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Research Article

A mixed *Bacillus gibsonii* and *Sphingomonas echinoides* infection in cultured rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) is a fish species with a long history of cultivation and bacterial pathogens are limiting the success rate. The aim of this study is the biochemical and molecular identification of two opportunistic pathogens detected in the rainbow trout cultured in net cages in a dam lake located on the Kızılırmak river; revealing the pathological symptoms of them in the moribund fish samples; determination of their antimicrobial susceptibility profile and determination of the antagonistic effect of two probiotic-candidate strains against them. Depending on the results of the conventional bacteriologic and molecular identification studies, bacterial isolates recovered from the internal organs of the moribund fish samples, a mixed bacterial infection case of *Bacillus gibsonii* and *Sphingomonas echinoides* was identified in the moribund fish samples showing general bacterial hemorrhagic septicemia symptoms for the first time in rainbow trout. Despite it was not possible to identify these isolates at the species level using conventional bacteriological methods, our isolates separately showed similarities more than 99% with the above mentioned species in the 16s RNA sequence analysis. The results of this study showed that, long term water quality parameter determination and bacterial distribution monitoring studies which include molecular tools should be carried out in the aquaculture sites to increase the success in trout culture.

Keywords
Aquaculture
Fish diseases
Rainbow trout
Bacillus gibsonii
Sphingomonas echinoides

Introduction

Rainbow trout (*Oncorhynchus mykiss*) is a freshwater fish species with the longest history of aquaculture in Turkey, which is among the leading countries for the culture of this fish with a production amount of 113.678 tons in 2020 (TUİK, 2020).

To continuously satisfy the increasing food demand of the rising human population worldwide, agriculture and aquaculture activities are also increasing. During this increase, as the new technologies were introduced and new areas were used; new species were cultured, various chemicals were increasingly used, antibiotic resistance cases were developed and new emerging or opportunistic pathogens were detected. Various bacterial pathogens, both Gram-negative (representatives of the genera *Aeromonas, Vibrio, Pseudomonas* etc.) and Gram-positive (representatives of the genera *Staphylococcus, Streptococcus* and *Lactococcus* etc.) were identified in rainbow trout cultured in Turkey in a great number of previous studies (Muz et al., 1995; Kan and Sarıeyyüpoğlu, 2008; Türe et al., 2012; Öztürk et al., 2013; Balta and Balta, 2019; Akaylı et al., 2020; Akaylı et al., 2021). Besides widely distributed and repeatedly isolated pathogens, some bacterial inhabitants of the aquatic environment may cause infections as opportunistic pathogens in stressed fish under rapidly changing environmental conditions (Austin and Austin, 2016).

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The aim of this study is the biochemical and molecular identification of two opportunistic pathogens detected in the rainbow trout cultured in net cages in a dam lake located on the Kızılırmak river; revealing the pathological symptoms of them in the moribund fish samples; determination of their antimicrobial susceptibility profile and determination of the antagonistic effect of two probiotic-candidate strains against them.

Material and Methods

Field sampling

A rainbow trout cage farm in a dam lake located on Kızılırmak river, in Bafra-Samsun region was visited five times (April, May, June, September and late October 2017) for field sampling. A total of 10 rainbow trout samples (100-300 g) with various clinical symptoms were analyzed in each sampling visit. Bacteriological samples were taken from liver, spleen and kidney of moribund fish samples and streaked onto TSA (Trypticase Soy Agar, Merck) and incubated at 22ºC for 72h for bacterial growth (Roberts, 2012; Austin and Austin, 2016). In this article, the results of the late October sampling will be discussed.

Bacterial identification

Bacterial colonies that were isolated from the internal organs of moribund fish samples collected in late October were purified and identified with conventional discriminative laboratory tests (Yabuuchi and Kosako, 2005; Logan and De Vos, 2009; Roberts, 2012; Austin and Austin, 2016). Later, bacterial DNA samples were isolated from the pure bacterial cultures with High Pure PCR Template Preparation Kit (Roche, Switzerland). Universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 907R (5'-CCG TCA ATT CMT TTR AGT TT-3') targeting a part of 16S / 23S gene (Lane, 1991) and PCR Master Mix - 2X (Fermantas, K 0171) were used for the PCR amplification. PCR amplification steps were used as described by Lane (1991). An 880 bp region of 16s RNA sequence analysis was performed with ClustalX 2.1 (Larkin et al., 2007) and BLASTN 2.2.20 (Zhang et al., 2000) algorithms on Bioedit v7.0.0 software (Hall, 1999).

Histopathology

Tissue samples (spleen, kidney, liver etc.) were fixed in %10 buffered formalin and processed for paraffin embedding. Histological sections (4-5µm) were stained with hematoxylin and eosin (H&E) and analyzed under light microscope (Roberts, 2012).

Antibiogram susceptibility profile

Modified Kirby-Bauer disc diffusion method (Bhunia et al., 1988) was used for the determination of antibiotic resistance profile of bacterial isolates against sulphametaxozole-trimethoprim, amoxicillin, tetracycline, florfenicole, erythromycin, enrofloxacine, oxolinic acid and ciprofloxacine with three replicates. Three replicates of Mueller-Hinton medium containing petri dishes were incubated at 22ºC for 48 h and inhibition zone diameters were measured and evaluated according to the CLSI standards (Wikler, 2006). Inhibition zones with a diameter of 0-1.5 cm were regarded as resistant; 1.6-2.0 cm were regarded as semi-resistant; 2.1 cm and above were regarded as sensitive.

Antagonistic effect studies

Lyophilized *Bacillus subtilis* (ATCC 6633™) and *Lactobacillus rhamnosus* (ATCC 7469™) strains were used as probiotic candidates. Fresh cultures of these strains were used with the modified Kirby-Bauer disc diffusion method for the determination of antagonism against recovered pathogenic bacteria (Bhunia et al., 1988). Briefly, 200 µl of fresh cultures of pathogenic bacteria growth in Nutrient Broth were streaked onto TSA medium to cover all the surface. Later, blank antibiotic susceptibility paper-discs were dipped into fresh cultures of probiotic-candidates growth in Nutrient Broth and placed onto TSA medium. Three replicates of TSA medium containing petri dishes were incubated at 22ºC for 48 h and inhibition zone diameters were measured and evaluated according to the CLSI standards (Wikler, 2006). Inhibition zones with a diameter of 0-1.5 cm were regarded as resistant; 1.6-2.0 cm were regarded as semi-resistant; 2.1 cm and above were regarded as sensitive.

Results and Discussion

Many freshwater rivers flows through the northern part of Turkey (also called as the Black Sea Region), there are many dam lakes on these rivers, and most of them host aquaculture facilities. Previously motile Aeromonads (Muz et al., 1995), *Streptococcus faecalis* (Kan and Sarıeyyüpoğlu, 2008) and *Lactococcus garvieae* (Türe et al., 2012; Öztürk et al., 2013; Balta and Balta, 2019) were recovered and identified as bacterial pathogens of rainbow trout cultured in various dam lakes in Turkey. During this one year monitoring study, various other infection cases were also detected; A mixed infection case that was caused by *Frigoribacterium faeni* and *Lactococcus garvieae* was detected in April (Akaylı et al., 2020), a *Citrobacter freundii* infection case was detected

in June and a *Hafnia alvei* infection case was detected in September samples (Akaylı et al., 2021).

In moribund fish samples analyzed in the October sampling where the water temperature was 18°C, darkening of the skin and loss of scales was observed. As observed in many of the previous bacterial haemmorhagic septicemia cases of rainbow trout caused by various pathogens (Roberts, 2012; Austin and Austin, 2016) hemorrhages and exophthalmos in the eyes was a common phenomenon in these samples where in some of them loss of eyes was observed (Figure 1a). In many samples, internal organs (kidney and spleen) preserved their healthy appearance, but their pale livers were mostly covered with petechial hemorrhages (Figure 1b).

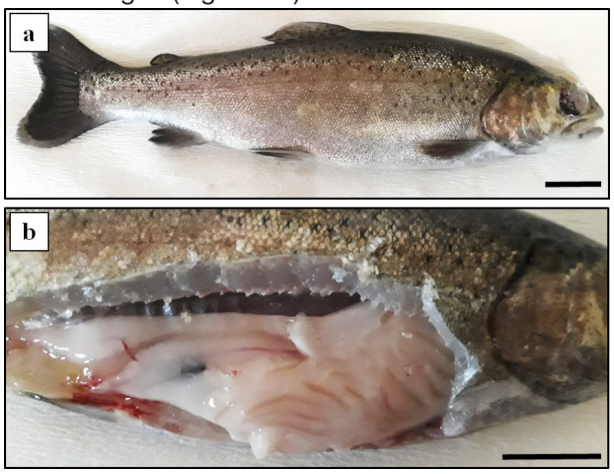

Figure 1. (a) Darkening of the skin, loss of scales and loss of eyes in moribund rainbow trout sample **(b)** Apparently healthy kidney and accumulation of fat tissue in the internal examination of a moribund rainbow trout sample. Scale bars: 2 cm

Depending on the results of the conventional bacteriologic and molecular identification studies, bacterial isolates recovered from the internal organs of the moribund fish samples, a mixed bacterial infection case of *Bacillus gibsonii* (Figure 2a) and *Sphingomonas echinoides* (Figure 2b) was identified in the October samples. Despite it was not possible to identify these isolates at the species level using conventional bacteriological methods (Table1), our isolates separately showed similarities more than 99% with the above mentioned species in the 16s RNA sequence analysis. Despite they are found to be sensitive or semi-sensitive against most of the antibiotic compounds that are commonly used in aquaculture, the probiotic candidates used in this study did not show a strong antagonistic effect against these opportunistic pathogens (Table 1). *B. subtilis* showed weak antagonistic effect against *B. gibsonii* and *S. echinoides* isolates and *L. rhamnosus* showed negative antagonistic effect against *B. gibsonii* and *S. echinoides* isolates (Figure 3).

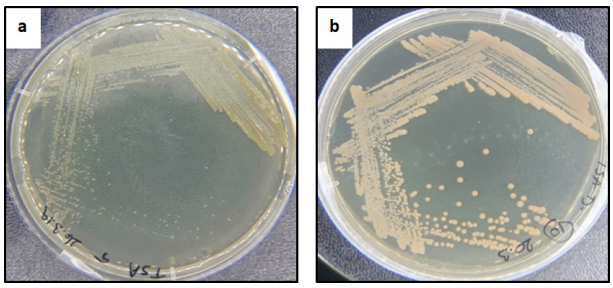

Figure 2. (a) Yellowish and round colonies of *Bacillus gibsonii* with a 2-3 mm diameter on TSA medium **(b)** Yellowish-creamy and round colonies of *Sphingomonas echinoides* with a 4-6 mm diameter on TSA medium

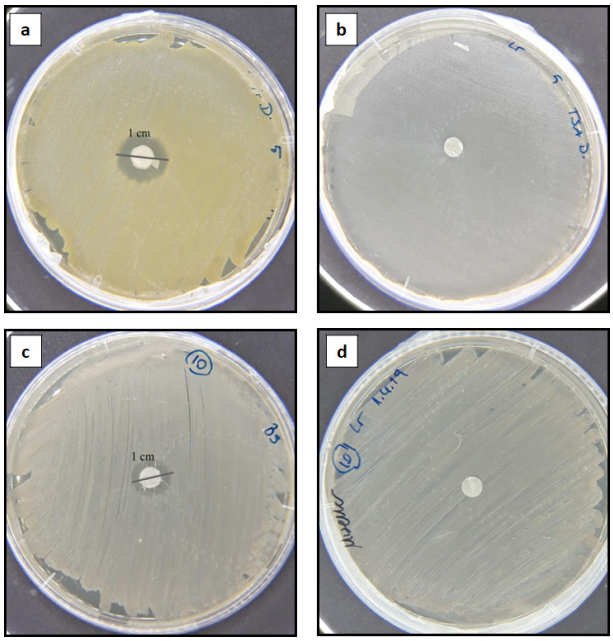

Figure 3. (a) Weak antagonistic effect of *Bacillus subtilis* against *Bacillus gibsonii* **(b)** Negative antagonistic effect of *Lactobacillus rhamnosus* against *Bacillus gibsonii* **(c)** Weak antagonistic effect of *Bacillus subtilis* against *Sphingomonas echinoides* **(d)** Negative antagonistic effect of *Lactobacillus rhamnosus* against *Sphingomonas echinoides*

In this study, histopathological effects of these opportunistic pathogens on the internal organs of moribund fish samples were revealed. Despite many organs such as kidney and spleen, looked apparently healthy during the clinical examination, histopathologic observations proved that this mixed infection caused a classical bacterial hemorrhagic septicemia. Histopathologically, samples co-infected with *B. gibsonii* and *S. echinoides* showed mass hyperemia in the liver

Table 1. Phenotypic and biochemical characteristics, antimicrobial profile and antagonistic effect profile of the bacterial isolates

Biochemical characteristics	*B. gibsonii* n=4	*S. echinoides* n=7	Antibiotic and antagonistic effect	*B. gibsonii* n=4	*S. echinoides* n=7
Colony colour	yellowish	light yellow	S.metax / Trimet	R	R
Gram	+	-	Ciprofloxacine	S	SR
Motility	+	+	Amoxicillin	S	SR
Oxidase	-	+	Tetracycline	S	S
Catalase	+	+	Florfenicole	S	S
O/F	F	O	Erythromycin	R	SR
Indole	-	-	Enrofloxacine	S	SR
Methyl Red	-	+	Oxolinic Acid	S	S
Vogues-Proskauer	-	-			
Citrate	-	+	*L. rhamnosus*	SR	R
Arginine	-	-	*B. subtilis*	SR	R
Lysine	-	-			
Ornithin	-	-			
Lactose	+	-	+: positive reaction - : negative reaction F: fermentative S: sensitive (> 21) SR: semi-resistant (14-20) R: resistant (0-13); (zone diameters in mm)		
Rhamnose	+	+			
Maltose	+	-			
Sorbitol	-	-			
İnositole	-	-			
16s RNA similarity	99%	99%			

(Figure 4a), small melanomachrophage centers and slight liquefactive necrosis areas in the kidney (Figure 4b), depletion of the pulps and small hemosiderin accumulation areas in the spleen (Figure 4c), mass hemorrhages around the necrotic hearth muscles (Figure 4d) similar to the general bacterial hemorrhagic septicemia symptoms (Roberts, 2012; Austin and Austin, 2016).

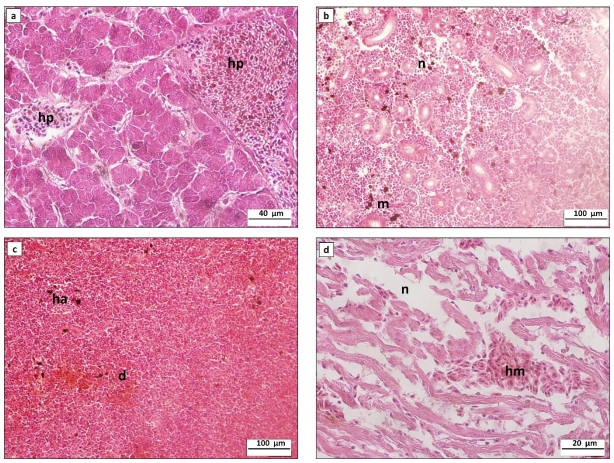

Figure 4. (a) Mass hyperemia [hp] in the liver **(b)** necrotic areas [n]and melanomacrophage centers [m] in the kidney **(c)** hemosiderin accumulation [ha] and depletion of the pulps [d] in the spleen **(d)** and necrotic areas [n] and hemorrhages [hm] in the heart tissues of rainbow trout samples co-infected with *B. gibsonii* and *S. echinoides*

When compared with a mix infection case of *Lactococcus garvieae* and *Frigoribacterium faeni* detected in the same sampling area (Akaylı et al., 2020), melanomacrophage centers were found to be smaller but distributed more frequently in the kidney. Also, despite mass hyperemia in a similar rate, the kidney and the spleen tissue cells looked healthier than the infection cases caused by *Citrobacter freundii* and *Hafnia alvei* in the same sampling area (Akaylı et al., 2021).

As the human and agricultural activities increase, the water chemistry of the rivers and lakes alternates (Taş, 2006; Tambekar and Dhundale, 2012) and the amount and deposit of organic compounds and chance of the occurrence of bacterial species which degrade these compounds also increases (Bakan et al., 2010; Engin et al., 2017). This may lead an increase in the diversity of the bacterial profile of water and fish and new disease agents are detected (Austin and Austin, 2016). Current research studies were generally focused on the molecular identification of clinical and environmental bacterial species that are sometimes hard to identify with the conventional laboratory methods that rely on the biochemical profile (Yabuuchi and Kosako, 2005; Logan and De Vos, 2009).

Representatives of *Bacillus* and *Sphingomonas* genera are distributed in various environments such as soil and water. Some of them are mostly

environmental species with some benefits by means of biotechnological use, but some species can be pathogenic to other organisms (Yabuuchi and Kosako, 2005; Logan and De Vos, 2009). Kızılırmak River receives substantial loads of nutrients, heavy metals (such as lead), trace metals and other organic compounds, resulting from anthropogenic and agriculture activities within its catchment (Bakan et al., 2010; Engin et al., 2017) which provide these bacteria a good source of carbon (Yabuuchi and Kosako, 2005; Logan and De Vos, 2009). The salinity and alkalinity of the water increases according to the agricultural activities and the dissolving of mineral salts present in the river bed (Taş, 2006).

Representatives of the genus *Bacillus*, including *B. gibsonii* is a major source for alkaline pectinase, an enzyme that catalyze the degradation of pectin polymers present in the plant cell walls (Li et al., 2005; Deng et al., 2014). Today, pectinases are the upcoming industrially important enzyme having major industrial importance and they hold a leading position among the commercially produced industrial enzymes (Deng et al., 2014; Kavuthodi and Sebastian, 2018). Tambekar and Dhundale (2012) identified *B. gibsonii* in Lonar Lake (India) with a saline and alkaline character similar to the sampling area of this study. Zhang et al. (2013) characterized a high lead (II) bio-sorption capacity of *B. gibsonii* and it may find potential low-coast application in industrial wastewater treatment.

B. gibsonii was detected in soil among the bacteria which play major roles in the mineralization of plant-derived materials, of humus, of pesticides, and of hydrocarbons in soil of the agriculture fields (Garbeva et al., 2003). Rafat et al., (2012) identified *B. gibsonii* as an endophytic bacterium in the aerial parts of gotu kola plant (*Centella asiatica*). *B. gibsonii* showed antagonistic effect against the fungal pathogen *Botrytis cinera* in tomato culture (Berrada et al., 2012) and the fungal pathogen *Fusarium moniliforme* in maize culture (Batool et al., 2019). Sezen et al. (2013) identified *B. gibsonii* from the European mole cricket, *Gryllotalpa gryllotalpa* collected from Tokat and Trabzon in Turkey and Eski et al. (2017) showed biopesticide effect of this bacterium against coleopterans, particularly against *Agelastica alni* (Coleoptera: Chrysomelidae) which is one of the serious pests of alder leaf (*Alnus sp.*) and hazelnut (*Corylus sp.*). Both of these trees, which can be regarded as a potential source of this bacterium, are commonly distributed along the northern parts of Turkey which includes the sampling area of this study (Dönmez, 2014). Besides, the benefits of various *Bacillus* species such as probiotic use of *B. marisflavi* (Akaylı et al., 2015), Orozova et al. (2018) recovered *Bacillus mycoides* and *B. pseudomycoides* along with *Aeromonas hydrophila* from cultured common carp (*Cyprinus carpio*) and cultured rainbow trout (*Oncorhynchus mykiss*) suffering from gill disease in Bulgaria.

Besides the other species of the genus *Bacillus*, the antagonistic properties of *B. gibsonii* was also evaluated against various pathogens. Antagonistic effect of *B. subtilis* was first reported against phytopathogenic fungi due to its ability to the production of antifungal lipopeptidases and good colonization aptitudes (Cazorla et al., 2007). Antagonistic effect of *B. subtilis* against clinical human isolates of *E. coli* due to its fibrinolytic activity (Jeong et al., 2015; Irkitova et al 2018) were also reported. Recently Nannan et al. (2021) reported the bacilycin production ability of *B. subtilis* which is an important antimicrobial peptide that gives this species the opportunity to be evaluated as a probiotic. Antagonistic effect of *B. subtilis* was also evaluated against fish pathogens and Das et al. (2014) reported a strong antagonistic effect against fish originated isolates of *Pseudomonas aeruginosa, Edwardsiella tarda, Vibrio parahaemolyticus, Flavobacterium columnare* and *Staphylococcus aureus*, but they also reported a negative antagonistic effect against *Aeromonas hydrophila*. Also a strong antagonistic effect of *B. subtilis* against fish pathogenic *Lactococcus garvieae* (Akaylı et al., 2020) and negative antagonistic effect against *Frigoribacterium faeni* (Akaylı et al., 2020), *Citrobacter freundii* and *Hafnia alvei* (Akaylı et al., 2021) recovered from the internal organs of moribund rainbow trout samples was reported. Antagonistic and inhibitory effect of *Lactobacillus rhamnosus* due to its intestinal cell monolayer colonization ability against *E. coli* (Gopal et al., 2001; Tuo et al., 2013), *Salmonella typhimurium* (Fayol-Messaoudi et al., 2007; Tuo et al., 2013) and *Shigella sonnei* (Tuo et al., 2013) and against an opportunistic fungal human pathogen, *Candida albicans* (Verdenelli et al., 2009) was reported. Recently, in addition to its colonization ability, Federova et al. (2018) reported the ability of this species to produce peptidoglycan hydrolases and endopeptidases and disrupting the peptidoglycan bacterial cell wall. Besides the human clinical isolates, *Lactocbacillus rhamnosus* was used as a probiotic bacterium especially against Gram-negative pathogens of marine fishes (Gomez-Gil et al., 2000; Ashraf, 2000; Katırcıoğlu, 2001) but it was insufficient to inhibit Gram-positive

pathogens (Ringo and Gatesoupe, 1998; Burr and Gathlin, 2005). Recently, negative antagonistic effect of *L. rhamnosus* against *L. garvieae, F. faeni* (Akaylı et al., 2020), *C. freundii* and *H. alvei* (Akaylı et al., 2021) recovered from the internal organs of moribund rainbow trout samples was reported.

Sphingonomas echinoides, which was previously classified as *Pseudomonas echinoides* (Denner et al., 1999) was first isolated as a laboratory contaminant (Heumann, 1960) and it is known to be metabolically versatile and can utilize a wide range of natural compounds as well as some types of environmental contaminants (Shin et al.2012). Although some *Sphingomonas* species (such as *S. paucimobilis*) are human pathogens, there are also some saprophytic species in the genus besides the ones used in the food, pharmaceutical and mining industries due to their extracellular products, enzymes and ability to degrade the organic compounds (Yabuuchi and Kosako, 2005; Balkwill et al., 2006). *S. echinoides* is the first marine microorganism exhibiting epoxide hydrolase activity, which is related with carbon assimilation and the metabolism of secondary metabolites from seawater (Kim et al., 2006).

In March 2003, *S. echinoides* dominated the microbial assemblages in both zones of the lagoon concomitantly with a bloom of filamentous cyanobacteria observed in the freshwater and brackish-water zones of a shallow coastal lagoon of the southwestern Atlantic Ocean, located in Uruguay (Piccini et al., 2006). Kimura et al (2011) detected *S. echinoides* in an extremely acidic and iron-enriched environment due to microbially accelerated oxidative dissolution of the sulfide mineral in a low-temperature (~8.5°C), long-abandoned (>90 years) underground pyrite mine in Wales. Pandey et al. (2021) identified *S. echinoides* from the microbiome of wheat in India. Singh et al (2015) recovered S. echinoides from the veterinary clinical cases of dogs in India. Demirci (2017) recovered *S. echinoides* from yogurt samples collected in Turkey. Besides the terrestrial plants, animals and their products, *S. echinoides* is also present in the aquatic environment and found to be associated with various aquatic organisms. It was previously identified in the intestinal microbiota of cultured grass carp (*Ctenopharyngodon idellus*) in China (Zhou et al., 2013; Yuan et al. 2015), among the surface-associated bacteria isolated from the sea cucumber *Stichopus badionotus* in Malaysia (Alipiah et al., 2016), in the microbiota of abalone (*Haliotis discus hannai*) in South Korea (Lee et al., 2016), among the intestinal microbiota of juvenile sea cucumber, *Apostichopus japonicus* (Ma et al., 2018) and among the surface-associated bacteria isolated from the sea urchin (*Tripneustes gratilla*) in South Africa (Brink et al., 2019). Besides, *S. paucimobilis* was also identified in the water released from a rainbow trout farm in Mersin region of Turkey (Özer et al., 2008) and a resistant strain of this species against various heavy metals and antibiotics was identified from the water column of Kızılırmak river (Özer et al., 2013; İçgen and Yılmaz, 2014).

Both the genera *Bacillus* and *Sphingomonas* include a high number of bacterial species which can be sometimes difficult to distinguish at the species level by using conventional laboratory methods. Almost all of the above mentioned citations used various molecular tools for the identification of *B. gibsonii* and *S. echinoides* as used in this study.

Conclusion

As discussed above, the interactions of these and other species of the genera *Bacillus* and *Sphingomonas* with various environments and organisms were shown and this is the first report of a mixed *B. gibsonii* and *S. echinoides* infection in cultured rainbow trout (*O. mykiss*). Various environmental bacterial species, including *B. gibsonii* and *S. echinoides* which can be brought to the sampling area from various sources such as plants, soil, agricultural activities, insects etc. by the water of Kızılırmak River that has a high input of organic compounds and various other elements (Ayaz et al., 2012) and these elements provide bacterial growth which may become pathogenic when suitable conditions are found. When considered the economic effects of diseases, the rising antimicrobial resistance problem and other fish welfare and environmental treats, the results of this study showed that, long term water quality parameter determination and bacterial distribution monitoring studies which include molecular tools should be carried out in the aquaculture sites to increase the success in trout culture.

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COMPLIANCE WITH ETHICAL STANDARDS

Authors' Contributions

Some parts of the data obtained from this long-term project was previously presented as an MSc thesis at İstanbul University Institute of Science Fish Diseases by Dilek ÖKMEN (Baraj gölü kültür gökkuşağı alabalıklarındaki (*O. mykiss*, W.) patojen bakterilere probiyotiklerin antagonistik etkisi). Authors contributed equally to this paper.

Conflict of Interest

The authors declare that for this article they have no actual, potential or perceived conflict of interests.

Ethical Approval

This study was conducted with the permission of Istanbul University Animal Experiments Local Ethical Committee (approved on 23.02.2017).

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