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Isolation and Identification of *Enterococcus faecium* from the Gastrointestinal Tract of the Common Carp (*Cyprinus carpio*) and the Nile Tilapia (*Oreochromis niloticus*)

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ABSTRACT

Enterococcus faecium, a member of the *Enterococcus* genus, has emerged as a significant concern in aquaculture due to its potential pathogenicity and antibiotic resistance. This study aimed to investigate the molecular diagnosis and characterization of six different *Enterococcus* species isolated from the gastrointestinal tracts of the common carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) and to evaluate their genetic diversity, antibiotic resistance profiles, and potential virulence factors. Among the isolates, 65.3% of the common carp and 60.8% of the tilapia were identified as *E. faecium*. All six species demonstrated the ability to metabolize various carbohydrates, indicating a broad metabolic capacity. Some species showed variability in their utilization of specific carbohydrates. For example, *E. faecium* and *E. faecalis* uniquely fermented adonitol, while *E. avium* and *E. hirae* were the only ones capable of fermenting D-arabitol. Additionally, Voges-Proskauer positivity was exclusively observed in *E. faecalis*. In terms of growth conditions, all species thrived at both 4°C and 45°C, except for *E. faecalis*, which failed to grow at 10°C. *E. faecium* and *E. faecalis* grew well at pH 9.6. Hemolysis tests revealed differences among the species: *E. faecalis* displayed beta-hemolysis, while *E. gallinarum* exhibited alpha-hemolysis. Motility was observed only in *E. gallinarum*, and esculin hydrolysis was exclusive to *E. faecalis*. Environmental adaptability varied among the species. *E. avium* showed limited growth in 6.5% NaCl, and some species exhibited little to no growth in 0.1% methylene blue milk. *E. faecalis* and *E. faecium* demonstrated survival at 60°C for 15 minutes, with E. faecium showing limited survival at 30 minutes, distinguishing them from the other species. The strains isolated from *Cyprinus carpio* and *Oreochromis niloticus* collected from local fish farms in Basrah City were confirmed as *E. faecium* through 16S rRNA gene sequencing. A PCR study using specific primers identified all isolates as *E. faecalis*.

INTRODUCTION

The global importance of aquaculture has increased, accounting for around 44% (74 million tons) of fish production, with a value of $160 billion. Two dominant fish species, the common carp (*Cyprinus carpio*) and the Nile tilapia (*Oreochromis niloticus*), contribute over 50% of the total farmed fish production worldwide **(FAO, 2016; FOA, 2020; Bartley, 2022)**. *C. carpio*, with a hardy nature, have been farmed for millennia, cherished for their ease of culture and ability to thrive in ponds and aquaculture systems

(Chirwa *et al.*, 2017). On the other hand, *O. niloticus* are valued for their rapid growth, efficient feed conversion, and suitability for intensive pond and tank culture **(FAO, 2013)**. Both species offer affordable, nutritious food security in regions struggling with malnutrition and poverty **(FAO, 2018)**. However, their success in aquaculture presents a potential quandary – the risk of disease outbreaks.

As the primary etiological agents of diseases in freshwater fishes globally, bacteria play a central role in this context. Major genera of fish pathogens, such as *Aeromonas, Edwardsiella, Pseudomonas, Flavobacterium, Vibrio,* and *Streptococcus,* are responsible for various diseases in distinct tropical freshwater fish species **(Plumb, 1997)**. In recent years, several opportunistic bacterial fish pathogens have been recognized as the primary causative agents of severe outbreaks within aquaculture facilities. Among these, *Enterococcus* sp. has emerged as a significant fish pathogen, exerting a profound impact on aquaculture practices on a global scale **(Martins *et al.*, 2008)**. *E. faecium* is a commensal bacterium that predominantly inhabits the gastrointestinal tracts of various animals, including fish **(Fisher & Phillips, 2009)**. While traditionally considered benign residents of the gut microbiota, recent research has unveiled a more complex picture of *E. faecium,* indicating its potential to turn into a pathogen and its growing resistance to antibiotics. These emerging concerns have prompted a closer examination of this microorganism's role in aquatic ecosystems and its implications for food safety. The gastrointestinal tracts of fish serve as dynamic microbial ecosystems that play a vital role in digestion and overall health. *E. faecium* is among the microbial species commonly found in these environments **(Talwar *et al.*, 2018)**. However, the transition of *E. faecium* from a commensal organism to a pathogen capable of causing diseases in fish has raised significant concerns. Moreover, the alarming rise in antibiotic resistance among *E. faecium* strains further exacerbates these concerns **(Bender *et al.*, 2016)**.

In this study, we embark on a comprehensive investigation into diagnozing and characterizing *E. faecium* strains isolated from the internal guts of *C. carpio* and *O. niloticus* fish. By analyzing the genetic and phenotypic traits of these strains, we aimed to shed light on their potential pathogenicity, antibiotic resistance profiles, and overall impact on aquatic health and food safety. This research seeked to provide valuable insights into the ecology and biology of *E. faecium* in fish, addressing the urgent need for a deeper understanding of this bacterium's role in aquatic ecosystems.

MATERIALS AND METHODS

1. Sampling period

This study was conducted between May 2019 and June 2019. Fish were collected from the common carp (*Cyprinus carpio*) and tilapia (*Oreochromus niloticus*), weighing 50-250g from some the local markets in Basrah province. A total of 27 fish, *C. carpio* (n=9) and *O. niloticus* (n=18) were obtained. Samples were transported to the laboratory in a box ice for 20 to 30min.

2. Bacterial isolation

The ventral surface of the fish was sterilized by 70% ethanol. Under sterile conditions, gut was dissected out and washed with sterile saline. After washing, the gastrointestinal was homogenized in peptone water (0.4%). Further dilution was carried out as deemed necessary. From the diluted samples, 0.1 ml was spread-plated onto bile esculin agar (BEA) (Difco, U.S.A.). The plates were incubated at 37°C for 24 hours, following the method of **Singh and Chauhan (2019)**.

After incubating fish samples on BEA at 37˚C for 24 hours, colonies displaying brown halos were considered indicative of Enterococcus colonies. At least four such colonies were selected, subsequently transferred to fresh BEA plates, and incubated again at 37˚C for another 24 hours. Gram staining was performed for each isolate to assess purity and Gram reaction. Isolates consisting of Gram-positive *cocci* forming short chains in pairs were subcultured onto nutrient agar slants and then incubated at 37˚C for 24 hours. The isolates were maintained at 48˚C with regular subculturing. The *Enterococcus* isolates were diagnozed to the genus level by using the biochemical tests presented in Table (1) in addition to molecular identification.

3. Molecular identification of *Enterococcus faecium*

3.1. DNA extraction

The DNA extraction process for *Enterococcus* spp. involved selecting an individual colony using a sterile loop from each sample. This colony was subsequently introduced into sterile 5ml brain heart broth tubes, where it was subjected to incubation at 37˚C for 24 hours. The DNA of the isolated enterococcal strains was then extracted using a DNA purification kit, following the manufacturer's instructions as provided by Jena Bioscience, Germany.

3.2 Polymerase chain reaction (PCR)

The primers utilized for the amplification of the ddI (D-Ala-D-Ala Ligase) chromosomal genes were the following: Forward primer (F) 5'-TTGAGGCAGACCAGATTGACG-3' and Reverse primer (R) 5'-TATGACAGCGACTCCGATTCC-3' **(Chabuck *et al*., 2012; Kafil & Asgharzadeh, 2014)**, which were provided in a lyophilized form (IDT, USA). A working stock solution was prepared by reconstituting them with PCR grade water to the recommended concentration by the supplier. The PCR reaction mixture was set up in 25µl volumes, comprising 2µl of template DNA, 2µl of specific primers for *E. faecium* (ddlE) (both R and F), 10µl of 2x Taq master mix, and 11µl of PCR grade water. PCR amplification was carried out using a thermal cycler (Optimus 96G, QLS, UK) employing a standard PCR protocol. This protocol began with an initial denaturation step of 5 minutes at 94°C, followed by 35 cycles, each consisting of denaturation at 94°C for 1 minute, annealing at

54°C for 1 minute, and extension at 72°C for 1 minute. The PCR reaction was concluded with a final extension step at 72°C for 7 minutes. Subsequently, DNA samples were visualized using agarose gel electrophoresis (Jena Bioscience, Germany), following the procedure described by **Mohanty *et al.* (2017)**. The electrophoresis was conducted in 1x TBE buffer (Genet Bio, Korea) with a 100bp DNA ladder (Jena Bioscience, Germany) used as a molecular weight marker. Electrophoresis was powered by an MP 300V power supply (Major Science, UK), and the agarose gel was then placed in a documentation system (Bio Doc Analyze, Germany) for UV light examination, enabling the documentation and determination of the expected DNA bands.

4. Statistical analysis

Physiological characterisations of phages were repeated in triplicate for both double-layer agar assays and spread plates. All readings were analyzed based on the one-way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) Statistics version 22 (IBM, USA).

RESULTS AND DUSSICATION

1. Bacterial isolation

From 293 isolates, 68 (65.3%) were identified as *E. faecium* from *C. carpio* and 115 (60.8%) isolates from *O. niloticus*, as shown in Table (1).

Table 1. Number and percentage of *Enterococcus* spp. isolated from the common carp and tilapia

No	*Enterococcus* sp.	Common carp	Tilapia
1	***E. faecium***	**65.3 (68/104)**	**60.8 (115/189)**
2	***E. faecalis***	**21.1 (22 /104)**	**23.8 (45/189)**
3	***E. gallinarum***	**5.7 (6/104)**	**4.2 (8/189)**
4	***E. avium***	**2.8 (3/104)**	**3.7 (7/189)**
5	***E. durans***	**1.9 (2/104)**	**2.6 (5/189)**
6	***E. hirae***	**2.8 (3/104)**	**4.7 (9/189)**

2. Biochemical test and molecular identification of *Enterococcus faecium*

Table (2) presents the results of 36 tests conducted to assess the metabolic capabilities and growth conditions of six different species. All six species demonstrated their proficiency in metabolizing N-Acetylglucosamine, Amygdalin, Cellobiose, Dextrin, D-Fructose, D-Glucose, Lactose, Maltose, and esculin hydrolysis, indicated by the presence of a plus (+) sign. This collective utilization showcases their diverse carbohydrate metabolic potential **(Day *et al.*, 2001)**. However, it is noteworthy that

certain species displayed variable (V) utilization of specific carbohydrates like Galactose and D-Tagatose. In contrast, *E. faecium* and *E. faecalis* uniquely exhibited the ability to ferment Adonito, while *E. avium* and *E. hirae* were the sole species capable of fermenting D-Arabitol. Moreover, Voges-Proskauer positivity, signifying acetoin production, was exclusively observed in *E. faecalis*, setting it apart from the other species. This results agree with those of **Lavová *et al*. (2014)**, **de Jesús Cortés-Sánchez and Barrón-Sosa (2017)** and **Atiyah and Alkhafaji (2020)**. Turning to growth conditions, all species exhibited the ability to thrive at both 4 and 45°C, except for *E. faecalis*, which failed to grow at 10°C. *E. faecium* and *E. faecalis* stood out again by demonstrating growth at pH 9.6, a feature not shared by the other species. *E. gallinarum* and *E. avium* displayed limited growth at both 10 and 45°C, with the latter showing a unique survival at 60°C for 15 minutes **(Ruoff *et al*., 1990; Valenzuela *et al*., 2010; Abdulrazzaq & Faisal, 2022)**. However, it is noteworthy that certain species displayed variable (V) utilization of specific carbohydrates viz. Galactose and D-Tagatose. In contrast, *E. faecium* and *E. faecalis* uniquely exhibited the ability to ferment Adonito, while *E. avium* and *E. hirae* were the sole species capable of fermenting D-Arabitol. Moreover, Voges-Proskauer positivity, signifying acetoin production, was exclusively observed in *E. faecalis*, setting it apart from the other species. This results coincide with those of **Cai (1999)**, **Lavová *et al*. (2014)** and **de Jesús Cortés-Sánchez and Barrón-Sosa (2017)**. Turning to growth conditions, all species exhibited the ability to thrive at both 4 and 45°C, except for *E. faecalis*, which failed to grow at 10°C. *E. faecium* and *E. faecalis* stood out again by demonstrating growth at pH 9.6, a feature not shared by the other species.

Table 2. Consensus matrix of biochemical tests for identification of *Enterococcus* spp.[a]

Test or characteristic	*E. faecium*	*E. faecalis*	*E. gallinarum*	*E. avium*	*E. durans*	*E. hirae*
N-Acetylglucosamine	+	+	+	+	+	+
Adonito	-	-	-	+	-	-
Amygdalin	+	+	+	+	+	+
L-Arabinose	+	-	+	+	-	-
D-Arabitol	-	-	-	+	-	-
Cellobiose	+	+	+	+	+	+
Dextrin	V	+	+	+	ND	ND
D-Fructose	-	+	+	+	+	+
Galactose	V	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
Glycerol	D	+	D	D	-	D
Inulin	-	-	D	D	-	-
Lactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Mannitol	+	+	+	+	-	-

Starch	D	D	D	-	D	-
Sucrose	+	+	+	+	D	(+)
D-Tagatose	-	+	+	+	-	(-)
L-Xylose	-	-	-	D	-	-
H_2S production	-	-	-	D	-	ND
Alpha hemolysis	D	V	D	+	V	D
Beta hemolysis	D	V	D	-	V	-
Motility	-	-	+	-	-	-
Voges-Proskauer	-	+	D	D	+	+
Esculin hydrolysis	+	+	+	+	+	+
Growth at: 4°C	+	-	-	-	ND	ND
10°C	+	+	+	D	+	+
45°C	+	+	+	+	+	+
50°C	V	-	-	-	-	-
pH 9.6	+	+	+	+	+	+
Growth in: 6.5% NaCl	+	+	+	D	+	+
0.1% Methylene blue milk	+	+	V	-	+	ND
0.04% Tellurite	-	+	D	-	-	-
Survival at 60°C for 15 min	+	+	(+)	+	ND	ND
30 min	+	+	D	+	ND	ND
1 h	+	+	-	(-)	ND	ND

[a] +, 90% or more of the strains of isolates are positive; (+), 75 to 89% are positive; V, 26 to 74% are positive; (-), 11 to 25% are positive; -, 10 or less are positive; ND, no data; d, discrepancies among reference studies.

E. gallinarum and *E. avium* displayed limited growth at both 10 and 45°C, with the latter showing a unique survival at 60°C for 15 minutes **(Rypka *et al*., 1967; Schleifer & Kilpper-Bälz, 1984; Ruoff *et al*., 1990)**. Hemolysis tests revealed further distinctions among the species. *E. faecalis* and *E. hirae* exhibited alpha hemolysis, while *E. faecium*, *E. gallinarum*, and *E. durans* produced variable results in this category. Conversely, *E. faecalis* displayed beta hemolysis, with *E. faecium*, *E. gallinarum*, and *E. durans* again showing variable outcomes **(Fifadara *et al*., 2003; Vidana *et al*., 2016)**. Interestingly, motility was exclusively observed in *E. gallinarum*, denoting its capacity to move in a liquid medium **(Facklam *et al*., 2002)**. Furthermore, Esculin hydrolysis was solely attributed to *E. faecalis*, setting it apart from the other species. Regarding environmental adaptability, all species demonstrated growth in 6.5% NaCl, except for *E. avium*, which exhibited a limited growth. *E. faecalis* and *E. hirae* exhibited growth in 0.1% methylene blue milk, while the other species displayed variable or no growth. Lastly, *E. faecalis* and *E. faecium* showcased the ability to survive at 60°C for 15 minutes, with *E. faecium* displaying a limited survival at 30 minutes, a trait not shared by the other species. Some of the isolates of *E. faecium* in the present study were exposed to confirm return by PCR technique, based on the detection of a specific gene that encodes D-alanine, namely D-

alanine Ligase for *E. faecium* (*ddlE*). Based on PCR results, sex *E. faecium* isolates (100%) were detected as *E. faecium*. The results confirm the presence of a 1022bp PCR product when compared with the DNA ladder, as shown in Fig. (1).

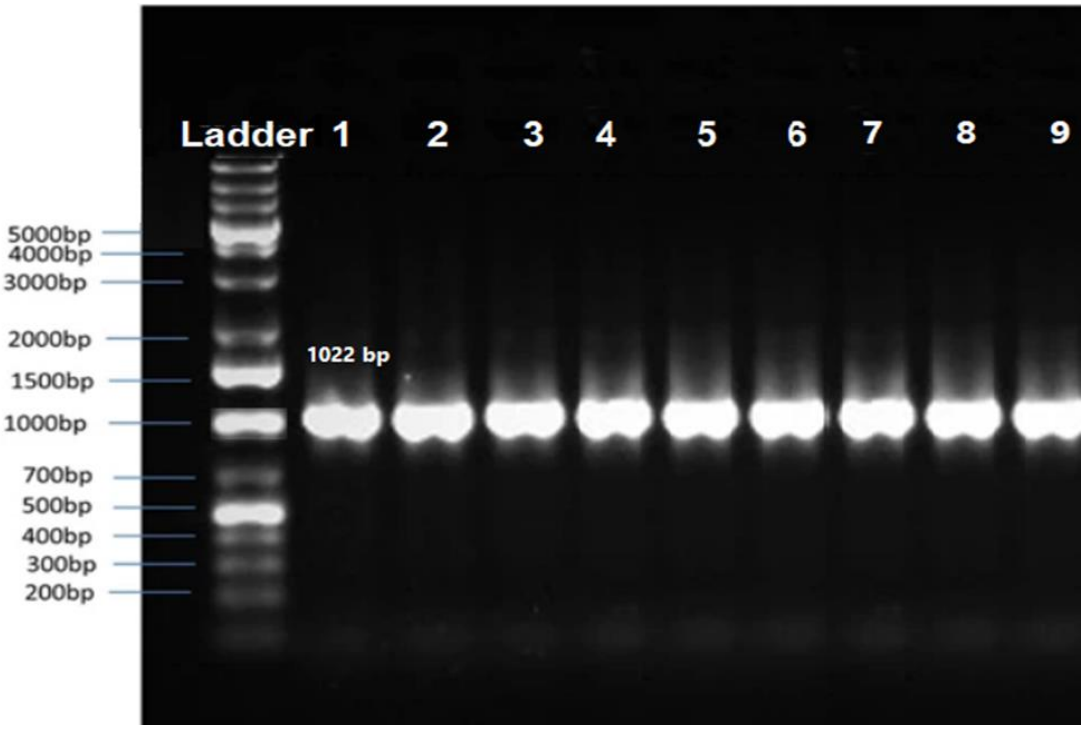

Fig. 1. Representative photograph of PCR targeting *E. faecium* specific *ddlE gene*

CONCLUSION

The molecular identification and characterization of *Enterococcus faecium* isolated from the gastrointestinal tract of the common carp (*Cyprinus carpio*) and tilapia (*Oreochromis niloticus*) have provided valuable insights into the presence and potential implications of this bacterium in aquatic environments. Our findings underscore the importance of understanding the microbial composition of fish gastrointestinal tracts, as *Enterococcus faecium* is known to be an opportunistic pathogen in both animals and humans. Furthermore, the detection of antibiotic resistance genes in some isolates highlights the potential for transmission of antimicrobial resistance through aquatic ecosystems. Moving forward, further research is warranted to investigate the ecological significance of *Enterococcus faecium* in fish populations and its potential impact on human health through foodborne transmission. Additionally, efforts should be exerted to implement strategies for the surveillance and control of antibiotic-resistant bacteria in aquaculture settings to mitigate the risk of dissemination into the environment and human populations. Overall, this study contributes to our understanding of the microbial ecology of aquatic environments and underscores the importance of continued research in this field.

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