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Natural Bacterial Co-Infection in Farmed European Sea Bass Intended for Experimental Research in Sicily, Southern Italy: Pathological Findings

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Abstract: In mariculture facilities, bacterial infections pose significant production challenges, with potentially catastrophic impacts on fish species. Bacterial co-infections are a widespread phenomenon in the natural marine environment, although their impact on aquatic organisms remains poorly investigated. This study aimed to detail the pathological findings associated with a natural bacterial co-infection caused by three different pathogens, namely *Photobacterium damselae* subsp. *piscicida*, *Tenacibaculum maritimum* and *Vibrio* sp., as the cause of mass mortality in European sea bass. The fish had been reared in open-net cages in Sicily and later transferred for experimental research purposes to a user establishment after immunization with an inactivated vaccine. Macroscopic, cytological and histopathological examinations were performed on 109 animals, and bacterial species were identified by the 16S rRNA gene. Overall, ulcerative skin lesions, necrotizing myositis and tail rot with occasional tail loss were associated with tenacibaculosis and vibriosis, while *P. damselae* subsp. *piscicida* mainly caused granulomatous inflammation in the spleen and head kidney. Finally, an injection site reaction due to the oil-adjuvanted vaccine administered intraperitoneally was observed in the abdominal fat. Understanding the impact of bacterial pathogens is essential to manage the health and welfare of farmed fish, and the importance of a good health monitoring program cannot be overstated to avoid outbreaks and the possible emergence of new pathogens due to the intensification of the production systems, antibiotic resistance and climate changes. The study would also highlight the importance of the quarantine period when animals supplied for research come from aquaculture farms and how the main goal in the near future should be to better define the procedures to provide completely pathogen-free animals.

Keywords: European sea bass; co-infections; *Dicentrarchus labrax*; *Vibrio* sp.; *Tenacibaculum maritimum*; *Photobacterium damselae* subsp. *piscicida*

Key Contribution: The present study details the pathological findings associated with a natural bacterial co-infection in European sea bass in Sicily. The findings would spur a better understanding of the potential biological risks affecting non-pathogen-free animals, especially when considering aquaculture as a source of fish species to be enrolled in experimental studies.

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1. Introduction

European sea bass (*Dicentrarchus labrax*, Linnaeus 1758) is a marine fish species of considerable importance in the European aquaculture sector, with significant economic, commercial and cultural value. In recent decades, the European sea bass aquaculture industry has experienced exponential growth, representing one of the most important farmed fish species today, with Turkey being the main producer [1,2]. Indeed, based on the 2020 report of the Federation of Greek Maricultures (FMG), European sea bass production represents almost 25% of the total European aquaculture production volume [3]. However, due to high production levels and high population densities under farmed conditions, the European sea bass is highly susceptible to several infectious diseases, which have a significant impact on overall commercial production [4–7].

In the aquaculture industry, bacterial infections alone pose significant challenges to production and have been found to kill over 10% of all farmed aquatic animal species [8]. In contrast, viral agents and parasitic infestations have a lower impact than bacterial diseases in sea bass, resulting in considerably lower economic losses [9]. Among bacteria, *Vibrio* spp. are ubiquitous members of the marine environment, representing important pathogens that severely affect the development of fish farming [10,11]. In particular, vibriosis still represents a major threat with a significant economic impact in the European sea bass aquaculture sector, being responsible alone for high morbidity and mortality rates [12]. Several *Vibrio* species have been isolated in seabass infections (e.g., *V. anguillarum*; *V. parahaemolyticus*; *V. harvey*; *V. alginolyticus*; *V. vulnificus*; and *V. ordalii*) [13], and while *V. anguillarum* has been recognized for decades as the major pathogen responsible for vibriosis in sea bass [14], *V. harvey* has recently emerged as a major concern for seabass aquaculture in the Mediterranean [12,15] and is currently also reported as a common bacterial species in Asian sea bass farmed in open net-cages [12,16,17]. In addition, several other bacteria are widespread in marine ecosystems with significant impacts on overall aquaculture productivity, including *Tenacibaculum maritimum*, *Aeromonas veronii*, *Mycobacterium marinum* and *Photobacterium damselae* subsp. *piscicida* [18–20]. In the natural marine environment, bacterial co-infections are a widespread phenomenon and result from the invasion of multiple pathogens into a single host through simultaneous or secondary infections [5]. In the marine ecosystem, fish commonly encounter several distinct opportunistic pathogens; those with a broader host range are more likely to locate suitable hosts than single-host specific pathogens, and concomitant infections can significantly alter the progression and severity of the disease, resulting in increased economic losses [5,21].

The European sea bass is a fish species commonly used for experimental research [18,22]. Directive 2010/63/EU established measures to protect animals used for scientific purposes, also including other fishes and cephalopods, together with the commonly used zebrafish as aquatic model organisms. However, the European sea bass is not included in Annex 1 of Article 10 of the Directive 2010/63/EU, which lists all animal species that may only be used in research procedures where those animals have been bred for use in those procedures. Specifically, the only fish species mentioned in Annex I is the zebrafish (*Danio rerio*), which is widely used as a model for human diseases in experimental trials due to its genes and gene structures being related to orthologous human ones [23]. Conversely, other fish species that can be used for research purposes may, by way of derogation to Annex I, originate from aquaculture farms. Therefore, health monitoring in aquaculture facilities, especially for animals intended for research, is essential, since infectious diseases have a catastrophic effect on the results of experimental studies, including the risk of sacrificing animals that could not provide the expected results for the purposes of the experiment [18]. Indeed, one of the main objectives in defining the procedures is to continue working to have and provide completely pathogen-free animals [24]. In European sea bass aquaculture, strategies for the prevention of infectious diseases include the application of biosecurity measures, vaccination and the use of probiotics and immunostimulants. In particular, immunization is currently one of the crucial methods for preventing the onset of bacterial diseases in aquaculture species and represents the most effective alternative to the use of

antibiotic drugs [25–29]. Moreover, intraperitoneal vaccination of juvenile sea bass is a widespread method for disease prevention in farmed animals, with commercially available products predominantly featuring an oil-adjuvanted antigen formulation [7]. Furthermore, significant progress has been made in recent years in breeding programs aimed at improving genetic resistance to common pathogens in European sea bass aquaculture, with the aim of reducing mortality rates and improving overall productivity in the near future [30].

In this study, we primarily aimed to describe the macroscopic and histopathological findings of a natural bacterial co-infection with *Photobacterium damselae* subsp. *piscicida*, *Tenacibaculum maritimum* and *Vibrio* sp. as a cause of mass mortality in European sea bass (*Dicentrarchus labrax*) reared in open-net cages in Sicily (Sothern Italy) and received for experimental research purposes. The impact of co-infections in aquatic animals remains poorly studied and effective monitoring of the health status of fish species aims to minimize the impact of pathologies on experimental trials and to ensure the reliability and reproducibility of research results [5]. To the best of the authors' knowledge, this study documents, for the first time, a case of mass mortality in European sea bass caused by co-infections of three different bacterial pathogens. Co-infection involving *Photobacterium damselae* and other pathogens is occasional, with co-infection with *V. harveyi* reported in spotted sea bass and in cobia (*Rachycentron canadum*) in Asia [31,32], and one suspected case of co-infection of *P. damselae* subsp. *piscicida*, *Ureaplasma* spp. and *Actinomyces*-like organisms observed in a bottlenose dolphin (*Tursiops truncatus*) [33].

2. Materials and Methods

2.1. Animals and Clinical Signs

One thousand European sea bass (*Dicentrarchus labrax*) specimens were purchased from an aquaculture farm in Sicily, Southern Italy, and transported to the Institute for Comparative, Experimental, Forensic, and Aquatic Pathology "Slavko Bambir", at the Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences of the University of Messina. The fish were transferred using a refrigerated truck equipped with tanks filled with marine water supplied by an air pump, at T 25 °C, salinity 1035 and DO 6 mg/L. Upon arrival at the university facility, the fish were placed in quarantine 400 L tanks at 19–20 °C, salinity 33‰, pH 8.0 and dissolved oxygen (DO) 7 mg/L. The animals were fed twice a day with commercial feed (Skretting, Italy) at 1.5% of body weight and maintained in a 12 h light/dark cycle.

During the quarantine period, most fish showed several nonspecific clinical signs, including inappropriate swimming behavior, with difficulties in coordination, anorexia and apathy. External examination also revealed ulcerative skin lesions and tail rot, often with tail loss. Fish showing severe clinical signs and gross lesions were humanely euthanized for health monitoring purposes using a lethal dose of MS-222 (0.7 mL/L) following spinal transection and sampled for further diagnostic investigations.

2.2. Postmortem Investigation

Postmortem examination was performed on a representative number of specimens (109 out of 1000) showing clinical signs and macroscopic skin lesions. In particular, all fish underwent a complete macroscopic examination and morphometric parameters (e.g., body weight [BW] and total length [TL]) were recorded for each animal. Bacteriology was performed on 15 fish out of 109, sampling gross lesions observed during necropsy in the skin/skeletal muscle, spleen and head kidney. Cytological preparations were obtained, and representative portions of each organ were sampled and either fixed in 10% neutral buffered formalin for histopathology or frozen at −80 °C for further molecular investigations.

2.3. Pathological Examination

Cytological preparations, including blood smears, impression smears and cell scraping were obtained from skin ulcerative lesions and representative organs (e.g., gills and organs of coelomic cavity). A fresh examination was performed on some of them, putting a drop

of water, to exclude the presence of ectoparasites. The other cytological preparations were air-dried and routinely stained using May Grunwald–Giemsa quick stain (Bio-Optica; Milano, Italy).

All tissues after necropsy were fixed in 10% neutral buffered formalin, washed in tap water and routinely processed for histology. Three µm thick tissue sections were stained with hematoxylin and eosin (HE). Ziehl–Neelsen and Gram stains were performed on 4 µm thick tissue sections. Cytological preparations and histological slides were visualized using a Leica DM6B microscope (Leica Camera, Wetzlar, Germany); the Leica Application Suite X software (version 1.4.6.110) and Leica DFC 7000 T were used for image acquisition.

2.4. Microbiology

Bacteriology was performed on 15 animals by sampling lesions of the spleen, head kidney and skin/skeletal muscle observed during necropsy. Isolation procedures were performed on freshly harvested fish. Briefly, samples were collected by rubbing sterile swabs on the skin ulcers, spleen and head kidney, after an incision with a sterile blade. Swabs were then streaked on Blood Agar (BA) (Biolife, Milan, Italy) and *Flexibacter maritimus* medium (FMM) (Biolife, Milan, Italy). Plates were incubated aerobically at 28 °C for 72 h. A representative number of colonies were then individually subcultured on plates of the same medium and incubated at 28 °C for 48 h until pure isolates were obtained. Finally, a Gram stain was performed.

2.5. Molecular Analysis

Genomic DNA from bacterial isolates was obtained using the GeneJET Genomic DNA Purification Kit (Catalog number: K0721; Thermo Scientific, Milan, Italy), following the manufacturer's instructions.

The 16S rRNA gene was amplified using the primer set 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). PCR was performed using GoTaq® Colorless Master Mix (Promega, Madison, WI, USA) in a final volume of 50 µL. The cycling conditions were set as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 45 s, annealing at 55 °C for 45 s, elongation at 72 °C for 60 s and a final extension step at 72 °C for 5 min [34–36]. PCR-amplified products were assessed on a 1.5% (*w*/*v*) agarose gel, and the concentration of nuclei acids (DNA) and purity were measured using the Nanodrop Spectrophotometer (NanoPhotometer N50, IMPLEN, Westlake Village, CA 91362, USA).

DNA sequencing of the purified fragments was performed by Genechron (Rome, Italy) using the same forward and reverse primers in both directions. Sequence alignments were performed using the ClustalW algorithm (https://www.genome.jp/tools-bin/clustalw; accessed on 10 September 2024). Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/) for similarity searches against the National Center for Biotechnology Information (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) database to calculate the statistical significance of the matches.

3. Results

3.1. Study Population and Gross Pathological Findings

A complete gross and histopathological evaluation was performed on 109 sea bass specimens (body weight: 23.45 ± 6.77 g [mean ± standard deviation]; length: 11.15 ± 1.54 cm). On gross examination, severe, multifocal ulcerative dermatitis was evident in all necropsied animals, involving the skin and reaching also the deeper layers, including the skeletal muscles, and producing an extensive ulcerative and necrotizing myositis. The ulcerative lesions were mostly located on the lateral side of the body and on the tail, and occasionally, disseminated cutaneous hemorrhages were observed throughout the fish body. In the most severe cases, the tail was completely absent due to the deep ulcerative lesions (Figure 1).

Figure 1. Macroscopic skin lesions. Severe and deep skin ulceration, mainly located on the flank and tail, also involving the deep skeletal muscle. In some cases, complete tail loss was evident.

On examination of the coelomic cavity, numerous brownish/reddish to yellowish nodules (1–5 mm) were observed in the mesentery abdominal fat in most of the animals, compatible with a granulomatous inflammatory process (Figure 2).

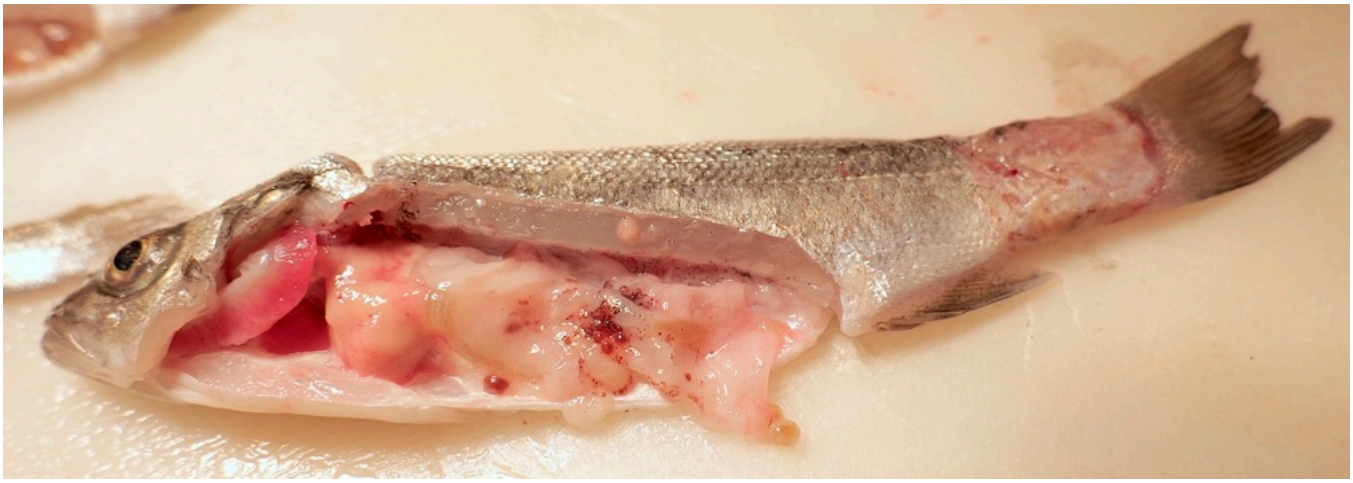


Figure 2. Coelomic cavity. Numerous brownish to yellowish nodules multifocally observed within the mesentery abdominal fat.

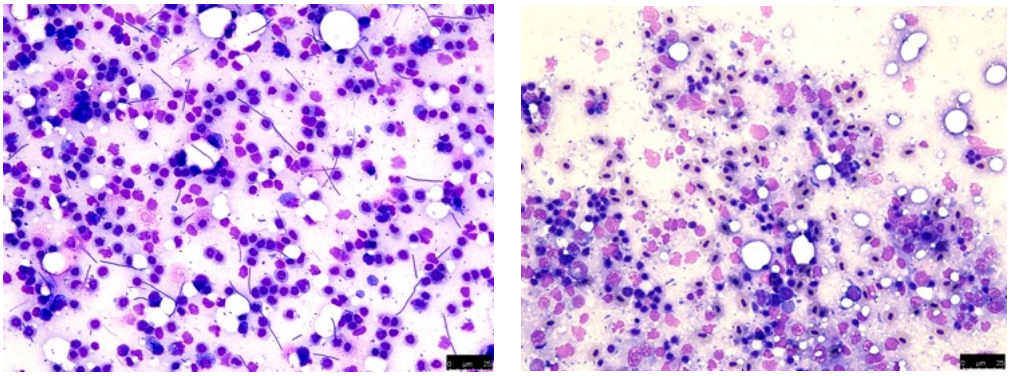
Splenomegaly was evident in most of the diseased animals, with granulomatous splenitis occasionally observed on gross examination (Figure 3). Mild and diffuse hepatic lipidosis was evident in all the fish (Figure 3), and most animals had overdistended gallbladders filled with abundant bile. Finally, most of the animals showed a moderate and diffuse cerebral congestion.

Figure 3. Coelomic cavity. Splenomegaly was evident in most of the diseased sea bass, with occasional granulomatous lesions (with inset) observed on gross examination.

3.2. Cytology

Cytological preparations obtained from ulcerative cutaneous lesions showed a mixture of inflammatory cells with low numbers of necrotic tissue cells. Neutrophils were the predominant inflammatory cell type (>50%), with many of them degenerated. Other inflammatory cell populations included macrophages (>30%), often with large foamy cytoplasm, and lymphocytes (20%), especially small lymphocytes. Bacterial aggregates with varying morphology were evident both extracellularly among the inflammatory cells and often found phagocytized within neutrophils, or occasionally within macrophages. Most microorganisms were long (0.7 µm × 2.1–7.2 µm), slender, filamentous rods, showing varying lengths based on different stages of cohesion between organisms and strongly suggestive of the species *Tenacibaculum maritimum* (Figure 4a). A second bacterial population was observed, as phagocyted bacterial rods or scattered extracellular bipolar bacterial rods, 0.7–1.4 × 1.3–4.0 µm, referable to the species *Photobacterium damselae*. These bipolar bacteria were also observed in cytological preparations obtained from the head kidney and spleen, and in blood smears (Figure 4b).

In addition to bacterial rods, impression smears obtained from macroscopic lesions of the spleen and head kidney showed and admixture of inflammatory cells, with a predominance of macrophages (>80%), fewer lymphocytes and occasional neutrophils. Finally, occasional curved rod-shaped (comma shape) bacteria (1.5 × 0.2–0.4 µm) suggestive of *Vibrio* spp. were observed in blood smears or skin cytological impressions (Figure 4a). Impression smears of the liver revealed most of the hepatocytes with large portions of the cytoplasm completely occupied by a single large optic empty vacuole, with peripheral displacement of the nucleus (macrovesicular lipidosis) and with scattered interspersed macrophages and lymphocytes.



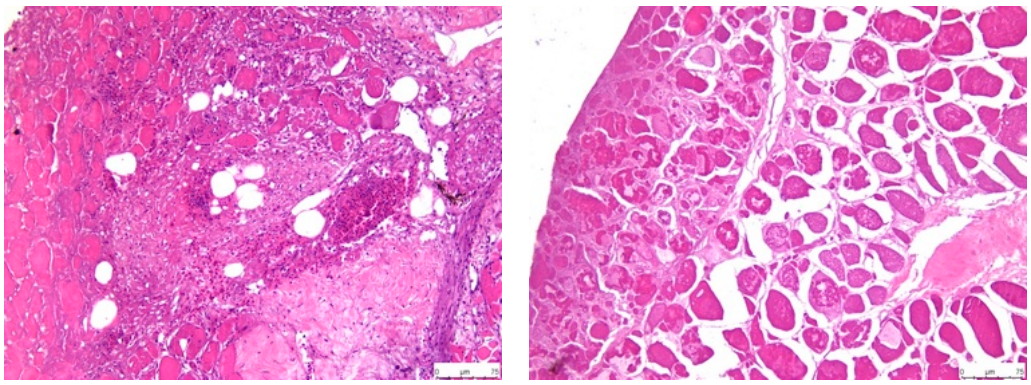
(**a**) (**b**)

Figure 4. Representative cytological preparations: (**a**) Long, thin, filamentous rods suggestive of the species *Tenacibaculum maritimum* and scattered comma-shaped bacteria suggestive of *Vibrio* spp. evident extracellularly among the inflammatory cells and often found phagocytized within macrophages; skin, impression smear. (**b**) Bipolar bacterial rods suggestive of the species *Photobacterium damselae*; Head kidney, impression smear.

3.3. Histopathology

3.3.1. Scaled Skin and Skeletal Muscle

Scaled skin was characterized by extensive ulceration and necrosis of the epidermis, with exposition of the underling dermis and/or hypodermis. A severe, multifocal, necrotizing myositis was observed in the skeletal muscle underlying the cutaneous lesions with lesions ranging from acute to subacute. Multifocally, the endomysium and perimysium were enlarged by extensive edema, occasional hemorrhages and a moderate inflammatory cell infiltrate, mainly composed of macrophages and neutrophils, with fewer lymphocytes and plasma cells. In most cases, 50–60% of the myofibers were characterized by monophasic necrotic changes, showing intensely eosinophilic sarcoplasm with loss of cross-striation, occasional disrupted sarcolemma and karyorrhectic or pyknotic nuclei (coagulative–colliquative necrosis). In some fish, polyphasic reactions were observed with multifocal dystrophic mineralization and occasional muscle regeneration, together with mild to moderate fibrosis. Scattered throughout the myofibers, there were aggregates of multiple, 1–2 microns in diameter, round to rod-shaped basophilic bacterial aggregates (Figure 5).

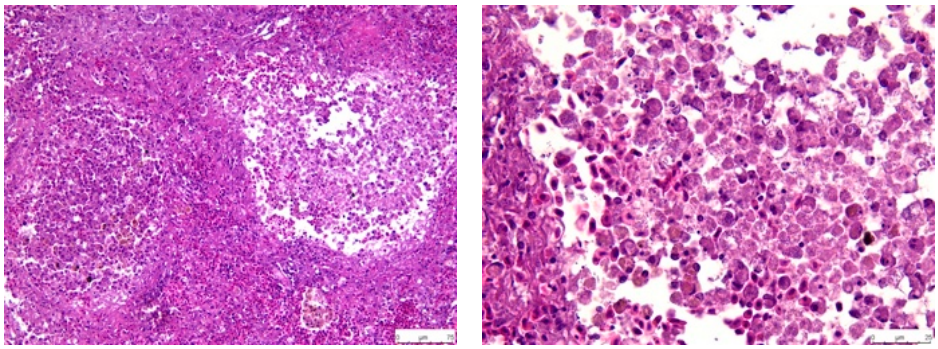


(**a**) (**b**)

Figure 5. Representative histopathology of muscle lesions: (**a**) severe, multifocal to coalescent, subacute, necrotizing, neutrophilic and histiocytic myositis; and (**b**) extensive coagulative and colliquative myonecrosis.

3.3.2. Spleen

In most of the diseased European sea bass, a moderate to severe, multifocal granulomatous splenitis was observed, with often well-defined granulomas. In particular, the splenic parenchyma was enlarged by abundant aggregates of macrophages with cytoplasm laden with abundant cellular debris or containing phagocyted basophilic bacteria and melano-macrophages arranged in clusters or loosely dispersed within the white pulp. Macrophages were admixed with moderate numbers of lymphocytes and multifocally embedded in abundant eosinophilic, necrotic cellular debris. Multifocal hemorrhages were occasionally observed (Figure 6).



(**a**) (**b**)

Figure 6. Representative histopathology of splenic lesions: (**a**) Multifocally, the splenic parenchyma was enlarged by abundant aggregates of macrophages with cytoplasm laden with cellular debris or containing phagocyted basophilic bacteria. (**b**) Higher magnification of macrophages laden with abundant basophilic rods, occasionally found extracellularly.

All splenic granulomas were Ziehl–Neelsen negative, showing the presence of Gram-negative intralesional bacteria.

3.3.3. Head Kidney

In most cases, markedly expanding and replacing the parenchyma were multifocal macrophage aggregates, often with large foamy cytoplasm (foamy macrophages) or with large, eosinophilic cytoplasm (epithelioid macrophages), or were macrophages laden with yellowish-brownish granular pigments (hemosiderin-ceroid). Scattered Gram-negative rod-shaped bacteria were observed among macrophages or intercellularly. Moderate numbers of lymphocytes and occasional plasma cells were also observed, embedded in a variable amount of eosinophilic cellular debris. Occasionally, some fish showed more chronic lesions with well-defined granulomas (Figure 7), negative with the Ziehl–Neelsen stain.

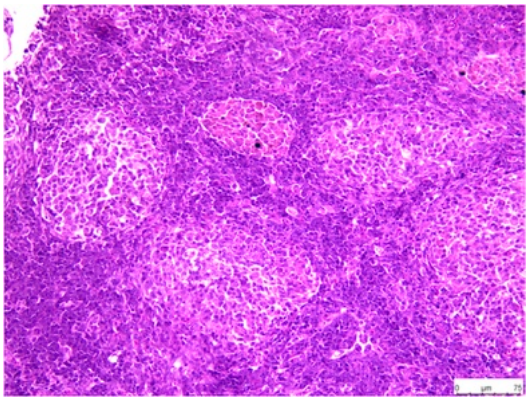


Figure 7. Representative histopathology of the head kidney. Multifocal aggregates of epithelioid macrophages with scattered interspersed rod-shaped bacteria.

3.3.4. Abdominal Mesentery Fat

The interlobular connective tissue septa were multifocally expanded by numerous optic empty vacuoles (oil droplets) with an even margin and found in the center of loose granulomas, or infiltrated by large, nodular aggregates of foamy macrophages, also infiltrated between the fat lobules, and characterized by a large cytoplasm filled with abundant blue-grey foreign material (vaccine product) also found free extracellularly. Moderate numbers of lymphocytes and plasma cells were also observed, together with mild fibrosis and multifocal coagulative necrosis of the adipocytes. The abundant blue-grey foreign material within macrophages, together with oil droplets in the context of the granulomatous inflammation, has been interpreted as an injection site reaction due to the intraperitoneally administered oil-adjuvanted vaccine.

3.3.5. Liver

Diffusely, hepatocytes were characterized by a large cytoplasm filled with a single optic empty vacuole, with peripheral displacement of the nucleus (macrovesicular lipidosis). Occasionally, multifocal single cell necrosis was observed together with mild infiltration of lymphocytes and macrophages. The liver was diffusely and moderately congested.

3.4. Isolation and Identification of the Bacterial Pathogens

After incubation of the plates at 28 °C for 72 h, colonies were subcultured individually and incubated at 28 °C for 48 h until pure isolates were obtained. Following these procedures, three pure Gram-negative isolates with distinct morphological and colony size characteristics were obtained from naturally infected European sea bass.

Tenacibaculum maritimum bacteria cultured on FMM (48 h, 28 °C) formed round colonies with irregular edges and a yellowish color and were mainly isolated from the skin/skeletal muscle and spleen. *Vibrio* sp. isolates were mainly from the skin/skeletal muscle and spleen; cultured on BA (48 h, 28 °C), they showed white/grey, diffuse and hemolytic colonies. *Photobacterium damselae* subsp. *piscicida* grown on BA (48 h, 28 °C) produced small white colonies without hemolytic activity and were grown from sampled splenic and renal lesions.

The nucleotide sequences of the amplified products were identical between biological replicates. BLAST analysis (NCBI) of the three sequences obtained from amplification with primers 27F and 1492R yielded significant alignments with reference sequences of *T. maritimum* (100% similarity, Query Cover 100%, E value 0; accession number: AB078057) [37] and *P. damselae* subsp. *piscicida* (100% similarity, Query Cover 100%, E value 0; PP053096). The 16S rRNA sequence obtained from *Vibrio* sp. revealed >99% sequence identity and 99% sequence coverage with reference sequences of *V. harveyi* (accession number MT860351). Representative 16S rDNA sequences of the three colonies were submitted to GenBank (Accession Numbers: PP981398, PP981400, PQ302566).

4. Discussion

To the best of the authors' knowledge, this study documents, for the first time, a concomitant natural infection of three different bacterial pathogens as the cause of mass mortality in *D. labrax* housed in a quarantine system of a user establishment. In detail, the co-infection involved *Photobacterium damselae* subsp. *piscicida*, together with *Tenacibaculum maritimum* and *Vibrio* sp. Furthermore, this study highlights the potential economic impact of bacterial co-infections in aquatic organisms, and therefore the importance of health monitoring programs in the mariculture facilities. Moreover, the findings would spur a better understanding of the potential biological risks affecting non-pathogen-free animals when considering aquaculture as a source of fish species to be enrolled in experimental studies. This is essential to ensure the reliability and reproducibility of the research findings, also reducing the risk of sacrificing animals that cannot provide reliable results. The findings also highlight the critical importance of the quarantine period in detecting possible infectious diseases in an animal facility, to minimize the risk of introducing pathogenic

or simply opportunistic microorganisms, even with potential zoonotic risk. Notably, the recent report of an increasing number of human infections caused by *Vibrio* species such as *V. harveyi* highlights the ever-increasing need for health monitoring programs and increases awareness of the possibility that wound contamination in humans may become more likely [4].

Infections and mortality observed in European sea bass often result from a complex interplay among multiple factors and are generally triggered by the combined action of various intrinsic and extrinsic factors, such as age-related host susceptibility and pathogen virulence, as well as seasonality, stress and poor water quality [10,19]. Moreover, interactions between invading pathogens have been found to synergistically alter hosts' susceptibility and duration of infection, resulting in an overall increase in the pathogenicity of individual pathogens [38]. Regarding pathogens, bacteria of the genus *Vibrio* are ubiquitous in marine ecosystems, with *Vibrio harveyi* reported as the principal heterotrophic bacterial species in the Western Mediterranean, representing one of the main pathogenic bacteria negatively affecting the development of the aquaculture sector and reported as the most frequent *Vibrio* species isolated from diseased fish [6,10,11]. Of note, vibriosis outbreaks caused by *V. harveyi* show a clear seasonal variation in incidence with a markedly increased prevalence during warm seasons when the temperature exceeds 20 °C, and for this reason, *Vibrio*-associated infections are expected to increase in the near future due to global warming [39]. It is well-known that fish physiology is directly influenced by several environmental factors, with temperature having the most crucial effect [40]. In particular, the increase in water temperature linked to climate change influences the basal metabolic processes of aquatic organisms, inducing a stress response and differentially impacting on various components of the immune system [41]. It is noteworthy that the vibriosis outbreak documented in this study occurred in European sea bass purchased from the mariculture facility during the summer period, during which a marked increase in the prevalence of the *Vibrio* species is expected. Furthermore, the increased prevalence of *Vibrio*-associated infections may lead to the increased occurrence of co-infections with other pathogenic or opportunistic bacteria [42].

In this study, widespread skin ulcerations along with necrotizing myositis, tail rot and complete tail loss could be associated with vibriosis, and also, the occasional identification of bacteria of the genus *Vibrio* in the blood smear and spleen by molecular investigations reflects the systemic spread of the pathogen in some individuals. Typically, for vibriosis, the incubation period is very short, reaching approximately 3 days, although its duration can be influenced by the susceptibility of the host and the virulence of the specific pathogen [7]. The main lesions in natural *Vibrio* infections include skin ulcerative lesions which subsequently extend to the underlying muscle layers, with necrosis that usually involved the tail with complete tail loss [13]. In the *Vibrio* genus, the Harveyi clade, especially *V. harveyi*, *V. alginolyticus*, *V. rotiferianus* and *V. owensii*, are the most frequently isolated species in sea bass aquaculture [15], although *V. harveyi* remains the main pathogen responsible for recurrent vibriosis outbreaks [15]. In Asian sea bass in Vietnam, similar necrotic and ulcerative lesions have been described and associated with *Vibrio harveyi* infection, recognized as the causative agent of "Scale Drop and Muscle Necrosis disease (SDMND)" [43]. In addition, consistent with our findings, disease outbreaks in Asian sea bass occurred more frequently in smaller fish, although outbreaks have been reported in fish of all sizes after stocking in open-net cages [43]. Gill necrosis and abdominal distension have also been reported as the major external lesions, along with anorexia, lethargy and abnormal swimming patterns as the main behavioral changes, with the possible systemic spread of infection and high mortality [44,45]. Also, *V. anguillarum* may be responsible for ulcerative skin lesions and necrotizing myositis in acutely affected fish [14], although the fish investigated were vaccinated intraperitoneally against *Vibrio anguillarum*, with vaccines having generally been shown to be effective in preventing disease [46].

In this study, pathological examination of the skin, blood smears, as well as microbiology, confirm the presence of the bacterium *Tenacibaculum maritimum*, which is generally

described as an opportunistic bacterial pathogen and, in the case of concomitant infections, has the ability to increase the severity of other diseases, with a rapid worsening of the clinical course [20,43,47]. Interestingly, *T. maritimum* has previously been reported to cause fin rot and muscle necrosis in various marine fish species, resulting in gross and histopathological lesions in skeletal muscle similar to those produced by *Vibrio* spp. [20,48]. Additionally, the lesions associated with tenacibaculosis include deep necrotizing and ulcerative skin lesions with numerous intralesional bacterial aggregates [20,47]. Therefore, it would be likely that the external lesions observed here in European sea bass could be the result of the combined action of *T. maritimum* and *Vibrio* sp., and eventually *V. harveyi*, with concomitant infections causing high mortality and increased overall disease severity. However, in Asian sea bass, although co-infections with several culturable and non-culturable bacteria, including *Tenacibaculum* spp., have been isolated in the case of "Scale Drop and Muscle Necrosis Disease", only the in vivo infection with a pathogenic strain of *V. harveyi* reproduced the main clinical signs and lesions of the disease, while other bacteria alone did not cause disease but only served as opportunistic pathogens [43]. Furthermore, as in the case of *Vibrio* spp. infections, *Tenacibaculum*-associated disease is more common and severe in smaller fish (2–80 g), with the occasional occurrence in larger animals [3,20,49]. Moreover, previous studies stated that the pathogenesis and morbidity are accelerated with water temperatures above 15 °C and high salinity (higher than 30‰) [3,20,49]. Finally, the pathogenicity and development of lesions typical of tenacibaculosis are strongly influenced by its moderate hydrophobicity and rapid ability to form biofilms [47,50].

Finally, the third bacterial species identified in the European sea bass examined in this study is *Photobacterium damselae* subsp. *piscicida*, considered a primary pathogen responsible for acute fish photobacteriosis. Photobacteriosis, also known as pseudotuberculosis due to the necrotic/granulomatous lesions which characterize the disease, represents a serious bacterial disease in mariculture facilities worldwide [51]. The disease still represents a serious threat in aquaculture, especially because *P. damselae* subsp. *piscicida* recognizes a wide host range and has a widespread distribution, causing massive mortality due to the lack of effective vaccines and widespread antibiotic resistance [51]. As for the vibriosis and tenacibaculosis outbreaks discussed above, the prevalence of photobacteriosis is higher during the summer season, thus reflecting the occurrence of the infection outbreak in the cultured sea bass investigated in the present study. Of note, for *Vibrio*-associated infections, the occurrence of photobacteriosis outbreaks has been associated with immune-suppression positively linked to sudden increases in water temperature [52].

Severe skin ulcers have been reported in sea bass exhibiting high mortalities, as well as hepatomegaly, engorged gall bladders and necrotic to granulomatous inflammation in subacute and chronic clinical forms, respectively, predominantly involving the spleen and kidney [53,54]. Therefore, in this study, *P. damselae* subsp. *piscicida* produced subacute to chronic granulomatous lesions in internal organs (i.e., spleen and head kidney), while ulcerative skin lesions and necrotizing myositis were associated with acute or subacute tenacibaculosis and vibriosis.

In European sea bass mariculture facilities, immunization represents a key prevention strategy for most bacterial and viral infectious diseases and is considered the most effective alternative treatment to the use of antimicrobial drugs [25–29]. Most commercially available vaccines are prepared with the whole pathogen inactivated using formaldehyde or heat, with or without the addition of adjuvants [55,56]. Especially in juveniles, vaccination using an oil-adjuvanted antigenic formulation administered intraperitoneally represents a widespread immunization strategy, and the combination of bacterins and oils stimulates a local inflammatory immune response, allowing protective effects together with the long-lasting stimulation of the immune system due to the slow and sustained release of antigens from the oil (depot effect) [7,57,58]. The inactivated oil-adjuvanted vaccine for *Vibrio anguillarum* and *Photobacterium damselae* subsp. *piscicida* has been intraperitoneally administered in the fish investigated in this study, as also demonstrated by the oil droplets found in the center of loose granulomas in the mesentery abdominal fat, together with

numerous macrophages laden with blue-grey foreign material, interpreted as the vaccine product. Many studies have been conducted to develop effective vaccines for a variety of infectious diseases, but the results are not repeatable. In particular, Hamaguchi and Kusuda [59] compared the efficacy of formalin-inactivated *P. damselae* subsp. *piscicida* vaccine preparations at different time points during culture and obtained a significant difference in efficacy based on culture periods. Failures in vaccine development can be explained by the inadequate growth phase of the bacteria to prepare vaccines and infection inocula [60], and nowadays, although commercially available, there are no effective formulations against photobacteriosis [55–66], whereas for *Vibrio anguillarum*, commercially available vaccines have been extensively and effectively applied [46].

In addition, adjuvanted vaccines may cause several side effects in fish, in particular, associated with its administration with incorrect injection sites or techniques which can often result in visceral adhesions and chronic inflammation, with defined granulomas and pigmentation [3,11,58,61–63]. Therefore, ongoing research aims to improve vaccine formulations and delivery methods to increase their effectiveness while minimizing potential adverse effects.

In recent years, alternative strategies for infectious disease control have been explored. Interestingly, probiotic treatments have emerged as the preferred environmentally friendly prophylactic approach in marine larviculture, and the use of beneficial indigenous bacteria isolated from aquatic organisms is gaining attention for pathogen control in the aquaculture sector. Noteworthy, several *Vibrio* species have shown potential probiotic activity by improving the survival of cultured fish after challenge with pathogenic *Vibrio* species [64,65]. Furthermore, the host microbiota plays an important role in disease control, being able to prevent and control infections by mechanisms such as niche exclusion and competition for nutrients or antagonism, and altered microbial communities may imply a loss of the ability to control pathogen populations [66]. Interestingly, the skin mucus microbiome and the immune response at both the local and systemic level have been studied in European sea bass after a natural vibriosis outbreak sustained by *V. harveyi*, in order to develop correct measures to reduce the incidence of the disease [67]. Of note, *Vibrio*-associated infections produced a clear dysbiosis in the skin mucus microbiota, along with a decrease in protease activity, as well as a concomitant reduction in lysozyme and an increase in serum peroxidase activity [67]. Another interesting study observed that a combination of culturable bacteria from the rainbow trout microbiota was able to confer protection against *Flavobacterium columnare* infections by inhibiting the pathogen population [68]. Furthermore, the host microbiota has been attributed a role in modulating the immune response against pathogens, capable of inducing inflammation to face pathogens in fish [66].

Finally, the selection of genetically resistant fish breeding stocks to infectious diseases, such as photobacteriosis, is a potential strategy to be pursued to reduce the likelihood of this disease in farms and avoid economic losses [56]. In addition, efforts in an innovative preventive strategy will aim to further improve the health and welfare of farmed European sea bass and support the growth and productivity of the aquaculture sector, and when the prophylaxis is not effective and/or adequately applied, an early diagnosis is essential to control disease outbreaks.

The limitations of this study include use of 16S rRNA gene sequencing to ultimately assign species-level classification and its limited ability to differentiate various *Vibrio* species among the *Vibrionaceae* family. However, the 16S rRNA-based sequencing technique remains a widely accepted method for genus-level identification. Given the primary goal of this study to describe the gross and histopathological findings of an outbreak caused by multiple bacterial pathogens, we focused on the 16S rRNA gene, which provides a valuable insight into the microbial community.

5. Conclusions

This study documents, for the first time, pathological findings associated with a natural co-infection caused by three different bacterial pathogens, as the cause of mass

mortality in European sea bass originating from a mariculture facility and intended for experimental research. The results would contribute to new insights into the pathogenicity of *P. damselae* subsp. *piscicida*, *T. maritimum* and *Vibrio* sp., providing valuable knowledge for the diagnosis, prevention and management of fish diseases induced by co-infection with these bacteria. The role of stressors, such as the poor quality of water, high density, transportation, vaccination and further manipulations, is here underlined. Furthermore, the findings would emphasize the importance of the quarantine period when animals supplied for research originate from mariculture. One of the main goals in the near future is to better define the procedures to have completely pathogen-free animals, which can be provided by a mariculture facility, to be involved in experimental trials. Alternative disease prevention strategies are therefore desirable, as an effective way to improve water quality and reduce stress, inhibit pathogen growth and boost the immune response of fish.

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Institutional Review Board Statement: The manuscript describes the pathological findings on sea bass that died naturally following a co-infection sustained by three different bacterial pathogens. Therefore, the animals were not enrolled in any experimental study or euthanized for research purposes. Thus, ethical approval is not required for this type of study.

Data Availability Statement: The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author.

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