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Fatty Liver Disease and Bacterial Co-Infection in Cultured Marine Fish

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Abstract: In the present study, we sampled sea bream and sea bass, weighing 400-450g, for routine microbiological, hematological, macroscopical and histopathological examinations. Samples were taken from seemingly healthy fish, which displayed increased feed conversion rates, signs of cold shock and slow movement. Routine bacteriological analyses included basic microbiological analyses for oxidase and catalase activity, Gram staining and O/F fermentation using API test kits. Furthermore, 16S rRNA gene sequencing was performed for identification. Serum biochemical and hematological values were determined using a Vetscan®VS2 analyzer and blood smears. Internal organs were examined by routine histopathological techniques. Hepatic fat accumulation and necrosis were noted, and liver damage was observed to be associated with significant alterations in liver enzymes. Due to multi-organ dysfunction and serious hematological disorders, primarily *Vibrio alginolyticus* also *Alteromonas* and *Pseudoalteromonas* species were isolated from the affected fish and co-infections were detected.
Keywords: Fatty liver syndrome, hematology in marine fish, hepatic lipidosis, *V. alginolyticus*

Kültürü Yapılan Deniz Balıklarında Karaciğer Yağlanması ve Bakteriyel Koenfeksiyon

Öz: Bu çalışmada rutin mikrobiyolojik, hematolojik, makroskopik ve histopatolojik incelemeler için 400-450 gr ağırlığında çipura ve levrek balıkları örneklenmiştir. Örnekler morfolojik olarak sağlıklı görünen ancak artan yem dönüşüm oranı, hasat sırasında şoklanma (soğuk şoku) sorunu gösteren ve hareketlerde yavaşlık gibi semptom gösteren balıklardan örnekler alınmıştır. Gram boyama, hareketlilik, oksidaz-katalaz aktivitesi ve O/F fermentasyonu gibi rutin bakteriyolojik analizler ve API test kitleri kullanılarak temel mikrobiyolojik analizlerle bakterilerin mikrobiyolojik teşhisleri yapılmıştır. Ön teşhisi yapılan türler16S rRNA gen bölgesi sekans analizi ile tür bazında identifiye edilmiştir. Serum biyokimyasal ve hematolojik değerleri, Vetscan® VS2 cihazı ve kan yayma frotileri kullanılarak analiz edilmiştir. Hastalıktan etkilenen iç organların incelenmesi için rutin histopatolojik incelemeler yapılmıştır. Hepatik yağ birikimi ve nekroz görünen en belirgin bulgulardan olmuştur ve karaciğer enzimlerinde önemli değişikliklerin karaciğer hasarıyla ilişkili olduğu tespit edilmiştir. Etkilenen balıklardan çoklu organ yetmezliği ve hematolojik bozukluklar nedeniyle başta *Vibrio alginolyticus* olmak üzere *Alteromonas* ve *Pseudoalteromonas* türleri izole edilmiş ve hastalık belirtileri gösteren balıkların bakteriyel koenfeksiyon taşıdığı tespit edilmiştir.
Anahtar kelimeler: Deniz balıklarında hematoloji, hepatik lipidosis, *V. alginolyticus,* yağlı karaciğer sendromu

Introduction

Globally, food resources, including both plant- and meat-derived proteins, are decreasing and production costs have increased at least two-fold in the last two years, particularly during the Covid-19 pandemic because of economic instability. Owing to its high protein content and digestibility, fishmeal is the best protein source for carnivorous fish, which need at least 40% of the fish-based protein in their diet (Monge-Ortiz et al., 2016). More than 3.3% of the total fish products are used for the production of fishmeal, but the remarkable increase in cultured fish stocks has brought about insufficiency of fishmeal protein. The increasing demand for fishmeal (FM), elevated prices and fluctuations in world supply have emphasized the need for alternative protein sources for fish. Thus, research on new plant-based protein sources has accelerated. Researchers have discovered several proteins, apart from animals that can replace fishmeal, and most of them enable fast growth, stimulate immunity and support non-specific defense in the short-term (Geay et al., 2011). However, the long-term effects of these plant proteins on fish, especially on their liver, spleen, kidneys, and immune system, remain unknown. Fishmeal is also a primary source of essential amino acids and fatty acids for carnivorous fish, and plants do not contain any of them. Fatty liver syndrome is encountered primarily in fish fed on an imbalanced diet, a carbohydrate-rich diet or

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higher plant-derived proteins instead of fishmeal (De Francesco et al., 2004). Moreover, histomorphological gut and liver alterations, immune status disorders and gut microbial imbalances have been reported to be associated with the provision of plant proteins instead of fishmeal (Egerton et al., 2020). Thus, the use of certain agricultural by-products seems to ultimately lead to a lower feed conversion efficiency and an increase in susceptibility to diseases and bacterial and parasitic infections, which may be induced by immunodeficiency or disrupted inflammatory response. Along with other physiological processes, in fish, the gut plays a key role in the immune response to potential pathogenic invasions besides liver, spleen and erythrocytes whose roles in the immune status and allow the early detection of impaired malnutrition and health, blood biochemistry is usually not used as a diagnostic tool in fisheries (Figure 1). This is due to the scarcity of reliable information on hematologic values and blood biochemistry parameters in well-nourished, healthy and stress-free fish. This issue is, however, very opportune, as fish reared under intensive conditions are subjected, on a daily basis, to different stressors that compromise their nutritional and immune competence; thus, an early and reliable diagnosis of these conditions would help to improve management practices (Peres et al., 2014).

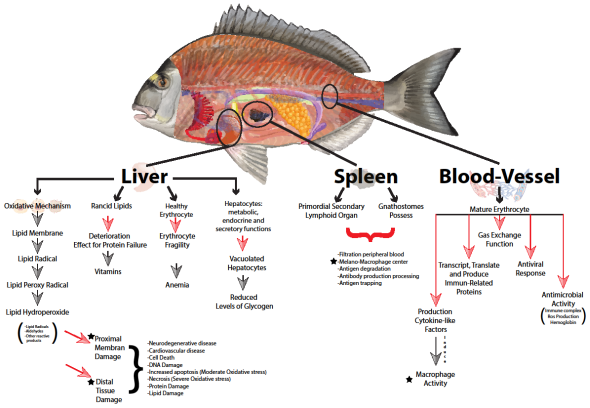

Figure 1. The role of the liver, spleen and erythrocytes in the immune system is illustrated.

In the presence of stress conditions, diseases caused by opportunistic pathogens, including primarily species of the genera *Vibrio* and *Alteromonas*, or other members of the microbiota gaining dominance, is only the tip of the iceberg (Balebona et al., 1998; Skovhus et al., 2007; Duman et al., 2020). *V. alginolyticus*, a prominent and ubiquitous organism found in seawater has been isolated from different marine organisms as part of the saprophytic microbiota (Carli et al., 1993). There is controversy about the precise role of *V. alginolyticus* as a fish pathogen (Balebona et al., 1998), and while extensive information is available on other fish-pathogenic *Vibrio* species, the epidemiological, physiological, and virulence characteristics of *V. alginolyticus* have not been established yet. *Alteromonas* and *Pseudoalteromonas* species have been isolated from marine habitats and sea water and are not considered to be fish pathogens. Hence, the isolation of these species from marine fish indicates exposure to severe stresses conditions and imbalance of the digestive and mucosal microbiota (Skovhus et al., 2007; Pujalte et al., 2007).

In the present study, we aimed to determine the results of fatty liver syndrome based on blood biochemistry, microbial status, morphology, and histopathology. Sampled fish were selected among those that displayed key symptoms, including cold shock (absence of rigor mortis or rapid progress to algor mortis), anemia, yellowish (fatty) liver and coagulopathy. The present study sheds light on contributing disease agents that may be overlooked in marine fish diagnosed with disease.

Material and methods

Fish samples

In total, 50 sea bream (*Sparus aurata*) and 50 sea bass (*Dicentrarchus labrax*), weighing 400-450g, were sampled for routine bacteriology after blood drawing in 2017. Cage farms were located in the Aegean region of Turkey. Blood, histopathological and bacteriological samplings were made from fish displaying absence of rigor mortis after death and very quick death after being taken out of water (Duman et al., 2019). No morphological lesions were detected in the sampled fish and clinical observations showed that the fish displayed lethargy, slow movement and reduced feed intake. The fish ration was presented in Table 1. This research was approved by the Local Ethics Commission (report 2012-14/04)

Table 1. Fish rations used in the study

Dietary ingredients (g/kg dry diet)*	
Total protein (%)	45
Total Fat (%)	20
Crude Cellulose (%)	2.5
Ash (%)	11
Vitamin A (IU)	12000
Vitamin D3 (IU)	2500
Vitamin E (mg)	200
Vitamin C (mg)	200

*Raw materials presented by firm: Fish meal, soybean meal, fish oil, wheat flour, wheat gluten, full fat soy, vitamin and mineral with unknown amount for Vitamin (K3, B1, B2, B6, B12, Niacin, D-pantothenic acid, Folic Acid, Biotin, Choline) and Mineral (Copper, Manganese, Cobalt, Iodine, Zinc, Selenium)

Bacterial isolation

Samplings were carried out according to the guidelines for the diagnosis of fish diseases and in compliance with the international guidelines for animal welfare and guidelines for aquatic animal health surveillance (Austin and Newaj-Fyzul 2017). Diseased fish were sampled aseptically with sterile loops and swabs from the liver, kidneys, spleen and acidic fluid. Samples were placed on tryptic soy agar containing 1.5% NaCl (Merck, 105458, TSA); TSA containing 1.5% marine salts (Sigma, S9883);marine agar (Sigma, 76448) and blood agar (BA; with 5% sheep blood). All isolates were sub-cultured in tryptic soy broth with 1.5% marine salts and marine broth for 2-7 days at 28°C to ensure purity. Pure cultures were supplemented with 20% of glycerol and kept at -80°C for long-term storage. The biochemical characteristics of the isolates were determined using conventional microbial tests, including the assessment of colony morphology, Gram staining, motility, oxidase and catalase activities, and sucrose fermentation (Garrity, 2007; Farmer et al.,2015). Detailed phenotypic characterization was performed using API ID 32E (Biomerieux, France) test kits in accordance with the manufacturer's instructions. The only modification to these instructions was the performance of incubation at 28°C for 24-48h.

Molecular identification and phylogeny

According to the manufacturer's instructions, the genomic DNA of the bacterial isolates was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). PCR amplification and sequence analysis were performed using the universal 16S rRNA primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR reactions were performed according to previously described methods (Duman et al., 2020). Based on 16S rRNA sequence similarities, the isolates were identified in the GenBank 16S RefSeq database, and all of the sequences were deposited in GenBank under the accession number MW513495-MW513504.

The evolutionary history was inferred using the maximum likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-4465.91) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model and then selecting the topology with a superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). This analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1553 positions in the final data set. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Blood and serum analysis

Blood samples were drawn from the caudal vein of the fish, as described before (Duman et al., 2019). Once collected, blood samples were immediately subjected to hematological analysis. The blood was diluted with appropriate diluting fluids for RBC and WBC counts, which were calculated using an improved Neubauer hemocytometer (Mgbenkaet et al., 2003; Shah and Altindag 2005). Replicated counts were made for each blood sample. Blood collected in dry tubes was centrifuged for serum extraction. Serum samples were analyzed for biochemical parameters (creatine kinase, CK;aspartate aminotransferase, AST;alanine aminotransferase, ALT;alkaline phosphatase, ALP;lactate dehydrogenase, bilirubin, BIL; sodium, NA; potassium, K; calcium, CA; urea, BUN;

creatine, CRE; total bilirubin, TBIL;albumin, ALB; glucose, GLU; Gamma-glutamyltransferase, GGT; total protein, TP; total globulin, GLOB; tCO_2; Amylase and Lipase) with a VETSCAN® VS2 chemistry analyzer (Zoetis US, UK).

Histopathology

Tissue samples taken from the liver, kidneys, heart, and gills were fixed in 10% buffered formalin solution, embedded in paraffin blocks, cut into 4-µ-thick sections, and stained with hematoxylin, and eosin (H&E).

Antimicrobial susceptibility testing

Resistance to different groups of antibiotics was determined using the disc diffusion method by complying with the performance standards for the antimicrobial susceptibility testing of bacteria isolated from aquatic animals (Clinical and Laboratory Standards Institute, CLSI guidelines VET03/VET04-S2) (CLSI-Clinical and Laboratory Standards Institute 2014). All antimicrobial susceptibility discs were supplied from Oxoid (Thermo Scientific, US). The antibiotics used in the susceptibility tests included sulfonamides (trimethoprim/sulfamethoxazole, SXT, 1/19; CT0052B), aminopenicillins (amoxicillin, AML; CT0223B), β-lactams (amoxicillin/clavulanic acid, AMC; CT0223B), tetracyclines (doxycycline, DO; CT0018B; oxytetracycline, OT; CT0041B), fluoroquinolones (enrofloxacin, ENR; CT0639B), quinolones (oxolinic acid, OA; CT0017) aminoglycosides (gentamicin, CN; CT0794B), macrolides (erythromycin, E; CT0020B), clindamycin (lincomycin, MY; CT0028B), and chloramphenicol (florfenicol, FFC; CT1754B). Antibiotic discs were placed on Mueller-Hinton agar (MHA)+NaCl (1%) plates, previously inoculated with a pure culture of the strains. After incubation at 28ºC (24-28 h), inhibition zones were measured in mm and compared with the critical values of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) to evaluate whether a strain was sensitive or resistant. *E. coli* ATCC 25922 was used as the quality control (QC) strain, and the acceptable ranges set by the CLSI for this strain were applied (CLSI-Clinical and Laboratory Standards Institute 2014).

Results

Bacterial Identification

In total, three different genera were phenotypically identified, including *Vibrio*, *Alteromonas*, and *Pseudoalteromonas*, which displayed glucose fermentation on oxidative-fermentative medium (OF basal medium). *Vibrio* species were observed as Gram-negative, motile, oxidase, and catalase-positive, sucrose-fermenting colonies on thiosulfate-citrate-bile salts-sucrose agar (TCBS). All isolates were primarily identified as either *Vibrio metschnikovii* (API ID 32E profile number: 11455361072; 00051221043; 20040321040) or *Vibrio alginolyticus* (API ID 32E profile number: 00071321050 and 10071321050) with the API tests. Ornithin decarboxylase activity was the only difference between the *V. alginolyticus* strains, which were at least 99% identical, whilst the test results were more variable for the *V. metschnikovii* isolates, which displayed a similarity rate lower than 90% in the API database.

According to genomic analyses, the isolates from the sampled sea bass were identified as *V. alginolyticus*, whilst the isolates from the sampled sea bream were identified as *V. alginolyticus, V. chagasii, Pseudoalteromonas gelatinilytica, Pseudoalteromonas* sp., and *Alteromonas mediterranea.* Sequences were compared in the NCBI database, and accession numbers were presented in the phylogenetic tree (Figure2).

Molecular characterization

The strains identified at genus level were confirmed by 16S rRNA gene sequencing, and the bacteria isolated from the sea bass and sea bream were identified as *Vibrio alginolyticus* (Seq1), MW513495 and (Seq3), MW513497, respectively. The other *Vibrio alginolyticus* isolates were grouped in a distant branch (Seq4, MW513498; Seq2 MW513496). *V. chagasii* (Seq8, MW513501) was closely related to *V. chagasii* type strain LMG 21353. Different from *V. alginolyticus* (Seq1, MW513495) isolated from sea bass, the *V. alginolyticus* strains isolated from sea bream were not all placed in the same geno group (branch).Furthermore, (Seq4), MW513498 was determined to be the most distant strain. The *P. gelatinilytica* strains (Seq5), MW513499 and (Seq9), MW513502 were in the same group with the reference strains of *P. gelatinilytica* and *P. shioyasakiensis*, NR 152003.1 and NR 125458.1, respectively, and were 100% identical to *P. gelatinilytica* (Reference RNA sequences, RefSeq_rna) in the NCBI database. Other *Pseudoalteromonas* strains did not present an identical profile at species level. Hence, they were identified as *Pseudoalteromonas* sp. in view of their close homology with *P. carrageenovora* and *P. hodoensis*, the reference accession numbers of which are NR 113605.1 and NR 126232.1, respectively. The last strain isolated from the sea bream was *A. mediterranea,* Seq6, which showed close homology with the reference strain (NR 148756.1). Detailed phylogenetic relationships are presented in Figure 2.

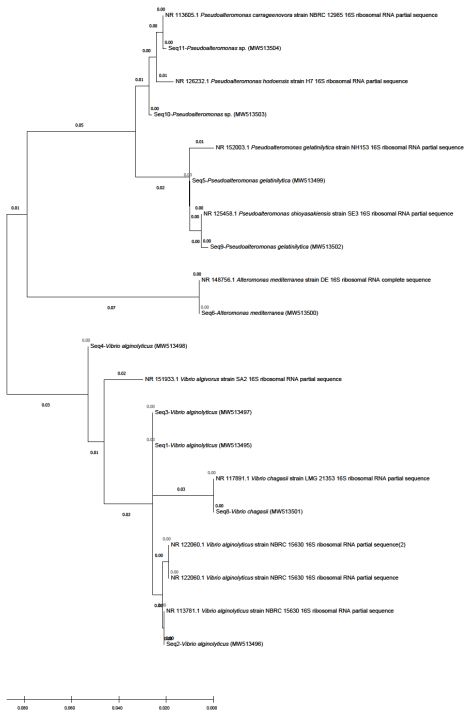

Figure 2. Bacterial phylogeny based on 16S rRNA gene sequencing.

Blood-serum analysis

The hematological values and serum biochemical parameters of the affected fish were compared to the results reported in a wide range of literature, in view of normal blood values being highly variable and depending on water temperature, feeding rate and regime, stock conditions and fish weight (Table 2). While the enzyme CKi was found at very low levels in sea bass, it was found at ten times higher levels in sea bream, similar to the case with AST values. ALT levels fell within the suggested range in the sampled sea bass, and were higher in the sampled sea bream. Contrary to the sampled sea bream, most values were lower than the reference values in the sea bass. While lipase and GGT levels were low in the majority of the samples, amylase levels were high in almost all of the samples.

Leukocytosis and monocytosis were observed in both fish species. Yet, eosinophil and basophil counts fell within the reference ranges in the sea bream. Lymphocytopenia, severe thrombocytosis and high numbers of immature erythrocytes were also noted (Table 2 and 3).

Macroscopic and histopathological findings

It was observed that the death of the fish occurred faster, rigor mortis developed more quickly, and 30 seconds after being caught from the cages, the fish remained motionless.

After being harvested, the fish suffered mainly from cold shock. Cold shock meaning is quick freezing after harvest. If an animal shows rigor mortis symptoms after quick freezing stage, this is a normal condition for foods; if it is not, the product has a problem in quick-freezing stage, naming cold shock problem. This was associated with the very rapid development of rigor mortis and algor mortis, such that some fish did not even show any rigor mortis indicator such as muscle stiffening or loss of tissue elasticity. There were no macroscopic lesions in the body. However, severe hemorrhage in the gills and operculum, and hyperemia, anemia and increased mucus secretion in the gill filaments were observed. Necropsy findings included severe fat accumulation in the liver, and anemia in the liver and kidneys. Another prominent finding was the very long coagulation time or absence of coagulation in some of the fish under anesthesia. The macroscopic findings are presented in Figure 3.

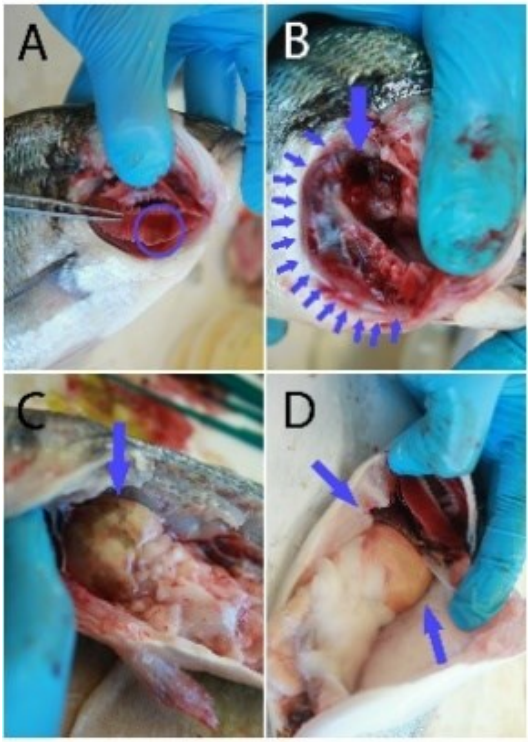

Figure 3. A: Haemorrhage on gill and gill arc (circle); **B:** Haemorrhage in the bottom of operculum (arrows); **C:** Severe fatty accumulation and anemia in the liver; **D:** Fatty and anemia in the liver (short arrow) and hematoma in the transverse septum.

While microscopic examination demonstrated multifocal coagulation necrosis and siderosis in the spleen, hyperemia in the blood vessels, severe diffuse fat accumulation and vacuolar degeneration in the liver, shrinkage of the pancreatic cells, hemorrhage and mononuclear cell infiltration in the heart, anemia, severe mononuclear cell infiltration, necrotic areas, increased mucus secretion and desquamation in the gills, the muscles appeared to be healthy (Figures4-6).

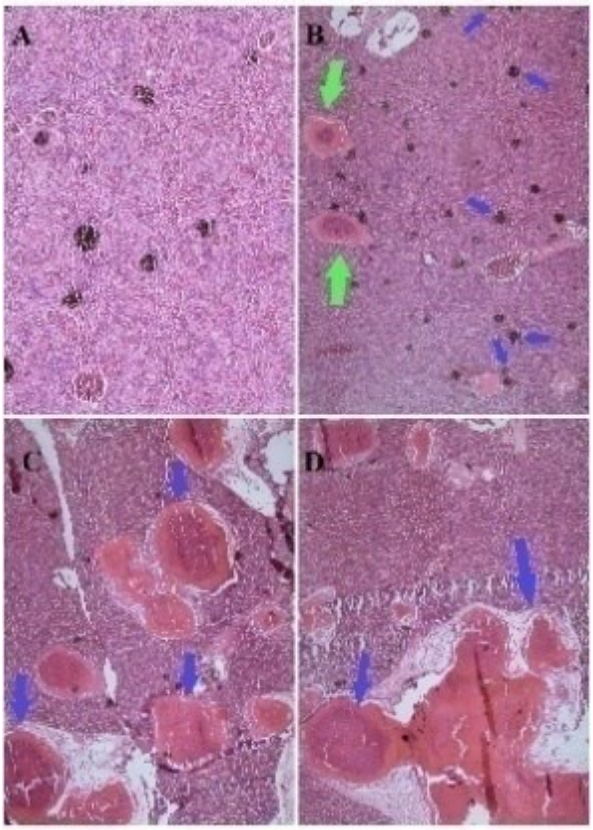

Figure 4. A: Healthy spleen (H&E X40); **B:** diseased spleen (H&E X10) includes mild necrotic area (green arrow) and siderosis (blue arrow); **C** and **D:** Severe diffuse coagulation necrotic area in the spleen (arrows) (C, H&E X10), (D, H&E X10).

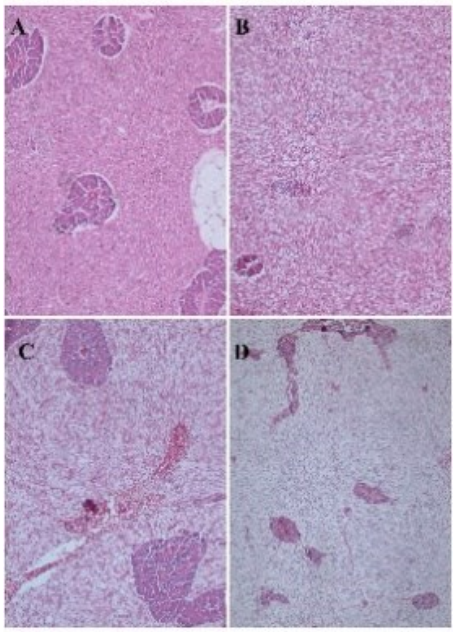

Figure 5. A: Healthy liver tissue (H&E X40); **B:** mild severity fatty accumulation in the liver (H&E X10); **C:** Moderate (H&E X40) and **D:** severe fatty accumulation in the liver (H&E X10).

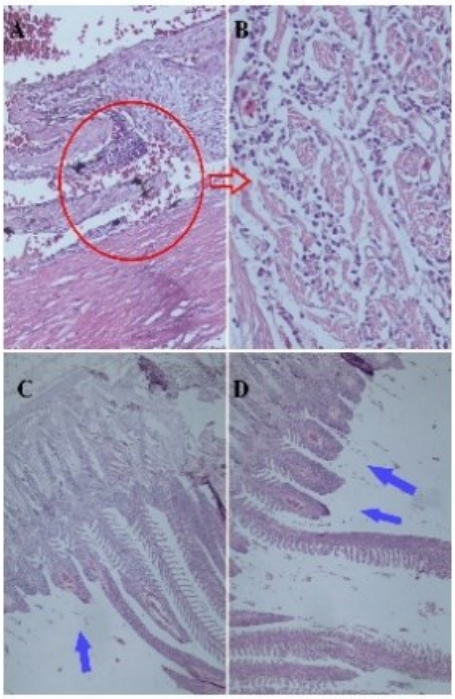

Figure 6. A and **B:** Haemorrhage and mononuclear cell infiltration in heart tissue (A, H&E X10; B, H&E X40); **C** and **D:** Necrosis and desquamation in primer and seconder lamella (arrows, H&E X10).

Antimicrobial susceptibility testing

All isolates were resistant to lincomycin and oxytetracycline with no growth zone. The isolated bacteria could not be evaluated for antimicrobial susceptibility or resistance as there was no breaking point published by the EUCAST or CLSI for these species (Accessed date 25.06.2021). For all isolates, the largest inhibition zones were noted for florfenicol, followed by amoxicillin-clavulanic acid in the second place (Table 4).

Table 4. Antimicrobial susceptibility results of isolated bacteria*

Family	Sulfonamides	Aminopenicillins	Tetracyclines		Fluoroquinolones	Quinolone	Aminoglycoside	β-lactam	Macrolides	Clindamycin	Chloramphenicol
Antibiotics	SXT	AML	DO	OT	ENR	OA	CN	AMC	E	MY	FFC
Disk content (µg)	1/19	25	30	30	5	10	10	30	15	15	30
A. mediterranea	21	0 (R)	0 (R)	0 (R)	15	10	23	20	15	0 (R)	25
P. gelatinilytica	22	0 (R)	0 (R)	0 (R)	0 (R)	0 (R)	20	11	12	0 (R)	25
Pseudoalteromonas	25	18	0 (R)	0 (R)	20	12	28	24	30	0 (R)	35
V. anguillarum	20	23	0 (R)	0 (R)	20	13	27	23	25	0 (R)	23
V. chagasii	25	0 (R)	30	0 (R)	30	23	30	12	20	0 (R)	40

*Zone diameter is reported in millimeters (mm). Grey cells represent resistant values

Discussion and Conclusion

In the present study, we discussed severe fatty liver disease that triggered bacterial infection in fish from a morphological, histopathological and molecular standpoint. The present study was conducted on a fish farm located in the Aegean region of Turkey, where fish were cultured in cages and given a commercial feed produced by the owner company. Macroscopic findings, anamnesis and farmer feedback pointed out to the slow movement of fish, increased FCR, rapid development of rigor mortis, shortened period of fluttering of the fish when taken out of the water and very little or no blood clotting. Yellowish and anemic liver tissue was another distinct finding.

Similar to the present study, previous studies have reported fat accumulation to be associated with a bronzed color of the liver, darkening of the body color and splenic deformations (Roberts, 2001; Weisman and Miller, 2006). In terrestrial animals, hematological findings such as ALT, AST, GGT and protein levels can be used as indicators of nutritional imbalance and liver damage, whilst a correlation between fatty liver disease and hematology has not been established in fish. This is because routine hematology instruments used at veterinary clinics or in human medicine cannot offer an accurate evaluation of fish blood samples due to the fact that fish erythrocytes are nucleated and blood cells vary among fish species (Yildiz, 2009; Fazio et al., 2012). In addition, the reference ranges of many hematological parameters are yet to be determined for a large number of fish species; and the reliability of available reference values is questionable as there are multiple stress factors that affect cultured fish (Fazio et al., 2012).

High percentages of neutrophils and monocytes were associated with lymphocytopenia in all groups. Severe anemia outstood in sea bream, compared to sea bass and previous literature reports (Esteban et al., 2000; Roncarati et al., 2006; Yildiz, 2009; Peres et al., 2013; Peres et al., 2014; Monge-Ortiz et al., 2016; Fazio et al., 2018). Almost all groups presented with severe thrombocytosis and high numbers of immature erythrocytes in the blood smears. Previous research has shown that, in fish, the immune response is predominantly lymphocyte-mediated; thus, lymphocytes play a primary role in acute infection, whilst firstly monocytes and secondarily neutrophils are involved in chronic infections. The high number of neutrophils and monocytes detected in our study showed that the fish were chronically infected.

Significantly altered ALT, AST, GGT, ALP and lipase activities are directly related to liver dysfunction or abnormalities, and high CK activity is proof of muscle damage that prevents the stiffening required to start rigor mortis. The underlying mechanism involves the catalysis of creatine conversion by creatine kinase, and the use of ATP by this enzyme to generate phosphocreatine and ADP. ATP separates the actin-myosin cross-bridges and enables muscle relaxation. When ATP is not produced in tissues, the body enters rigor mortis because it is unable to break these bridges. As high CK activity in fish causes ATP depletion, tissue and muscle ATP levels could be very low (Watabe et al., 1991). Thus, upon death, these fish cannot enter rigor mortis or quickly progress to algor mortis. To enlarge upon the process, the synthesis of ATP is initially by creatine kinase (CK), but subsequently by glycogenolysis and glycolysis. CK usually drops to low levels within 1-2h postharvest. Once CK levels drop to 75% of the initial levels, ATP starts to decline. As CK disappears, Pi increases in the cells postharvest, and lactate accumulates concomitantly with an increasing hydrogen ion (H+) con-

centration. Once ATP reaches low levels, muscle extensibility disappears, and both pH level and lactic acid concentration reach their final values (in a well-fed, rested animal). However, very high CK levels in live fish prevent postmortem ATP decrease and muscle relaxation does not occur. Therefore, the high CK levels detected in the present study played a major role in the absence of rigor mortis.

Liver damage also affects the Vitamin K cycle, activation of clotting factors, and blood coagulation mechanism (Roberts, 2001). We observed that severe fat accumulation and hepatocyte dysfunction caused coagulation abnormalities in the sampled fish, which hindered the evaluation of the hemogram. Increased white blood counts indicate inflammation in tissues also. The role of the liver and spleen in erythrocyte production and maturation involves a complex mechanism, which has been explained by Fánge (1992) and Roberts (2001). Fish erythrocytes are known to produce cytokine like-factors that are involved in macrophage activation (Passantino et al., 2004). Immature erythrocytes, which do not function properly, cause phagocyte inhibition, which eventually renders fish vulnerable to microorganisms, even those that are commensal.

The diffuse necrotic areas in the spleen and the liver damage detected upon histopathological examination were considered to have caused a high level of immature erythrocytes in the blood circulation. Our findings related to blood clotting, rigor mortis and tissue hemorrhage were linked to the tissue damage detected in the histopathological examination and were in agreement with previous research. Although these findings were directly suggestive of multiple organ dysfunction, and primarily of hepatic lipidosis, given that *V. alginolyticus* has pathogenic effects on marine fish (Balebona et al., 1998), bacterial findings may mask the primary cause and mislead clinicians. There is only weak evidence on the genera *Alteromonas* and *Pseudoalteromonas* including pathogenic species (Pujalte et al., 2007), and species belonging to these genera are considered to be natural members of marine habitats and the microbiota of most marine fish (Skovhus et al., 2007; Garrity, 2007; Farmer et al., 2015; Yan et al., 2016). Thus, these bacteria having been isolated in the present study demonstrated that the cause of death was a hidden stress factor. If we had isolated only *V. alginolyticus* and no other species, and had not examined the liver and blood profile of the fish, we would have assumed the presence of a bacterial infection and directly applied antimicrobial treatment. The isolation of both potential pathogens (*V. alginolyticus* and *V. chagasii*) and commensals (*Alteromonas* and *Pseudoalteromonas*) were attributed to multiple points; (I) primary immunosuppression by multiple organ dysfunction, (II) phagocytic cell maturation failures associated with liver and spleen necrosis, (III) coagulation problems, thus, hemorrhage in all tissues permitting bacterial invasion and proliferation, (IV) immature erythrocytes not producing cytokines, which contribute to immune defense (Figure 1). Egerton et al. (2020) have reported that plant proteins significantly alter gut microbial composition and intestine histology, and thus, directly affect fish immunity by altering the members of the microbial community. Changes in intestine histology and gut microbiome balance explain how opportunistic pathogens or members of microbiota become pathogenic organisms.

Zhenyu (2014) reported five major causes of fatty liver in farmed fish, including nutritional imbalance, environmental pollution, physiological processes, inter-species differences and genetic mutation. We took notice of all these factors, such that the possibility of environmental pollution was eliminated by screening other farms located near the sampling site, the possible effect of physiological processes was eliminated by sampling seemingly healthier smaller sized fish at the same farm (with short-term exposure to imbalanced diet), and the possibility of inter-species differences and genetic mutations were eliminated by inspecting other fish groups on the same farm and at different farms in the same region.

In conclusion, the main (primary) problem detected in the present study was fat accumulation in the liver, which is referred to as hepatic lipidosis. Thus, secondary microbial infection due to cellular immune suppression stemmed from fat accumulation in the liver, diffuse necrotic areas in the spleen and hematological impairment. As fatty liver syndrome is mainly diet-based and dietary imbalance is common in animals, this disease can be avoided by optimizing fish rations. Before initiating antimicrobial treatment, an assessment should be made for possible multi-organ damage and severe anemia, which increases the number of immature erythrocytes in the blood circulation. Thereby, the contribution can be made to preventing the spread of antimicrobial resistance in the marine environment.

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