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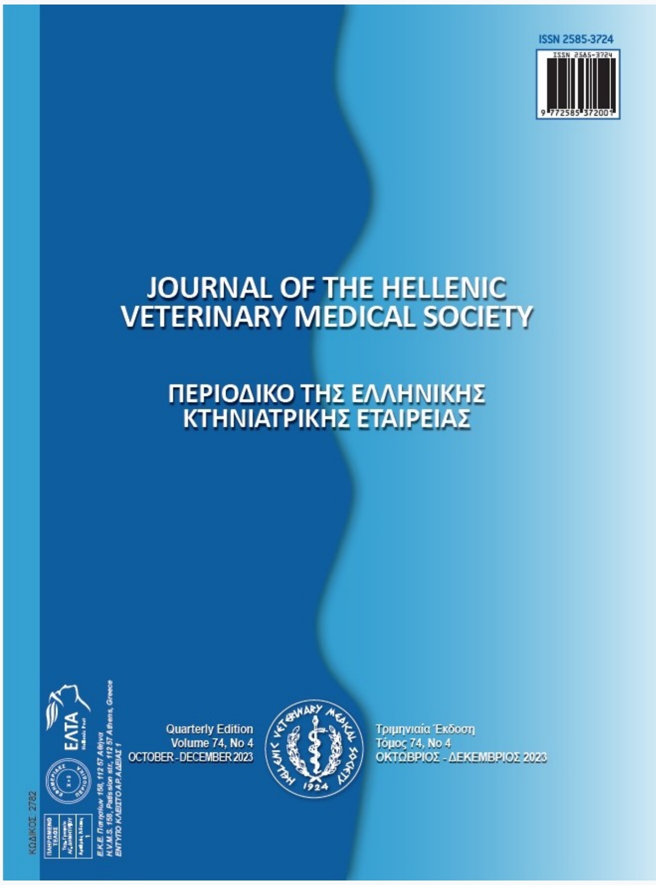

Presence of genes encoding aminoglycoside-modifying enzyme (AME) and virulence factors in high-level aminoglycoside-resistant (HLAR) Enterococcus strains isolated from retail chicken meat in Turkey

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Presence of genes encoding aminoglycoside-modifying enzyme (AME) and virulence factors in high-level aminoglycoside-resistant (HLAR) *Enterococcus* strains isolated from retail chicken meat in Türkiye

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ABSTRACT: In this study, the presence of aminoglycoside-modifying enzyme (AME) and virulence factor genes were investigated in previously isolated 32 high-level aminoglycoside-resistant (HLAR) *Enterococcus* strains isolated from retail chicken meat in Türkiye. At least one AME-encoding gene was detected in HLAR enterococci by polymerase chain reaction (PCR). The *ant(6')-Ia* was identified as the most prevalent (87.5%, 28/32) AME gene. The *aph(3')-IIIa* (78.13%, 25/32), *ant(4')-Ia* (68.75%, 22/32), *aph(2'')-Ib* (62.5%, 20/32), *aac(6')-Ie-aph(2'')-Ia* (21.88%, 7/32) and *aph(2'')-Ic* (9.38%, 3/32) are the other detected AME-encoding genes in strains. The *aph(2'')-Id* was found in none of the HLAR strains. The *aph(2'')-Ib* and *ant(6')-Ia* were identified as the most frequently AME-encoding genes in high-level gentamicin-resistant (HLGR) and high-level streptomycin-resistant (HLSR) strains, respectively. All HLAR strains showed α-hemolytic activity except *E. durans* MG13.4 and *E. casseliflavus* MGM111.1, which were exhibited β- and γ-hemolytic activity, respectively. Among the 32 HLAR strains, only *E. faecalis* MSE61.1 and *E. avium* MSE63.1 were found capable of hydrolyzing gelatine. It was determined that all HLAR strains, except *E. durans* MGE13.1 and MGE63.1, contain at least one virulence factor gene. The $efaA_{fm}$ (87.5%, 28/32), *acm* (65.63%, 21/32) and *gelE* (37.5%, 12/32) were found to be the most prevalent virulence factor genes. HLAR enterococci strains that have the virulence factor genes may pose a risk to consumer health.

Keywords: Chicken meat; *Enterococcus*; high-level aminoglycoside resistant**;** aminoglycoside-modifying enzyme; virulence factor gene

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INTRODUCTION

Enterococci are lactic acid bacteria that are present in the natural microflora of the human and animal gastrointestinal tracts (Guzman Prieto et al., 2016). In addition, they have been used as starter or adjunct cultures for centuries in the production of various fermented foods (Hugas et al., 2003; M'hir et al., 2012). However, they have also been known as important nosocomial pathogens since the 1970s, are multi-drug resistant, and have a wide range of virulence factors (Arias and Murray, 2012). Enterococci show natural resistance to many antibiotics, as well as easily gain resistance to clinically important different groups of antibiotics, increasing the pathogenicity of these bacteria (Yoğurtçu and Tuncer, 2013; Abauelnaga et al., 2016). As a result of the inability of aminoglycosides to penetrate the cell wall of enterococci, these bacteria have a moderate intrinsic low-level resistance to them (Hollenbeck and Rice, 2012). In clinical practice, aminoglycoside antibiotics such as gentamicin and streptomycin are frequently utilized (Özdemir and Tuncer, 2020). In recent years, it has been reported that high-level of gentamicin (HLGR) (MIC≥500 µg/mL) and streptomycin-resistant (HLSR) (MIC≥2000 µg/mL) enterococci have been isolated from clinical samples (Niu et al., 2016), ready-to-eat meat samples (Chajęcka-Wierzchowska et al., 2016), raw milk (Özdemir and Tuncer, 2020; Kang et al., 2021), dairy products (Chajęcka-Wierzchowska et al., 2020; Özdemir and Tuncer, 2020) and retail chicken meat (Choi and Woo, 2013; Kim et al., 2018; Kim et al., 2019; Yalçın and Tuncer, 2021). In enterococci, gains of genes that code for AMEs such as phosphotransferases, acetyltransferases, and nucleotidyltransferases result in high-level acquired resistance to aminoglycosides (Guzman Prieto et al., 2016). AAC(6′)-APH(2″), a bifunctional AME encoded by the *aac(6')-Ie-aph(2'')-Ia* gene, mediates high-level gentamicin resistance. The monofunctional AME encoding genes *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id* are also responsible for high-level gentamicin resistance. The *ant(6')-Ia* gene encoding Ant(6')-Ia, an adenylyltransferase, is responsible for high-level streptomycin resistance (Hollenbeck and Rice, 2012). Other monofunctional AME-encoding genes found in enterococci include *aph(3')-IIIa* and *ant(4')-Ia*. The *aph(3')-IIIa* gene encodes an aminoglycoside phosphotransferase, Aph(3')-IIIa, which confers resistance to kanamycin and neomycin, whereas the *ant(4')-Ia* gene encodes a nucleotidyltransferase, Ant(4'')-Ia, which confers resistance to tobramycin, amikacin, neomycin, and kanamycin (Hauschild et al., 2008; Hollenbeck and Rice, 2012).

The cause of infections due to enterococci is the presence of surface factors that affect the colonisation of host cells and secretory agents that damage the tissues. Virulence factors have a role in the pathogenesis of enterococcal infections by mediating adhesion, colonization, and invasion into host tissues, as well as modulating host immunity, which increase the infection's severity (Sava et al., 2010; Chajęcka-Wierzchowska et al., 2017). The main virulence factors identified in enterococci species include i) surface factors; aggregation protein (*agg*), collagen-binding protein (*ace*, *acm*), cell wall adhesins ($efaA_{fm}$, $efaA_{fs}$), extracellular surface protein (esp_{fm}, esp_{fs}), ii) secretory agents; cytolysin (*cylM*, *cylB*, *cylA*), gelatinase (*gelE*), hyaluronidase (*hyl*) and iii) sex pheromones (*cpd*, *cob*, *ccf*, *cad*) (Chajęcka-Wierzchowska et al., 2017; Akpınar and Tuncer, 2022).

There is limited information on the prevalence of AME and virulence factor genes in HLAR *Enterococcus* strains isolated from retail chicken meat in Türkiye. Therefore, this study aimed to investigate the genes encoding AMEs (*aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *ant(4')-Ia*, *ant(6')-I*, aph*(3')-IIIa*) and virulence factors (*agg*, $efaA_{fm}$, $efaA_{fs}$, esp_{fm}, esp_{fs}, *ace*, *acm*, *cylM*, *cylB*, *cylA*, *gelE*, *hyl*, *cpd, cob, ccf*, *cad*) in HLAR *Enterococcus* strains isolated from retail chicken meat in Türkiye.

MATERIAL AND METHODS

HLAR *Enterococcus* strains

A total of 32 HLAR *Enterococcus* strains previously isolated from 112 retail chicken meat samples (39 chicken wings, 37 chicken breasts, 20 chicken legs, 16 chicken drumsticks) were used in this study. According to MIC test results for gentamicin and streptomycin, one, 13 and 18 of 32 HLAR *Enterococcus* strains were found to be HLGR, HLSR and both HLGR and HLSR, respectively. The 32 HLAR isolates were identified as *Enterococcus* species by conventional tests and genus-specific polymerase chain reaction (PCR). The identification of the 18 *E. faecium*, five *E. faecalis*, five *E. durans,* and one *E. casseliflavus* strains were done species-specific by PCR while three *E. avium* strains were identified by 16S rDNA sequence analysis (Yalçın and Tuncer, 2021). Stock cultures of 32 HLAR strains were stored in antibiotic-containing de man Rogosa and Sharpe (MRS) broth with the addition of sterile glycerol at -32 °C.

Genomic DNA extraction

Genomic DNA from HLAR strains was extracted according to the method of Cancilla et al. (1992). The 500 µL of overnight cultures of HLAR isolates were centrifuged at 15.493 x *g* for 5 minutes in 2 mL microcentrifuge tubes. The pellets were resuspended in the same volume of lysis buffer and incubated at 37 °C for 30 minutes. Then 30 µL of SDS (10%, w/v) was added to the tubes and incubated at 80 °C for 5 minutes. After incubation, 700 µL of phenol: chloroform (1:10, v/v) was added to the tubes and centrifuged at 15.493 x *g* for 5 minutes. The upper phase was taken by micropipette and transferred to new tube. The 700 µL of propan2-ol (Merck, Darmstadt, Germany) was added to the tubes for the precipitation of nucleic acids. Nucleic acids were pelleted by centrifugation at 15.493 x *g* for 5 minutes and dissolved in 50 µL of Tris-EDTA buffer (pH 8.0).

Detection of AME-encoding genes

Detection of AME-encoding genes in HLAR *Enterococcus* was done using specific primers by PCR according to Vakulenko et al. (2003) and Niu et al. (2016). AME genes, primers, product sizes and PCR protocols are given in Table 1. PCR was performed in 50 µL PCR mixture prepared by adding 25 µL of PCR master mix (Thermo Scientific, USA), 20 µL nuclease-free water, 3 µL of template DNA and 1 µL of each primer to 0.2 mL of PCR tube (Thermo Scientific). PCR was carried out in gradient thermal cycler (TurboCyler 2 Blue-Ray Biotech Ltd., Taiwan). The agarose gel electrophoresis of amplified products was done on agarose gels (2%, w/v) in Tris-acetate-EDTA buffer at 85 V for 90 minutes. The gels were stained with ethidium bromide (0.2 µg/mL), visualized on a UV transliminator (Vilber Lourmat, France), and photographed via a digital camera (D5100 Nikon Inc., Japan). The aminoglycoside-resistant strains *E. faecalis* ATCC 51299 (*aac(6')-Ie-aph(2'')-Ia*$^{+}$, *ant(6')-Ia*$^{+}$, *aph(3')-IIIa*$^{+}$) and *E. faecium* ATCC 51599 (*ant(6')-Ia*$^{+}$, *aph(3')-IIIa*$^{+}$) were used as positive controls. The aminoglycoside-susceptible strain *E. faecalis* ATCC 29212 was used as a negative control.

Hemolytic and gelatinase activities

Hemolytic activity of HLAR *Enterococcus* strains was detected on sheep blood agar (Liofilchem, Roseto degli Abruzzi, Italy). Petri dishes were incubated at 37 °C for 48 hours. The hemolytic reaction was evaluated as β (clear zone formation around the colony), α (fuzzy greenish zone formation) or γ (non-zone formation) hemolytic activity (Cariolato et al., 2008). β-hemolytic *S. aureus* ATCC 25923 was used as a control strain.

HLAR *Enterococcus* strains were cultured overnight and then transferred on Todd-Hewitt agar medium (Liofilchem) containing 3% gelatine (Merck) and incubated at 37 °C for 24 hours. After, Petri dishes were kept refrigerated at 4 °C for 5 hours. The presence of opaque zones surrounding the colonies was evaluated as a positive result (Eaton and Gasson, 2001). Gelatinase positive *E faecalis* NYE7 was used as a control strain (Inoğlu and Tuncer, 2013).

Detection of virulence factor genes

The virulence factor genes encoding aggregation protein (*agg*), cell wall adhesins ($efaA_{fm}$, $efaA_{fs}$), cell wall-associated protein (esp_{fm}, esp_{fs}), collagen-binding protein (*ace*, *acm*), cytolysin (*cylM, cylB*, *cylA*), gelatinase (*gelE*), hyaluronidase (*hyl*) and sex pheromones (*cpd, cob, ccf*, *cad*) were investigated in HLAR *Enterococcus* strains by PCR (Eaton and Gasson, 2001; Vankerckhoven et al., 2004; Reviriego et al., 2005; Camargo et al., 2006; Ben Belgacem et al., 2010). Virulence factor genes, primers, product sizes and PCR protocols are given in Table 1. PCR was performed in 50 µL reaction mixtures prepared as described above. The PCR products were verified on 2% (w/v) agarose gel and stained as described above. The *E. faecalis* ATCC 29212 (*agg*$^{+}$, $efaA_{fm}{}^{+}$, $efaA_{fs}{}^{+}$, $esp_{fm}{}^{+}$, $esp_{fs}{}^{+}$, *ace*$^{+}$, *acm*$^{+}$, *cylM*$^{+}$, *cylB*$^{+}$, *cylA*$^{+}$, *gelE*$^{+}$, *hyl*$^{+}$, *cpd*$^{+}$, *cob*$^{+}$, *ccf*$^{+}$, *cad*$^{+}$) was used as a positive control strain.

RESULTS

Detection of AME-encoding genes

In this study, it has been determined that HLAR *Enterococcus* strains contain between one and five AME-encoding genes (Table 2). The most prevalent AME-encoding genes in HLAR *Enterococcus* strains was found to be *aph(3')-IIIa* (78.13%, 25/32) and *ant(6')-Ia* (75.00%, 24/32) (Figure 1). These genes were followed by *ant(4')-Ia* (68.75%, 22/32), *aph(2'')-Ib* (62.50%, 20/32), *aac(6')-Ie-aph(2'')-Ia* (21.88%, 7/32) and *aph(2'')-Ic* (9.38%, 3/32). However, *aph(2'')-Id* gene was not detected in any of the HLAR strains. The distribution of AME-encoding genes in HLAR *Enterococcus* strains is given in Table 3. Twelve distinct AME-encoding gene pattern types were discovered in HLAR *Enterococcus* strains using PCR amplification products. The most common

AME-encoding gene pattern type of HLAR *Enterococcus* strains was type I (34.38%, 11/32) included *aph(3')-IIIa*, *ant(4')-Ia*, *ant(6')-Ia*, and *aph(2'')-Ib* genes.

Hemolytic and gelatinase activities

The hemolytic and gelatinase activities of strains were phenotypically tested. The results of the hemolytic activity test revealed that the majority (93.75%, 29/31) of the HLAR *Enterococcus* strains exhibited α-hemolytic activity on sheep blood agar. On the other hand, *E. durans* MGE13.4 and *E. casseliflavus* MGM111.1 strains showed β- and γ-hemolytic activities, respectively. Among the 32 HLAR *Enterococcus* strains, only *E. faecalis* MSE61.1 and *E. avium* MSE63.1 showed gelatinase activity on Todd-Hewitt agar.

Detection of virulence factor genes

The presence of virulence factor genes in HLAR enterococci strains were investigated by PCR using specific primers. The results of the PCR detection

Table 1. Primers sequences and PCR protocols used for detection of AME* and virulence factor** genes

Genes	Primers sequence (5′ to 3′)	Product size (bp)	PCR protocol	References
aph(3′)-IIIa*	f: GGCTAAAATGAGAATATCACCGG r: CTTTAAAAAATCATACAGCTCGCG	523	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
ant(4')-Ia*	f: CAAACTGCTAAATCGGTAGAAGCC r: GGAAAGTTGACCAGACATTACGAACT	294	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
ant(6')-Ia*	f: ACTGGCTTAATCAATTTGGG r: GCCTTTCCGCCACCTCACCG	577	94 °C for 3 min x1; 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s x35; 72 °C for 5 min x1	Niu et al. (2016)
aac(6')-Ie-aph(2'')-Ia*	f: CAGGAATTTATCGAAAATGGTAGAAAAG r: CACAATCGACTAAAGAGTACCAATC	369	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
aph(2'')-Ib*	f: CTTGGACGCTGAGATATATGAGCAC r: GTTTGTAGCAATTCAGAAACACCCTT	867	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
aph(2'')-Ic*	f: CCACAATGATAATGACTCAGTTCCC r: CCACAGCTTCCGATAGCAAGAG	444	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
aph(2'')-Id*	f: GTGGTTTTTACAGGAATGCCATC r: CCCTCTTCATACCAATCCATATAACC	641	94 °C for 3 min x1; 94 °C for 40 s, 55 °C for 40 s, 72 °C for 40 s x35; 72 °C for 2 min x1	Vakulenko et al. (2003)
$efaA_{fm}^{**}$	f: AACAGATCCGCATGAATA r: CATTTCATCATCTGATAGTA	735	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
$efaA_{fs}^{**}$	f:GACAGACCCTCACGAATA r: AGTTCATCATGCTGTAGTA	705	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
cad**	f: TGCTTTGTCATTGACAATCCG r: ACTTTTTCCCAACCCCTCAA	1299	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
ccf**	f: GGGAATTGAGTAGTGAAGAAG r: AGCCGCTAAAATCGGTAAAAT	543	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
cpd**	f: TGGTGGGTTATTTTTCAATTC r: TACGGCTCTGGCTTACTA	782	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
cob**	f: AACATTCAGCAAACAAAGC r: GCGTCATAAAGAGTGGTCAT	1405	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
esp_{fm}^{**}	f: TTGCTAATGCAAGTCACGTCC r: GCATCAACACTTGCATTACCGAA	955	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
esp_{fs}^{**}	f: TTGCTAATGCTAGTCCACGACC r: GCGTCAACACTTGCATTGCCGAA	933	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
ace**	f: AAAGTAGAATTAGATCCACAC r: TCTATCACATTCGGTTGCG	350	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Ben Belgacem et al. (2010)
acm**	f: GGCCAGAAACGTAACCGATA r: CGCTGGGGAAATCTTGTAAA	353	95 °C for 5 min x1; 95 °C for 30 s, 52 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Camargo et al. (2006)
gelE**	f: ACCCCGTATCATTGGTTT r: ACGCATTGCTTTTCCATC	419	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
agg**	f: AAGAAAAAGAAGTAGACCAAC r: AAACGGCAAGACAAGTAAATA	1553	95 °C for 5 min x1; 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s x35 ; 72 °C for 10 min x1	Eaton and Gasson, (2001)
cylM**	f: CTGATGGAAAGAAGATAGTAT r: TGAGTTGGTCTGATTACATTT	742	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
cylB**	f: ATTCCTACCTATGTTCTGTTA r: AATAAACTCTTCTTTTCCAAC	843	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
cylA**	f: TGGATGATAGTGATAGGAAGT r: TCTACAGTAAATCTTTCGTCA	517	95 °C for 5 min x1; 95 °C for 30 s, 54 °C for 30 s, 72 °C for 60 s x35; 72 °C for 10 min x1	Reviriego et al. (2005)
hyl**	f: ACAGAAGAGCTGCAGGAAATG r: GACTGACGTCCAAGTTTCCAA	276	95 °C for 2 min x1; 95 °C for 30 s, 56 °C for 90 s, 72 °C for 90 s x35; 72 °C for 10 min x1	Vankerckhoven et al. (2004)

Table 2. Isolation material, antibiotic resistance patterns, AME-encoding genes and virulence factor genes in HLAR *Enterococcus* strains

Strain	Isolation material	Antibiotic resistance[a]	MIC[a,b]		AME genes	Virulence factor genes
			CN	S		
E. durans MGE13.1	Chicken breast	DO, E, CN, QD, MH, F, S, TE	>4096^{R}	4096^{R}	*aac(6′)-Ie-aph(2″)-Ia*	-
E. durans MGE13.2	Chicken breast	E, CN, QD, MH, F, S, TE	4096^{R}	4096^{R}	*ant(4′)-Ia*, *aac(6′)-Ie-aph(2″)-Ia*	*gelE*, *efaA*$_{fm}$, *efaA*$_{fs}$, *ccf*, *cad*, *cylA*
E. durans MGE13.3	Chicken breast	E, CN, QD, MH, F, S, TE	>4096^{R}	2048^{R}	*aac(6′)-Ie-aph(2″)-Ia*	*efaA*$_{fm}$, *cad*
E. durans MGE13.4	Chicken breast	E, CN, QD, MH, F, S, TE	>4096^{R}	2048^{R}	*aac(6′)-Ie-aph(2″)-Ia*	*efaA*$_{fm}$, *acm*, *cad*
*E. faecium*MSM14.1	Chicken breast	DO, E, CN, LEV, MH, RD, S, TE	4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$
E. faecium MSE31.1	Chicken wing	DO, E, CN, MH, F, NOR, P, CIP, S, TE	1024^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. faecium MSE53.1	Chicken breast	DO, E, CN, C, MH, S, TE	4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*	*efaA*$_{fm}$, *acm*, *agg*
E. faecalis MSM53.1	Chicken breast	DO, E, CN, LEV, QD, MH, NOR, CIP, S, TE	4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fs}$, *cpd*, *cob*, *ccf*, *cad*
E. faecium MSE58.1	Chicken breast	DO, E, CN, C, LEV, MH, NOR, CIP, S, TE	>4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *ace*, *acm*
E. faecalis MGE58.1	Chicken breast	DO, E, CN, C, MH, NOR, P, CIP, S, TE	>4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecalis MSE61.1	Chicken leg	DO, E, CN, QD, MH, S, TE	>4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fs}$, *cpd*, *cob*, *ccf*, *cad*
E. durans MGE63.1	Chicken wing	E, CN, C, QD, S	>4096^{R}	>4096^{R}	*ant(4′)-Ia*	-
E. avium ME63.1	Chicken wing	E, CN, C, LEV, QD, MH, F, NOR, CIP, S, TE	4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aac(6′)-Ie-aph(2″)-Ia*, *aph(2″)-Ic*	*gelE*, *efaA*$_{fm}$, *efaA*$_{fs}$, *ccf*, *cad*, *cylA*
E. avium MSE63.2	Chicken wing	CN, MH, S, TEC	2048^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *aph(2″)-Ib*, *aph(2″)-Ic*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSM76.1	Chicken wing	DO, E, LEV, MH, NOR, CIP, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecalis MSM 93.1	Chicken wing	AMP, DO, E, C, LEV, LZD, QD, MH, NOR, P, RD, CIP, S,TEC, TE,VA	128	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*,	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSM95.1	Chicken wing	DO, E, LEV, MH, CIP, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSM103.1	Chicken leg	AMP, DO, E, MH, RD, S, TE	64	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSM104.1	Chicken leg	DO, E, MH, S, TE	64	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. faecium MSE104.1	Chicken leg	DO, E, CN, LEV, MH, S, TE	>4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSE104.2	Chicken leg	DO, E, CN, LEV, MH, NOR, CIP, S, TE	>4096^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*	*efaA*$_{fm}$, *acm*
E. faecium MSM105.1	Chicken leg	DO, E, QD, MH, CIP, S, TE	64	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fm}$, *acm*
E. faecium MSM106.1	Chicken wing	DO, E, MH, RD, S, TE	64	>4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. faecium MSM107.1	Chicken leg	DO, E, MH, CIP, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*gelE*, *efaA*$_{fm}$, *acm*, *cylA*
E. faecium MSM108.1	Chicken leg	DO, E, MH, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. faecium MSM109.1	Chicken breast	DO, E, MH, RD, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*	*efaA*$_{fm}$, *cpd*, *ccf*, *acm*, *cylA*
E. faecium MSM110.1	Chicken wing	DO, E, MH, NOR, RD, CIP, S, TE	32	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. casseliflavus MGM111.1	Chicken wing	E, CN, C, MH, TE	4096^{R}	64	*ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$
E. avium MSE111.1	Chicken wing	E, CN, MH, S, TE	512^{R}	4096^{R}	*aph(3′)-IIIa*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$
E. faecium MSE111.2	Chicken wing	E, LEV, QD, MH, S, TE	256^{R}	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*	*efaA*$_{fm}$, *acm*
E. faecalis MSM112.1	Chicken drumstick	DO, E, QD, MH, NOR, CIP, S, TE	16	2048^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$, *acm*
E. faecium MSM112.2	Chicken drumstick	DO, E, LEV, MH, RD, CIP, S, TE	64	>4096^{R}	*aph(3′)-IIIa*, *ant(4′)-Ia*, *ant(6′)-Ia*, *aph(2″)-Ib*	*efaA*$_{fm}$

[a] These data were taken from a previous study by Yalçın & Tuncer (2021). AMP, Ampicillin; C, Chloramphenicol; CIP, Ciprofloxacin; DO, Doxycycline; E, Erythromycin; CN, Gentamicin; LEV, Levofloxacin; LZD, Linezolid; MH, Minocycline; NOR, Norfloxacin; P, Penicillin G; QD, Quinupristin/dalfopristin; RD, Rifampin; S, Streptomycin; TE, Tetracycline; TEC, Teicoplanin; VA, Vancomycin.
[b] Antibiotics were diluted in a concentration range of 0.125 to 4096 µg/mL. Susceptibility or resistance of HLAR *Enterococcus* were determined according to the guidelines of CLSI (2016) and EUCAST (2018).

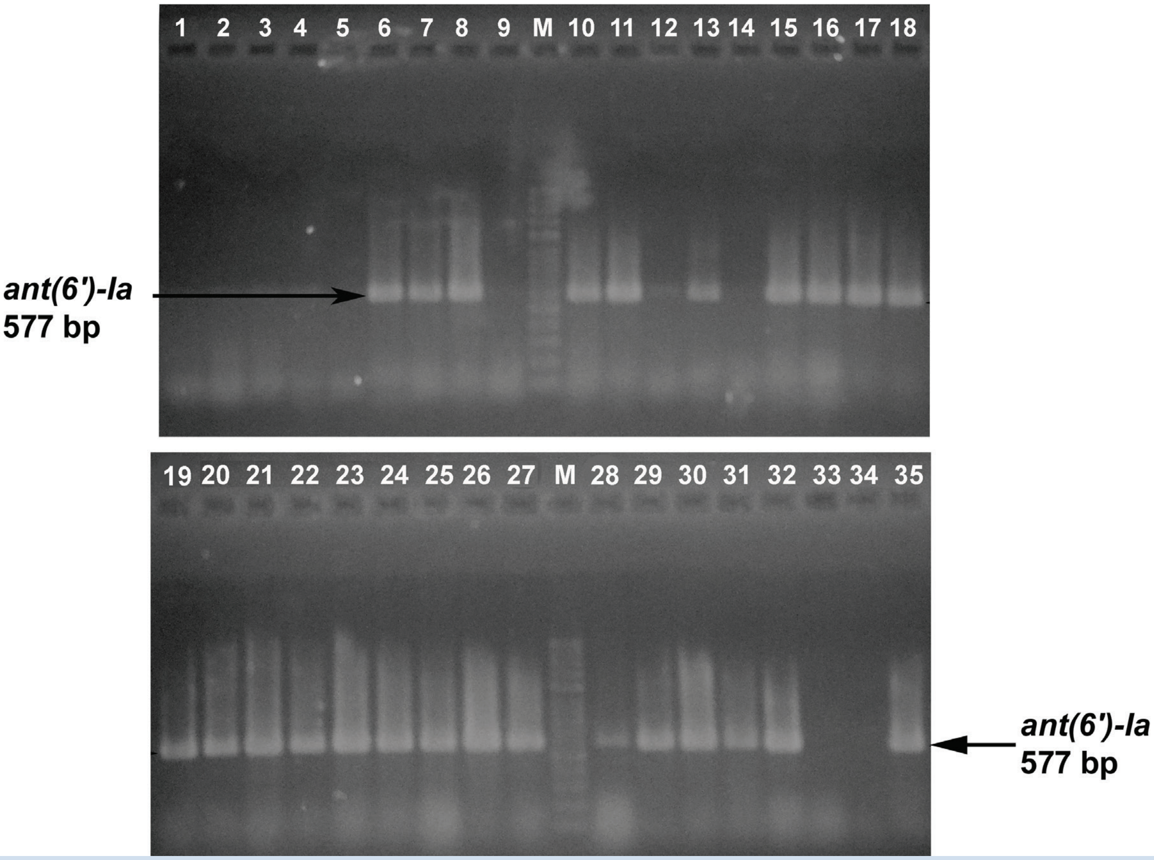

Figure 1. PCR amplification of *ant(6')-Ia* in HLAR *Enterococcus* strains. Line 1: *E. durans* MGE13.1; line 2: *E. durans* MGE13.2; line 3: *E. durans* MGE13.3; line 4: *E. durans* MGE13.4; line 5: *E. faecium* MSM14.1; line 6: *E. faecium* MSE31.1; line 7: *E. faecium* MSE53.1; line 8: *E. faecalis* MSM53.1; line 9: *E. faecium* MSE58.1; line 10: *E. faecalis* MGE58.1; line 11: *E. faecalis* MSE61.1; line 12: *E. durans* MGE63.1; line 13: *E. avium* MSE63.1; line 14: *E. avium* MSE63.2; line 15: *E. faecium* MSM76.1; line 16: *E. faecalis* MSM 93.1; line 17: *E. faecium* MSM95.1; line 18: *E. faecium* MSM103.1; line 19: *E. faecium* MSM104.1; line 20: *E. faecium* MSE104.1; line 21: *E. faecium* MSE104.2; line 22: *E. faecium* MSM105.1; line 23: *E. faecium* MSM106.1; line 24: *E. faecium* MSM107.1; line 25: *E. faecium* MSM108.1; line 26: *E. faecium* MSM109.1; line 27: *E. faecium* MSM110.1; line 28: *E. casseliflavus* MGM111.1; line 29: *E. avium* MSE111.1; line 30: *E. faecium* MSE111.2; line 31: *E. faecalis* MSM112.1; line 32: *E. faecium* MSM112.2; line 33: *E. faecalis* ATCC 29212 (negative control); line 34: negative control (water) (negative control); line 35: *E. faecium* ATCC 51559 (positive control); line M: 100 bp DNA ladder (Thermo Scientific)

Table 3. AME-encoding gene pattern types and distributions of AME-encoding gene patterns in HLAR *Enterococcus* strains

AME-encoding gene pattern type	**AME-encoding genes**	***E. faecium* (*n*= 18)**	***E. faecalis* (*n*= 5)**	***E. durans* (*n*= 5)**	***E. avium* (*n*= 3)**	***E. casseliflavus* (*n*= 1)**	**Total (*n*= 32)**
		***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**	***n* (%)**
I	*aph(3')-IIIa+ant(4')-Ia+ant(6')-Ia+aph(2'')-Ib*	8 (44.44%)	3 (60%)				11 (34.38%)
II	*aph(3')-IIIa+ant(4')-Ia+ant(6')-Ia*	5 (27.78%)					5 (15.63%)
III	*aph(3')-IIIa+ant(6')-Ia+aph(2'')-Ib*	3 (16.67%)	1 (20%)		1 (33.33%)		5 (15.63%)
IV	*ant(6')-Ia+aac(6')-Ie-aph(2'')-Ia*			3 (60%)			3 (9.38%)
V	*ant(4')-Ia*			1 (20%)			1 (3.13%)
VI	*aph(3')-IIIa+ant(6')-Ia*		1 (20%)				1 (3.13%)
VII	*ant(4')-Ia+ant(6')-Ia+aph(2'')-Ib*					1 (100%)	1 (3.13%)
VIII	*ant(4')-Ia+ant(6')-Ia+aac(6')-Ie-aph(2'')-Ia*			1 (20%)			1 (3.13%)
IX	*aph(3')-IIIa+aac(6')-Ie-aph(2'')-Ia+aph(2'')-Ib*	1 (5.56%)					1 (3.13%)
X	*aph(3')-IIIa+ant(4')-Ia+aac(6')-Ie-aph(2'')-Ia+aph(2'')-Ib*	1 (5.56%)					1 (3.13%)
XI	*aph(3')-IIIa+ant(4')-Ia+aph(2'')-Ib+aph(2'')-Ic*				1 (33.33%)		1 (3.13%)
XII	*ant(4')-Ia+ant(6')-Ia+aac(6')-Ie-aph(2'')-Ia+aph(2'')-Ic*				1 (33.33%)		1 (3.13%)

of virulence factor genes in HLAR enterococci are shown in Table 2. HLAR strains, except *E. durans* MGE13.1 and MGE63.1, contain at least one virulence factor gene. The *efaA*$_{fm}$ (87.5%, 28/32), *acm* (65.63%, 21/32) and *gelE* (37.5%, 12/32) were found to be the most prevalent virulence factor genes in HLAR strains. In addition, the *cad*, *ccf*, *cylA*, *efaA*$_{fs}$, *cpd*, *cob*, *agg* and *ace* were found in 18.75% (6/32), 16.63% (5/32), 12.5% (4/32), 12.5% (4/32), 9.38% (3/32), 6.25% (2/32), 3.13% (1/32) and 3.13% (1/32) of strains, respectively. The other virulence factor genes, *esp*$_{fm}$, *esp*$_{fs}$, *cylM*, *cylB* and *hyl*, were not detected in any of the strains used in this study.

DISCUSSION

Enterococci are frequent contaminants of poultry meat due to the lower hygienic standards in poultry slaughtering (Bortolaia et al., 2016). Previous studies reported that different *Enterococcus* species are isolated from retail chicken meat samples in USA (Donabedian et al., 2003), South Korea (Kim et al., 2018; Kim et al., 2019) and Türkiye (Yılmaz et al., 2016; Onaran et al., 2019; Yalçın and Tuncer, 2021). It was showed that some of these strains are HLAR (Donabedian et al., 2003; Kim et al., 2019; Yalçın and Tuncer, 2021). The presence of AME-encoding genes is the primary cause of high-level aminoglycoside resistance (Niu et al., 2016). The high-level streptomycin resistance in enterococci is generally associated with *ant(6')-Ia* and *aph(3')-IIIa* genes (Niu et al., 2016; Özdemir and Tuncer, 2020). In our study, 27 of 31 HLSR *Enterococcus* strains carried at least one of these two AME-encoding genes. Similar to our results, the *ant(6')-Ia* and/or *aph(3')-IIIa* are the most commonly detected in HLSR enterococci isolated from clinical samples (Niu et al., 2016), ready-to-eat meat samples (Chajęcka-Wierzchowska et al., 2016), retail chicken meat (Kim et al., 2019), and raw milk and traditional Turkish cheeses (Özdemir and Tuncer, 2020). On the other hand, the presence of *ant(6')-Ia* and/or *aph(3')-IIIa* genes was not detected in four *E. durans* MGE13.1, MGE13.2, MGE13.3 and MGE13.4 strains that were phenotypically high-level streptomycin resistant. The high-level streptomycin resistance in these strains is thought to result from a different AME-encoding mechanism (Ramirez and Tolmasky, 2010; Peyvasti et al., 2020). Moreover, the *ant(6')-Ia* gene was found in HLGR *E. casseliflavus* MGM111.1 strain although it was phenotypically susceptible to streptomycin. Similar to our result, Choi and Woo (2013) reported that one of their HLGR enterococci isolates was susceptible to streptomycin even though it was carrying *ant(6')-Ia*.

The *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id* genes are responsible for high-level gentamicin resistance in enterococci (Niu et al., 2016; Shete et al., 2017). In our study, it was determined that 16 out of 19 HLGR *Enterococcus* strains contained at least one of the *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib* and *aph(2'')-Ic* genes. The most common detected AME-encoding gene among these three genes in HLGR *Enterococcus* strains was found as the *aph(2'')-Ib* (11/19, 57.90%). The *aph(2'')-Ib* gene was followed by *aac(6')-Ie-aph(2'')-Ia* (7/19, 36.84%) and *aph(2'')-Ic* (3/19, 15.79%) genes. Contrary to our results, recent research has shown that high-level gentamicin resistance in enterococci is predominantly associated with the presence of bifunctional *aac(6')-Ie-aph(2'')-Ia* gene (Choi and Woo, 2013; Li et al., 2015; Jaimee and Halami, 2016; Niu et al., 2016; Shete et al., 2017; Amini et al., 2018; Ramin et al., 2018; Kim et al., 2019; Peyvasti et al., 2020). On the other hand, Özdemir and Tuncer (2020) indicated that none of the 54 HLGR enterococci strains did contain *aac(6')-Ie-aph(2'')-Ia* or *aph(2'')-Ib* genes but 10 of 54 HLGR strains contained *aph(2'')-Ic* gene. The *aph(2'')-Id* gene was detected none of the 32 HLAR *Enterococcus* strains, as previously reported by Choi and Woo (2013), Padmasini et al. (2014), Shete et al. (2017), Amini et al. (2018) and Özdemir and Tuncer (2020). However, the presence of a low rate of *aph(2'')-Id* gene in HLAR enterococci isolated from various sources was reported by different researchers (Donabedian et al., 2003; Harada et al., 2004; Jackson et al., 2004; Li et al., 2015; Chajęcka-Wierzchowska et al., 2016; Nowakiewicz et al., 2017).

Gelatinase is an extracellular metalloendopeptidase encoded by the *gelE* gene located on chromosome. Potentially contributing to virulence, this enzyme hydrolyzes gelatin, elastin, collagen, hemoglobin and other bioactive peptides (Chajęcka-Wierzchowska et al., 2017). Previous researches showed that gelatinase activity is more commonly detected in both clinical and food isolates of *E. faecalis* than other enterococci species (Eaton and Gasson, 2001; Semedo et al., 2003). In this study, gelatinase activity was detected in only two HLAR *Enterococcus* strains. To our knowledge, there are limited study investigating gelatinase activity in HLAR enterococci strains (Han et al., 2011; Adifon and Tuncer, 2019). Contrary to our results, Han et al. (2011) high frequently detected gelatinase activity in HLAR enterococci isolated from broiler feces in South Korea. However, Adifon and Tuncer (2019) reported that only three *E. faecalis* strains among 54 HLAR enterococci isolated from traditional Turkish Tulum cheeses showed gelatinase activity, as confirmed in this study.

Hemolysin/cytolysin, a bacterial toxin, is one of the virulence factors secreted by enterococci. Hemolytic activity, which can be encoded by plasmid or chromosomal DNA, plays an important role in increasing the severity of infection. β-hemolytic activity is mostly observed in clinical isolates of *E. faecium* and *E. faecalis* species. It is not desirable to isolate enterococci with β-hemolytic activity from foods (Semedo et al., 2003). In this study, only the *E. durans* MGE13.4 strain was found to have β-hemolytic activity, while the majority of the HLAR *Enterococcus* strains had α-hemolytic activity (93.75%). Similar to our results, Adifon and Tuncer (2019) reported that only three of 54 (5.55%) HLAR enterococci strains from traditional Turkish cheeses were shown β-hemolytic activity. Researchers also stated that 46.30% (25/54) and 48.15% (26/54) of their HLAR isolates were γ- and α-hemolytic, respectively.

The most common virulence factor genes in HLAR *Enterococcus* strains were found to be *efaA*$_{fm}$ (87.5%, 28/32), *acm* (65.63%, 21/32) and *gelE* (37.5%, 12/32). Similar to our results, Kim et al. (2019) reported that HLAR *E. faecium* and *E. faecalis* strains isolated from retail chicken meat in South Korea contain mostly *efaA* and *gelE* genes. Researchers also stated that *ace* (a collagen-binding protein) and *asa1* (aggregation substance) were the other most common detected virulence genes in their HLAR isolates, as conversely to our results. In our study, although the *ace* gene was detected very low rate in HLAR enterococci strains, distinct collagen-binding protein-encoding gene *acm* was detected in 21 of 32 strains. Also, different researchers stated that *efaA* (26.7% and 85.19%) and *gelE* (33.3% and 59.2%) genes were commonly found in HLAR enterococci isolated from clinical samples (Niu et al., 2016) and traditional cheeses (Adifon and Tuncer, 2019), respectively. In addition, Adifon and Tuncer (2019) indicated that *ccf* (88.89%), *acm* (77.78%), *cpd* (51.85%) and *esp*$_{fs}$ (50%) were other most prevalent virulence factor genes in their HLAR enterococci isolates. In another similar study, Kang et al. (2021) reported that HLAR *E. faecalis* strains isolated from bulk tank milk in Korea exhibited a high prevalence of virulence genes such as *ace* (99.5%), *efaA* (98.9%), *cad1* (98.4%), *gelE* (85.9%), and *asa1* (61.6%).

CONCLUSION

This study indicates that HLAR *Enterococcus* strains isolated from retail chicken meat in Türkiye contain various AME-encoding genes and virulence factor genes. The *aph(2'')-Ib* and *aph(3')-IIIa/ant(6')*-Ia genes were found to be the most common AME-encoding genes in HLGR and HLSR enterococci strains, respectively. At least one virulence factor gene was detected in HLAR enterococci strains, except *E. durans* MGE13.1 and MGE63.1. The most prevalent virulence factor genes in HLAR strains were detected as *efaA*$_{fm}$, *acm* and *gelE*. In conclusion, HLAR enterococci strains containing the AME-encoding genes may serve as reservoirs for the spread of high-level aminoglycoside resistance among bacteria. In addition, HLAR enterococci strains that have the virulence factor genes may pose a risk to consumer health.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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