



## Antimicrobial Resistance and Diversity of *Lactococcus garvieae* by Genomic Approaches

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Geliş/Received: 28.07.2021

Kabul/Accepted: 04.11.2021

Yayın/Published: 31.12.2021

How to cite: Kumru, S. (2021). Antimicrobial Resistance and Diversity of *Lactococcus garvieae* by Genomic Approaches. *J. Anatolian Env. and Anim. Sciences*, 6(4), 506-513.

Atf yapmak için: Kumru, S. (2021). *Lactococcus garvieae*'nin Genomik Yaklaşımlarla Çeşitliliği ve Antimikrobiyal Direnci. *Anadolu Çev. ve Hay. Dergisi*, 6(4), 506-513.

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**Abstract:** *Lactococcus garvieae* is the Gram-positive cocci bacteria known as the causative agent of infectious systemic disease. It causes fatal hemorrhagic septicemia in mainly cultured fish species, animals, and humans worldwide. Comparative genome analyses provide valuable information about genome identification and unique genomic features. In this study, the available *L. garvieae* genomes are evaluated by comparative genomics approaches. The results indicated that there are four distinct genetic groups of *L. garvieae* based on the Average Nucleotide Identity (ANI) value. The phylogenetic tree that was produced using the 16S rRNA sequence has similar genetic variances with the publicly available *L. garvieae* genome data on NCBI and supports the ANI value. *In silico* analysis of antimicrobial resistance revealed that each *L. garvieae* genome groups have unique antimicrobial resistance class genes, even though, all genomes have a common antibiotic-resistant class. Analyzing results of the antimicrobial resistance supports the *L. garvieae* genetic variations. This extensively comparative approach will provide new insights into the understanding of *L. garvieae* genomic diversity and antimicrobial resistance

**Keywords:** Antibiotic resistance, comparative genomics, *Lactococcus garvieae*, phylogenetic tree.

## *Lactococcus garvieae*'nin Genomik Yaklaşımlarla Çeşitliliği ve Antimikrobiyal Direnci

**Öz:** *Lactococcus garvieae*, bulaşıcı sistemik hastalığın etkeni olarak bilinen Gram pozitif kok bir bakteridir. Dünya çapında ağırlıklı olarak yetiştiriciliği yapılan balık türlerinde, hayvanlarda ve insanlarda ölümcül hemorajik sepsisemiye neden olmaktadır. Karşılaştırmalı genom analizleri, genomların tanımlanması ve benzersiz genomik özellikler hakkında bilgiler sağlarlar. Bu çalışmada, mevcut *L. garvieae* genomları karşılaştırmalı genomik yaklaşımlarla değerlendirilmiştir. Sonuçlar, Ortalama Nükleotid Kimliği (ANI) değerine dayalı olarak *L. garvieae*'nin dört farklı genetik gruba sahip olduğunu göstermektedir. Filogenetik ağaç, 16S rRNA genom bölgesi dizisi kullanılarak üretilmiş, NCBI üzerindeki halka açık *L. garvieae* genom verileriyle benzer genetik farklılıklara sahip olarak belirlenmiş ve ANI değerini desteklemiş olacak şekilde oluşmuştur. Yapılan antimikrobiyal dirençli genlerin *in silico* analizi ile tüm genomların ortak antimikrobiyal dirençli gen sınıfına sahip olmasına rağmen, her *L. garvieae* genom grubunun benzersiz antimikrobiyal direnç sınıfı genlerine sahip olduğunu ortaya çıkarmıştır. Yapılan antimikrobiyal direnç analiz sonuçları, *L. garvieae* genetik varyasyonlarını desteklemektedir. Bu kapsamlı karşılaştırmalı genom analizi yaklaşımı, *L. garvieae*'nin genomik çeşitliliği ve antimikrobiyal direncin anlaşılmasına yönelik yeni anlayışlar ortaya koymaktadır.

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**Anahtar kelimeler:** Antibiyotik direnci, karşılaştırmalı genomik, *Lactococcus garvieae*, filogenetik ağaç.

## INTRODUCTION

*Lactococcus garvieae* is the Gram-positive cocci bacteria that were previously considered part of the *Streptococcus* genus. It is the causative agent of Lactococcosis, which is known as an infectious systemic disease (Vendrell, 2006; Varsha, 2016). *L. garvieae* was firstly isolated from cows with mastitis and later characterized phenotypically and genetically as *L. garvieae* (Teixeira, 1996). “*Lactococcus*” genus has substantial importance to dairy and farm industry; it was reported that *L. garvieae* was isolated from goat cheese, raw cow milk, poultry, pork meat, and beef (Gibello, 2016; Sahu, 2019). In the 1950s, *L. garvieae* was determined on rainbow trout (*Oncorhynchus mykiss*) farms in Japan as well (Hoshina, 1958). It is a well-known pathogen for aquatic animals like fish, crustaceans, and mollusks; it has mainly devastated the cultured salmonid species and other fish species such as yellowtail (*Seriola quinqueradiata*) and grey mullet (*Mugil cephalus*) (Vendrell, 2006). Likewise, in different countries, human infections have been stated due to *L. garvieae* by ingestion of raw seafood, and *L. garvieae* has been isolated from human skin ulcer, blood, and urine (Vela, 2000; Wang, 2007; Wilbring, 2011). Thus, *L. garvieae* should be considered as a potential zoonotic agent because of causing disease in humans and different animals (Vela, 2000; Kawanishi, 2006; Tsai, 2013).

Even though first *L. garvieae* cases had been reported earlier, the *L. garvieae* strain 21881 genome, isolated from humans, was firstly sequenced and released in 2011 (Aguado-Urda, 2011). Recently, fish, humans, animals, and environmental isolate *L. garvieae* genomes have been released on the National Center for Biotechnology Information (NCBI). Comparative analysis of whole genomes makes available valuable information to identify unique genomic features (Zhang et al., 2014; de Vries et al., 2017). Thus, the availability of several *L. garvieae* genomes, isolated from different sources and locations, provides an excellent opportunity to determine genome features and biological structures (Morita, 2011).

Marker genes' phylogenetic inference are mainly used for microbial taxonomy activities and classification; on the other hand, with the improved whole genome sequencing technology, the genomic sequence has been suggested for species identification and antimicrobial susceptibility testing (Ellington, 2017; Tsai, 2019) Identification of predicted antibiotic-resistant elements has important to understand the control/treatment of infection. In this study, 29 *L. garvieae* genomes that were publicly available on NCBI as of February 2020 are evaluated. Even though there are valuable studies on antibiotic-resistant elements of *L. garvieae*, this is the first report of an extensive genomic approach on all *L. garvieae* strains. This study provides new insights into the

understanding of *L. garvieae*' genomic variations and antibiotic resistance elements.

## MATERIAL AND METHODS

**Bacterial genomes and annotation:** *Lactococcus garvieae* genomes' data were obtained from the National Center for Biotechnology Information (NCBI). 29 genomes have been reported as *L. garvieae* (as of 02/02/2020). NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP) and Rapid Annotation using Subsystem Technology (RAST) annotation pipeline were used to conduct genome analysis (Angiuoli, 2008; Overbeek, 2014)

**The average nucleotide identity (ANI) value and phylogenetic tree analysis:** To show the genetic similarity between genomes, the Average Nucleotide Identity (ANI) value (Goris, 2007) was calculated by using ANI calculator software (Rodriguez-R, 2016; Yoon, 2017) with default features. Based on genomic BLAST of the 29 *L. garvieae* genomes, a dendrogram was obtained from the NCBI genome database. In addition, MEGA X (Kumar, 2018) was used to construct a phylogenetic tree with 16S rRNA region by using the neighbor-joining method (Saitou, 1987). The 16S rRNA sequence of *L. garvieae* genomes was downloaded from NCBI. *L. garvieae* strain UBA5784 and strain UBA11300 genomes do not include the 16S rRNA sequence region, so they are missing in constructed 16S rRNA phylogenetic tree.

**Antimicrobial resistant elements:** Antibiotic-resistant elements of 29 *L. garvieae* genomes were identified by using ARG-ANNOT (Antibiotic Resistance Gene-Annotation) (Gupta, 2014) with uploading annotated protein files. Duplicates were removed. A cutoff E value of  $10^{-20}$  was considered important (Kumru, 2020; Tekedar, 2019).

## RESULTS

**Genome features:** *Lactococcus garvieae* genomes are used in this study, and their features are listed in Table 1. Genome sizes of *L. garvieae* strains range between 1.950 Mb to 2.239 Mb, and G+C ratios are from 37.7% to 38.9%.

**The average nucleotide identity (ANI) value and phylogenetic tree analysis:** The ANI values between the 29 *L. garvieae* genomes are listed in Table 2. Based on ANI value, there are four different genetic groups. In the first group, ANI values between strains KS1546, 21881, M14, TB25, Lg-ilsanpaik-gs201105, MGYG-HGUT-00230, PAQ102015-99, and 8831 are bigger than 97%. In the second group, ANI values between strains 122061 and I113 are bigger than 97%. In the third group, ANI values between strains RTCLI04, M79, LG9, UNIUD074, UBA5784,

IPLA31405, UBA11300, Tac2, ATCC 49156, ATCC 49156 acyDJ, ATCC 49156 acpMN, JJN1, Lg2, DSM 20684, NBRC 100934, and CCUG 32208T are bigger than 97%. In the fourth group, ANI values between strains A1 and DCC43 are bigger than 98%. Between the groups, ANI values are less than 95%. The groups include subgroups as well. Obtained two phylogenetic tree were shown in Figure 1. ANI values supported the phylogenetic tree, which was obtained

from NCBI based on genomic BLAST. The phylogenetic tree, which was constructed with 16S rRNA region, showed similar results with ANI value and another phylogenetic tree.

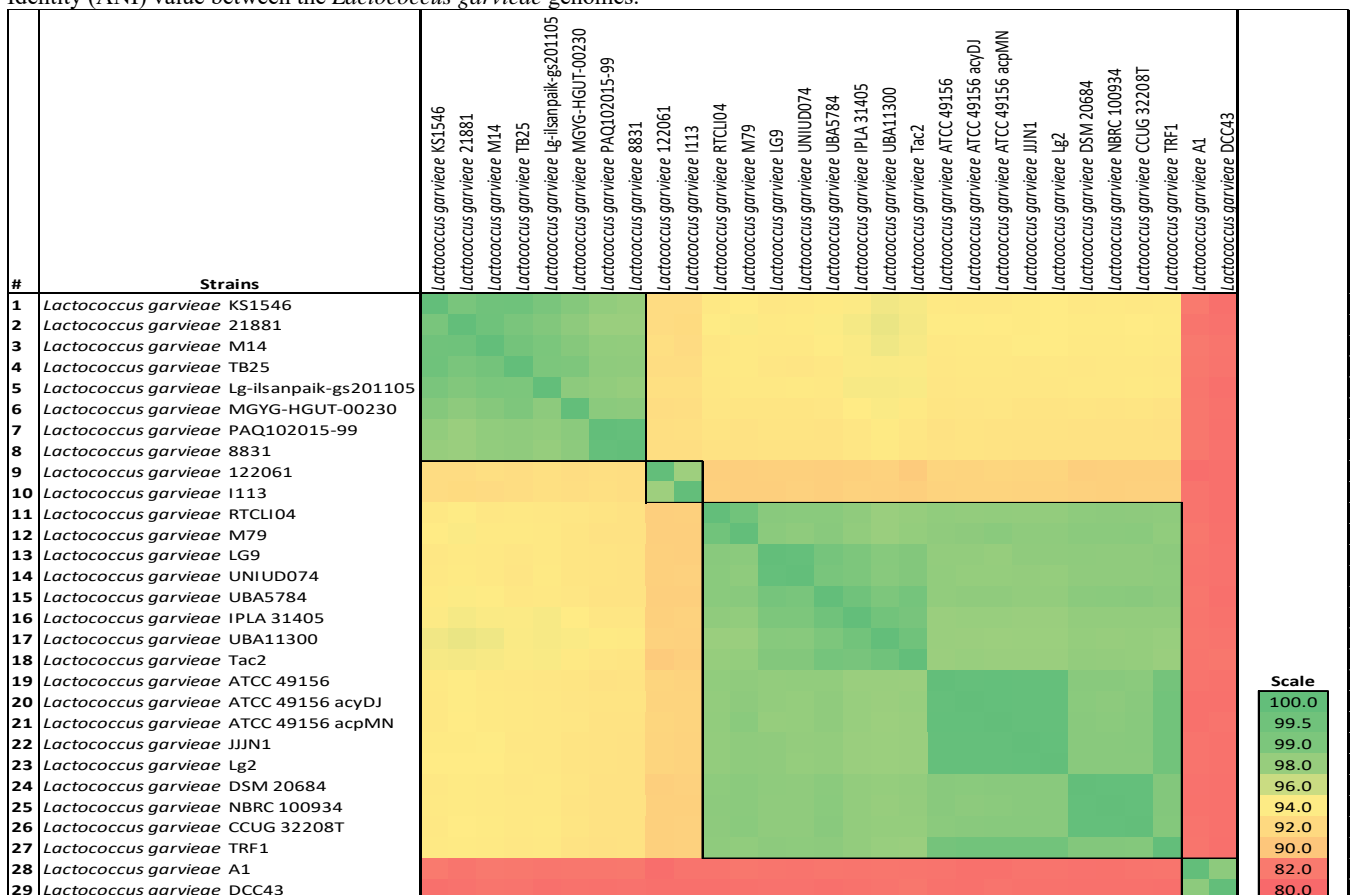
**Antimicrobial-resistant elements:** In evaluated *L. garvieae* genomes, 66 proteins divided into 13 antibiotic resistance classes were determined, and 17 of the antibiotic resistance proteins were common in all strains (Table 3).

**Table 1.** Genome features of *Lactococcus garvieae* strains.

#	Organism Name	Location	Source	Level	Scaffolds	Size(Mb)	GC%	Accession
1	<i>L. garvieae</i> KS1546	Kosovo	Milk	Contig	31	2.175	37.8	NZ_LTDA01000000
2	<i>L. garvieae</i> 21881	Spain	Homo Sapiens	Contig	91	2.164	37.9	NZ_AFCC01000000
3	<i>L. garvieae</i> M14	Algeria	Milk	Contig	13	2.254	37.7	NZ_CXCX00000000.1
4	<i>L. garvieae</i> TB25	Italy	Cheese	Contig	91	2.009	38.1	NZ_AGQX01000000
5	<i>L. garvieae</i> Lg-ilsanpaik-gs201105	South Korea	Homo Sapiens	Contig	53	1.959	38.1	NZ_JPUJ01000000
6	<i>L. garvieae</i> MGYG-HGUT-00230	N/A	Homo Sapiens	Scaffold	19	1.982	38.3	NZ_CABJEF01000000
7	<i>L. garvieae</i> PAQ102015-99	USA	Trout	Contig	14	2.068	38.0	NZ_LXWL01000000
8	<i>L. garvieae</i> 8831	Spain	Trout	Contig	87	2.086	38.0	NZ_AFCD01000000
9	<i>L. garvieae</i> 122061	Japan	Yellowtail	Complete	2	2.000	38.2	NZ_AP017373
10	<i>L. garvieae</i> I113	Italy	Meat	Contig	49	2.179	37.9	NZ_AMFD000000000.1
11	<i>L. garvieae</i> RTCLI04	India	Trout	Contig	35	2.055	38.6	NZ_RSFD00000000
12	<i>L. garvieae</i> M79	N/A	Camel	Scaffold	36	2.160	38.6	NZ_FOTJ01000000
13	<i>L. garvieae</i> LG9	Italy	Trout	Contig	139	2.082	38.5	NZ_AGQY00000000.1
14	<i>L. garvieae</i> UNIUD074	Italy	Trout	Contig	25	2.171	38.7	NZ_APHF01000000
15	<i>L. garvieae</i> UBA5784	USA	Environmental	Scaffold	22	2.026	38.6	DIEC00000000
16	<i>L. garvieae</i> IPLA 31405	Spain	Cheese	Contig	23	2.052	38.5	NZ_AKFO00000000.1
17	<i>L. garvieae</i> UBA11300	N/A	Environmental	Scaffold	22	1.953	38.6	DQHM00000000
18	<i>L. garvieae</i> Tac2	Italy	Meat	Contig	97	2.243	38.2	NZ_AMFE00000000.1
19	<i>L. garvieae</i> ATCC 49156	Japan	Yellowtail	Complete	1	1.950	38.8	NC_015930
20	<i>L. garvieae</i> ATCC 49156 acyDJ	N/A	N/A	Contig	6	1.932	38.7	NZ_AMSW00000000.1
21	<i>L. garvieae</i> ATCC 49156 acpMN	N/A	N/A	Scaffold	5	1.951	38.9	NZ_AHYM00000000.1
22	<i>L. garvieae</i> JJN1	South Korea	Mullet	Complete	1	1.966	38.8	NZ_CP026502
23	<i>L. garvieae</i> Lg2	Japan	Yellowtail	Complete	1	1.964	38.8	NC_017490
24	<i>L. garvieae</i> DSM 20684	United Kingdom	Mastitis	Contig	71	2.023	38.5	NZ_JXJV01000000
25	<i>L. garvieae</i> NBRC 100934	N/A	Mastitis	Contig	56	2.028	38.5	NZ_BBJW00000000.1
26	<i>L. garvieae</i> CCUG 32208T	N/A	Mastitis	Contig	48	2.044	38.5	NZ_VXKD01000004
27	<i>L. garvieae</i> TRF1	USA	Snake	Contig	112	2.205	38.5	NZ_AVFE00000000.1
28	<i>L. garvieae</i> A1	USA	Soil	Contig	25	2.039	38.0	NZ_NBBK01000000
29	<i>L. garvieae</i> DCC43	Spain	Duck	Contig	67	2.239	37.8	NZ_AMQS00000000.1

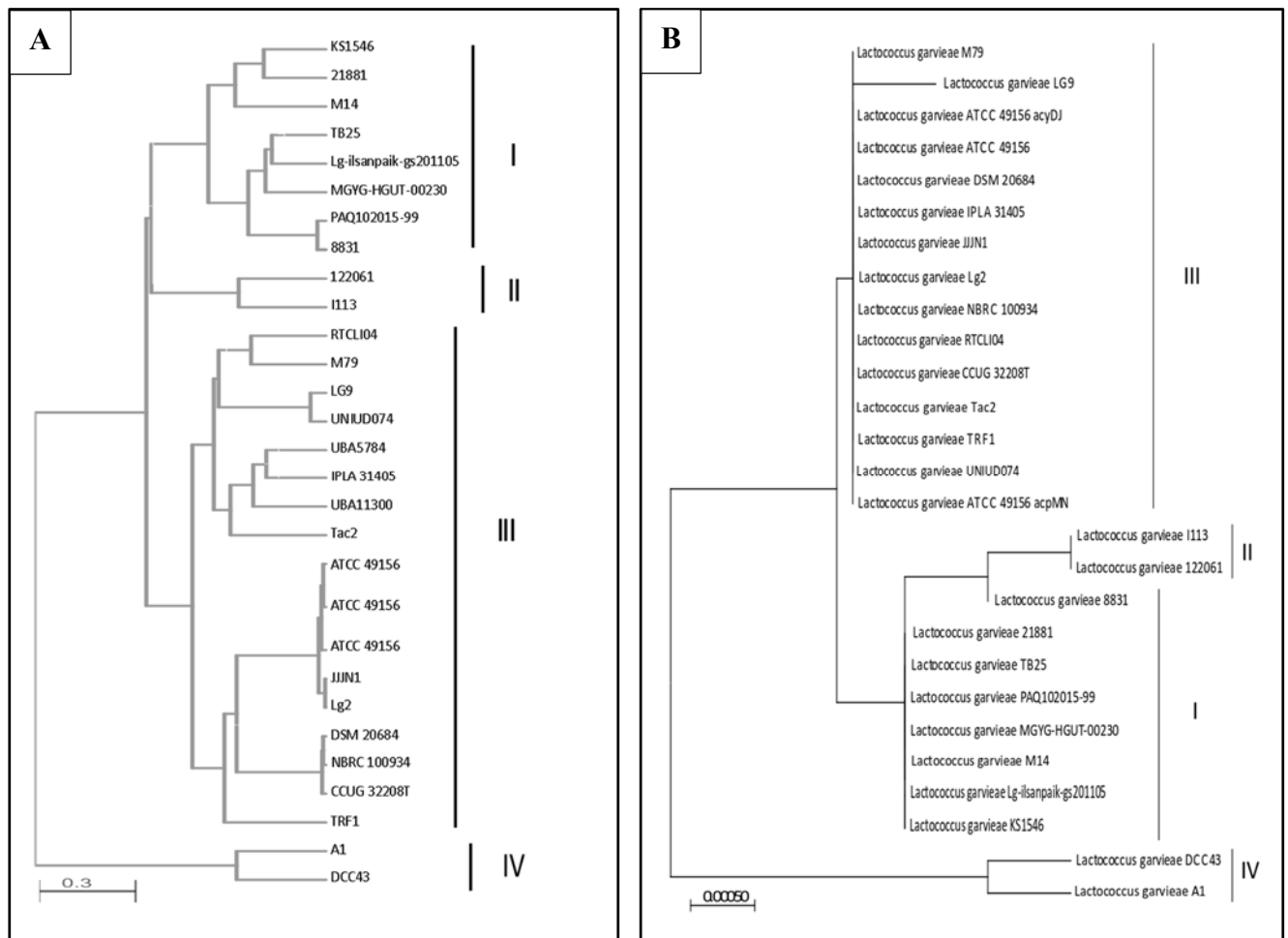
N/A: Not Available

**Table 2.** The Average Nucleotide Identity (ANI) values of *Lactococcus garvieae* genomes. Scale and color indicate the Average Nucleotide Identity (ANI) value between the *Lactococcus garvieae* genomes.



**Table 3.** Antibiotic resistance class and genes of *Lactococcus garvieae* genomes. AGly: Aminoglycosides, Bla: Beta-lactamases, Fcd: Fusidic acid resistance, Flq: Fluoroquinolones, MLS: Macrolide-Lincosamide-Streptogramin, Gly: Glycopeptides, Oxxzn: Oxazolidinones, Phe: Phenicol, Tet: Tetracyclines, Sul: Sulfonamides, and Tmt: Trimethoprim. Pen\_Ecoli\*: Penicillin Binding Protein Ecoli. Red color indicated the absence genes; green color indicated the presence genes in the genome.

Antibiotic Resistance		<i>Lactococcus garvieae</i> KS1546	<i>Lactococcus garvieae</i> 21881	<i>Lactococcus garvieae</i> M14	<i>Lactococcus garvieae</i> TB25	<i>Lactococcus garvieae</i> Lg-ilsampaik-gs201105	<i>Lactococcus garvieae</i> MGYG-HGUT-00230	<i>Lactococcus garvieae</i> PAQ102015-99	<i>Lactococcus garvieae</i> 8831	<i>Lactococcus garvieae</i> 122061	<i>Lactococcus garvieae</i> 1113	<i>Lactococcus garvieae</i> RTCl04	<i>Lactococcus garvieae</i> M79	<i>Lactococcus garvieae</i> LG9	<i>Lactococcus garvieae</i> UNIU074	<i>Lactococcus garvieae</i> UBA5784	<i>Lactococcus garvieae</i> IPLA 31405	<i>Lactococcus garvieae</i> UBA11300	<i>Lactococcus garvieae</i> Tac2	<i>Lactococcus garvieae</i> ATCC 49156	<i>Lactococcus garvieae</i> ATCC 49156 acyDj	<i>Lactococcus garvieae</i> ATCC 49156 acpMN	<i>Lactococcus garvieae</i> JJJN1	<i>Lactococcus garvieae</i> Lg2	<i>Lactococcus garvieae</i> DSM 20684	<i>Lactococcus garvieae</i> NBRC 100934	<i>Lactococcus garvieae</i> CCUG 32208T	<i>Lactococcus garvieae</i> TRF1	<i>Lactococcus garvieae</i> A1	<i>Lactococcus garvieae</i> DCC43
Classes	Genes																													
AGly	apH-Stph																													
	aac6-Aph2																													
	ant6-la																													
	spw																													
Bla	AmpC1_Ecoli																													
	PBP1a																													
	PBP1b																													
	penA																													
	Pen_Ecoli *																													
Fcd	fusB																													
	fusD																													
Flq	norA																													
Gly	vanRc4																													
	vanR-C																													
	vanR-Pt																													
	vanYF-Pp																													
	vanS-F																													
	vanZ-A																													
	vanS-L																													
	vanS-M																													
	vanL																													
	vanR-B																													
	vanRc3																													
	vanR-F																													
	vanR-M																													
	vanS-D																													
	vanTr-L																													
	vanY-B																													
vanR-A																														
vanS-Pt																														
MLS	cfr(A)																													
	erm(36)																													
	Lmr(B)																													
	mph(D)																													
	lmr(A)																													
	lsa(A)																													
	mef(A)																													
	ole(C)																													
	cfr(B)																													
	erm(32)																													
	erm(K)																													
	mef(B)																													
	msr(C)																													
vgb(B)																														
Mupirocin	mupB																													
Oxxzn	OptrA																													
Phe	bcr1																													
	catQ																													
	catA1																													
	catA3																													
	cmlV																													
Rif	arr2																													
	arr5																													
Sul	sul4																													
Tet	otrA																													
	tetB-P																													
	tetM																													
	Tet(58)																													
	tetS																													
	otrB																													
	tet(32)																													
	tet(38)																													
	tet(42)																													
Tet(60)																														
Tmt	tetT																													
	dfrE																													



**Figure 1.** Phylogenetic tree of *Lactococcus garvieae* genomes obtained from NCBI (A) and created with 16S rRNA sequence by using MEGA X and the neighbor-joining method (B).

## DISCUSSION AND CONCLUSION

In this study, as reported *L. garvieae* genomes were evaluated by comparative genomics approaches. This is the first extensive assessment of 29 whole *L. garvieae* genomes that were isolated from 9 different locations and 13 different sources (Table1).

Multi-locus sequence typing (MLST) and 16S rRNA gene sequence have been actively used in prokaryotic microorganism's classification, but using only 16S rRNA gene sequence cannot show differentiation of some species because of 16S rRNA gene sequence similarities (>99%). Thus, ANI value has been advised for prokaryotic organisms' classification and phylogenetic analysis (Maiden, 2006; Figueras, 2014; Kim, 2014). For the same species, the ANI value should be considered higher than 95% (Goris, 2007). For identification of *L. garvieae*, API 32 strep kit, MALDI-TOF, 16S rRNA PCR, and BD Automated Phoenix System have been used (Malek, 2019). By increasing the availability of next-generation sequencing technology, species identification might be possible with genomic sequence (Ellington, 2017). It was reported that pig, cow,

and trout isolate *L. garvieae* strains showed a genetic variation by PFGE analysis (Tejedor, 2011). In addition, it was recently reported that local fish and human isolate *L. garvieae* had the six novel sequence types by MLST (Lin, 2020). The *L. garvieae* genomes' phylogenetic tree was obtained from NCBI, and built the phylogenetic tree by using the 16S rRNA gene sequence (Figure 1). The ANI values between all the *L. garvieae* genomes were identified (Table 2). The results indicated that the phylogenetic tree of *L. garvieae* obtained from NCBI and ANI values correlate very well. The generated phylogenetic tree by using 16S rRNA sequence and ANI values correlate as well. The results supported previous studies and showed that there is four distinct *L. garvieae* genetic groups. *L. garvieae* genetic groups 1 closes to genetic group 2 with 92% ANI value and group 1 and 3 are closer by 94% ANI value. The ANI values between genetic group 4 and others are nearly 81%. In addition, groups include sub-groups as well. Group 2 and 4 have only two genomes each, so more genomes need for understanding sub-group variations. Consequently, it seems that there should be considered reclassification of *L. garvieae* species based on ANI value.

To treat infectious diseases, antimicrobials have been used, but bacteria may develop antimicrobial resistance by mediating horizontal mobile gene transfer or chromosomal acquisitions that make them non-sensitive to convinced antimicrobial treatments (Holmes, 2016; Nguyen, 2018). It was reported that *L. garvieae* strains are confirmed or predicted to resistant different antibiotics including tetracycline, clindamycin, lincomycin, streptomycin, trimethoprim, nalidixic acid, rifampin, sulfamethoxazole, tobramycin, penicillin, oxacillin, sulfamethoxazole/trimethoprim, erythromycin, beta-lactam, vancomycin, nitrofurantoin, sulfonamide, and fluoroquinolone (Walther, 2008; Ture, 2015; Meyburgh, 2017; Eraclio, 2018; Balta and Dengiz Balta, 2019; Shahi, 2020). In this study, 66 proteins in 13 antimicrobial resistances' classes were predicted in the assessed *L. garvieae* genomes (Table 3). There are four distinct genomic groups of *L. garvieae*, and the results showed that there are variations in the encoded antimicrobial-resistant elements between *L. garvieae* genomes group. Increased antimicrobial-resistance causes the limitation on helpful choices for infectious treatment. Thus, detecting antibiotic resistance related gene distribution is necessary to develop control strategies against *L. garvieae* pathogens.

In conclusion, this study will provide valuable information about the *L. garvieae* genomes' features by genomics approaches. Based on the ANI value between all *L. garvieae* genomes and phylogenetic trees will expand our awareness of *L. garvieae* strains situation in taxonomic groups because there are four different genetic groups and subgroups. Even though all *L. garvieae* groups have common antibiotic resistance elements, the *L. garvieae* groups and subgroups show variation by encoding antibiotic-resistant elements. Thus, this genomic approach of *L. garvieae* is crucial and provides valuable knowledge for further studies to understand the *L. garvieae* genomes variation and antibiotic resistance elements.

#### Funding information

This research did not receive any grant from a specific funding agency.

#### ACKNOWLEDGEMENTS

This work was supported by the Faculty of Fisheries, Recep Tayyip Erdogan University, Rize, 53100, Turkey.

#### Conflicts of interest

The author state there are no conflicts of interest.

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