerum, 2012)**. In general, due to the presence of *Enterococcus* sp. in human and animal feces, its capability to adapt to varied environmental circumstances (freshwater and marine settings), and their relevance to human health, the surveillance of *Enterococcus* sp. is utilized as sentinels for changes in water quality **(Lebreton *et al.*, 2014)**.

Since conventional cultural identification methods have been proved to have several limitations with respect to microbiological diagnosis, species-specific (PCR) primers such as *ddI E. faecalis* and *ddI E. faecium* were documented as competent method for *Enterococcus* species identification. The use of PCR with these primers may be the simplest molecular approach for the effective identification of distinct *Enterococcus* species **(Kafil & Asgharzadeh, 2014)**.

Several virulence factors in enterococci have been reported, including aggregation substance (*asa1*), gelatinase (*gelE*), cytolysin (*cylA*), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*), with *asa1*, *gelE*, *cylA* and *esp* virulence factors mostly found in

E. faecalis, while *hyl* and *esp* are more frequent in *E. faecium* **(Rice *et al.*, 2003, Savaşan *et al.*, 2016)**.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is widely used in microbiological laboratories because of its reliability, speed, low cost, and accurate capacity in the identification of many pathogenic bacteria. However, this approach has not been properly tested in unusual *Enterococcus* species **(Quintela- Baluja *et al.*, 2013)**.

The current study aimed to investigate the occurrence and virulence of *Enterococcus* sp. in the Nile tilapia and humans that may result in nosocomial infection, in addition to assessing the accuracy of MALDI-TOF MS in *Enterococcus* sp. identification compared to conventional PCR technique.

2. MATERIALS AND METHODS

2.1. Samples

Protocol of sample collection and examination for this study was reviewed and approved by the ethical committee of the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (approval No. 201826).

A total of 70 fresh water *Oreochromis niloticus*, showing signs of septicemia (exophthalmia, distended abdomen, hemorrhage on the operculum, mouth, anal opening and fins, as well as, dark pigmentation in the whole body) were collected from Al Abbassah Farm, Sharkia Governorate, Egypt during the period from August 2019 to February 2021. Collected fish were transferred in an icebox within 2 hours to the microbiology laboratory, Faculty of Veterinary Medicine, Suez Canal University for bacteriological examination.

In addition, 45 samples (21 urine, 17 pus swabs from wound infection, and 7 blood samples) were collected from patients who have different forms of health care associated infections in Suez Canal University Hospital at Ismailia Governorate. Patients were of of both sexes, from different age groups and from different wards, including ICU, Urology, and Surgery. Patients received antibiotics within 48hrs. prior to the collection of samples excluded from participation in the study. Consent was obtained from all patients to use their data in the current research work. Samples were collected under the supervision of each ward's medical doctors, placed directly in sterile labeled containers and transferred in an icebox to microbiology laboratory in the Faculty of Medicine, Suez Canal University in 2hrs time for bacteriological examination.

2.2. Isolation and biochemical identification of *Enterococcus* species

The external body surface of every fish was cleansed with 70% ethyl alcohol, and postmortem inspection was performed according to **Austin and Austin (2007)**. A loopful was taken from the ascetic fluid of every fish, as well as, deep loopfuls from internal organs (liver, kidney, and spleen), after the surface of each organ has been sterilized using hot spatula. Loopfuls were enriched in Tryptone soya broth (TSB, DIFCO, USA), containing 6.5% salt and incubated at 29-30°C for 18-24hrs. Selective plating was performed by inoculating a loopful from each enrichment broth on M-Enterococcus agar (APHA, Washington) and incubated at 37°C for 24-48hrs **(Peter *et al.*, 2012)**. Human samples were cultured directly on M-Enterococcus agar and incubated at 37°C for 24-48 hrs. **(Hajia *et al.*, 2012)**.

The suspected smooth, pink or red colonies were examined with the following tests: Gram stain, catalase test, oxidase test, motility testing, and investigated for Esculin production by culture on Bile Esculin Agar (BEA) (Himedia, India) **(Švec & Devriese, 2009)**. Identified isolates were preserved in brain heart broth (Oxoid, UK), with 20% (*v*/v) glycerol at –80°C for further investigations.

2.3. Molecular identification and detection of virulence of enterococci isolates.

DNA was extracted from 24hrs refreshed isolates on blood agar medium (Oxoid, UK) using QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's guidlines. *Enterococcus* species specific primers (Table 1) were used for the amplification of *ddl E. faecalis* and *ddl E. faecium* genes (Biobasic, Canada) using conventional PCR. The amplification was performed using T3 Thermal cycler (Biometra, Germany) in a 50µl total reaction volume consisted of 25µl Emerald Amp GT PCR mastermix (2× premix) (Takara, Code No. RR310, India), 6 µl DNA template, 1.0 µl of each primer (20 pmoL concentration) and 15 µl molecular grade water. Positive controls for *E. faecalis* (ATCC 19433) and *E. faecium* (ATCC 35667) and negative control (PCR grade water) were included in each run. Moreover, a multiplex PCR was performed for the simultaneous detection of five enterococcal virulence genes as previously described (Table 1). Amplified products were photographed using Gel Documentation System (Alpha Innotech) after being electrophoresed through 1.5% agarose gel (Sigma, USA), containing ethedium bromide (0.5 µg ml^{-1}) (Sigma, USA) in 1× TBE buffer.

2.4. Identification of *Enterococcus* species isolates by MALDI-TOF MS

Single pin point-sized pure colony was picked up with the tip of a sterile toothpick from freshly grown overnight *Enterococcus* species on blood agar media. The colony was placed on the polished steel of MSP-96 target plate (Bruker Daltonics, Germany) of MALDI-TOF MS. After about 15 minutes of dryness at 25°C, 1.0 µl of matrix [a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA) in 50%

acetonitrile 2.5% trifluoroacetic acid (2.5 mg) 70 matrix solutions (Bruker Daltonics)] was added to the plate in a unique deposition and left to dry at ambient temperature.

Mass spectra were measured using the Ultraflex LT mass spectrometer (Bruker Daltonics) and analyzed using the MALDI Biotyper Software package (version 3.1) with the reference database (version 3.4.119.0, Bruker Daltonics). As recommended by the manufacturer, a log score identification value of 2.00-3.00 means that the tested samples features high confidence, secure genus and species identification (perfect identity), and a score value ranging in between 1.7-1.99 means that the tested sample features identified as secure genus identification with low confidence species identification. However, a score value of 0.00-1.69 means that the tested sample has no reliable identification **(Cherkaoui *et al.*, 2010)**.

Table 1. Target genes, oligonucleotide primers sequences, and cycling conditions for identification and virulence genes of *Enterococcus* species

Target genes	Nucleotide sequence (5'→3')	Cycling conditions	Amplicon size (bp)	References
ddl E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	Initial denaturation 94°C/2 min 30 cycles: Denaturation at 94°C/1 min Annealing at 54°C/1 min Extension at 72°C/1 min Final extension at 72°C/10 min	941	**(Dutka-Malen *et al.*, 1995)**
ddl E. faecium	ACGATTCAAAGCTAACTG TCGAATGTGCTACAATC		550	
asa1	GCACGCTATTACGAACTATGA TAAGAAAGAACATCACCACGA	Initial denaturation 94°C/1 min 30 cycles: Denaturation at 94°C/1 min Annealing at 56°C/1 min Extension at 72°C/1 min Final extension at 72°C/10 min	375	**(Coque *et al.*, 1995)**
gelE	TATGACAATGCTTTTTGGGAT AGATGCACCCGAAATAATATA		213	**(Willems *et al.*, 2002)**
cylA	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT		688	**(Galli *et al.*, 1990)**
Esp	AGATTTCATCTTTGATTCTTGG AATTGATTCTTTAGCATCTGG		510	**(Su *et al.*, 1991)**
Hyl	ACAGAAGAGCTGCAGGAAATG GACTGACGTCCAAGTTTCCAA		502	**(Rice *et al.*, 2003)**

3. RESULTS

3.1. Isolation, identification, and virulence of enterococci isolated from fish and human samples

Based on cultural examination, biochemical identification and molecular confirmation, *Enterococcus* species were detected in samples of the Nile tilapia (32.9%, 23/70) and human (28.9%, 13/45). All isolates showed typical characteristics of colony morphology on M-Enterococcus agar (smooth, pink or red colonies), a black zone was noticed around the colonies on Bile Esculin Agar, and isolates were oxidase and catalase negative and appeared as Gram-positive non-spore-forming diplococci on stained Gram

smears. All recovered isolates belonged to 2 species of enterococci; *E. faecalis* and *E. faecium* (Table 2).

Molecularly, tested isolates showed the expected amplification band either for ddl *E. faecalis* (941 bp) or ddl *E. faecium* (550 bp) that was relevant to its phenotypic and bacteriological characterization. As shown in Table (2), isolates showed variable virulence profile for the occurrence of *gelE*, *asa*, *hyl*, *cylA*, and *esp* genes. At least, each isolate carried one of the virulence genes; however, six isolates from fish and one isolate from human carried all virulence genes.

3.2. Spectroscopic identification of enterococci isolated from fish and human using MALDI-TOF MS

Spectroscopic identification using MALDI-TOF- MS (Bruker Daltonis Ultraflex spectroscopy) of ten representatives *Enterococcus* sp. isolates from fish and human that are formerly phenotypically and molecularly identified, are presented in (Table 3). MALDI-TOF MS failed to identify *Enterococcus* sp. isolates in 5 out of 10 investigated isolates. Overall, 4/5 (80%) of human isolates and 1/5 (20%) of fishes isolates matched their phenotypic and genotypic identification. Two human isolates, phenotypically and molecularly identified as *E. faecalis*, were identified up to the *Enterococcus* genus level using MALDI-TOF MS. Nevertheless, their species level were identified as *E. hirae*, with identification score of (2.46). Identification of two human isolates of *E. faecium* (Fig. 1) and one *E. faecalis* from fish (Fig. 2) by MALDI-TOF mass spectroscopy matched its phenotypic and molecular identification.

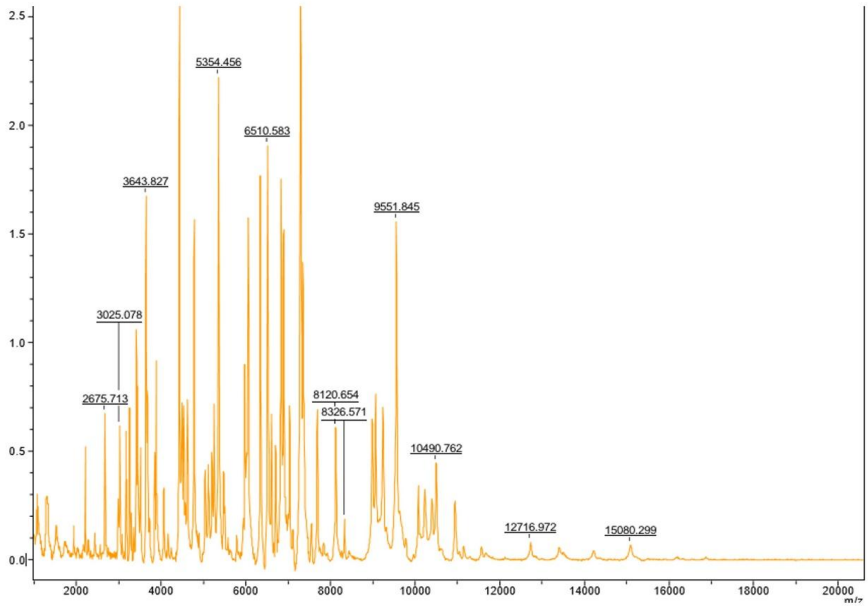

Fig. 1. Obtained spectra based on MALDI-TOF-MS analysis specific to *E. faecium* isolated from human (score 2.47)

Table 2. Virulence profile of enterococci isolated from fish and human samples

Host	*Enterococcus* Species	Source of the isolate	Virulence genes				
			gelE	***asa1***	***hyl***	***cylA***	***Esp***
O. niloticus	*E. faecalis*	Kidney	+	+	+	+	+
	E. faecalis	Liver	+	+	-	+	+
	E. faecalis	Kidney	+	+	-	+	+
	E. faecalis	Ascetic fluid	+	-	-	+	+
	E. faecium	Ascetic fluid	+	+	+	+	+
	E. faecium	Ascetic fluid	+	+	+	+	+
	E. faecium	Spleen	+	+	+	+	+
	E. faecium	Spleen	+	+	+	+	+
	E. faecalis	Ascetic fluid	+	+	-	+	+
	E. faecalis	Liver	+	+	-	+	+
	E. faecalis	Ascetic fluid	+	+	-	+	+
	E. faecalis	Spleen	+	+	-	+	-
	E. faecalis	Ascetic fluid	+	+	-	-	-
	E. faecalis	Liver	+	+	-	+	+
	E. faecalis	Ascetic fluid	+	+	-	+	-
	E. faecalis	Liver	+	+	-	+	+
	E. faecalis	Liver	+	+	-	+	+
	E. faecalis	Ascetic fluid	+	+	+	+	+
	E. faecalis	Spleen	+	+	-	+	+
	E. faecalis	Ascetic fluid	+	+	-	+	+
	E. faecium	Spleen	-	-	-	+	-
	E. faecium	Liver	-	-	-	+	-
	E. faecium	Spleen	-	-	-	+	-
Human	*E. faecalis*	Urine	+	-	-	-	-
	E. faecalis	Urine	+	-	-	-	-
	E. faecalis	Urine	+	+	-	+	+
	E. faecalis	Urine	+	+	-	+	+
	E. faecalis	Urine	+	+	+	+	+
	E. faecium	Urine	-	-	-	+	+
	E. faecium	Blood	-	-	-	+	+
	E. faecium	Blood	+	+	-	+	+
	E. faecium	Blood	+	+	-	-	-
	E. faecium	Blood	+	+	-	-	-
	E. faecium	Wound	+	+	-	+	+
	E. faecium	Wound	+	+	-	+	+
	E. faecium	Wound	+	+	-	+	+
Total No. (%)			31 (86.1)	28 (77.8)	7 (19.4)	31 (86.1)	26 (72.2)

Table 3. MALDI-TOF MS spectroscopic identification of *Enterococcus* sp. isolates from fish and human compared to phenotypic and molecular identification

Phenotypically and molecularly identified isolates	Host	MALDI-TOF MS identification	Score	NCBI Identifier
E. faecalis	Human	*Enterococcus hirae* LMG 12286 LMG	2.46	1354
E. faecalis	Human	*Enterococcus hirae* LMG 12286 LMG	2.46	1354
E. faecalis	Fish	*Bacillus cereus* 994000168 LBK	1.54	1396
E. faecalis	Fish	*Enterococcus faecalis* DSM20371	2.33	145391
E. faecalis	Fish	*Staphylococcus hominis ssp hominis* DSM 20330 DSM	2.28	145391
E. faecalis	Fish	*Staphylococcus hominis ssp hominis* DSM 20330 DSM	2.20	145391
E. faecium	Fish	*Bacillus licheniformis* CS 54_1 BRB	1.70	1402
E. faecium	Human	*Enterococcus faecium* 11037 CHB	2.47	1352
E. faecium	Human	*Staphylococcus hominis ssp hominis* DSM 20330 DSM	2.19	145391
E. faecium	Human	*Enterococcus faecium* 11037 CHB	2.47	145391

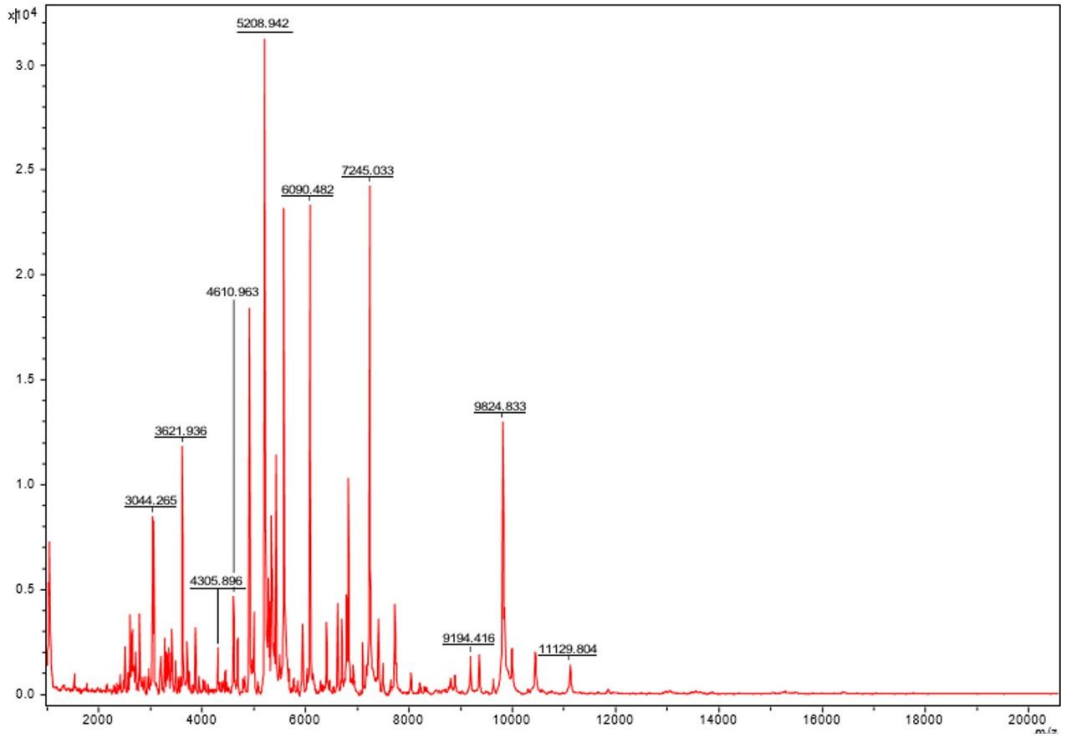

Fig. 2. Obtained spectra based on MALDI-TOF-MS analysis specific to *E. faecalis* isolated from fish (score 2.33)

4. DISCUSSION

In few recent years, some opportunistic bacterial fish bacteria have been recognized as the causal agents of major outbreaks in aquaculture systems. Among these, *Enterococcus* sp. has emerged as one of the most serious fish bacterial infection, impacting aquaculture techniques throughout the world **(Paganelli *et al.*, 2017)**.

In the present study, bacteriologically and molecularly, enterococci were identified in *Oreochromis niloticus* (32.9%) and human samples (28.9%). Only two species of *Enterococcus*; *E. faecalis* and *E. faecium* were identified in the present study, with *E. faecalis* being the most prevalent species in both the Nile tilapia and human samples. This was higher than the occurrence of enterococci (23.76%) reported from *O. niloticus* in an earlier study in Egypt **(Khafagy *et al.*, 2009)**. Nonetheless recently, *E. faecalis* was isolated at a lower level (2.7%) from the farm-raised Nile tilapia, implicated in seasonal summer mortalities at Kafr El-Sheikh and Dakahlia Governorates, Egypt **(El-Kader & Mousa-Balabel, 2017)**. Such differences could be due to different farm raising conditions such as using poultry manure, waste water contaminated by feces of animals and humans, and using agriculture irrigation as main source of water in fish farms.

In health care settings, enterococci are one of the most prevalent nosocomial pathogens associated with higher mortality rates and treatment costs. Similar to our findings, but at lower levels, enterococci were isolated from exudates (5.54%), urine (1.98%) and blood (1.40%) in an earlier study **(Bhakare *et al.*, 2014)**. These differences may be due to variation in the number of the examined samples. Generally, in recent years, the incidence of urinary tract infection (UTI) in human caused by *E. faecalis* has been estimated to be five times greater than that of *E. faecium* **(Kline & Lewis, 2016)**. Moreover, it's considered the second etiologic agent of UTIs and the third of nosocomial bacteremia **(Simmons & Larson, 2015)**. However, the most frequent nosocomial disorders caused by *E. faecium* are urinary tract and bloodstream infections, which can progress to total organ sepsis. Furthermore, it is frequently encountered in patients following operations, producing postoperative wound infections that may necessitate reoperation **(Messler *et al.*, 2019)**. This was clearly noticed in the present study, regarding the distribution of *E. faecalis* and *E. faecium* from human clinical samples.

Many factors can influence the pathogenicity of *Enterococcus* species, such as their capacity to colonize the GIT or their adhesion to a variety of extracellular matrix proteins or epithelial cells. Many enterococcal virulence genes may be implicated in the initiation or worsening of illness symptoms in humans and fish **(Anderson *et al.*, 2016)**. In the present study, all isolates carried at least one enterococcal virulence gene, with higher occurrence of gelatinase (*gelE*) (86.1%), cytolysin (*cylA*) (86.1%), aggregation substance (*asa1*) (77.8%), and enterococcus surface protein (*esp*) of 72.2%, compared to hyaluronidase (*hyl*) (19.4%). Similarly in another study, but with lower percentage, *E. faecium* and *E. faecalis* from diseased fishes harbored *asa1* (27%), *gelE* (11.5%), and *esp*

(17%), while *cylA* can't be detected **(Savaşan *et al.*, 2016)**. In addition, enterococci strains isolated from carp and the Nile tilapia in southern Brazil harbored *gelE* (64.6%), *asa1* (13.9%), and *cylA* (3.8%) genes, but none of the isolates carried *esp* gene **(Araújo *et al.*, 2020)**. However, *esp* failed to be detected in *E. faecium* isolated from human in an earlier study **(Shankar *et al.*, 2001)**. Moreover, *E. faecalis* from human clinical samples carried *gelE* (77.2%), *asa1* (57.9%), *cylA* (54.4%), and *esp* (68.4%) virulence genes **(Medeiros *et al.*, 2014)**. The presence of such virulent *Enterococcus* strains in fish may be a risk for workers in fish farming industry who are in contact with infected fish or contaminated water especially those having skin abrasion.

MALDI-TOF MS is widely used in microbiological laboratories due to its reliability, speed, low cost, and accuracy in the identification of many pathogenic bacteria. However, this approach has not been properly tested in unusual *Enterococcus* sp. **(Suarez *et al.*, 2013; Sauget *et al.*, 2017; Wang *et al.*, 2018)**. In the current study, results of MALDI-TOF-MS were compared to the currently used phenotypic and molecular identification methods for *Enterococcus* sp. isolates. As presented in Table "2", MALDI-TOF MS analysis doesn't match with *Enterococcus* sp. identification as obtained by phenotypic and molecular methods in five out of the ten investigated isolates. Similarly, this conflict was observed in other studies. MALDI-TOF MS identified 101/199 (50.0%) as 37 *E. faecium* and 64 *E. faecalis* isolates, which were confirmed by species-specific PCR **(Werner *et al.*, 2012)**. Although MALDI-TOF was thought to be a rapid and simple approach for routine pathogenic bacteria identification, the physical breakdown of peptidoglycan in the cell wall of Gram-positive bacteria makes the direct colony method inferior to the extracted protein method for identification by MALDI-TOF **(Smole *et al.*, 2002)**. The use of whole-cell method in MALDI-TOF MS for reliable strain typing was still debatable, and the MALDI-TOF MS proteomic profiles of outbreak isolates were not similar enough to indicate a single strain outbreak **(Schlebusch *et al.*, 2017)**.

Overall, using MALDI-TOF-MS as a diagnostic tool for enterococci identification isn't advisable to be instead of other conventional methods (culture based methodologies) and PCR assay because of the conflicting results which might be due to the software and models used to analyze the data, where results of MALDI-TOF-MS are built based on peak intensities. Furthermore, one explanation could be that MALDI-TOF-MS is able to detect only a rather random bacterial sub proteome in a restricted mass range (m/z 2000-12000), which is linked to the fact that analytically only a specific number of proteins is preferentially ionized, limiting the number of signals accessible for strain identification **(Suarez *et al.*, 2013)**.

Findings in this study add to the available data about enterococci from fish and humans. Virulent *Enterococcus* species (*E. faecalis* and *E. faecium*) are widely distributed in the farmed Nile tilapia and incriminated in septicemia, urinary tract and wound infections in humans. Using MALDI-TOF-MS as the only diagnostic tool for

enterococci identification isn't advisable as it has conflicting results, and the phenotypic and molecular identification are the gold standard methods.

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