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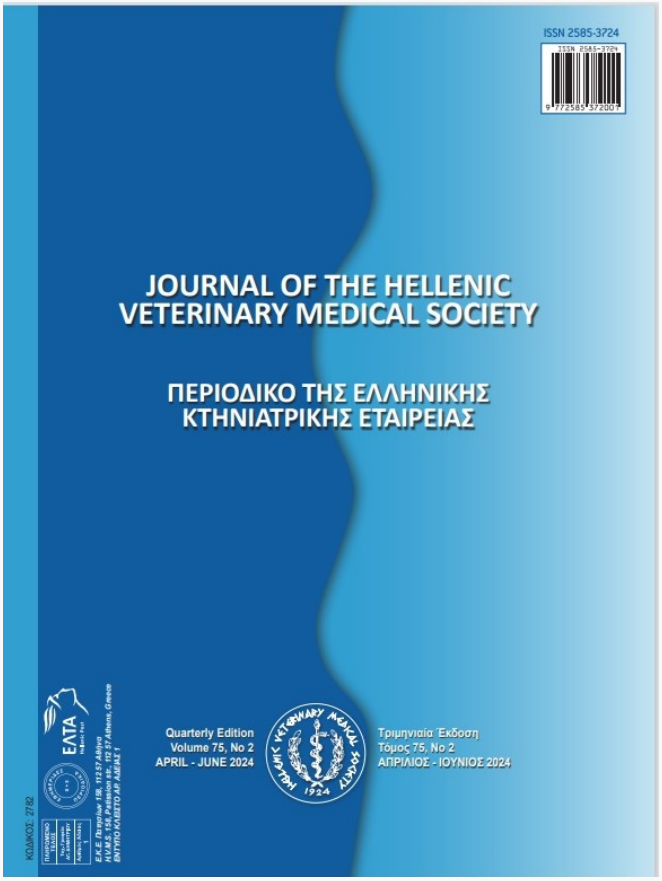

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Bacterial and Parasitic Co-Infection in *Carassius gibelio* Bloch, 1782 Caught in the Onaç Reservoir, Türkiye

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ABSTRACT: This study investigates the bacterial and parasitic co-infection of Prussian carp (*Carassius gibelio* Bloch, 1782). *C. gibelio* belongs to the Cyprinidae family, an alien species in the freshwater systems of Türkiye. Alien species can cause the emergence of new diseases in native species that constitute the ichthyofauna. *C. gibelio* specimens were examined from Onaç Reservoir in Burdur, Türkiye. Symptoms were skin lesions, exophthalmos, darkening in color, rupture, and hemorrhage in the radius of the caudal fin, and loss of scale of the fish. *Gyrodactylus sprostonae* and *Trichodina* sp. were also observed. Additionally, *Shewanella putrefaciens* and *Aeromonas sobria* were isolated from the fish specimens. Erosive and ulcerative lesions were frequently seen during the histopathological investigation. Both isolates were resistant to penicillin, oxacillin, lincomycin, and ampicillin. In conclusion, the presence of various bacterial strains, a ciliate parasite, and a monogenic parasite was determined in *C. gibelio*. We report that the aforementioned fish pathogens created a co-infection in the region. In the future, more study is needed to a clearer awareness of the *C. gibelio* dealing with co-infection and the details of the interaction between bacterial and parasitic diseases. This will be beneficial for fish disease management.

Keywords: *Carassius gibelio*; *Gyrodactylus sprostonae*; *Trichodina* sp.; *Shewanella putrefaciens*; *Aeromonas sobria*

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NTRODUCTION

Türkiye has 25 alien fish species (İnnal and Erk'akan, 2006). One of these exotic species is *C. gibelio*, reported from 46 different freshwater systems in Türkiye. The first record of *C. gibelio* was reported from Lake Gala in the Thrace Region, Turkey. *C. gibelio* is a dangerous fish species for native fish communities (Baran and Ongan, 1988; Arslan and Emiroğlu, 2011). As any alien species can transport alien organisms such as parasites, they cause new diseases in the seriously threatened habitats of native species (Uğurlu and Polat, 2006).

Generally, seasonal diseases recur in the natural environment due to regular seasonal environmental changes. Seasonal diseases in natural habitats were often parasitic or bacterial infections. Bacterial infections generally develop secondary to parasitic infections (Plumb, 1999). One of the most important problems encountered in aquatic products is parasitic diseases. Parasites have a lethal effect on fish, whereas their growth, behavior, and resistance to stress factors were affected. At the same time, it causes the fish to be deprived of vitamins and minerals by exploiting the nutrient from the fish (Timur and Timur, 2003; Austin and Austin, 2016). In addition, parasites form wounds, incisions, and similar damage in muscles and other internal organs by adhering to the adhesion organs in their hosts (Demir, 2014).

Parasites of the genus *Gyrodactylus* can be seen in freshwater and marine fish. Approximately 409 *Gyrodactylus* species have been identified so far, and it is predicted that 20,000 species can be identified (Özer et al., 2011). There are no eyes or eye spots. The parasite is generally located in the skin, fins, and nostrils. Fish-to-fish transmission occurs through direct contact (Stoskopf, 2015).

There is a great number of Trichodinid ectoparasites species reported in nature, and they are a group of widely distributed ectoparasitic ciliates causing serious diseases (Wang et al. 2018). Trichodinid ciliates are one of the most common ectoparasites in wild fish (Basson and As, 2006; Martins and Ghiraldelli, 2008; Martins et al., 2012). With the rapid increase in the number of trichodinids in aquatic environments with high stocking density, the circular movements of the parasite can cause abrasive lesions on the host's body surface (Wang et al., 2022). Secondary bacterial infections that develop from lesions on the body surface can increase fish losses (Iqbal and Haroon, 2014; Valladao et al., 2014).

The damage caused by parasites creates potential port antre for bacterial pathogens (Cusack and Cone, 1985; Cusack and Cone, 1986). There withal, the development of bacteria causes a reduction in the immune system of the fish, which makes the host body suitable for parasitic infestation. Co-infection has often been seen in natural habitats (Gorgoglione et al., 2020). Co-infections can frequently occur in fish. The potential impact of co-infections is much more significant than developing a single disease.

Shewanella putrefaciens (formerly *Pseudomonas putrefaciens, Alteromonas putrefaciens*) is a Gram-negative facultatively anaerobic bacteria in Vibrionaceae (MacDonell and Colwel, 1985; Koziñska and Pêkala, 2004; Varalakshmi et al., 2022). In recent years, the new agent of the disease called Shewanellosis in freshwater fish has been identified as *S. putrefaciens*. This infection has been seen in aquaculture conditions and in many marine and freshwater fish found in nature (Koziñska and Pêkala, 2004; Qin et al., 2012a; Rusev et al., 2016; Pękala et al., 2015; Mohammed and Peatman, 2018)

Aeromonas species are motile or non-motile and generally give positive results in glucose tests, catalase, and oxidase (Martinez-Murcia et al., 2005). The motile Aeromonas-caused infections (*Aeromonas hydrophila, A. sobria,* and *A. caviae*) are mostly known as the reason for hemorrhagic septicemia, mostly associated with fish diseases (Austin and Austin, 2016). Diseases are progressing with high mortality and significant economic losses in freshwater fish, crustaceans, and sometimes marine fish.

In this study, disease-related symptoms were seen in the natural environment on *C. gibelio*. Therefore, we have conducted an investigation on Prussian carp, *C. gibelio* for bacterial and parasitic agents in the Onaç Reservoir, Burdur, Türkiye.

MATERIAL AND METHODS

Study area and fish sampling

Onaç Reservoir was built on the Onaç Stream (37°30'22.17'' N; 30°34'31.71'' E) near Bucak (Burdur) in 2006 for irrigation purposes (Figure 1). The water level rises considerably, especially in spring, due to the runoff waters. In addition to the *C. gibelio* species, alien species *Pseudorasbora parva, Gambusia holbrooki,* and the endemic species *Pseudophoxinus ninae* are distributed at the subject lake.

Figure 1. The study area (Onaç Reservoir) and the sampling point (asterisk).

C. gibelio individuals (n=20) were examined in April 2022 from Onaç Reservoir in Burdur, Türkiye. Fish samples were taken with an electroshocking device. Diseased fish were transported to the laboratory alive (n=20) with ice and oxygen assistance for isolation, diagnosis, and pathogen identification. Firstly, the length-weight measurements and sexuality of the moribund fish were determined. Moribund Prussian carp was 10.96 cm in mean length and 25.48 gr in live weight. The water temperature at the sampling date was 16.9°C, and dissolved oxygen was measured as 4.44 mg/l.

Bacterial Isolation and Identification

The examination was performed in 20 individuals. Samplings were gathered from the head kidney, spleen, and liver and streaked onto tryptic soy agar (TSA) and TSA with 2% NaCl added (TTSA).

Incubation of the plates was done at 24°C for 48 hours. Colonies were identified based on morphological characteristics, physiological and biochemical. All procedures were performed in accordance with guidelines for diagnosing fish diseases and international and national animal welfare guidelines (OIE, 2003; Buller, 2004; Austin and Austin, 2016).

Biochemical, physiological, and morphological characteristics of the strains were determined with the following tests: cytochrome oxidase test kit (Merck), catalase-peroxidase (EC 1.11.1.21) test, motility observed under light microscopy (Olympus CX21), oxidative and fermentative degradation of glucose with O/F basal medium (Merck) supplemented with 1% glucose, resistance to vibriostatic agent O/129 (Buller, 2004; Austin and Austin, 2016).

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed to determine the antimicrobial resistance profile of the isolated bacterial strains. For this purpose, the specification of the antimicrobial susceptibility of colonies was done according to the Kirby-Bauer disc diffusion method (Oxoid) (Biemer, 1973). Results were assessed by the instructions of the Clinical and Laboratory Standards Institute (CLSI, 2014).

Molecular Identification

Bacterial colonies were taken individually from the first isolates, and then serial streaking to an agar plate was done for purification. The differentiated two colonies from biochemical, morphologic, and physiological tests were identified by sequencing the 16S rRNA (16s ribosomal Ribonucleic Acid) gene. According to the manufacturer's protocol, the genomic DNA from selected isolates was extracted using the GeneMatrix Bacterial and Yeast Genomic Purification Kit (EURx, Gdansk, Poland). The 16S rRNA gene was amplified by the following primers:

27F (5'AGAGTTTGATCMTGGCTCAG-3')

1492R (5' TACGGYTACCTTGTTACGACTT-3').

PCR reactions were prepared with the following ingredients: 1X PCR buffer, 1.5 mM MgCl2, 0.2 mM dNTP mix, 0.3 µM forward primer, 0.3 µM reverse primer, 2U of Taq DNA polymerase from 5U/µl stock, and 3 µl of DNA template. The reaction mixture was prepared to be 35 µl for each reaction. Thermal cycles were set to 95°C 5 min. - initial denaturation, 30 cycles of "95°C for 45 s - denaturation, 57°C for 45 s - annealing, 72°C for 60 s - extension", and 72°C for 5 min for a final extension. PCR products were controlled with the Gel electrophoresis (1x TAE buffer, %1.5 Agarose, 100 volts, 90 min., EtBr stained). PCR products were purified with MAGBIO "HighPrep^{TM} PCR clean-up System" following the manufacturer's protocols. ABI 3730XL Sanger sequencing instrument (Applied Biosystems, Foster City, CA) and BigDye Terminator v3.1 Cycle Sequencing Kit were used for Sanger Sequencing samples (Applied Biosystems, Foster City, CA). Reads obtained with primers 27F and 1492R were contiguous to form a consensus sequence. CAP contig assembly algorithm was used in BioEdit software to perform this process. Obtained final sequences were submitted to NCBI nucleotide search for identification.

Parasitical Examination

Each fish's external and internal organs were examined for parasites at the Faculty of Eğirdir Fisheries in Isparta, Türkiye. Parasite species were identified using an Olympus light microscope (Olympus CX21). Diagnosis of the present parasites was made morphologically using various references (Basson and As, 2006; Buchmann and Bresciani, 2006; Schäperclaus, 1984; Stoskopf, 1993).

Histopathological Analysis

Lesioned skin and visceral organ samples were taken during the necropsy and fixed in a 10% buffered formalin solution for histopathological examination. An automatic tissue processor (Leica ASP300S, Wet-

Figure 2. External lesions in *Carassius gibelio* individuals from Onaç Reservoir (a- 6.6 cm SL, b- 8 cm SL, c-12.7 cm SL, d-19.4 cm SL, e- 10.8 cm SL, f- 113.3 cm SL, g- 1016.6 cm SL)

zlar, Germany) was used to process the tissue samples, then embedded in paraffin wax. A fully automatic rotary microtome (Leica RM2155, Wetzlar, Germany) was used to cut paraffin blocks with a thickness of 5 µm. Then sections were stained with Hematoxylin-eosin (H&E) staining and analyzed under a light microscope (Luna, 1968). The Database Manual Cell Sens Life Science Imaging Software System created microphotographs and evaluated morphometric data (Olympus Corporation, Tokyo, Japan).

RESULTS

Gross findings

Common symptoms seen in infected fish (Figure 2) at the gross examination were ulcerative skin lesions (n=20), exophthalmos (n=15), darkening in color (n=15), rupture and hemorrhage in the radius of the caudal fin (n=20), loss of scale (n=18), hemorrhage (n=18), and hyperemia in the tail and mouth (n=18), excessive amount of mucus (n=18), hyperemia of the gills (n=20), and swelling of the gill filaments (n=18), local hemorrhages (n=18), and prolapse of the anus (n=18).

Parasitic Findings

In the parasite examinations, *Gyrodactylus sprostonae* from the monogenic group and *Trichodina* sp. from protozoan ciliate parasites were detected (Figure 3). Morphological features were used to define the *G. sprostonae* species. *Gyrodactylus* are small, spindle-shaped, ectoparasitic trematodes. The body length of the parasites we caught varied between 0.3-0.6 mm (The body of a normal *Gyrodactlus* varies between 0.2-1.0 mm). The front end of the body had 2 contractile suckers and it didn't have the eye. The attachment organ had 18 peripheral hooks and two connecting bars. It had anchor-shaped hooks.

The presence of both parasites was observed intensively in all fishes. During the study period, 20 Prussian carp were examined, and parasites were found in all (100%).

Bacteriologic Findings

In bacteriologic examinations, inoculations made from the head kidney, spleen, and liver have resulted in morphologically different two colonies only on the TSA plate for all samples. Analysis of pure colonies revealed that stain 1 (n=18) was Gram-negative, motile, catalase and cytochrome oxidase positive, and fermentative. Stain 2 (n= 20) was Gram-negative, catalase and cytochrome oxidase-positive, non-fermentative, and O/129 sensitive.

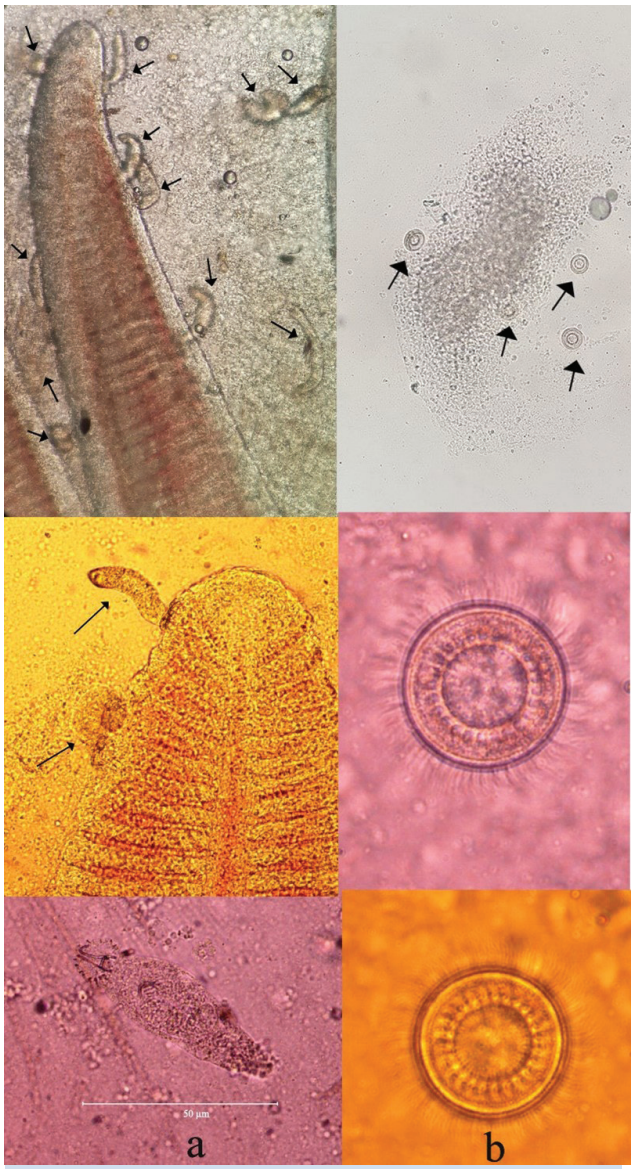

Figure 3 *G. sprostonae* (a) and *Tricodina* sp. (b) on gill filaments in *C. gibelio* individuals from Onaç Reservoir.

The partial 16S rRNA sequence (ca. 1.4 kb) of the *S. putrefaciens* isolate was submitted to the GenBank database with accession no: OR468003. Similarities between its 16S rRNA sequence and those of *S. putrefaciens* strains in the GenBank database were to 100%.

The partial 16S rRNA sequence (ca. 1.4 kb) of the *Aeromonas sobria* isolate was submitted to the GenBank database with accession no: OR468004. Similarities between its 16S rRNA sequence and those of *A. sobria* strains in the GenBank database was to 100%.

The antibiogram test was performed in 3 parallels. The diameters of the inhibition zones averages are recorded in Table 1. According to the antibiogram test results, both isolates were susceptible to Oxolinic Acid, Florfenicol, Enrofloxacin, Norfloxacin, Tetracycline, Doxycycline, Kanamycin, Spectinomycin, Chloramphenicol, Flumequine, Nitrofurantoin, and Erythromycin. However, both isolates were resistant to Penicillin, Oxacillin, Lincomycin, and Ampicillin.

Histopathological Findings

In the affected skin areas, erosive and ulcerative lesions were frequently seen during the histopathological investigation. These areas had completely necrotic skin layers, and some muscle tissues were significantly affected. In locations with lesions, inflammatory cells infiltration was moderate to noticeable. Additionally, hemorrhages and edema were also noted. Although melanomacrophages were also seen, heterophile leukocytes and lymphocytes make up the majority of the inflammatory cells (Figure 4).

In addition to skin lesions, numerous parasites were seen in the gills and visceral organs. Pathological findings, including inflammatory cell infiltrations or gill fusions, were brought on by parasites. Subepithelial necrotic cells, subepithelial leukocyte-based inflammation, and adaptive hyperplastic alterations involving mucus and epithelial cells were common changes in gills. Around the parasites, the other locations had a slight inflammatory reaction (Figure 5)

DISCUSSION

It is known that the resistance of fish against diseases decreases at the end of the winter period and at the beginning of spring in fish in natural aquatic environments. When it was reduced resistance to diseases, bacterial and parasitic diseases commonly relapse, and co-infection occur (Schade et al., 2016; Akaylı et al., 2019).

Parasites of the genus *Gyrodactylus* have the capability to rapidly widen their population in a relatively short period due to high breeding rates and direct transmission between hosts (Rawson and Rogers, 1973). Thus, the parasites reproduce rapidly in a short time, take their place in the hosts' skin, gills, and fins, and cause significant damage to the fish. Wounds

Table 1 The diameters of the inhibition zones (mm) of *S. putrefaciens* and *A. sobria* isolates

Antibiotic Disc	**Isolate, *S. putrefaciens***	**Isolate, *A. sobria***
Gentamicin	16 (S)	15 (I)
Oxolinic acid	26 (S)	31 (S)
Penicillin	6 (R)	0 (R)
Oxacillin	6 (R)	0 (R)
Florfenicol	35 (S)	34 (S)
Apramycin	16 (S)	10 (R)
Enrofloxacin	29 (S)	40 (S)
Streptomycin	9 (R)	15 (I)
Norfloxacin	23 (S)	38 (S)
Tylosin	19 (S)	0 (R)
Tetracycline	21 (S)	30 (S)
Doxycycline	22 (S)	27 (S)
Kanamycin	19 (S)	17 (S)
Spectinomycin	18 (S)	20 (S)
Pristinamycin	24 (S)	10 (R)
Lincomycin	0 (R)	0 (R)
Chloramphenicol	31 (S)	34 (S)
Ampicillin	0 (R)	0 (R)
Clindamycin	19 (S)	0 (R)
Flumequine	29 (S)	41 (S)
Nitrofurantoin	20 (S)	23 (S)
Vancomycin	21 (S)	11 (I)
Erythromycin	30 (S)	20 (S)

"S" = Sensitive (d > 15 mm); "I" = Intermediate (10 mm < d ≤ 15 mm); "R" = Resistant (d ≤ 10 mm)

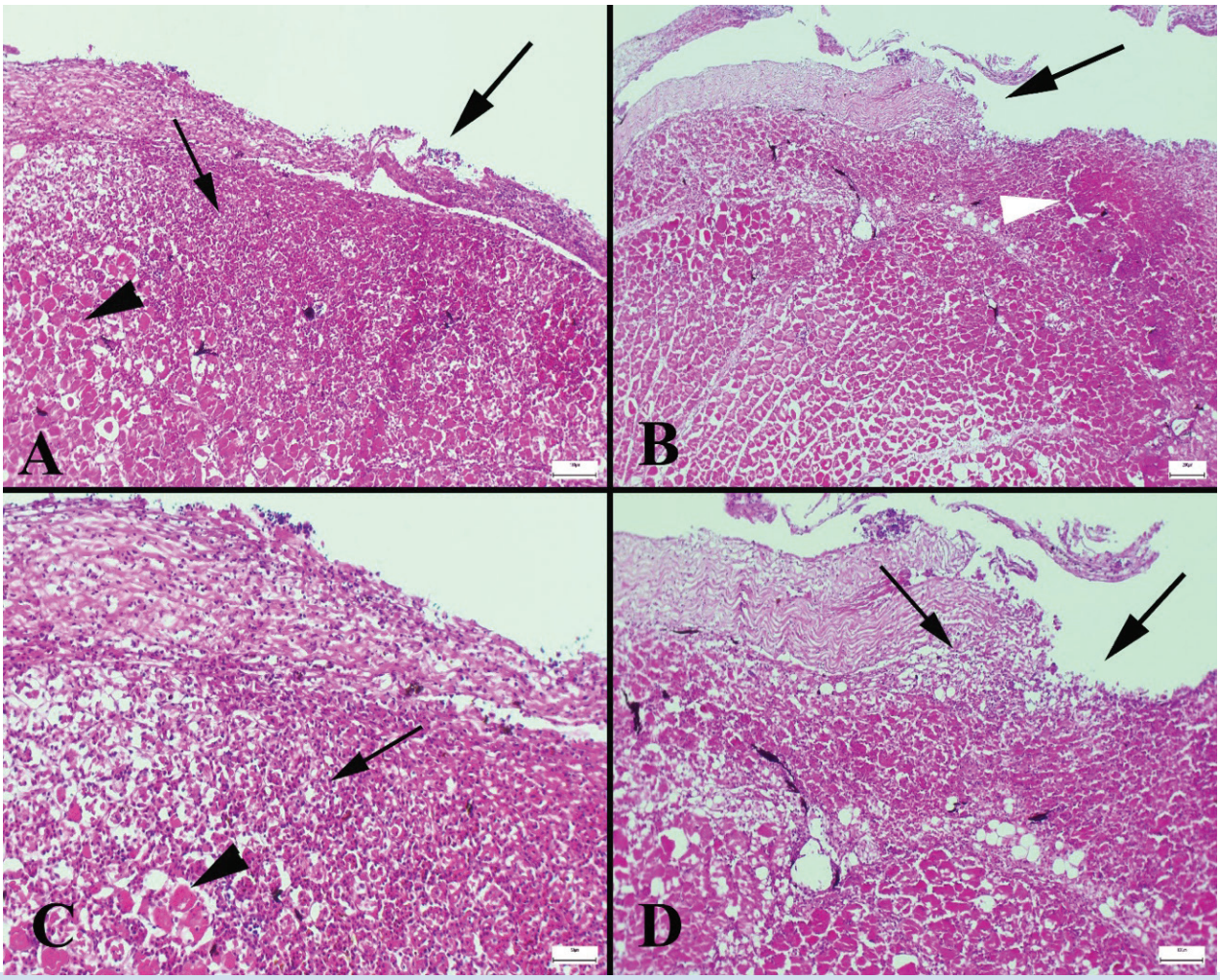

Figure 4 Histopathological appearance of the lesioned skin areas. (a) Totally ulcerated skin (thick arrow) and the inflamed muscle area, inflammatory cell infiltrations (thin arrow), and degenerated muscle cells (arrowhead), H&E, Scale bar=100μm. (b) Totally ulcerated skin and hypodermis (thick arrow) and a big necrotic area in the muscle (white arrowhead), H&E, Scale bar=200μm. (c) Higher magnification of the inflammatory reaction (thin arrow) and degenerated muscle cells (arrowhead), H&E, Scale bar=50μm. (d) Higher magnification of the lesioned area in another fish inflammatory reaction (thin arrow) and skin ulcer (thick arrow), H&E, Scale bar=100μm

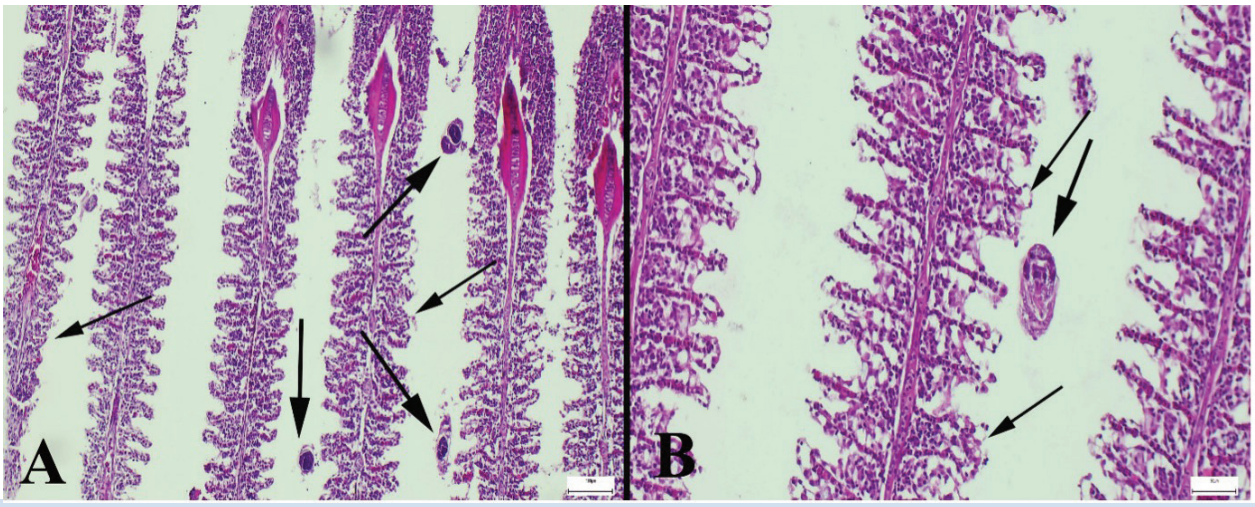

Figure 5 The microscopical appearance of the parasites. (a) Numerous parasites (thick arrows) in the gills of a fish and fusions in gill lamellas (thin arrows), H&E, Scale bar=100μm, (b) Another gill parasite (thick arrow) and lamellar fusions (thin arrow) in a fish, H&E, Scale bar=50μm.

inflicted by parasites create a port antre for bacteria (Cusack and Cone, 1985; Cusack and Cone, 1986).

Kayış et al. (2018) detected parasitic and bacterial pathogens in naturally distributed fish species in Deriner Dam Lake. Similar to our study, it was observed that both bacterial agents, along with the *Trichodina* and *Gyrodactylus* parasites, caused co-infection in different fish species. Despite co-infections, the presence of different types of parasites was determined. Akaylı et al. (2019), *Gyrodactylus* and *Trichodina* parasites were reported in Seven khramulya (*Capoeta capoeta*). Bacterial infections *S. capitis*, *Vibrio fluvialis*, *Staphylococcus warneri*, and *A. hydrophila* were also observed, which caused co-infection in the host.

Studies have shown that parasites of the genus *Gyrodactylus* are generally located on the fish skin and fins (Schäperclaus, 1984; Stoskopf, 1993). However, in the examinations we have made in Prussian carp, it has been seen that *G. sprostanae* was dominantly located in the gill tissue.

Trichodina has been studied for almost 200 years. Species identification of this genus and methods based on morphological features for identification remain challenging due to their high-level interspecific similarity, variation, and low host specificity (Tang et al., 2017; Wang et al., 2019; Wang et al., 2022). Therefore, the parasite was identified as *Trichodina* sp in the present study.

In bacteriologic examinations, two different pure colonies were found, and molecular studies showed that there were *S. putrefaciens* and *A. sobria* species. The bacterial fish pathogen *S. putrefaciens* poses a serious problem because it is facultative and can cause disease under unfavorable environmental conditions (Paździor, 2016). This pathogen was isolated from *Carassius* spp by Qin et al. (2012b). However, for *C. gibelio*, it is the first isolation in Türkiye. There are plenty of reports declare that *S. putrefaciens* was isolated together with other species, e.g., *Pseudomonas* sp. and *Aeromonas* sp. *(*Pêkala et al., 2015), *Listonella* sp. (Qin et al., 2012a; b), and *S. warneri* (Rusev et al., 2016). Our study is similar to other researchers'; the pathogen was isolated with *A sobria*. The disease usually occurs in spring when the water temperature is 7-10 °C. (Pêkala et al., 2015). Furthermore, other studies have shown that *S. putrefaciens* grows in pond water with temperatures between 5 and 15 °C. In this study, diseased fish were found in spring at 16.9 °C. This shows a correlation between fish infection and the spring season. Symptoms of Shewanellosis infection included ulcers and lethargy on the dorsal part of the body, hemorrhage in the kidney and spleen, and petechiae in the swim bladder. The observed symptoms were similar to other research reports (Jiang et al., 2022). *S. putrefaciens* was first observed in *Siganus rivulatus* reared in the Red Sea in 1985 (Saeed et al., 1987). *S. putrefaciens* was also isolated in 2004 from *Cyprinus carpio* and *Oncorhynchus mykiss* farms in Poland (Kozińska and Pêkala, 2004). First isolation from marine finfish was reported in *Dicentrarchus labrax* in the Aegean region of Türkiye (Korun et al., 2009), from the Black Sea region of Türkiye by Kayış et al. (2009), and *Carassius auratus auratus* by Altun et al. (2014). *S. putrefaciens* infection is also seen in humans (Bulut et al., 2004).

Antibiotic resistance of *S. putrefaciens* varies by source. Nowadays, studies have been conducted on the susceptibility of not only humans but also freshwater fish and marine fish to various chemotherapeutic agents. *Shewanella* spp are generally resistant to penicillins, also found in the present study. Most isolates showed sensitivity to tetracycline as in this study (Heritier et al., 2004) (Table 1).

Bacterial infections caused by motile members of the genus *Aeromonas* are known as motile Aeromonas septicemia (Haemorrhagic septicemia). It is among the common diseases in fish farms and natural populations. The *Aeromonas* bacteria that cause these infections are called aeromonads. Motile *Aeromonas* were responsible for significant financial losses each year, whether they moved alone or co-infections with other organisms. All members of the *Aeromonas* genus are small, motile, Gram-negative, and rod-shaped bacteria (Camus et al., 1998; Austin et al., 2007). *A. sobria* was isolated from wild egg-laying *Dorosoma cepedianum* in Maryland (Toranzo et al., 1989) and cultured *Perca fluviatilis* in Switzerland (Wahli et al., 2005; Austin et al., 2007). In Türkiye, the Elazığ region by Muz et al. (1995). Hemorrhagic septicemia is characterized by small surface lesions (lead to scale shedding), local hemorrhages in the gills and vent, ulcers, abscesses, exophthalmia, and abdominal distension (Austin et al., 2007). Our study, found that sepsis infection was concentrated in the abdominal cavity. Moribund fish showed loss of scales and occasional skin bleeding on the abdomen and fin bases. Additionally, there was some evidence of abdominal distension. It has been reported in many studies that *S. putrefaciens* bacteria cause co-infection with *Aer-*

omonas species in fish (Yi et al., 2012; Kotob et al., 2017).

CONCLUSIONS

This may contribute to the development of infection prevention methods. In recent years, *S. putrefaciens* infection has been common in freshwater fish. Therefore, there is a need to characterize this bacterium for the future protection of fish health, the environment, and humans. Co-infections are very common in natural aquatic environments. Co-infection can have serious consequences, altering the course and severity of diseases in different fish. This study determined the presence of various bacterial strains in Prussian carp, a ciliate parasite, and a monogenic parasite. Co-infection of fish has been shown to cause severe disease and tissue damage. Accordingly, the synergistic effects of pathogens cause more damage to fish. In this study, it was observed that *S. putrefaciens* co-infected with *A. sobria* for the first time. Investigation of the pathogenesis and epidemiology of such co-infections in fish is essential in taking necessary precautions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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