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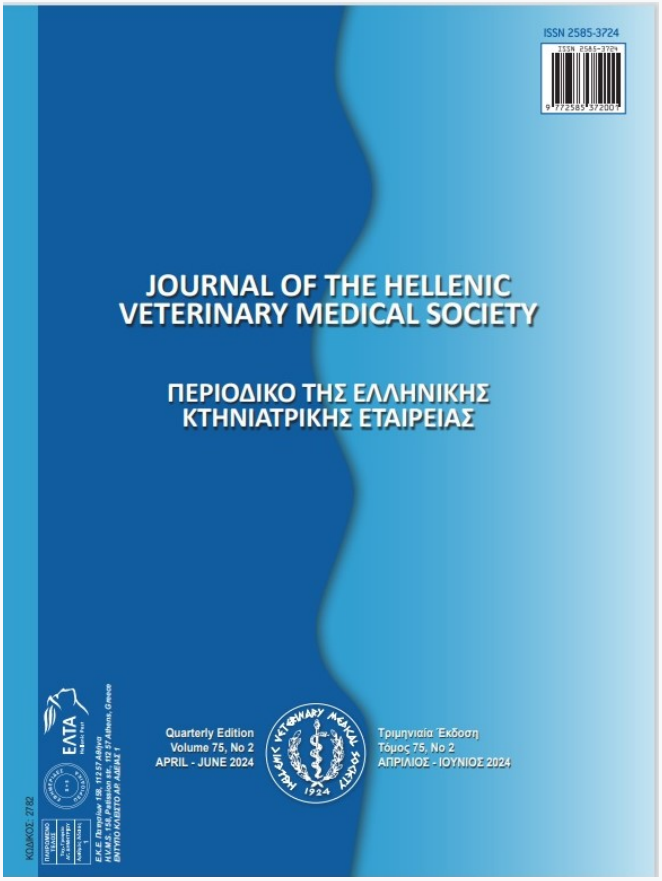

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Research article
Ερευνητικό άρθρο

Identification of Enterococcus Species Isolated from Commercial Fish Feeds and Infected Fish Specimens

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ABSTRACT: *The genus of lactic acid bacteria Enterococci* are Gram-positive cocci bacterium that can survive in different environmental conditions such as water, plants, and soil. They are also bacteriological signs of fecal contamination. In aquaculture facilities, *Enterococcus* species have appeared as one of the crucial opportunistic fish pathogens. *Enterococcus-caused* fish diseases have been reported in different fish species like yellow tail, turbot, and tilapia. Even though *Enterococcus* species are used as probiotics and are members of the gastrointestinal flora, they also have pathogenic potential to produce septicemia, wound infections, urinary tract infections, and others. In this study, we isolated bacterial strains from affected rainbow trout and trout feed specimens. Based on the API 20 strep test kit, they were determined as *Enterococcus faecium*. While fish-isolated samples had 74.4%-99.9% similarity to *E. faecium*, trout feed isolated samples had 98.4%-99.9% similarity to *E. faecium*. In order to identify the isolates of the trout feed, PCR was performed using universal 16S rRNA primers. Sequence results indicate that the samples were *E. faecium* and *E. faecalis*. The phylogenetic tree was constructed with other *Enterococcus* species of 16S rRNA, and our samples were located in the *E. faecium* and *E. faecalis* species. In conclusion, there may be contamination of *Enterococcus* with food or other factors. Enterococcus sp. strains are opportunistic microorganisms and cause pathogenicity when the host immunity weakens. Even though all samples with API 20 strep test kit were identified as E. faecium, they had the lower percentage similarity, so they may be E. faecalis and other Enterococcus species. Thus, further studies are needed to understand their probiotic and pathogenicity functions in aquaculture production.

Keywords: *Enterococcus*; *Enterococcus faecalis*; *Enterococcus faecium*; trout; fish feeds

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INTRODUCTION

The *Enterococcus* genus members are Gram-positive cocci bacteria that can survive anywhere in nature and be found in a variety of environments including soil, sediments, marine water, freshwater, and different plants. They are generally isolated from contaminated water via fecal wastes or sewage so they are commonly used as bacteriological indicators for fecal contamination. *Enterococcus* species are typically known members of the regular gastrointestinal flora of both humans and livestock as well. There is a new insight that free-living birds may be a vector and reservoir of *Enterococcus* species, which impact animal and human health (Kwit et al. 2023). Currently the genus comprises of 66 species and many undefined groups. *Enterococcus faecalis* is one of the most common species that is isolated primarily from clinical cases (Jha et al., 2005; Lebreton et al., 2014; Zaheer et al., 2020). *E. faecalis* causes disease in humans and animals like *Streptococcus* (Akter et al., 2023).

In aquaculture facilities, opportunistic fish pathogens have been recently recognized as the causative mediators for several epidemics. *Enterococcus* species have appeared as essential fish pathogens (Martins et al., 2008). *Enterococcus* species have been reported as pathogens from important fish species such as yellow tail (*Seriola quinqueradiata*) and turbot (*Scophthalmus maximus*) (Nieto et al., 1995). Moreover, *E. faecalis* were isolated from streptococcosis like infection in tilapia (*Oreochromis niloticus*) in Egypt, India, and Bangladesh (Akter et al., 2023). *Enterococcus* spp. were isolated from infected and healthy fish in Bangladesh (Rahman et al., 2017). On the other hand, it was reported that *E. faecium* was used as a possible probiotic for ornamental cichlid fish (*Pterophyllum scalare*) and it facilitates nutrient uptakes from the feed (Dias et al., 2019).

Antimicrobial-resistant bacterial species have been identified in fish farming systems and water (Novais et al., 2018). *Enterococci* are members of the highlighted water-isolated bacteria among resistant microorganisms (Lebreton et al., 2014). Even though *Enterococcus* species are used as probiotic and are members of the gastrointestinal flora, they can cause the pathogenicity like septicemia, wound infections, urinary tract infections, and others as opportunistic microorganisms when the host immunity weakens (Kwit et al., 2023). Currently, bacterial species have been identified with sequencing approach. In this study, bacterial species isolated from infected rainbow trout and commercial fish feed were identified as *E. faecalis* and *E. faecium* with sequencing and API test.

MATERIAL AND METHODS

Bacterial culture

From trout food, bacteria were isolated from five trout food samples including different sizes and brands. Three replicates were used from each sample and nearly 1-gram (gr) food was transferred to 10 milliliter (ml) distilled water into 15 ml centrifuge tubes. The centrifuge tubes were vortexed and then samples were streaked on tryptic soy agar (TSA) via a loop. Similarly, bacterial samples were isolated from 20 kidneys of affected rainbow trouts, exhibiting signs described in detail in section "Clinical Symptoms and Gross findings of infected fish". The fish weights were between 100 and 150 grams and the fish age was 6-7 months. The bacterial samples were streaked on the surface of TSA with the help of a loop. They were incubated for 1-3 days at 20 °C. Bacteria showing colony characteristics of *Enterococcus* members, which occur singly or settled in pairs, in short chains, or as small irregular clusters (de la Maza et al., 2020; Qamer et al., 2003), were transferred to tryptic soy broth (TSB), and were incubated for 1 day at 20 °C. Then, enrichment culture was streaked on TSA agar again to see pure colonies.

Bacterial identification tests

To identify the bacterial species, conventional biochemical tests were performed on all purified colonies motility test, Gram staining, catalase, and oxidase activity (Balta, 2016). Also, rapid API 20 strep (bioMérieux, France) kit systems were used for Gram-positive bacteria. For API test kits and bacterial hemolysis activity, bacteria were inoculated on blood agar supplemented with 5% sheep blood. They were incubated on blood agar for 24-48 hours at 25 °C. They were checked whether the isolates formed hemolysis on blood agar. Isolates grown on blood agar were inoculated conferring to API 20 strep test kit instructions and incubated for 24 and 48 hours in an incubator at 25 °C. API 20 strep test kits were evaluated by adding reagents after 24 hours. The results of the biochemical tests for isolates were evaluated by making the final reading at the end of 48 hours for sugar tests (Balta and Karatay, 2021).

DNA extraction and PCR

From determined colonies, enrichment overnight

culture was prepared, and then DNA extraction was performed using the previously described protocol (Dashti et al., 2009). Briefly, bacterial cells were gathered from 1 ml of TSB culture, and then the cells were washed with distilled water three times. The cells were suspended in 1 ml distilled water, and then the suspensions were heated at 99 °C for 10 minutes. Finally, the suspension was centrifuged at 10.000 rpm for 5 minutes, and the supernatants were used as DNA templates for PCR. The PCR was carried out using Taq PCR Master Mix kit (Qiagen, Germany) with a pair of universal 16S rRNA bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-GGTTACCTTGTTACGACTT-3') (Srinivasan et al., 2015). The thermal cycling conditions were as follows: Initial denaturation at 95 °C for 5 minutes followed by 30 cycles of 95 °C for 1 minute, 60 °C for 1 minute, 72 °C for 1 minute, then final extension at 72 °C for 10 minutes. PCR products then underwent an electrophoresis step using 1% agarose gel to identify specific bands of nearly 1450 bp for 16S RNA.

DNA sequencing and Phylogenetic tree

PCR products were sequenced by a sequencing company using Sanger sequencing (Macrogen-Europe, Netherlands). The sequence results were trimmed using the Sequencher 5.4.6 (Gene Code Corporation). Then, the sequences were analyzed via BLAST searches against *Enterococcus* genomes on the National Center for Biotechnology Information (NCBI). A phylogenetic tree was made using BLAST pairwise alignments via the Neighbor-Joining method (Zhang et al., 2000).

RESULTS

Clinical Symptoms and Gross findings of infected fish

The fish were collected at the water outlet and pool sides, and they were swimming lethargically on the water surface. Darkening of the skin and exophthalmia were determined. In the autopsy of the fish, the liver was pale and the peritoneum and air sacs had petechial hemorrhages. The stomach was generally empty and filled with liquid, and the intestines were hemorrhagic and filled with yellow exudate. The symptoms of the external and internal organs of the diseased fish were similar to previous studies (Athanassopoulou and Roberts, 2004) and are shown in Figure 1.

Bacterial isolates

Based on the API test results, 11 *E. faecium* isolates were identified. 5 of them (1BYB, 1KYB, 9BYGD, 11YGD, and 12YGD) were isolated from trout food. Additionally, 6 of them (B368, B369, B370, B384, B387, and B388) were isolated from diseased fish. Bacterial isolates' positive and negative reactions on the API 20 strep kit are shown in Table 1. Also, based on the API test kit company, bacterial isolates similarity is given in Table 2. From trout food isolates, 11YGD has the highest similarity (99.9%) to *E. faecium* while 1KYB has the lowest similarity (96.4%) to *E. faecium*. On the other hand, from diseased fish isolates, B387 and B388 have the highest similarity (99.9%), and B368 has the lowest similarity (74.4%) to *E. faecium.* From samples, an assessment of negative and positive results of the API 20 strep test kit for B384 identification is given in Figure 2.

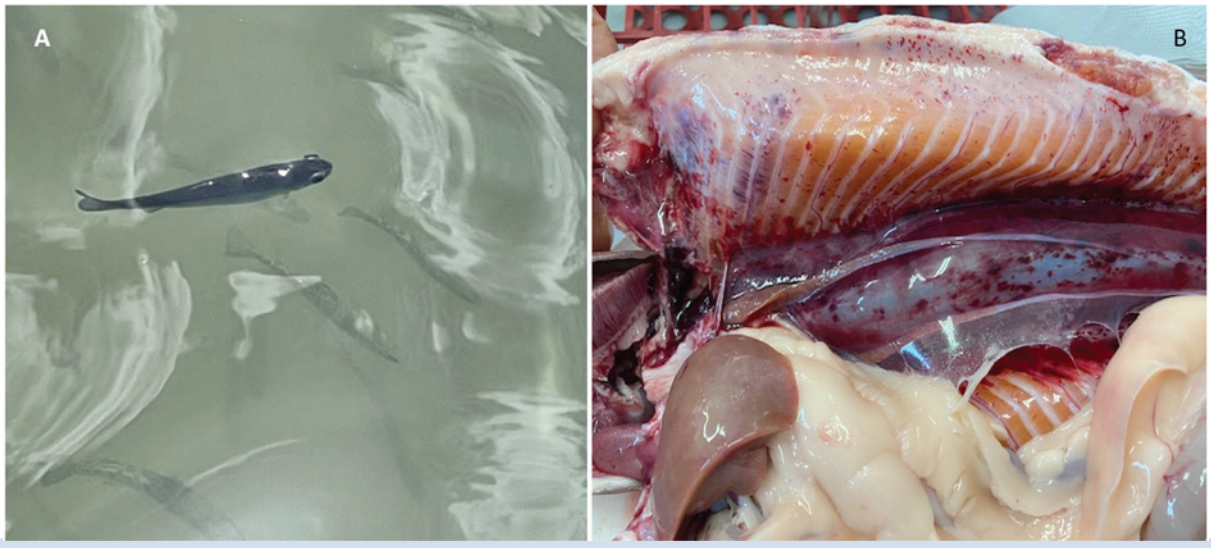

Figure 1. Darkening of the skin and exophthalmia (A), Liver pale, petechial hemorrhage on peritoneal membrane and air sac (B).

Table 1. Assessment of positive and negative results of API 20 strep to identify bacterial samples from food and diseased fish isolates.

API 20	Bacteria isolates										
Strep	1BYB	1KYB	9BYGD	11YGD	12YGD	B368	B369	B370	B384	B387	B388
VP	-	+	+	+	+	+	+	+	+	+	+
HIP	+	+	+	+	+	+	-	+	+	-	-
ESC	+	+	+	+	+	+	+	+	+	+	+
PYRA	+	+	+	+	+	+	+	+	+	+	+
αGAL	-	+	+	+	+	+	+	+	+	+	+
βGUR	-	-	-	-	-	-	-	-	-	-	-
βGAL	+	+	+	+	+	+	+	+	+	+	+
PAL	-	-	-	-	+	+	+	+	+	-	-
LAP	+	-	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+	+	+
RIB	+	+	+	+	+	+	+	-	+	-	-
ARA	+	+	+	+	+	-	-	+	+	+	+
MAN	+	+	+	+	+	+	+	-	+	+	+
SOR	-	+	+	-	+	+	+	+	+	-	-
LAC	+	+	+	+	+	+	-	+	+	+	+
TRE	+	+	+	+	+	+	+	-	+	+	+
INU	-	-	+	-	-	-	-	-	-	+	+
RAF	-	+	+	+	+	+	+	+	+	+	+
AMD	-	+	+	-	+	+	+	+	+	+	+
GLYG	-	-	+	-	-	-	-	-	-	-	-
βHEM	-	-	-	-	-	-	-	-	-	-	-

VP: Pyruvate, HIP: Hippurate hydrolysis, ESC: Esculin, PYRA: Pyrrolidonyl arylamidase, αGAL: α-galactosidase, βGUR: β-glucuronidase, βGAL: Galactosidase, PAL: Alkaline phosphatase, LAP: Leucine arylamidase, DH: Arginine dihydrolase, RIB: Ribose, ARA: L-Arabinose MAN: Mannitol, SOR: Sorbito, LAC: Lactose, TRE: Trehalose, IN U: Inulin, RAF: Raffinose, AMD: Starch, GLYG: Glycogen, βHEM: Beta hemolysis.

Table 2. Bacterial samples and their percentage similarity against the company database.

Bacterial Samples	Sum of Positive	Matching Bacterial Species	Similarity (%)
1BYB	6157510	*Enterococcus faecium*	98.4
1KYB	7317751	*Enterococcus faecium*	96.4
9BYGD	7357773	*Enterococcus faecium*	98.4
11YGD	7357550	*Enterococcus faecium*	99.9
12YGD	7377751	*Enterococcus faecium*	99.8
B368	7373751	*Enterococcus faecium*	74.4
B369	5373551	*Enterococcus faecium*	98.9
B370	5373551	*Enterococcus faecium*	94.9
B384	7377751	*Enterococcus faecium*	99.8
B387	5355571	*Enterococcus faecium*	99.9
B388	5355571	*Enterococcus faecium*	99.9

Figure 2. Assessment of positive and negative results API 20 strep for sample B384.

Phylogenetic tree

Bacterial samples, isolated from trout food, were identified as *E. faecium* and *E. faecalis* according to 16S rRNA sequence similarity. Their percentage identity similarities against the NCBI database are listed in table 3. All samples were submitted to NCBI. From samples, 1BYB (accession#: OQ13113.1) and 1KYB (accession#: OQ131137.1) were submitted as *Enterococcus* sp.. Moreover, 12YGD (accession#: OQ132535.1), 11YGD (accession#: OQ132524.1), and 9BYGD (accession#: OQ131217.1) were submitted to NCBI as *E. faecalis*. To show the relationship between samples and other *Enterococcus* species, the phylogenetic tree is shown in Figure 3.

Table 3. Bacterial samples and their percentage similarity against the NCBI database.

	E. faecium			*E. faecalis*			
	DSM 20477	**ATCC 19434**	**LMG 11423**	**ATCC 19433**	**JCM 5803**	**LMG 7937**	**EGM 183**
1BYB	98.5	98.4	97.9				
1KYB	95.7	95.6	95.1				
9BYGD				99.9	99.6	99.7	99.9
11YGD				98.9	98.5	98.7	99.0
12YGD				99.0	98.7	98.8	99.1

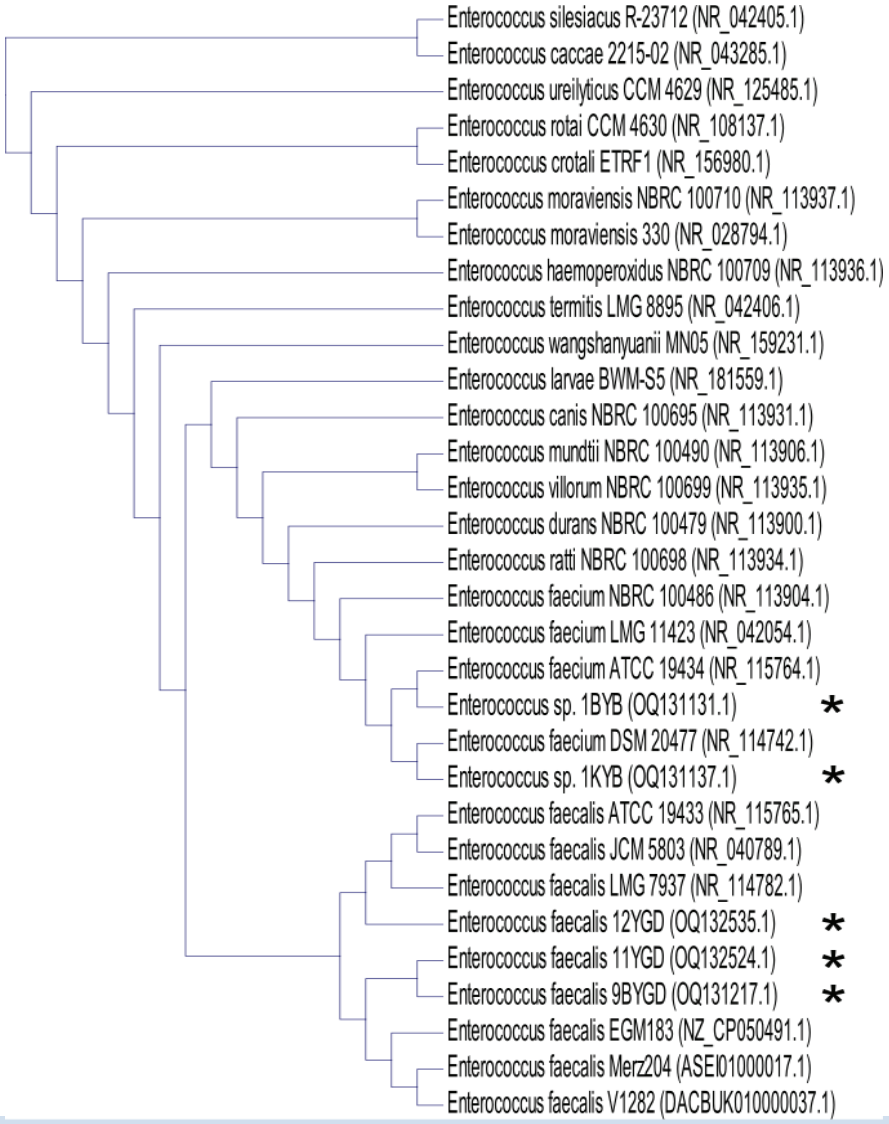

Figure 3. The phylogenetic tree was constructed with a 16S rRNA sequence of bacterial samples from feed and other *Enterococcus* species (* indicates bacterial samples used in this study).

DISCUSSION

Aquaculture is influenced by bacterial agents that cause economic losses and reduce the efficiency of production worldwide (Kotob et al., 2016; Zorrilla et al., 2003). Recently, some bacterial fish pathogens in aquaculture facilities have been determined as contributory agents for severe outbreaks. *Enterococci* are important opportunistic fish pathogens that affect the aquaculture industry (Martins et al., 2008). *Enterococcus* sp. was previously described in yellow tail (*Seriola quinqueradiata*) as a fish pathogen in Japan and was determined as enterococcal septicemia in turbot (*Scophthalmus maximus*) in Spain (Nieto et al., 1995). Then, *E. faecalis* was stated as a tilapia pathogen causing streptococcal infection in Thailand and Egypt (Petersen and Dalsgaard, 2003). It was reported that *Enterococcus* sp. was often isolated from infected and healthy fish in Bangladesh (Rahman et al., 2017). Moreover, it was reported that the pathogenic *E. faecalis* was isolated from a tilapia suffering from streptococcosis in Bangladesh (Akter et al., 2020). On the other hand, in red tilapia (*Oreochromis hybrid*), experimental infection of *E. faecalis* showed low pathogenicity in producing streptococcosis (Rizkiantino et al., 2021). *E. faecalis* and *E. faecium* species, isolated from trout food and diseased fish, might be pathogenic to trout, and this pathogenicity may have been transferred from food contamination.

In Brazil, multi-resistant and virulent *Enterococcus* spp. were isolated from fish farming environments, and it was suggested that multi-resistant may be related to environmental pollution and aquaculture may be a reservoir for virulent and resistant *Enterococci* (Araujo et al., 2021). *E. faecium* was also isolated from fish mucus (El Jeni et al., 2020), so it may be related to environmental contamination as well. On the other hand, *in vitro* and *in vivo*, studies on trout showed that *E. faecalis* and its enterocin had a protective effect against the fish pathogen *Lactococcus garvieae*. In aquaculture, the enterocin may have a potential for alternatives to antibiotics to control diseases (Banos et al., 2019). Similarly, it was reported that heat-killed *E. faecalis* had stimulatory effects on cell-mediated immunity in crucian carp (*Carassius auratus*) (Matsuura et al., 2017). Even though, it was stated *E. faecalis* had protective effects against the fish pathogens in previous studies, further studies are needed to explore these bacterial species' protective effects against other pathogens.

It was reported that *E. faecium*, which was isolated from the gastrointestinal tract of tilapia, had quorum sensing potential so it had a protective effect in controlling *Aeromonas hydrophila* infection in goldfish (*Carassius auratus*) when it was used as a probiotic (Vadassery and Pillai, 2020). *E. faecium* was used as a probiotic for angelfish (*Pterophyllum scalare*). The result was an improvement in juvenile angelfish growth when supplemented with feed (Dias et al., 2019). Additionally, *E. faecium* supplemented diet improved the immunological response, growth performance, and disease resistance to *A. hydrophila* in *Cirrhinus mrigala* production (Tilwani et al., 2022). The microencapsulated and herbal hydrogel-based encapsulated feed with *E. faecium* increases the resistance of tilapia against *Streptococcus iniae* and *Streptococcus agalactiae* infection (Kahieshesfandiari et al., 2021; Nami et al., 2022; Suphoronski et al., 2021). It was determined that *E. hirae* was isolated from the intestine of juvenile seabass, and it had new potential as a probiotic against pathogenic *vibriosis* (Masduki et al., 2020). It is not clear that isolated bacterial species, *E. faecalis* and *E. faecium*, have probiotic features against other fish pathogens. They may have new potential as protective effects and probiotics for other fish pathogens.

The pathogenicity of enterococcal infection is not clearly understood, and the infection is horizontally transferred via direct contact with infected fish or contaminated fish food (Athanassopoulou and Roberts, 2004). Multidrug-resistant *E. faecium* was isolated from feed, trout tanks, and upstream samples. It was reported that feed was an additional contamination source in aquaculture production (Novais et al., 2018). Similarly, rainbow trout feed and background environments was suitable sources to isolate enterococci as probable probiotics for aquaculture (Araujo et al., 2015). Even though the isolation of enterococci from trout feed, tanks, and rearing environments was reported as contamination and a possible probiotic for sustainable aquaculture, we isolated enterococci from trout food and infected fish. Our results indicate that the identified enterococci species may originate from contaminated feed.

CONCLUSION

In conclusion, *Enterococcus* spp., *E. faecalis*, and *E. faecium* were isolated from infected trout and commercial fish food and were identified via 16S rRNA sequence and API strep kit test results. Based on the previous studies, *E. faecium* can be used as a beneficial probiotic supplement while *E. faecalis* may be

determined as a fish pathogen. In addition, *Enterococcus* species are mostly resistant to antimicrobial agents and they may cause pathogenicity when the host immunity weakens because of stress and other infections. There may be a contamination of farm water and food via free-living birds or others. All samples were determined as *E. faecium* via API 20 strep test kit but some of them, isolated from fish and food, had lower coverage so they may be fish pathogen *E. faecalis* or other *Enterococcus* species as well. Thus, further studies are needed to identify the *enterococci* species isolated from fish food and infected fish to improve our understanding of their pathogenicity or probiotic functions in aquaculture production.

CONFLICT OF INTEREST

The author state there are no conflicts of interest.

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