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Research article

Characterization of pathogenic *Lactococcus garvieae* isolated from farmed mullet (*Mugil cephalus*)

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Abstract

This study aimed to characterize pathogenic *Lactococcus garvieae* isolated from farmed mullets (*Mugil cephalus*) by studying their virulence, antimicrobial resistance, and heavy metal resistance properties and their significance for aquaculture production. A total of 95 strains of *L. garvieae* were isolated from 154 diseased mullets (*M. cephalus*). Virulence genes, including *hly1* (99.00%), *ENO* (98.95%), *hly3* (97.90%), *NADH oxidase* (97.90%), *LPxTG* 3 (95.80%), *AdhC11* (94.80%), *AdhPsav* (94.80%), *SOD* (92.70%), *PG* (92.64%), and *AdhPav* (90.60%) were detected. In the disc diffusion test, resistance to streptomycin (93.69%), nalidixic acid (80.10%), rifampicin (66.33%), amikacin (41.66%), cefoxitin (28.42%), and ampicillin (20.00%) was observed. A statistically significant difference was found in the variation frequency of antimicrobial resistance in *L. garvieae* isolates from different areas. Heavy metal tolerance testing of Cr, Pb, Cu, and Cd metals showed resistance in 22.10%, 21.00%, 20.00%, and 16.80% of isolates. Antimicrobial and heavy metal resistance genes, such as *aac*(3')-*IIa* (72.70%), *aac*(6')-*Ib* (56.80%), bla_{SHV} (4.20%), and *CzcA* (90.00%) were identified. According to our study, *L. garvieae* from farmed mullets exhibit significant virulence, antimicrobial resistance, and heavy metal resistance properties, which should not be disregarded.

Keywords: Antimicrobial resistance, Aquaculture, Fish pathogen, Lactococcosis, Virulence.

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INTRODUCTION

Mullet (*Mugil cephalus*) is a worldwide coastal species cultivated widely in Korea, Japan, Taiwan, and the Mediterranean areas. In South Korea, mullets are mostly eaten as sliced raw fish and as a substitute for sashimi. Mullets are also consumed as dried salted fish in Japan, Taiwan, and several other countries (Kim et al., 2013). Grey mullet (*M. cephalus*) and Red lip mullet (*Chelon haematocheilus*) can be found all along Korea's coastline, mainly in the Hadong and Yesou areas (Kim et al., 2013; Han et al., 2015). They have been frequently caught in different regions of Korea for human consumption. Mullets are also cultivated on farms to fulfill the high demand and are among the most treasured species in the aquaculture industry in Korea (FAO, 2020).

Lactococcus garvieae is a Gram-positive, non-motile bacterium and the etiological agent of lactococcosis, hyperacute, and hemorrhagic septicemia in fish (Altun et al., 2013). Symptoms of lactococcosis include erratic swimming, dark pigmentation, exophthalmos, internal congestion, and meningoencephalitis in fish (Vendrell et al., 2006). Damage induced by *L. garvieae* in aquaculture has been associated with high mortality, decreased growth and production rate, and increased treatment expenditures (Raissy and Ansari, 2011).

An outbreak of *L. garvieae* infection in red lip mullet has been reported in Korea (Han et al., 2015). There were epizootic outbreaks of lactococcosis in farmed rainbow trout (*Oncorhynchus mykiss*) in Greece, Iran, and Mexico in 2007, 2008, and 2020, respectively (Savvidis et al., 2007; Soltani et al., 2008; Ortega et al., 2020). A mortality outbreak of lactococcosis in farmed rainbow trout and brook trout (*Salvelinus fontinalis*) was also reported in Italy in 2019 (Pastorino et al., 2019). *L. garvieae* has also been reported to cause opportunistic infections in the circulatory, respiratory, and urinary systems of humans after consumption of seafood, suggesting that this bacterium has zoonotic potential (Wang et al., 2007; Li et al., 2008; Pérez-Sánchez et al., 2011).

In earlier studies, the presence of the exopolysaccharide (EPS) capsule is considered to play a central part in the virulence of *L. garvieae* (Morita et al., 2011; Soltani et al., 2014). However, recent studies indicate that several other virulence factors are also engaged in the pathogenicity of *L. garvieae* (Meyburgh et al., 2018). For example, *L. garvieae* secrete a set of putative virulence factors, in particular hemolysins, adhesion surface proteins, NADH (nicotinamide adenine dinucleotide phosphate) oxidase, and resistance to antimicrobials. These virulence factors have crucial functions in the pathogenesis of this bacterium (Ture and Altinok, 2016; Eraclio et al., 2018).

In aquaculture farms, antimicrobials are extensively applied to cure bacterial infections (Miyauchi et al., 2012). Frequently used antimicrobials to control *L. garvieae* infections in aquaculture are oxytetracycline, erythromycin, doxycycline, and amoxicillin. However, L. garvieae showed different degrees of sensitivity to these antimicrobials (Raissy and Ansari, 2011; Al Khaziri et al., 2018). Antimicrobial drugs in veterinary and human therapeutic practices can lead to antimicrobial contamination in coastal water environments in Korea, which can increase the number of antimicrobial-resistant bacteria in that specific region (Kang et al., 2016). Additionally, the release of agriculture, industrial, livestock, and human wastewater can pollute aquatic environments with heavy metals such as copper, zinc, cadmium, cobalt, and nickel (Trajanovska et al., 1997). These Heavy metals can accumulate in aquatic organisms, including fish pathogens (Ture et al., 2021). In response to accumulation, fish pathogenic bacteria may develop resistance to heavy metals and cause adverse effects on fish health (Cervantes et al., 1994). Therefore, monitoring heavy metal levels in fish pathogenic bacteria is crucial for protecting fish health.

In the scientific literature, no evidence exists regarding the virulence, antimicrobial resistance, and heavy metal resistance properties of pathogenic *L.*

garvieae isolated from mullets in Korea. Therefore, this study characterizes the genotypic and phenotypic properties of bacterial fish pathogen *L. garvieae* from farmed mullets.

MATERIALS AND METHODS

Collection of fish samples

From 2020 to 2021, 154 diseased mullet (*M. cephalus*) samples were collected during regular pathological examinations from fish farms in Korea's Hadong, Namhae, and Yeosu regions (Figure 1). Every fish was packed independently in poly bags and delivered to the laboratory in chilled conditions.

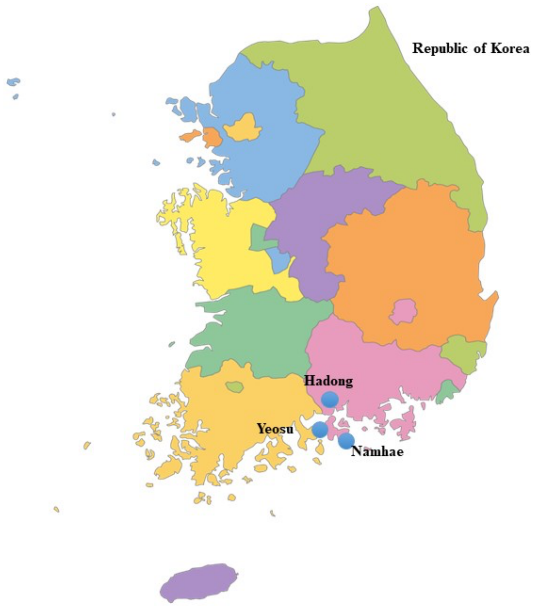

Figure 1 Sample collection areas of *L. garvieae*

Isolation and biochemical identification of *L. garvieae*

Bacteria were isolated from kidney and spleen tissues by direct aseptic streaking of organs on Trypticase soy agar (TSA; MB cell, Seoul, Korea) and 5% sheep blood agar (MB Cell, California, USA). The agar plates were incubated at 25°C for 24-48 h (Altun et al., 2013). Medium-sized single colonies from agar plates with pure cultured growth were restreaked again on brain heart infusion agar (BHIA; MB cell, Seoul, Korea) and incubated under the same conditions to obtain pure isolates (Karami et al., 2019).

Pure isolates were identified performing the following biochemical tests, including Gram staining and motility: the production of gelatinase, catalase, Simmons citrate, oxidase, indole, methyl red, and H2S, hydrolysis of starch, nitrate reduction, and oxidation-fermentation. Growth in brain heart infusion agar, nutrient agar, MacConkey agar, and TSA were examined. In addition, the ability to grow in 1%, 2.5%, 6.5%, and 8% NaCl and pH 9.6 was observed. Moreover, hemolysis in 5% of sheep blood agar was also measured (Altun et al., 2013).

Molecular identification of *L. garvieae* by PCR

Biochemically identified isolates were confirmed by species-specific PCR assay described by Duman et al. (2020). Each isolate's genomic DNA (gDNA) was

extracted using the AccuPrep® genomic DNA extraction kit (Bioneer, Seoul, Korea) following the manufacturer's procedure. 16S- rRNA gene (1100bp) was amplified by using species-specific primer pair pLG-1(F) (CATAACAATGAGAATCGC) and pLG-2(R) (GCACCCTCGCGGGTTG). The PCR assay was performed in a 50µL reaction mixture comprised of 1µM of each primer, 1µL of template DNA, 0.25 mM concentration of each deoxynucleoside triphosphate (dNTP), 0.5 U of Taq DNA polymerase (GeneAll, Seoul, South Korea), 5 µL of 10X Taq buffer with $MgCl_2$ and distilled water upto final volume (50 µL). The amplifications were performed in a MultiGene^{TM} Optimax thermal cycler (Labnet) with the following conditions: initial denaturation at 94°C for 2 min, denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 90 s (25 cycles). The final extension was done at 72°C for 5 min. Agarose gel 1.5% (W/V) was utilized to electrophorese in 1X TAE buffer with RedSafe™ (Intron Biotechnology) that was visualized under ultraviolet (UV) light. A DNA ladder (Cosmo Genetech, South Korea) of 100-bp was utilized as the molecular weight marker. This study considered the *L. garvieae* ATCC 49156 strain as a positive control strain (Kawanishi et al., 2005).

PCR amplification of virulence genes

Ten virulence-related genes of *L. garvieae* were retrieved using the conventional PCR method. The PCR test was conducted in the final volume of 20µL consisting of 2.0 µL of 10× Taq polymerase buffer, 1.6 µL of dNTP mix, 0.2 µL of AmpOne Taq DNA polymerase, 1.0 µL of each forward and reverse primers, 1.0 µL of template DNA, and 13.2 µL of PCR water. Gel electrophoresis was used to visualize the amplified PCR products on 1.5% (w/v) agarose gels.

The specific primers pair nucleotide sequences of each virulence gene are mentioned in Table 1. The following conditions were used for the amplification process. Pre-denaturation at 95°C for 2 min was followed by 28 cycles of denaturation at 95°C for 45 s, annealing at 53-60°C for 45 s, extension at 72°C for 1.5 min, and a final extension phase at 72°C for 7 min.

Antimicrobial resistance pattern

The disc diffusion test was performed to evaluate the antimicrobial resistance pattern of *L. garvieae* for the following antimicrobials: streptomycin (10 µg), gentamycin (10 µg), cefoxitin (30 µg), nalidixic acid (30 µg), meropenem (10 µg), rifampicin (5 mg), ceftriaxone (30 µg), colistin sulfate (10 µg), fosfomycin (50 µg), erythromycin (15 µg), ampicillin (10 µg), ofloxacin (5 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), amikacin (30 µg), oxytetracycline (30 µg), tetracycline (30 µg), amoxicillin (15 µg), and oxacillin (5 µg) (Oxoid LTD., Basingstoke, Hampshire, England). The test was performed on Muller-Hilton agar (Difco, Becton-Dickinson Co., Sparks, MD, USA). According to the method published by the Clinical and Laboratory Standards Institute (CLSI), the zone of inhibition and the resistance status of isolates were determined (CLSI, 2002; 2006; 2015). The number of antimicrobials to which isolates are resistant (a) was divided by the total number of antimicrobials tested (b) to determine the multiple antimicrobial resistance (MAR) index; MAR= a/b (Adenaike et al., 2016). *L. garvieae* ATCC 49156 and *L. garvieae* ATCC 49157 were quality control strains for antimicrobial resistance testing (Kawanishi et al., 2005).

Heavy metal tolerance testing

The isolates' heavy-metal resistance properties were evaluated against copper (Cu), chromium (Cr), cadmium (Cd), lead (Pb), and mercury (Hg) metals. $CuCl_2$, $CrCl_3$, $CdCl_2$, $PbCl_2$, and $HgCl_2$ metal chlorides (Samchun, Seoul, Korea) were used to acquire Cu, Cr, Cd, and Pb metal ions. The broth dilution testing method was performed to calculate the minimum inhibitory concentration (MIC) (microdilution) of heavy metal ions (He et al., 2016). Trypticase soy broth (TSB; MB cell, Seoul, Korea) was used to perform the test. Cu, Cr, Cd, and Pb concentration

series set out from 3,200 mg/mL to 6.25 mg/mL, while Hg concentration series ranged from 400 to 0.78 mg/mL were used. The results were measured after 24 h incubation at 37°C.

Detection of heavy metal resistance genes (HMRGs) and antimicrobial-resistance genes (ARGs)

Four heavy metal genes and thirteen antimicrobial resistance genes were used for evaluation. The primers pair nucleotide sequences and the annealing temperature of each gene are stated in Table 1. Each reaction mixture's PCR cycle conditions were created as follows: initial denaturation at 94°C for 2 min was followed by 35 amplification cycles. Each cycle included a 94°C denaturation phase lasting 30 s, an annealing phase lasting 50 s, and an extension phase lasting 1 min at 72°C. A 10-minute extension phase was used for the final cycle. Gel electrophoresis was performed on 1.5% (w/v) agarose gel to check the amplified PCR products.

Table 1 Oligo sequences and PCR conditions used for the investigation of virulence genes, antimicrobial-resistant genes, and heavy metal resistance genes

Category	Gene	Sequence (5′-3′)	Annealing temp (°C)	Amplicon size (bp)	Reference
Virulence genes					
Hemolysin	*hly I*	F: CCTCCTCCGACTAGGAACCA R: GAAAAGCCAGCTTCTCGTGC	54	521	Ture and Altinok (2016)
	hly III	F: TAGCACTTGTTTGGCTTTGTGC R: CCATAGATGGAGAACCACATCA	60	301	Eraclio et al. (2018)
Adhesin Pav	*adhPav*	F: GACACAGACCTTGCAGTCCA R: GATGACGGACTCATCAGGTG	59	1048	Teker et al. (2020)
Adhesin PsaA	*adhPsaA*	F: CGGGAAGGACCATGTTGATG R: AGTTGGGCTGGTGTACCTTG	59	552	Teker et al. (2020)
Adhesion Cluster2	*adhCII*	F: TGATTACACACCCAGCTCCA R: CTTTTTCCTAGCCCGAAAGC	57	732	Teker et al. (2020)
LPxTG-containing surface proteins	*LPxTG-3*	F: TTAAGCACAACGGCAACAGC R: CACGCGAAATGATGGTGCAT	55	231	Teker et al. (2020)
Superoxide dismutase	*SOD*	F: GCAGCGATTGAAAAACACCCA R: TCTTCTGGCAAACGGTCCAA	54	80	Ture and Altinok (2016)
Phosphoglucomutase	*PG*	F: AAGTTTACGGCGAAGACGGT R: TTTTCTGGTGCATTGGCACG	53	997	Ture and Altinok (2016)
Enolase	*ENO*	F: CAAGAGCGATCATTGCACGG R: CATTCGGACGCGGTATGGTA	54	201	Salighehzadeh et al. (2020)
NADH oxidase	*NADH oxidase*	F: TGCGATGGGTTCAAGACCAA R: GCCTTTAAAAGCCTCGGCAG	53	331	Salighehzadeh et al. (2020)
Antibiotic resistance genes					
Aminoglycoside resistance	*aphAI-IAB*	F: AAACGTCTTGCTCGA GGC R: CAAACCGTTATTCATTCGTGA	33	500	De Silva et al. (2022)
	aac(6′)-Ib	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	55	482	De Silva et al. (2022)
	strA	F: TTGATGTGGTGTCCCGCAATGC R: CCAATCGCAGATAGAAGGCAA	60	267	Raissy and Moumeni (2016)

Category	Gene	Sequence (5′-3′)	Annealing temp (°C)	Amplicon size (bp)	Reference
	aac(3′)-IIa	F: CGGAAGGCAATAACGGAG R: TCGAACAGGTAGCACTGAG	55	749	De Silva et al. (2022)
Plasmid-mediated quinolone resistance	*qnrB*	F: GATCGTGAAAGCCAGAAAGG R: ACGATGCCTGGTAGTTGTCC	53	496	De Silva et al. (2022)
	qnrS	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGCG	56	428	De Silva et al. (2022)
Tetracycline resistance	*tetA*	F: GTAATTCTGAGCACTGTCGC R: CTGCCTGGACAACATTGCTT	62	1000	Mouneimné et al. (1999)
	tetB	F: CTCAGTATTCCAAGCCTTTG R: CTAAGCACTTGTCTCCTGTT	58	400	De Silva et al. (2022)
	tetS	F: ATCAAGATATTAAGGAC R: TTCTCTATGTGGTAATC	60	590	Raissy and Moumeni (2016)
Extended-spectrum β-lactams	bla_{TEM}	F: ATGAGTATTCAACATTTCCG R: CAATGCTTAATCAGTGAGG	45	859	De Silva et al. (2022)
	bla_{SHV}	F: AGCCGCTTGAGCAAATTAAAC R: ATCCCGCAGATAAATCACCAC	58	795	De Silva et al. (2022)
	bla_{CTX-M}	F: GGTTAAAAAATCACTGCGTC R: TTGGTGACGATTTTAGCCGC	50	863	De Silva et al. (2022)
Macrolide resistance	*ermB*	F: AGACACCTCGTCTAACCTTCGCTC R: TCCATGTACTACCATGCCACAGG	60	640	Raissy and Moumeni (2016)
Heavy metal resistance genes					
Copper-transporting P-type ATPase	*CopA*	F: CGGTCTCTACGAATACCGCTT CAA R: GAAATAGCTCATTGCCGAGGC GT	55	1300	Bouskill et al. (2007)
Divalent metal cation efflux transporter	*CzcA*	F: GTTCACCTTGCTCTTCGCCATGTT R: ACAGGTTGCGGATGAAGGAGATCA	56	320	Bouskill et al. (2007)
Chromate resistance protein	*ChrA*	F: TGAAAAGCTGTTTACCCCACT R: TTACAGTGAAGGGTAGTCGGTATAA	54	350	Rahman et al. (2017)
Mercuric reductase	*merA*	F: GTGCCGTCCAAGATCATGAT R:TAGCCYACRGTSGCSACYTG	57	933	Dahanayake et al. (2019)

Statistical Analysis

The evaluation of the variation frequency of antibiotic resistance of *L. garvieae* isolates from fish farms located in Hadong, Namhae, and Yeosu areas in Korea was tested by ANOVA. The post hoc Tukey HSD test and LSD test were used for statistical evaluation of the incidence of the antibiotic resistance of *L. garvieae* strains among three different areas. Statistical analysis was performed in SPSS (IBM SPSS 29.0, USA), and the significance level was set at $P < 0.05$.

RESULTS

Biochemical and molecular identification of *L. garvieae*

From the 154 samples of mullet fish, 95 *L. garvieae* strains were identified and utilized in this study. All the isolates were non-motile, oxidase, and catalyze negative, Gram-positive cocci. Isolates showed no gelatin hydrolysis, citrate utilization, and indole production. The identified strains could grow at pH 9.6 and 6.5% NaCl. The phenotypic properties of biochemically identified *L. garvieae* isolated from mullet are mentioned in Table 2.

Isolates showed 1100-bp PCR amplification products were positive for the 16s RNA gene and were confirmed as *L. garvieae*.

Table 2 Phenotypic characteristics of *L. garvieae* strains isolated from cultured mullet in Korea

Characteristic	*L. garveaie*
Gram stain	+
Morphology	cocci
Motility	-
Gelatinase production	-
Catalase	-
Citrate utilization	-
Oxidase	-
Indole	-
Methyl red	+
H_2S	-
Starch hydrolysis	-
Nitrate reduction	-
Oxidation-fermentation	fermentation
Growth on	+
Brain heart infusion agar	+
Nutrient agar	+
TSA	+
MacConkey agar	+
Growth in	
1% NaCl	+
2.5% NaCl	+
6.5% NaCl	+
8% NaCl	-
Ph 9.6	+
Hemolysis in 5% sheep blood agar	α

Prevalence of virulence genes

Virulence-related genes, including *hly1* (99.00%), *ENO* (98.95%), *hly3* (97.90%), *NADH oxidase* (97.90%), *LPxTG* 3 (95.80%), *AdhC11* (94.80%), *AdhPsav*

(94.80%), *SOD* (92.70%), *PG* (92.64%), and *AdhPav* (90.60%) were detected (Table 3).

Table 3 Prevalence of virulence-related genes of *L. garvieae* isolated from cultured mullet in Korea

Virulence genes	No. of isolates	%
hly I	94	99.00
hly III	93	97.90
adhPav	86	90.60
adhPsaA	89	93.70
adhCII	90	94.80
LPxTG-3	91	95.80
SOD	88	92.70
PG	88	92.64
ENO	94	98.95
NADH oxidase	93	97.90

Antimicrobial resistance testing

Seventeen different antimicrobial drugs were used to assess antimicrobial resistance. *L. garvieae* isolates demonstrated resistance to several antimicrobial agents (The antimicrobial resistance profile of *L. garvieae* is shown in Figure 2). Resistance against streptomycin (93.69%), nalidixic acid (80.10%), rifampicin (66.33%), amikacin (41.66%), cefoxitin (28.42%), ampicillin (20.00%), fosfomycin (14.74%), gentamycin (12.63%), oxacillin (8.42%), ceftriaxone (5.20%), ciprofloxacin (4.21%), colistin sulfate (2.16%), and ofloxacin (2.10%) were observed. Meropenem and erythromycin showed susceptibility in all isolates. Streptomycin, nalidixic acid, and rifampicin were observed as the most resistant antimicrobials against *L. garvieae.* Cefoxitin showed intermediate resistance in 25.27% and complete resistance in 14.70% of isolates. Following the number of resistant antimicrobials by each isolate, 67% of isolates were recognized as multidrug-resistant (MAR index ≥0.2). The MAR index value ranged from 0.11 to 0.35 (Figure 3). A statistically significant difference was found in the variation frequency of antimicrobial resistance in *L. garvieae* isolates from three fish farms in Korea's Hadong, Namhae, and Yeosu areas ($P < 0.05$).

Prevalence of antimicrobial-resistant genes (ARGs)

The following antimicrobial-resistant genes belonging to the aminoglycoside group and extended-spectrum β-lactams group were found in PCR assays. The *aac*(3′)-*IIa* was the more dominant gene which was observed in 72.70% of isolates. Other antimicrobial-resistant genes identified in *L. garvieae* were *aac*(6′)-*Ib* (56.80%) and bla_{SHV} (4.20%).

Heavy-metal resistance and related genes (HMRGs)

Cr, Pb, Cu, and Cd metals showed resistance in 22.10%, 21.00%, 20.00%, and 16.80% of isolates. Hg resistance was not noticed in any of *L. garvieae* isolates. *CzcA* (cobalt/zinc/cadmium efflux protein) gene was identified in 90% of *L. garvieae* isolates.

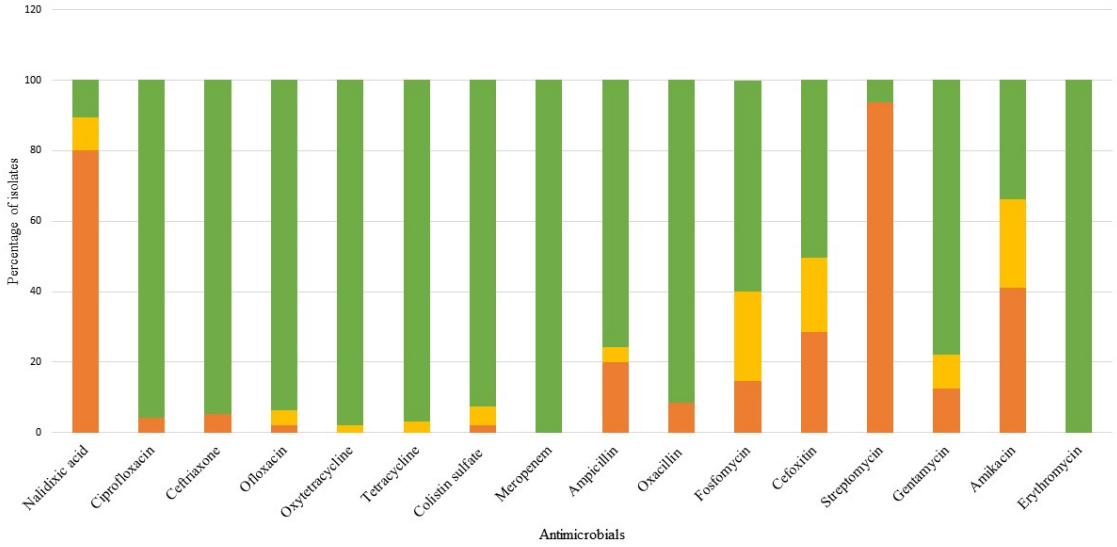

Figure 2 Antimicrobial susceptibility profile of *L. garvieae* isolated from cultured mullets (*Mugil cephalus*) in Korea. (Resistant Intermidediate resistant Suceptible)

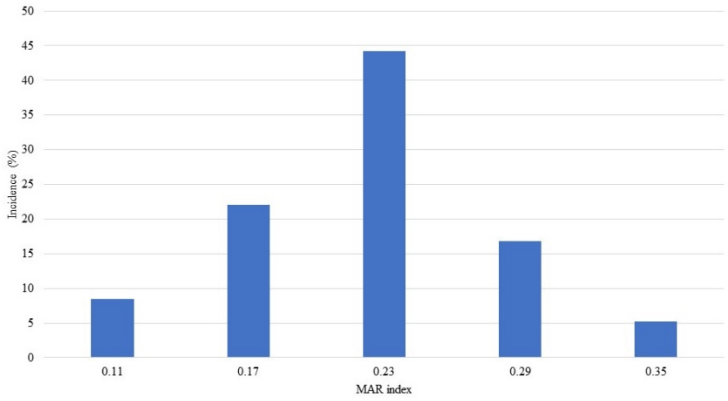

Figure 3 Antimicrobial resistance (MAR) index values in mullet-borne *L. garvieae*.

DISCUSSION

Mullets are economically important species but can harbor high levels of contaminants in their edible tissues. As fish are at the top of the aquatic food chain, environmental contaminants can transfer from water to fish and ultimately to humans, highlighting the critical need to monitor mullets to ensure public health (Ture et al., 2021). *L. garvieae* is a common fish pathogen that causes infection in cultured and wild fish species in many countries, including Spain, Taiwan, Turkey, and England (Vendrell et al., 2006). In Korea, an outbreak of *L. garvieae* infection was reported in red lip mullet displaying green liver syndrome (Han et al., 2015). In our study, a total of 95 *L. garvieae* strains were isolated from grey mullet raised in three regions of South Korea (Hadong, Namhae, and Yeosu). Their molecular

characterization was examined to determine health risks related to *L. garvieae* in mullets.

L. garvieae is a pathogenic bacterium with various virulence, antimicrobial, and heavy metal resistance genes (Ture et al., 2018; Teker et al., 2019). However, the pathogenic mechanisms of *L. garvieae* are not well known. The extracellular capsule is considered important for pathogenicity because it increases the bacterium's defiance to phagocytosis in fish (Ture and Altinok, 2016). The capsular gene cluster contains 17 genes, which is important for the virulence of *L. garvieae* (Miyauchi et al., 2012). In addition to the capsular gene cluster, *L. garvieae* possesses potential virulence factors, especially virulence genes that encode for hemolysin, NADH oxidase, adhesion surface proteins, and resistance to antimicrobials, which are essential to the pathogenicity of *L. garvieae* both in fish and humans (Eraclio et al., 2018). *L. garvieae* is an α-hemolytic bacterial species and harbors *hly1*, *hly2*, and *hly3* genes responsible for the hemolytic activity. Hemolysis plays an important role in pathogenesis due to its enzymatic activity. It causes the lysis of host erythrocytes and phagocytes by disrupting phospholipids' structure and establishing membrane pores (Goebel et al., 1988; Gibello et al., 2016). In this study, hly1 and hly2 genes were used to determine the hemolysis activity of isolates. The *hly1* gene was amplified in 99% of isolates, while the *hly3* gene was amplified in 97.90% of isolates, which agrees with a previous study where they detected the *hly1* gene in 99% of isolates. However, the *hly2* gene was observed in 76% of isolates (Teker et al., 2019). Shahi and Mallik (2020) reported the presence of *hly1* and *hly2* genes in *L. garvieae* isolated from rainbow trout. Adhesion properties are necessary for colonization and bacterial pathogenesis. Bacterial pathogens express different adhesions that are crucial in adhesion-based virulence (Klemm and Schembri, 2000). In the present study, *AdhPav*, *AdhC11*, and *AdhPsav* adhesion genes were expressed in more than 90% of the isolates. Ture and Altinok (2016) detected the *AdhPav*, and *AdhPsav* genes in all *L. garvieae* strains isolated in Turkey, while Teker et al. (2020) detected the *AdhPsav* gene in 100% and *AdhC11*, and *AdhPav* genes in 71% of *L. garvieae* isolates. Miyauchi et al. (2012) detected different types of adhesion genes in *L. garvieae* strains isolated from fish and humans.

LPxTG is a surface protein that covalently binds to peptidoglycan and plays an essential role in bacterial virulence (Mariscotti et al., 2012). We detect the *LPxTG3* gene in 95.80% of *L. garvieae* isolates. The presence of different types of *LPxTG* genes in *L. garvieae* was reported by Salighehzadeh et al. (2020) and Teker et al. (2020). PG and ENO (enolase) are immunogenic proteins found in the cell extract of *L. garvieae* and are also known as moonlighting proteins associated with the virulence of bacteria. Moonlighting proteins are subsets of multifunctional proteins that perform different biochemical functions in cells (Amblee and Jeffery, 2015). These proteins are also involved in plasminogen, a process of invading host tissue (Meyburgh et al., 2018). PG, ENO, and NADH oxidase are also considered metabolic enzymes (Salighehzadeh et al., 2020). In our study, *NADH oxidase*, *PG*, and *ENO* genes were expressed by more than 90% of isolates. Salighehzadeh et al. (2020) reported the existence of *NADH oxidase*, *PG*, and *ENO* genes in *L. garvieae* isolated from mugger crocodiles (*Crocodylus palustris*) and rainbow trout. Shin et al. (2009) found enolase and phosphoglucomutase in the cell extract of *L. garvieae*. Enolase has also appeared in *Streptococcus* spp. isolated from humans and fish (Bergmann et al., 2001; Kim et al., 2007).

SOD (Superoxide dismutase) are enzymes produced by the bacteria during infection to prevent them from being killed. In the present study, the *SOD* genes were produced by more than 90% of the isolates. Meyburgh et al. (2018) reported the presence of *NADH oxidase* and *SOD* genes in *L. garvieae* strains isolated from rainbow trout. The *SOD* gene was also identified in *L. garvieae* in two previous studies (Miyauchi et al., 2012; Shahi and Mallik, 2020). The presence of various virulence genes in *L. garvieae* isolated from mullet in our study indicates that these

strains are extremely virulent strains of *L. garvieae.* Moreover, the transfer of potentially virulent strains of *L. garvieae* to humans through seafood consumption can be a threat to public health because of its zoonotic potential (Wang et al., 2007; Lin et al., 2020).

L. garvieae infections in aquaculture resulted in economic losses (Colorni et al., 2003; Radosavljević et al., 2020). Different antimicrobials are used to decrease the occurrence of illnesses. However, the overuse of antimicrobials to control infections has resulted in antimicrobial resistance (Raissy and Moumeni, 2016). The results of our study provide relevant information about the development of antimicrobial resistance in *L. garvieae* and efficient and safe antimicrobials for the cure of *L. garvieae* infections. *Streptococcus* spp. showed susceptibility to β lactams (Tras et al., 2007). In this study, *L. garvieae* showed different percentages of susceptibility for β lactams. Meropenem showed susceptibility in all isolates. Kav and Erganis (2008) reported that *L. garvieae* was resistant to oxacillin. However, in our study, oxacillin and colistin were susceptible in more than 90% of isolates. Ampicillin showed susceptibility in 75% of isolates. Diler et al. (2002) and Karami et al. (2019) informed ampicillin susceptibility in *L. garvieae.* Cefoxitin and fosfomycin showed susceptibility. Kawanishi et al. (2005) also reported the susceptibility of *L. garvieae* against fosfomycin. However, in our study, fosfomycin also showed complete resistance in 28.42% and intermediate resistance in 21.28 % of isolates. The development of resistance in bacterial isolates suggests that if fosfomycin usage is not controlled in fish farms, *L. garvieae* may lose its susceptibility to fosfomycin in the future.

L. garvieae showed susceptibility to erythromycin. Similar results were reported by Diler et al. (2002), Kav and Erganis (2008), and Al Khaziri et al. (2018) in *L. garvieae* isolated from rainbow trout. Susceptibility to erythromycin in *L. garvieae* suggests that erythromycin can be considered to treat *L. garvieae* infection, but should be use with care. While Raissy and Ansari (2011) reported that *L. garvieae* isolated from rainbow trout showed resistance to erythromycin. This resistance might be due to the misuse of antimicrobials in that area and the difference among *L. garvieae* isolates (Raissy and Ansari, 2011).

When aminoglycosides were evaluated, *L. garvieae* resistance to streptomycin was observed in high frequency. Karami et al. (2019) reported streptomycin resistance in *L. garvieae.* High resistance to streptomycin showed that this might be due to its usage in high doses, unnecessary repetition of antimicrobial therapy, and failure to achieve the treatment period (Karami et al., 2019). Gentamycin showed sensitivity in 77.96% of isolates. The high sensitivity of *L. garvieae* to gentamycin demonstrates the value of this antimicrobial in the fight against lactococcosis. Al Khaziri et al. (2018) and Pérez-Sánchez et al. (2011) reported that *L. garvieae* is sensitive to gentamycin. In contrast, Kav and Erganis (2008) and Raissy and Ansari (2011) found that *L. garvieae* showed no susceptibility to gentamycin, which may be caused by differences in antimicrobial usage in the area, resulting in the development of resistance to gentamycin in those isolates (Kav and Erganis, 2008). Amikacin showed susceptibility in 33.68% of isolates and resistance in 41.06% of isolates. The development of resistance in some isolates suggests that misuse of amikacin may lead to failure of amikacin activity against *L. garvieae.*

L. garvieae showed susceptibility to tetracycline and oxytetracycline. Kav and Erganis (2008) and Al Khaziri et al. (2018) reported that *L. garvieae* was susceptible to oxytetracycline. Due to the susceptibility of oxytetracycline in *L. garvieae,* this drug is commonly used on aquaculture farms (Karami et al., 2019). AST (Antibiotic susceptibility testing) of quinolones against *L. garvieae* showed susceptibility for ceftriaxone, ciprofloxacin, and ofloxacin. In contrast, *L. garvieae* showed resistance against nalidixic acid. Pérez-Sánchez et al. (2011) reported resistance of *L. garvieae* to nalidixic acid in different species of lactococcus. Statistical analysis of the variation frequency of antimicrobial resistance of *L.*

garvieae isolates from three different areas showed significant differences, suggesting different antimicrobial treatments are often applied in aquaculture on a prophylactic basis in medicated feed. The use of medicated feed leaches antimicrobials into the sediment and water, leading to the horizontal transfer of antimicrobial resistance genes (Guglielmetti et al., 2009). This may account for antimicrobial-resistant *L. garvieae* isolates found in our study. The antimicrobial resistance of *L. garvieae* has been related to the unnecessary use of antimicrobials on aquaculture farms (Raissy and Moumeni, 2016). It may be proposed from the outcomes that the utilization of antimicrobials should be used with standard treatment guidelines in fish farms to prevent and control the spread of antimicrobial-resistant bacteria in aquaculture and humans.

Environmental contaminants pose a significant threat to bacterial populations, as they can develop resistance when exposed to high concentrations of heavy metals (Trajanovska et al., 1997). *L. garvieae* showed complete resistance against copper, chromium, lead, and cadmium in almost 20% of isolates. Bacteria develop resistance against copper and cadmium by developing an efflux system to remove these metals (Trevors, 1997). In contrast, all isolates show sensitivity against mercury. The discharge of wastewater mainly stimulates resistance development due to heavy metal contaminants (Hubeny et al., 2021). In our study, more than 75% of isolates showed sensitivity against heavy metals, suggesting that the water supply to the cultured mullet fish has a very low amount of wastewater addition.

Using antimicrobial agents against infection leads to the acquisition of antimicrobial resistance and the development of ARGs. ARGs can be shifted from non-pathogenic bacteria to pathogenic bacteria in fish and humans. ARG presence can limit the efficacy of antimicrobial treatment in fish disease (Shah et al., 2012). This study observed ARGs and HMRGs in *L. garvieae* isolated from fish. The *aac*(3′)-*IIa*, and *aac*(6′)-*Ib* genes encoding aminoglycosides were found more frequently than other ARGs. The bla_{SHV} gene, which encodes β lactams, was also found in a few isolates. Ture et al. (2018) reported β lactams genes in *L. garvieae* isolated from fish. The presence of different antimicrobial resistance genes in *L. garvieae* isolated from rainbow trout was also reported by Shahi and Mallik (2020).

Although *L. garvieae* isolate did not show resistance against heavy metals phenotypically, 90% of isolates revealed the presence of the *czcA* gene (which encodes for cadmium, zinc, and cobalt efflux pump). Deredjian et al. (2011) reported no association between the *czcA* gene presence and the phenotypic resistance to heavy metals in outdoor and hospital strains of *Pseudomonas aeruginosa*. Heavy metals like Cd, Cu, and Zn have been used in veterinary medicine as animal growth promoters (Liang et al., 2011; Yu et al., 2017). While bacteria also need these elements for growth, excessive quantities can be toxic to them. Bacteria can develop resistance to heavy metals in high contaminant levels in aquatic environments and transfer this resistance to others (Trajanovska et al., 1997; Zhang et al., 2018). Consequently, the therapeutic and prophylactic uses of metal ions may contribute to the acquisition of metal resistance genes (MRGs) in bacteria (Liang et al., 2011; Yu et al., 2017). The existence of virulence genes and the distribution of antimicrobial and heavy metal resistance in isolated *L. garvieae* is a great concern for aquaculture and public health. This also shows the significance of managing human activities to decrease the spread of antimicrobial and heavy metal resistance in aquaculture environments.

CONCLUSIONS

To the best of our knowledge, this is the first study to molecularly characterize *L. garvieae* isolated from cultivated mullet. Our findings highlight the presence of virulence genes in *L. garvieae* strains from farmed mullet, indicating its

potential virulence and relevance to aquaculture and human health. Given the public health importance of this bacterium, the observed antimicrobial and heavy metal resistance in *L. garvieae* suggests a need for ongoing attention in aquaculture, the seafood industry, and veterinary medicine. Therefore, further research is encouraged to enhance our understanding and management of *L. garvieae* infections and their transmission to fish and humans.

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AUTHOR CONTRIBUTIONS

Sana Majeed and Gang-Joon Heo contributed to the conception and design of the study. Material preparation, data collection, and analysis were performed by Sana Majeed, L.A.D.S. De Silva, P.M. Kumarage, and Gang-Joon Heo. The first draft of the manuscript was written by Sana Majeed, and all authors contributed to reviewing and editing the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

There is no conflict of interest to report on the part of the authors.

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