

## Determination of virulence associated immunogenic proteins in some of *Lactococcus garvieae* strains

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Article Info	Abstract
<b>Article history:</b> Received: 10 July 2018 Accepted: 25 September 2018 Available online: 15 June 2019	<p>Lactococcosis disease incident caused by <i>Lactococcus garvieae</i> has been increased with increasing aquaculture productions and outbreaks of the disease have become a threat on farmed species. To prevent lactococcosis, inactivated vaccine has been used, however, it only provides protection when given by injection. Other than inactivated vaccine, various vaccines such as subunit vaccines can be developed. In the present study, total protein profile of 43 strains of <i>L. garvieae</i> isolated from fish, milk and cheese by SDS-PAGE and virulence associated immunogenic proteins of <i>L. garvieae</i> strains using western blot with hyper-immune rabbit sera were determined. After analyzing whole-cell lysate protein of <i>L. garvieae</i> strains with SDS-PAGE, protein bands were ranged between 8.00 and 140.00 kDa. Among strains, variable protein bands were ranged between 17.00 and 48.00 kDa with some variability in the staining intensity of the protein bands and formed in 6 clusters. The immunogenic protein bands were ranged between 25.00 - 75.00 kDa. Only a variable and highly immunogenic protein band was observed between 40.00 and 45.00 kDa. Most of the strain including Lgper had 44.00 kDa immunogenic protein while nonvirulent ATCC strain had 42.50 kDa immunogenic protein. Predominant immuno-reactive proteins encoded by genes can be used as a subunit vaccine.</p>
<b>Key words:</b> Immunogenic protein Lactococcosis Rainbow trout Western blot Yellowtail	

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### تعیین حدت مرتبط با پروتئین های ایمونوژنیک در برخی از سویه های لاکتوکوکوس گارویه

#### چکیده

شیوع بیماری لاکتوکوکوز با عامل مولد لاکتوکوکوس گارویه با افزایش تولیدات آبی پروری رو به فزونی گذاشته است و وقوع بیماری در گونه های پرورشی به یک تهدید تبدیل شده است. به منظور جلوگیری از لاکتوکوکوز، واکسن غیر فعال مورد استفاده قرار می گیرد. اما این نوع واکسن صرفاً از طریق تزریقی ایمنی ایجاد می کند. واکسن های متفاوتی به غیر از واکسن غیر فعال نظیر واکسن های تحت واحد را می توان تهیه کرد. در مطالعه حاضر، پروفایل پروتئین نام ۴۳ گونه از لاکتوکوکوس گارویه جدا شده از ماهی، شیر و پنیر با استفاده از SDS-PAGE و پروتئین های ایمونوژنیک مرتبط با حدت گونه های لاکتوکوکوس گارویه از طریق وسترن بلات همراه با سرم های خردگوشی هیبرایمیون تعیین شدند. بعد از آنالیز پروتئین لیزات، سلول گونه های لاکتوکوکوس گارویه با SDS-PAGE، باند های پروتئینی بین ۸/۰۰ تا ۱۴۰/۰۰ کیلو دالتون و متغیر پروتئینی بین ۱۷/۰۰ تا ۴۸/۰۰ کیلو دالتون (با تنوع در شدت رنگ پذیری باندها) قرار گرفتند و شش گروه تشکیل دادند. باند های پروتئینی ایمونوژنیک بین ۲۵ تا ۷۵ کیلو دالتون قرار داشتند. صرفاً یک پروتئین متغیر و بسیار ایمونوژنیک بین ۴۰/۰۰ تا ۴۵/۰۰ کیلو دالتون قرار گرفت. بیشتر سویه های واجد Lgper دارای پروتئین ایمونوژنیک ۴۴/۰۰ کیلو دالتونی بودند، درحالیکه سویه غیر حد ATCC دارای پروتئین ایمونوژنیک ۴۲/۵۰ کیلو دالتونی بود. پروتئین های ایمنو راکتیو غالب که با ژن ها کدبندی شده اند را می توان به عنوان تحت واحد واکسن مورد استفاده قرار داد.

**واژه های کلیدی:** پروتئین ایمونوژنیک، دم زرد، قزل آالی رنگین کمان، لاکتوکوکوز، وسترن بلات

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## Introduction

*Lactococcus garvieae*, gram-positive cocci bacteria, is the causative agent of the lactococcosis, known as an infectious systemic disease.<sup>1</sup> Lactococcosis disease affects both freshwater and saltwater fish<sup>2-4</sup> including yellowtail (*Seriola quinqueradiata*),<sup>5</sup> rainbow trout (*Oncorhynchus mykiss*)<sup>6</sup> and black rockfish (*Sebastes schlegelii*).<sup>7</sup> *Lactococcus garvieae* is also considered as a zoonotic agent capable of causing disease in different animal species and humans.<sup>8</sup> In Turkey, lactococcosis was first reported in Aegean Region<sup>9</sup> and it started to spread across the country. The pathogen causes serious losses in cultured fish especially in farmed rainbow trout.<sup>1</sup>

Conventionally, diagnosis of the lactococcosis disease is achieved by agar cultivation and then biochemical and phenotypic properties of the bacteria.<sup>10,11</sup> Molecular techniques can also be used to identify *L. garvieae* directly from host tissue without culturing bacteria.<sup>12</sup> Serological characteristics of *L. garvieae* have been widely studied to characterize the strains. *L. garvieae* strains can be classified as: Capsulated (KG<sup>-</sup>) and non-capsulated (KG<sup>+</sup>) strains.<sup>13</sup> KG<sup>-</sup> strain is agglutinated by anti KG<sup>-</sup>serum. Meanwhile, KG<sup>+</sup> strain is agglutinated by both anti KG<sup>-</sup> and anti KG<sup>+</sup> sera.<sup>14</sup> Serotype KG<sup>-</sup> are hydrophilic, resistant to phagocytic ingestion and more virulent compared to serotype KG<sup>+</sup>.<sup>15</sup> On the other hand, presence of capsule gene is not directly related to virulence of *L. garvieae*. Ture and Altinok<sup>16</sup> found that all the Turkish *L. garvieae* isolates lacked capsule gene, however, they were virulent.

Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is fairly fast, easy and reproducible technique for whole cell protein pattern analysis.<sup>17</sup> The SDS-PAGE protein profile analyses can be used to identify inner membrane proteins,<sup>18</sup> outer membrane proteins<sup>19</sup> and quantification of allergen proteins.<sup>20</sup> For antigen expression or vaccine efficiency, antigenic compound of the bacteria should be recognized by fish. Limited research has been conducted on the whole cell protein and antigenic variety of *L. garvieae*.<sup>15,21</sup> The aims of the present study were to determine total protein profile of various *L. garvieae* strains by SDS-PAGE and to determine virulence associated immunogenic proteins of *L. garvieae* strains using western blot with hyper-immune rabbit sera.

## Materials and Methods

**Bacterial strains.** *Lactococcus garvieae* strains (n = 43) were obtained from different fish farms located in Aegean and Black Sea regions of Turkey and also from Spain, Italy, France and Japan (Table 1). Genetic and biochemical properties of the *L. garvieae* strains were determined prior to this study and none of the strains had capsule protein gene.<sup>16</sup> The ATCC49156 strain (avirulent) was used as a negative control and Lgper strain (virulent)

was used as a positive control. Prior to the experiment, rainbow trout (78.00 ± 8.50 g) were challenged with both strains and Lgper strain caused 74.00% mortality while ATCC 49156 strain did not cause any mortality. All the bacteria were subcultured on trypticase soy agar (TSA) to ensure purity of the colonies (Table 1).

**Rabbits.** Animals used in antibody generation were six months old male New Zealand rabbits (n = 3) weighing 2.50 - 3.00 kg each. The rabbits were obtained from Firat University and quarantined for 10 days prior to the study. Throughout the study, animals were held in Karadeniz Technical University, Faculty of Medicine. Animal studies were carried out with the guidelines of the Institutional Animal Care and Use Committee at the Karadeniz Technical University (approval #11/2013).

**Preparation of antigen and antibody generation.** Lgper strain was used to produce hyper-immune rabbit sera. Bacteria were incubated overnight at 29.00 °C on tryptic soy agar (TSA). Then single colony was subcultured in brain heart infusion broth (BHIB) at 29 °C for 5 hr. The bacteria concentration was adjusted to an optical density of 0.60 (OD<sub>630</sub> 0.60 × 10<sup>9</sup> CFU mL<sup>-1</sup>) using Shimadzu UV-2550 (Tokyo, Japan) spectrophotometer. Bacteria were inactivated by 0.70% (v/v) formaldehyde as described by Huang *et al.* with slight modifications.<sup>22</sup> After inactivation procedure, samples were centrifuged at 4,000 g for 5 min and then bacterial pellet was re-suspended in phosphate-buffered saline (Sigma Aldrich, Taufkirchen, Germany) at final concentration of 1.40 × 10<sup>9</sup> CFU mL<sup>-1</sup> and stored at 4 °C. For injection, 3.00 mL of Freund's Complete Adjuvant (Sigma-Aldrich) was mixed with equal amount of formalin-inactivated bacterial solution. Each rabbit was injected subcutaneously with 1.00 mL of the solution three times with a two-week interval. Blood samples were collected from marginal ear vein of the rabbits after two weeks post-injection and serum was obtained by centrifugation at 4000 g for 15 min at 4°C and then stored at - 80 °C.

**Extraction of bacterial proteins and protein profile by SDS-PAGE.** Bacteria were cultured in BHIB and incubated at 29 °C until bacterial optical density was reached to 2 (OD<sub>630</sub>). Bacteria culture was washed twice with PBS and optical density adjusted to 2 (OD<sub>630</sub>) for each strain. Bacteria were sonicated three times (Bandelin Electronic, Berlin, Germany) at 20 Watt on ice for 2 min and then bacterial cells were disrupted by boiling for 5 min. The cell debris were removed by centrifugation at 10.000 g for 10 min at 4°C. Protein concentrations were measured using bicinchoninic acid (BCA) protein assay kit (BioVision Inc., Milpitas, USA) according to the manufacturer's instructions. Proteins were separated on 4.00 - 12.00% SDS-PAGE gels by loading 15.00 µL samples per lane. The electrophoresis was performed at 125 V, 41 mA for 65 min. Protein fragments were visualized by silver staining according to Winkelstroter *et al.*<sup>23</sup>

**Table 1.** *Lactococcus garvieae* strains used in whole-cell protein profile and western blot, (T = Turkey).

No	Strain	Host	Origin	Isolated in
1	1684	Rainbow trout	Spain	1997
2	2398	Rainbow trout	France	1998
3	M1	Rainbow trout	Muğla (T)	2002
4	<i>L. lactis</i>	Cow milk	Isparta, (T)	2005
5	AA	Rainbow trout	Rize (T)	2008
6	Pap225-1	Rainbow trout	Artvin (T)	2008
7	K9	Rainbow trout	Gümüşhane (T)	2010
8	8053 C/02	Rainbow trout	Spain	2000
9	164 A/03	Rainbow trout	Spain	2000
10	04/8782 FTPI	Rainbow trout	Spain	2001
11	04/8782 (532)	Rainbow trout	Spain	2001
12	04/8782 (498)	Rainbow trout	Spain	2001
13	04/8782 ITP109	Rainbow trout	Spain	2002
14	8740/03	Rainbow trout	Spain	2003
15	1073/03C	Rainbow trout	Spain	2000
16	M300	Goat cheese	Italy	2001
17	A-58	Rainbow trout	Italy	2002
18	G-27	Cow milk	Italy	2003
19	AF-14	Rainbow trout	Rize (T)	2009
20	PP60	Rainbow trout	Italy	2004
21	M2	Rainbow trout	Muğla (T)	2002
22	M3	Rainbow trout	Muğla (T)	2003
23	M5	Rainbow trout	Muğla (T)	2009
24	M4	Rainbow trout	Muğla (T)	2011
25	216-6	Rainbow trout	Rize (T)	2007
26	AA-37	Rainbow trout	Rize (T)	2008
27	512-8	Rainbow trout	Rize (T)	2008
28	OME	Rainbow trout	Rize (T)	2008
29	511-15	Rainbow trout	Rize (T)	2008
30	Isfk	Rainbow trout	Rize (T)	2009
31	Sider-17	Rainbow trout	Rize (T)	2008
32	399-18	Rainbow trout	Artvin (T)	2008
33	BB	Rainbow trout	Artvin (T)	2008
34	636-16	Rainbow trout	Gümüşhane (T)	2008
35	671-14	Rainbow trout	Gümüşhane (T)	2008
36	670-20	Rainbow trout	Gümüşhane (T)	2008
37	673-5	Rainbow trout	Gümüşhane (T)	2008
38	235-16	Rainbow trout	İzmir (T)	2009
39	R-817	Rainbow trout	Trabzon (T)	2014
40	Ser114	Rainbow trout	Trabzon (T)	2009
41	Trb	Rainbow trout	Trabzon (T)	2011
42	A30	Rainbow trout	Rize (T)	2009
43	ATCC 49156	Yellowtail	Japan	1974
44	Lgper	Rainbow trout	Ordu (T)	2011

**Western blotting.** Proteins were again resolved by 5.00 - 12.00% SDS-PAGE gels by loading 15.00 µL samples per lane. The electrophoresis was performed at 80.00 V (500 mA, 150 W) for 10 min and at 110 V for 90 min. Proteins were transferred to 0.45 µm pore size nitrocellulose membranes (Bio-Rad, Hercules, USA) at 35 V (500 mA, 150 W) for 1 hr and blocked with 5.00% non-fat milk in tris-buffered saline with Tween 20 (TBST; 20 mM Tris-HCl (Sigma Aldrich), 150 mM NaCl (Sigma Aldrich) , 0.05%, 0.10% Tween 20 (Sigma Aldrich) for 1 hr on vertical shaker. The membranes were incubated with hyper-immune rabbit sera (primary antibodies) at 1:1000 dilutions for 1 hr at room

temperature and washed three times with TBST with 10 min intervals. Subsequently incubated with mouse anti-rabbit IgG-HRP secondary antibody (1:10000; Santa Cruz Biotechnology Inc., Dallas, USA) at room temperature for 1 hr and washed three times with TBST. Protein bands were visualized using ChemiDoc imaging system (Bio-Rad, Hercules, USA) with ECL Prime kit (Merck Millipore, Burlington, USA). For negative control nonimmune rabbit sera were used as primary antibodies. In order to determine specification of antibody or sera obtained from rabbits after exposing to *L. garvieae* Lgper strains, western blotting was performed with *Enterobacter cloacae*, *Aeromonas hydrophila*,

*Pseudomonas fluorescens*, *Streptococcus iniae*, *Yesinia ruckeri*, *Vibrio fluvialis*, *P. fluorescens*, *P. putida*, *Listonella anguillarum*, *Vibrio parahaemolyticus* and *P. luteola* whole cell proteins.

**Statistical Analyses.** Band patterns were analyzed with Bionumerics GelCompar II (Applied Maths, Brussels, Belgium). The similarities between band patterns of different isolates were calculated using the Dice similarity coefficient with a 2.50% tolerance and 1.00% optimization. The dendrogram was constructed using cluster analysis unweighted pair-group method with arithmetic mean (UPGMA). Discriminatory indices (DIs)

of the typing methods were calculated based on the formula developed by Hunter and Gaston.<sup>24</sup>

**Results**

After analyzing whole-cell lysate protein of *L. garvieae* strains with SDS-PAGE, protein bands were ranged between 8.00 and 140.00 kDa. Among strains, number of fragments were ranged between 7 - 17 and variable protein bands were ranged between 17.00 and 48.00 kDa with some variability in the staining intensity of the protein bands and formed in six clusters (Fig. 1).

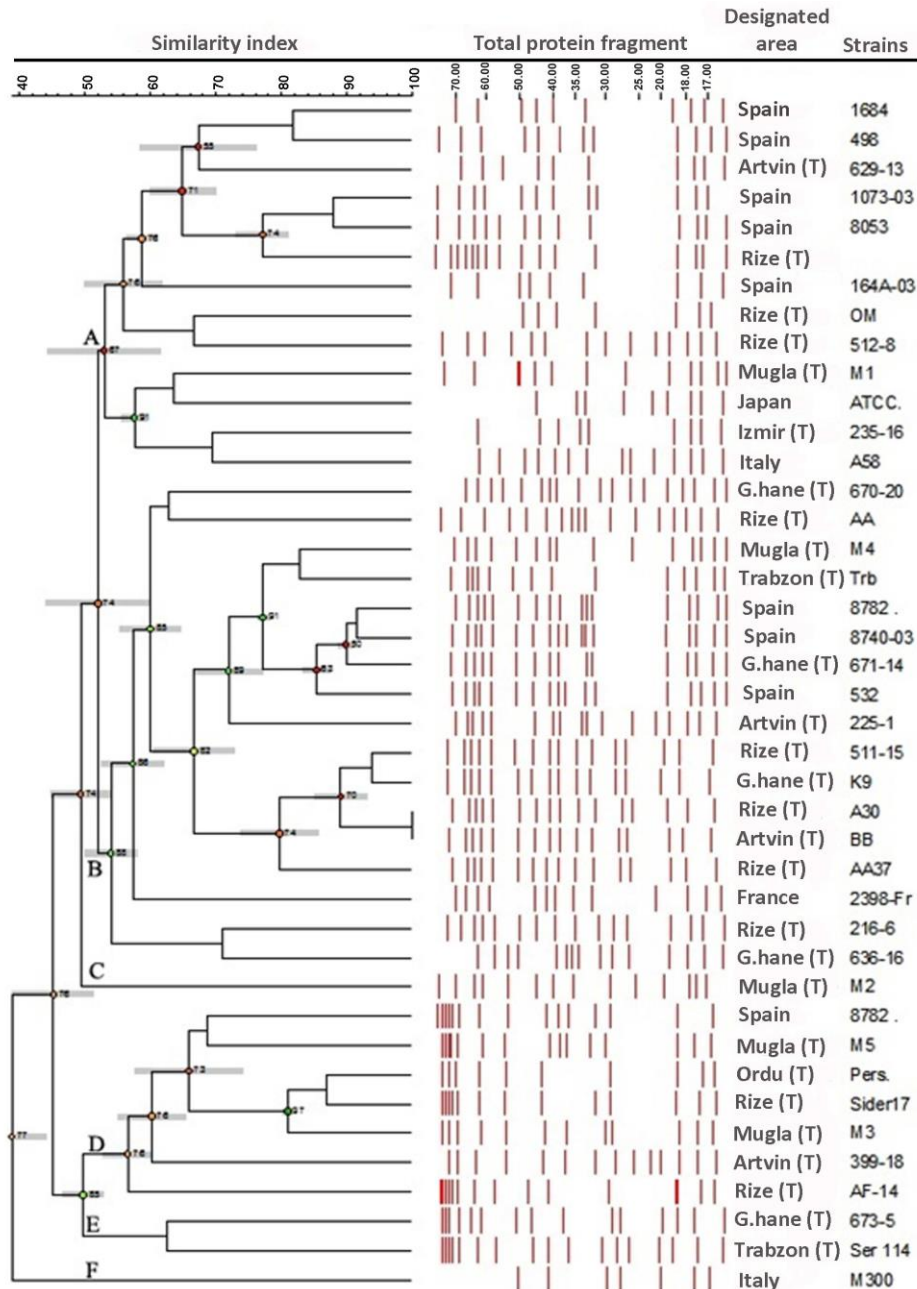


Fig. 1. Dendrogram obtained from SDS-PAGE whole-cell protein profiles of *Lactococcus garvieae*.

According to the Dice similarity coefficient, similarity rates among strains were found to be low ( $\leq 40.00\%$ ), (Fig. 2). Cluster A was composed of two sub-clusters; A1 and A2. Spain ( $n = 5$ ) and Eastern Black Sea region of Turkey ( $n = 4$ ) strains formed sub-cluster A1 with  $55.80 \pm 5.90\%$  similarity, meanwhile Mugla (M1), Izmir (235-16), ATCC 49156 and Italy (A58) strains formed sub-cluster A2 with  $57.80 \pm 1.80\%$  similarity. Similarity between sub-cluster A1 and A2 was determined as  $53.00 \pm 8.00\%$ . Cluster B consisted of 17 strains which were grouped in three sub-clusters with similarity of  $59.90 \pm 3.90\%$ . Similarity between Cluster A and B was determined as  $52.00 \pm 7.90\%$ . Cluster C consisted of only France (2398) strain and its similarity with Cluster A and B was  $49.40 \pm 4.50\%$ . Spain (8782), Mugla (M3, M5), Ordu (Lgper), Rize (Sider17, Af-14) and Artvin (399, 18) grouped in cluster D with  $56.60 \pm 3.80\%$  similarity. Gumushane (676-5) and Trabzon (Ser114) grouped in cluster E. Italy (M300) strain isolated from goat cheese formed cluster F with  $38.90 \pm 5.30\%$  similarity with other clusters (Fig. 1).

Molecular weights (MV) of the immunogenic proteins were ranged between 25.00 - 75.00 kDa. All of the *L. garvieae* strains had identical western blot patterns except for R-817 and Ser114 strains isolated from Trabzon, and only a single variable and highly immunogenic protein band were observed between 40.00 and 45.00 kDa. Most of the strain including Lgper had 44.00 kDa immunogenic

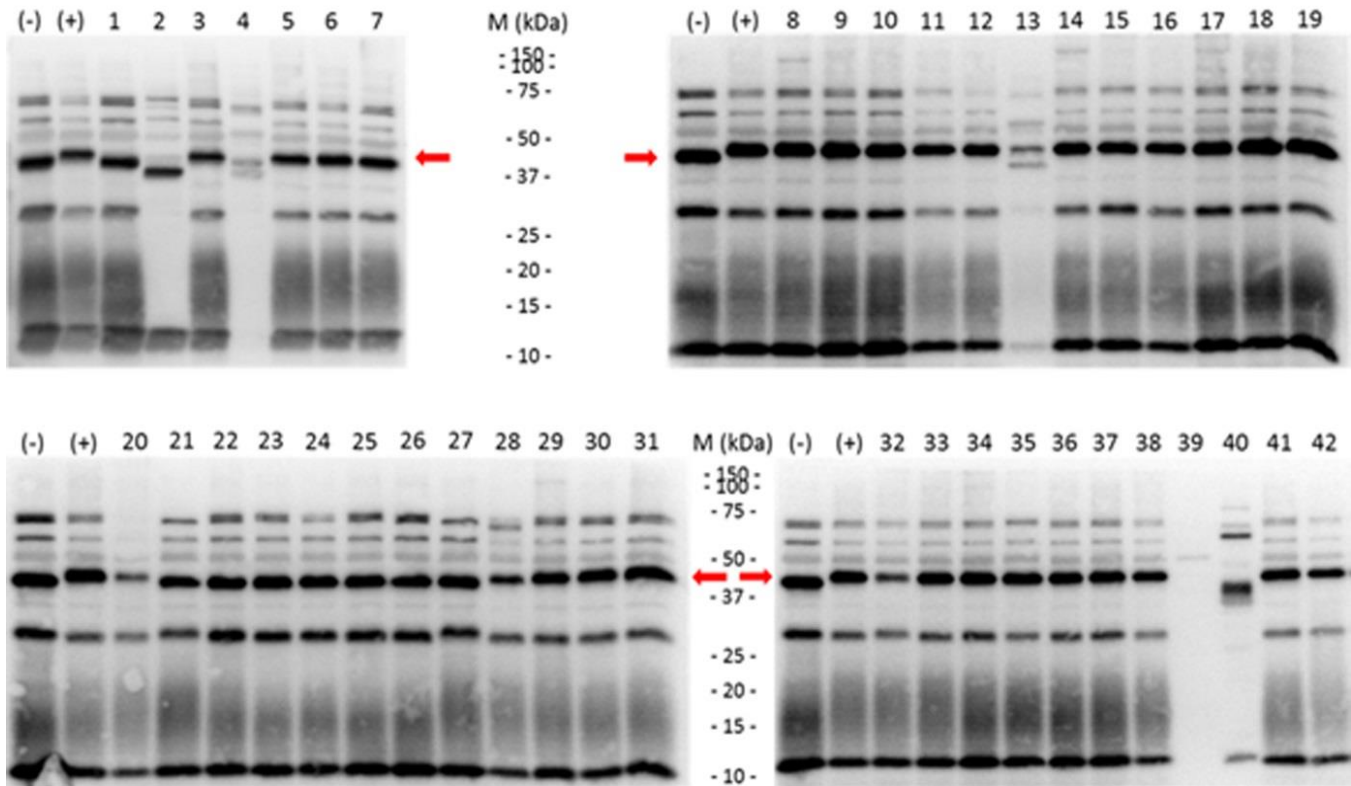
protein while nonvirulent ATCC strain had 42.50 kDa immunogenic protein.

*Lactococcus lactis* and 04/8782 ITP109 Spain strain had similar pattern that was completely different from the other *L. garvieae* strains (Fig. 2).

Specificity of the Lgper antibody was tested against 11 different bacteria. While *Enterobacter cloacae* and *A. hydrophila* had weak antigenic band similar to Lgper, the other bacteria did not have antigenic band indicating the specificity of Lgper serum obtained from rabbits (Fig. 3).



**Fig. 3.** Reaction of *Lactococcus garvieae* antibody with other bacteria species M: Marker (-): ATCC 49156, (+): Lgper, 1: *Enterobacter cloacae*, 2: *Aeromonas hydrophila*, 3: *Pseudomonas fluorescens* 4: *Streptococcus iniae*, 5: *Yesinia ruckeri*, 6: *Vibrio fluvialis*, 7: *P. fluorescens*, 8: *P. putida*, 9: *Listonella anguillarum* 10: *V. parahaemolyticus*, 11: *P. luteola*.



**Fig. 2.** Western blot images of *Lactococcus garvieae* and *L. lactis* strains. (-) and (+) indicate ATCC 49156 and Lgper strain, respectively. Please check the Table 1 for the strains.

## Discussion

The SDS-PAGE can be used to identify species and subspecies of bacteria.<sup>25,26</sup> *Lactococcus garvieae* strains used in the present study were confirmed by PCR and biochemical tests in previous studies.<sup>4,16</sup> Although all the strains were confirmed as *L. garvieae*, they had different whole protein profile based on analysis by SDS-PAGE. Six clusters were formed depending on strains isolated. *Lactococcus garvieae* M300 isolated from goat cheese in Italy had thoroughly different whole cell protein profile than the other *L. garvieae* strains; however, its immuno-genic protein profile was similar to that of the other strains. Furthermore, immunogenic properties of antigenic sera obtained from rabbit was specific to *L. garvieae*, because, it did not form cross reactivity to other bacterial species.

Lactococcosis is a systemic fish disease affecting many species. The disease agent was first isolated in the Aegean Region and then it was spread to every region where rainbow trout was cultured in a short time and it caused high mortalities.<sup>1</sup> Antibiotics are commonly used to treat the lactococcosis disease and most of the strains gain resistance to them.<sup>27,28</sup> Furthermore, to prevent lactococcosis, inactivated vaccine has been used, however, it usually only provides protection when applied by injection. Immersion or oral application does not provide remarkable protection.

In order to help prevent environmental pollution and reduce antibiotic usage, development of an efficient vaccine such as subunit vaccine against lactococcosis is vital. In light of the results obtained from this study, highly immunogenic protein band was observed between 40.00 and 45.00 kDa. Most of the strain including Lgper had 44.00 kDa protein while nonvirulent ATCC strain had 42.50 kDa protein. Predominant immunogenic proteins encoded by genes can be used as a subunit vaccine.

The GAPDH, a cytosolic glycolytic protein, is a path of the outer membrane proteins. The GAPDH is responsible for pathogenesis or it is a virulence associated immune-modulatory protein.<sup>29</sup> The GAPDH gene encodes 44.00 kDa protein and it has immunoprotective properties.<sup>30</sup> In the present study, antigenic protein band was observed between 37.00 and 50.00 kDa. Therefore, this protein could be responsible for the virulence of *L. garvieae* and it could be used as a subunit vaccine.

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## Conflict of interest

The authors declare that there is no conflicts of interest regarding the publication of this article.

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