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Comparison of Experimental Susceptibility of Rainbow Trout (*Oncorhynchus mykiss*), Turbot (*Psetta maxima*), Black Sea Trout (*Salmo trutta labrax*) and Sea Bass (*Dicentrarchus labrax*) to *Lactococcus garvieae*

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Abstract

Lactococcus garvieae is a major fish pathogen leads to haemorragic septicemia in many fish species. Present study was designed to determine and compare differences in susceptibilities of some important fish species to *L. garvieae*. Rainbow trout (*Oncorhynchus mykiss*), Black Sea trout (*Salmo trutta labrax*; synonym, *Salmo coruhensis*), turbot (*Psetta maxima*), and sea bass (*Dicentrarchus labrax*) were kept at different temperature ranges (12-23°C) for 30 days and challenged by intraperitoneal (i.p.) injection and immersion (imm.) with *L. garvieae* (Lgper), isolated from rainbow trout and ATCC49156 strain. Bacteria were ranged between 1.7×10^5 - 2×10^6 colonies forming unit (cfu) fish^{-1} in experiments. As a result of the experiments, Lgper strain had high virulence for rainbow trout, with 98% mortality when challenged by i.p. injection. Rainbow trout and Black Sea trout tested at the lower temperature (12ºC) were less susceptible (max. mortality 16%) to *L. garvieae*. These two fish species had similar susceptibilities to *L. garvieae* ($P>0.05$). There was no mortality or any clinical signs when turbot and sea bass were challenged with *L. garvieae*. The results of the study generate important source in controlling of possible lactococcosis outbreaks in aquaculture industry.

Keywords: *L. garvieae*, rainbow trout, turbot, Black Sea trout, sea bass, virulence

Gökkuşağı Alabalığı (Oncorhynchus mykiss), Kalkan Balığı (Psetta maxima),Karadeniz Alabalığı (*Salmo trutta labrax*) ve Deniz Levreğinin (*Dicentrarchus labrax*) *Lactococcus garvieae* Bakterisine Karşı Deneysel Duyarlılıklarının Karşılaştırılması

Özet

Lactococcus garvieae birçok balık türünde hemorajik septisemiye neden olan önemli bir balık patojenidir. Bu çalışma, bazı önemli balık türlerinin *Lactococcus garvieae'*ye karşı duyarlılıklarının belirlenmesi ve karşılaştırılması amacıyla yapılmıştır. Gökkuşağı alabalığı, Karadeniz alabalığı, kalkan balığı ve deniz levreği 30 gün boyunca farklı sıcaklık aralıklarında (12-23°C) tutularak, alabalıklardan izole edilen *L. garvieae* (Lgper) ve ATCC 49156 suşları ile periton içi (i.p.) ve daldırma (imm.) metotları ile deneysel enfeksiyona tabi tutulmuştur. Deneylerde, $1{,}7 \times 10^5$ - 2×10^6 cfu/balık aralığında bakteri kullanılmıştır. Deneylerin sonucunda, Lgper suşunun gökkuşağı alabalıklarında %98 ölüm oranı ile yüksek virülansa sahip olduğu görülmüştür. Düşük sıcaklıkta, *L. garvieae*'ye karşı test edilen gökkuşağı ve Karadeniz alabalıklarında ise ölümler düşük seviyede görülmüştür (max. mortalite %16). Bu iki balık türünün *L. garvieae*'ye karşı duyarlılıkları benzer düzeyde bulunmuştur ($P > 0{,}05$). Ayrıca, *L. garvieae* ile enfekte edilen kalkan balığı ve deniz levreklerinde ölüme ya da herhangi bir klinik bulguya rastlanmamıştır. Bu çalışmanın sonuçları, su ürünleri yetiştiriciliği alanında muhtemel laktokokosiz salgınlarının önlenmesi için önemli bir kaynak oluşturma potansiyeline sahiptir.

Anahtar Kelimeler: *L. garvieae*, gökkuşağı alabalığı, kalkan balığı, Karadeniz alabalığı, deniz levreği, virülans

Introduction

Lactococcus garvieae (former synonym, *Enterococcus serolicida*) is a causative agent of lactococcosis and a zoonotic pathogen has been isolated from various sources such as fish, terrestrial animals, humans, food products and also it causes great economic losses in the aquaculture industry in worldwide (Eldar *et al*., 1996; Kawanishi *et al*., 2006; Wang *et al*., 2007; Alegria *et al*., 2009; Tejedor *et al*., 2011). *L.garvieae* was first isolated from yellow tail in Japan (Kusuda *et al*., 1991) and has subsequently been found in the most area where salmonids are cultured in Turkey (Diler *et al*., 2002). In the last

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decade there has been an important awareness about the disease and it has been reported in Central Anatolian Region (Kav and Erganis, 2007), Aegean Region (Avci *et al*., 2010), Mediterranean Region (Tanrikul and Gultepe, 2011) and Black Sea Region (Ture *et al*., 2012) of Turkey.

Aquaculture industry mainly focused on aquatic carnivore species such as trout, sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*). Rainbow trout (*Oncorhynchus mykiss*) is an important salmonid species reared in fresh water and marine environment in Turkey. Its annual production is more than 100,000 tonnes. Sea bass is one of the most intensive reared fish species in marine environment in Turkey (TSI, 2013). Also, cultivation studies of turbot (*Psetta maxima*) and Black Sea trout (*Salmo trutta labrax*; synonym, *Salmo coruhensis*) are being proceed and these species have been recently introduced to intensive aquaculture. These two species have been acquired increasing importance in Turkish aquaculture industry for several years.

L. garvieae is excessively virulent for various fish species (Vendrell *et al*., 2006). However its virulence to some fish species including Black Sea trout, turbot and sea bass is not well known. Therefore, we aimed to investigate experimental susceptibilities of different fish species such as rainbow trout, sea bass, Black Sea trout and turbot to *L. garvieae* isolated from cultivated rainbow trout (Lgper) and reference strain (ATCC49156) in our study.

Material and Methods

Fish

In experiment 1, rainbow trout reared in fresh water (n= 480, mean weight ± SE, 7.39 ± 0.23 g) were obtained from a local trout farm (Trabzon, Turkey). In experiment 2, 240 fish of rainbow trout (24.17 ± 1.16 g) were obtained from same farm and 240 fish of Black Sea trout (19.45 ± 1.45 g) were obtained from another local trout farm. In experiment 3, turbot (n=240, 17.21 ± 0.49 g) were obtained from Central Fisheries Resarch Instutite (CFRI). In experiment 4, we used two different size of sea bass (n= 120, 6.63 ± 0.31 g and n=240, 13.14 ± 0.36 g) obtained from CFRI. All fish were obtained from farms and CFRI with known health status. These fish had no history of untoward abnormalities or mortalities causing from lactococcosis. Before the experimental trials, all fish were allowed to acclimatize for 20 days in sea water. After the adaptation period, fish were determined to be free of bacteria and external parasites.

Bacteria

L. garvieae (ATCC49156*)* isolated from yellowtail was used as a reference strain, kindly provided by Dr. Ilhan Altinok from Karadeniz Technical University in Trabzon, Turkey. Another strain of *L. garvieae* (named Lgper) was isolated from a diseased rainbow trout farm in Perşembe, Turkey (Ture *et al*., 2012). These strains were maintained as stock cultures at -80°C with 20% glycerol containing Nutrient Broth (NB, Merck) until experiments. Before experimental trials, bacteria were subcultured at 30ºC for 2 days on trypticase soy agar (TSA, Merck) to check purity and then pure colonies were inoculated on NB and incubated for 18 h at 30°C by shaking. These strains were used to infect rainbow trout by i.p. injection and reisolated to ensure that would reliably cause lactococcosis. This procedure was done in duplicate (Altinok *et al*., 2001).

Experimental Trials

Experiment 1 was set up to determine the virulence of Lgper strain and to compare with reference strain on rainbow trout. We aimed to detect susceptibility of Black Sea trout to *L. garvieae* and compare it with rainbow trout in experiment 2. In experiment 3 and 4, turbot and sea bass were challenged with *L. garvieae* ATCC strain. In experiment 4, two different sizes of sea bass were used. Experiments 1, 2, and 3 were performed at the temperature of 16, 12 and 16°C (± 0.5ºC) respectively for 30 days. Also experiment 4 was maintained for 15 days at 16°C, then temperature was raised to 23°C (accrual for each day is 1°C) and they were kept at final temperature for 15 days. Fish exposed to a certain number of bacteria in all experiments were shown in Table 1.

Infection Protocol

Before the experimental trials, 50% lethal dose (LD_{50}) were estimated for each fish species by Probit method (Finney, 1971). Briefly, 10 fish from each species were maintained in 50 L tanks at 16°C. These fish were injected with Lgper strain and three different dose rates were tested. LD_{50} was approximately calculated as $2x10^5$ colony forming units (cfu) fish^{-1} during 7 days for rainbow trout. By the reason of the tested turbot and sea bass no displayed mortality, LD_{50} value could not be calculated for individuals.

For each experiment, *L. garvieae* strains were recovered from frozen stocks for inoculation on TSA and incubated at 30°C for 18 h. Isolated pure colonies were inoculated on NB and incubated overnight by shaking. The cultures were harvested using Phosphate Buffered Saline (PBS) and the density was adjusted to prepare bacterial stock solutions. Serial 10-fold dilutions of each suspension were inoculated on TSA plates for estimating bacterial cfu. Bacterial cfu were counted by plating serial dilutions as described by (Altinok *et al*., 2001; Algöet *et al*., 2009).

Experimental challenges were performed in

Table 1. Summary of four experimental infection trial results

Experiment	Challenge method	Dose (cfu fish^{-1})	Species	NF	NS	NM	DRM	NSC
1	i.p.	$2x10^7_{ATCC}$	Rainbow trout	90	8	2	80	8
		$2x10^7_{Lgper}$		90	2	0	88	2
	imm.	$4x10^5_{ATCC}$		90	77	2	11	2
		$4x10^5_{Lgper}$		90	37	5	48	4
2	i.p.	$5x10^6_{ATCC}$	Rainbow trout	90	72	3	15	72
			Black sea trout	90	80	2	8	64
	imm.	$4x10^5_{ATCC}$	Rainbow trout	90	87	2	1	69
			Black sea trout	90	82	2	5	48
3	i.p.	$2x10^6_{ATCC}$	Turbot	90	90	0	0	81
	imm.	$8x10^5_{ATCC}$	Turbot	90	90	0	0	44
4	i.p.	$4.8x10^6_{ATCC}$	Seabass$_{finger}$	90	90	0	0	57
	imm.	$1.7x10^5_{ATCC}$	Sea bass$_{finger}$	90	90	0	0	24
	imm.		Sea bass$_{fry}$	90	90	0	0	42

NF: Number of fish at the beginning of the trial, NS: Number of surviving fish at the end of the trial, NM: Naturel mortality, DRM: Disease related mortality, NSC: Number of surviving fish carrying *L. garvieae*.

triplicate tanks, each holding 30 fish, were set up for each test group and supplied with ozonated and aerated sea water. For i.p. injections, fish were anaesthetized with benzocaine and injected intraperiteonally with 0.1 ml of the bacterial suspension. The same amount of NaCI solution (0.85%) was injected to fish in control tanks. The immersion challenges were performed by adding 100 ml relevant bacterial suspension to each test tanks except control tanks. Fish were exposed to *L. garvieae*, water flow was stopped for 5 h and then overnight culture of *L. garvieae* in NB was added to water to give a relevant final concentration (Altinok *et al*., 2001; King *et al*., 2001). During the experiments fish were fed twice a day with commercial fish diets. Dead or moribund fish were checked daily and removed immediately for bacterial examination.

Bacterial Recovery and Confirmation

Kidney and liver of dead and moribund fish were sampled to determine the disease related mortality (DRM) in all experiments. For this purpose, samples were inoculated on TSA plates. Characteristics such as gram staining, oxidation and fermentation test (O/F), fermentation of glucose, sucrose and lactose in triple sugar iron agar (TSI, Merck), activity of Pyrrolidonyl Arylamidaz (PYR), motility, cytochrome oxidase, catalase, lack of H_2S and gas production were used to identify *L. garvieae* (Vendrell *et al*., 2006).

Statistical Analysis

Statistical tests were evaluated by using SPSS packet program, version 11.5 (SPSS Inc., Chicago, IL, USA). Cumulative mortalities in each experiment were compared by a paired samples test. Experiments were at confidence interval of 95% ($P<0.05$). P value was regarded as significant if the difference was < 0.05.

Results

There was no DRM among control groups in any of the four challenge experiments. All fish species suffered from mortality by the exposure to *L. garvieae* by i.p. and imm. routes and survival rates of fish in each species were shown in Table 1.

Pathogenicity of Lgper and ATCC Strains

Percent DRM ranged between 90 to 98% (i.p.) and 12 to 53% (imm.) in rainbow trout for strains of ATCC and Lgper, respectively (Figure 1). There was no statistical difference between the level of mortalities for the rainbow trout which were challenged with strains of ATCC and Lgper by the i.p. route ($P>0.05$). However there was a significant difference between imm. groups ($P<0.01$). Affected fish had clinical signs indicating characteristic of lactococcosis infection such as exophthalmia, anorexia, darkening and haemorrhages at the base of the fins.

The symptoms were observed to be similar in fish suffered from mortality following exposure to different *L. garvieae* strains. Anorexia, lethargie and darkness in color on the skin were observed in the infected fish during the first few days. Haemorrhages were identified at the base of the fins, around the anus and occuler area. Bilateral exophthalmia was observed frequently with the opacity of the cornea. The petechial haemorrhages were also observed in some of the organs including stomach, pyloric caeca, liver and spleen (Figure 2). The liver and spleen were generally enlarged. In addition, the ulcer on the skin and ascites were observed in a few number of fish.

Susceptibility of Rainbow Trout and Black Sea Trout to *L. garvieae*

In the 30 days after infection, percentages of

DRM attributed to lactoccoccosis, were ranged between 1.1 to 5.5% (imm.) in rainbow trout and Black Sea trout, respectively. There were significant differences (P<0.05) between groups in this experiment. Also, mortality rates were determined as 16.6 and 8.8% in rainbow trout and Black Sea trout by i.p. route, respectively (Figure 3). There were no significant differences (P>0.05) between groups in this experiment. Haemorrhages around the mouth and ocular area and cachexia were observed in Black Sea

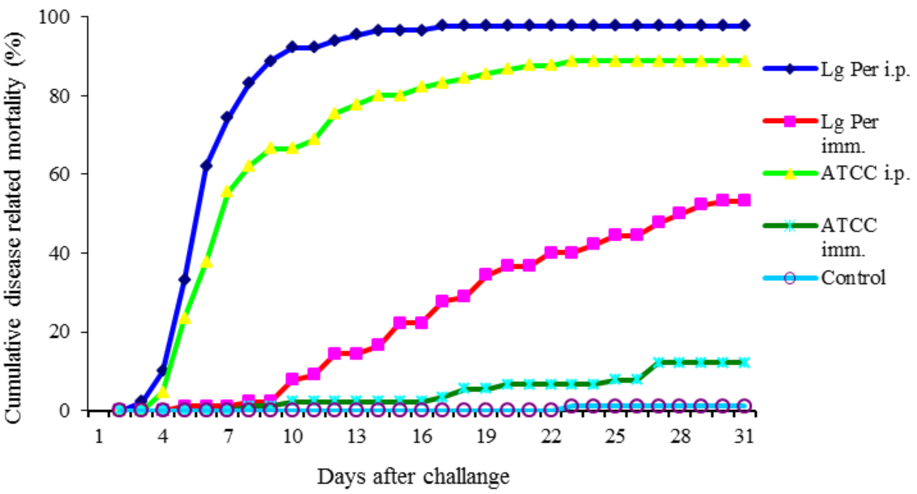

Figure 1. All fish were infected by i.p. and imm. routes, with ATCC and Lgper strains.

Figure 2. Gram staining of *L. garvieae* (A), bilateral exophthalmia (B), and haemorrhagic lesions on the liver and spleen (C).

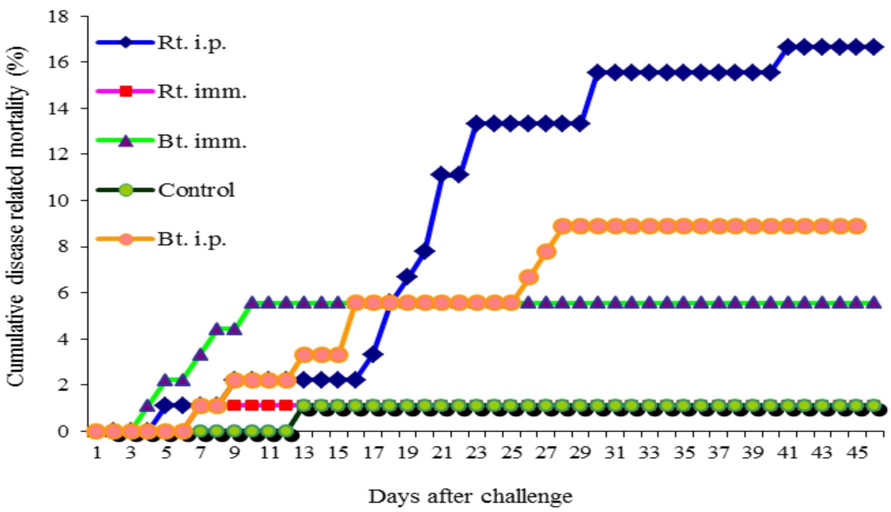

Figure 3. Cumulative percent lactococcosis mortalities in rainbow trout and Black Sea trout. Rt: Rainbow trout, Bt: Black Sea trout.

trout while bilateral exophthalmia was occured frequently with the opacity of the cornea in rainbow trout.

Susceptibility of Turbot and Sea Bass to *L. garvieae*

At the end of the experiment, none of the tested fish displayed mortality or clinical signs of disease. Viable *L. garvieae* cells were also recovered from the liver and kidney of surviving turbot and sea bass (Table 1).

Disscussion

Aquaculture is a relatively new industry for Turkey that started with rainbow trout culture in the 1970s. Today, Turkey is one of the most important aquaculture producer in the Mediterranean (Cagirgan, 2009; TSI, 2013). Main cultured fish species are rainbow trout, sea bass, sea bream and Black Sea trout in Turkey. Black Sea trout is now becoming popular for farms due to their fast growth rate and ability to adaptation. It also have higher market value than rainbow trout (Cakmak *et al*., 2007). In developed countries, the main objective is the production of high quality products for human consumption. In this regard, turbot which is economically important marine species distributed to all over the world should be evaluated due to its high value (Wheeler, 1987). On the other hand, turbot cultivation is being an important branch for Turkish aquaculture industry and amount of production is being scaled up year by year (Zengin *et al*., 2006).

L. garvieae is one of the most important infectious pathogen leads to significant economic losses in trout farms worldwide (Eyngor *et al*., 2004). *L. garvieae* has been isolated as a causative agent of disease from several species such as rainbow trout (Prieta *et al*., 1993), yellowtail, tilapia, Japanese eel (Kusuda *et al*., 1991), olive flounder (Lee *et al*., 2001), giant fresh water prawn (Chen *et al*., 2001) and grey mullet (Chen *et al*., 2002). However, rainbow trout is the most sensitive species when compared to other fish species (Vendrell *et al*., 2006). For this reason, many studies were carried out concerned with the pathogenicity and epidemiological characterization of *L. garvieae*. Besides, there are limited studies about susceptibility of different fish species to *L. garvieae* that have really economical importance. Therefore, in the present study we aimed to determine the possible detrimental effects of lactococcosis on the main fish species, so that probable economical losses can be avoided.

L. garvieae ATCC49156 was firstly isolated from diseased yellowtail in 1974 (Kusuda *et al*., 1991). It has been reported as non-pathogenic for the yellowtails due to phenotypic changes (Kawanishi *et al*., 2006). However, we found that the ATCC strain had high virulance for rainbow trout in experiment 1. A comparison study of the susceptibilities of Black Sea trout and rainbow trout were performed in experiment 2. Two salmonid species were suffered by mortality approximately in similar rates, following the exposure to *L. garvieae* ATCC strain (Figure 3). As reported in the previous studies, this study also demonstrated that temperature ranges of $\geq$ 16°C triggered fish mortality. Because, experimental infection was carried out at low water temperature (12 ± 0.5ºC), level of mortality was found low in the experiment 2. So this result contributes a new data to the literature. Algoet *et al*. (2009) performed a similar study determining the susceptibility of freshwater fish species such as grayling (*Thymallus thymallus*), rainbow trout, Atlantic salmon (*Salmo salar*), brown trout (*Salmo trutta*) and cyprinid species (*Cyprinus* spp.) in UK. They reported that, grayling and rainbow trout were highly susceptible to *L. garvieae* with the rates of mortalities 65% and 37%, respectively. In addition, brown trout was less susceptible than other fish species. Black Sea trout, closely related to the brown trout (Ogut and Altuntas, 2011). However, we found that Black Sea trout was more susceptible than rainbow trout in imm. trial with 5.5 and 1.1% mortality rates. Besides, rainbow trout was more susceptible than Black Sea trout to *L. garvieae* in i.p. injection trials with mortality rates of 16.7 and 8.9%, respectively (Experiment 2). Moreover, ATCC reference strain was used for the first time in the experiments to determine the susceptibility of sea bass, turbot and Black Sea trout to *L. garvieae*.

L. garvieae has only been isolated from rainbow trout farms in fresh water and marine environment in Turkey (Diler *et al*., 2002; Kav and Erganis, 2007; Tanrikul and Gultepe, 2011). However, its presence had not been reported for other fish species such as Black Sea trout, sea bass and turbot. Many bacteria, viruses and parasites such as *Aeromonas salmonicida* (Ewart *et al*., 2008), *Vibrio anguillarum* (Munro *et al*., 1995), *viral hemorrhagic septicemia virus* (Isidan and Bolat, 2011), and *Scuticociliate spp*. (Iglesias *et al*., 2001) were reported for reared turbot but *Lactococcus* has not been reported yet. In our study, seven-month old turbot were challenged with *L. garvieae* by i.p. and imm. routes and it was detected that there was no mortality among the fish. (Experiment 3). Also, none of the challenged fish displayed clinical signs. As a result, findings of the experiment 3 proved that turbot was a resistant fish species to *L. garvieae.*

Sea bass is also one of the most economic fish species which is reared in a widespread manner in Turkey. The main bacterial diseases of sea bass are vibriosis and pasteurellosis caused by *Vibrio anguillarum* and *Pasteurella piscicida* in Turkey (Cagirgan and Yurekliturk, 1996). While lactococcosis or streptococcosis caused by *L. garvieae* has not been reported in Europen countries, many parasites such as *Amyloodinium ocellatum, Cryptobia* sp., *Trichodina* sp., *Cryptocaryon irritans* and *Diplectanum aequans* were encountered in sea bass (Toksen, 1999). In experiment 4, two different sizes of sea bass (average weight: 6.5 and 13 g) were challenged with strain of ATCC at two different temperatures (16 and 23ºC). These temperatures are indicator for transferring the fish to the sea and maximum growth temperature for sea bass, respectively. At the end of the 30 days trial, none of the challenged fish displayed mortality or clinical signs.

All surviving fish for each experiments were tested to determine the presence of the bacteria at the end of trials (Table 1). It is clear that, diseases develop when environmental conditions are optimal for the agent and asymptomatic carrier fish are generally known to play a major role as the source of bacterium (Avci *et al*., 2010). Our results indicated that the bacterium could grow in turbot and sea bass without causing disease, making them a potential reservoir of this pathogen.

The results of the present study are crucial for the fast growing aquaculture sector including Black Sea trout, sea bass and turbot in Turkey. Lgper strain isolated from the local rainbow trout farm had high virulence in rainbow trout. There was no statistically significant difference between the two trout species in terms of susceptibility to *L. garvieae*. It was proved that *L. garvieae* is an important pathogen for Black Sea trout like rainbow trout. In addition, the turbot and sea bass were found to be resistant to *L. garvieae*.

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