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Photobacterium damselae subsp. *piscicida* Infection, a Persistent Cause of Mortalities in Old Hatchery-reared Gilthead Seabream (*Sparus aurata*) Broodstocks: Molecular-pathological Evidences and Control Strategy

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Abstract

ALMOST all Egyptian marine hatcheries collect their young Gilthead seabream brood stock from open water at Deeba Triangle or Damietta. Some of these wild broodstock could possibly harbour hidden mild *Photobacterium damselae subsp. piscicida* infection. In the current study, a total of 20 mortal/moribund 5-7-years old broodstocks were clinically examined and then subjected to microbiological and histopathological examination. Moribund brood stocks were lethargic and anorexic. Externally, broodstocks showed generalized skin darkening, haemorrhages at the base of fins together with pale gills. Internally, the spleen, liver, and kidney were remarkably infiltrated with several whitish various-sized nodules together with an unpleasant odor originating from the abdominal cavity of necropsied brood stocks. Pathology has revealed multiple granulomas together with lymphocytic and inflammatory cell infiltrations through the anterior kidney, spleen and liver tissues together with severe melano-macrophage center (MMC) activation. Bacteria-laden macrophages were also noticeable through stained tissue sections. *Photobacterium damselae subsp piscicida* was presumptively isolated from the internal organs of moribund and mortal fishes. Initial identification was based on bipolarity staining, API 20 E profile, and antibiotic susceptibility testing. The final identity was confirmed using partial sequencing of 16S rRNA gene. The control strategy was based initially on oral antibiotic therapy (florfenicol) to combat systemic infection and tank water disinfectant (Hydrogen peroxide) to minimize bacterial load followed by an immune-boosting feed additive program utilizing probiotics, B-glucan, nucleotides, vitamin C,

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organic zinc and selenium. Brood stock tanks' follow-up for 1 month after the initiation of the control strategy has revealed no mortalities among reared broodstocks.

Keywords: Gilthead seabream; broodstocks; hatchery; *Photobacterium damselae subsp. piscicida*; systemic granulomatous diseases; control strategy.

Introduction

By 2050, there will be more than nine billion people on the planet, meaning that an additional two billion people will require safe, sustainable food sources to support their diets. A major goal of the current UN Sustainable Development Goals (SDGs) Decade of Action is to meet each of these requirements [1]. Marine aquaculture is facing numerous challenges to its record of accomplishment as a sustainable ocean industry; however, Outbreaks of disease among farmed fish and worries about declining fish health make intensification of farming practices difficult. Biodiversity may threatened by rising infection pressure on wild species. Due to their large nutrient discharge, aquaculture of fed fish and crustacean species can pollute water [2]. Finding new fish feed sources that don't overfish wild fish stocks, compete with agricultural products, or take up space that could be used to grow human food is another difficulty associated with fed aquaculture [1].

Gilthead seabream (*Sparus aurata*) is one of the most cultured fish species in the Mediterranean region and ranks second among cultured marine fish [3, 4]. However, several infectious diseases jeopardize the further growth of marine aquaculture. Most of these are of bacterial cause, such as photobacteriosis, a septicemic disease triggered by *Photobacterium damselae subsp. piscicida*. This is probably the most dangerous disease for seabream (*Sparus aurata*) [5] and causes recurrent outbreaks associated with high mortalities. This persistent disease also affects other economically important species, such as seabass (*Dicentrarchus labrax*) [6], sole (*Solea senegalensis*) [7], yellowtail (*Seriola quinqueradiata*) [8], and cobia (*Rachycentron canadum*) [9].

Photobacteriosis occurs in both acute and chronic forms [10]. The chronic form usually occurs without clinically visible lesions, with ulcers on the skin, pelvic fins, caudal peduncle and around the gills, mild hemorrhagic areas in the head and gills, and skin darkening [11]. Internally, infected fish usually exhibit multifocal necrosis in the kidney, liver, and spleen, pale liver [12], splenomegaly and kidney enlargement, and the presence of numerous whitish nodules, scattering in the spleen and kidney tissue [13, 14] with diameters between 0.5 and 3.5 mm. Histologically, the nodules are necrotic lesions surrounded by macrophages engulfed by the bacteria [15].

The diagnosis of *P. damselae subsp. piscicida* carried out using conventional microbiological methods (culturing, isolation, and biochemical identification) [16]. The AIP20E miniaturized system is typically utilized for the presumptive identification of the bacteria [17]. The limitations of conventional microbiological techniques have been solved by the establishment of molecular diagnosis based on 16S rRNA gene sequencing, which allows the accurate and specific identification of *P. damselae subsp. piscicida* [18].

Controlling bacterial diseases with antibiotics is considered one of the most effective strategies for aquaculture producers to combat bacterial pathogens [19]. Antibiotics have been the first line of treatment to combat outbreaks of photobacteriosis, and there are several antibacterial agents, including florfenicol, oxytetracycline, sulphadiazine-trimethoprim, chloramphenicol, amoxicillin, ampicillin, flumequine, oxalinic acid, and tetracycline, have been applied to treat the pathogen [20, 21].

Although antibiotics are utilized for the treatment and control of fish diseases, remarkable negative impacts can be attached to the uncontrolled use of antibiotics, such as the potential development of antimicrobial drug-resistance genes in some pathogenic bacteria [22] and the risk of antibiotic residue accumulation in the food chain [23]. Therefore, a promising strategy for using biological control such as probiotics and immunostimulants can be implemented for disease control and prevention [22]. Furthermore, the implementation of biosecurity measures is essential for preventing outbreaks of diseases in marine aquaculture [24].

In aquaculture, probiotics can be used to control diseases and increase feed conversion rates [25]. Sanolife Pro-F ® (INVE Aquaculture, Belgium) is one of the commercial probiotics containing *Bacillus subtilis*, *B. licheniformis*, and *B. pumilus*. *Bacillus* species is one of the commonly used probiotics that inhibits pathogens, competing with them for adhesion sites, boosting the innate immune response in several aquatic organisms, facilitating enzymatic digestion, and enhancing water quality [25, 26].

β-glucan is regarded as an efficient immunostimulant because of its ability to bind to various receptors on leukocytes resulting in the activation of immunological reactions such as antibacterial activity and the production of cytokines and chemokines. These signalling proteins (cytokines and chemokines) have been shown to

trigger fish immune-competent cells, such as neutrophils, macrophages, and monocytes, as well as to kill pathogens by phagocytosis, oxidative burst, and cytotoxic killing mechanisms [27]. Furthermore, β- glucan stimulates T and B lymphocytes to produce specific antibodies and immunological memory [28].

The present research aims to investigate the hidden etiologies of recurrent mortalities in old Gilthead seabream broodstocks in a private marine hatchery in Ismailia province, Egypt. Further, our ultimate goal is to establish/implement a competent control strategy to prevent such mortalities and ensure the reliable sustainability of national mariculture.

Material and Methods

Case history

A total of 750 (5-7 years old) gilthead seabream *(Sparus aurata)* broodstocks were reared at a private marine hatchery located in Ismailia, Egypt in July 2022. Fish were reared in 5 concrete tanks; each tank is 50 m^3 in size and contains 150 broodstocks **(**average weight between 1.5 and 2 kg). Each tank was supplied with UV-treated saltwater pumped from an artesian well bordering the Suez Canal. Broodstocks were fed marine fish pellets (42% crude protein and 16% fats, Skretting®) during the regular/post-spawning rearing period and seafood during the spawning period. The seafood comprises sardine, mackerel, and squid which were imported from Sultanate Oman.

Fish sampling

A total of 20 mortal/moribund gilthead seabream (*Sparus aurata*) broodstocks were collected from the affected tanks. The gathered moribund/freshly dead fish were immediately transported in an ice box to the Aquatic Animal Medicine and Management Laboratory (AAMML) at the Faculty of Veterinary Medicine, Cairo University. Further clinical, microbiological, antibiogram, and molecular identification were performed according to the method described by Eissa [29], and Eissa *et al.* [3].

Water samples

Water samples were taken under complete aseptic conditions from broodstock tanks according to the standard methods outlined by APHA [30]. Water samples were physio-chemically analyzed for temperature, dissolved oxygen, pH, and salinity using a portable multi-parameter water quality meter (HI98194, Hanna Instruments) and ammonia (HI4101, Hanna Instruments).

Bacterial isolation and phenotypic characterization

Loopfuls from the liver, kidney, and spleen were taken under complete aseptic conditions and inoculated onto Brain Heart Infusion Agar (BHIA) (Himedia, India), Tryptic Soy Agar (TSA) (Himedia, India) supplemented with 2% (w/v) NaCl, and Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Oxoid). The inoculated plates were incubated at 25°C for 24-48 hrs. Randomly selected colonies were pure cultured on Tryptic Soy Agar (TSA) (Himedia, India) supplemented with 2% (w/v) NaCl. Traditional identification was performed using gram staining, susceptibility to 2, 4-diamino-6, 7-diisopropypteridin (O/129) disc (150 µg concentration), and conventional biochemical tests, according to Buller [31]. Presumptive characterization was completed using a commercial miniaturized API 20E Kit (BioMérieux, France) in compliance with the manufacturer's guidelines. Hemolysis test was carried out by selecting a pure colony, inoculating it on blood agar plates supplied with 5% sheep blood (Oxoid), and incubating the plates for 24 h at 25°C. For the motility test, an 18-h pure single colony was inserted into motility agar (TSB + 0.3% agar) using a sterile plastic tip and then incubated at 25°C for 24 h [3]. Ultimately, the purified isolates were preserved in tryptic soy broth supplied with 2% NaCl and 15% (vol/ vol) glycerol and maintained at -80^0 C for further molecular characterization.

16S rRNA gene sequencing

Genomic DNA was extracted from purified bacterial isolates using QIAamp DNA Mini Kit (Qiagen) in compliance with the manufacturer's guidelines. The extracted DNA was maintained at -20 °C for further molecular identification. PCR for the 16S rRNA gene was done using the universal primers: forward (5-AGA GTTTGATCCTGGCTCAG-3) and reverse (5-GGTTACCTTGTTACGACTT-3) [32]. The PCR reaction was carried out with a final volume of 25 µL using 1X PCR mix containing 250 ng of genomic DNA, 200 mM dNTP, 1 U Taq polymerase, and 0.25 µM of both primers. The thermal cycling conditions comprised an initial DNA denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 50 s, extension at 72 °C for 1 min, and a final extension phase at 72 °C for 10 min.

A QIAquick PCR purification kit was used to purify the PCR product, and a 3500/3500xL Genetic Analyzer (Applied Biosystems, at Faculty of Agriculture Research Park, Faculty of Agriculture, Cairo University) was then used to sequence the PCR product in the two directions. This 16S rRNA gene sequence was compared to other 16S rRNA gene sequences in the GenBank database utilizing the BLAST® search (National Center for Biotechnology

Information, NCBI).

Phylogenetic analysis

The raw sequence of the 16S rRNA gene of the bacterial isolate was assembled using Bio-Edit version 7.0 [33**]** and submitted to GenBank to estimate the genetic distance. The phylogenetic tree was generated with MEGA X 11 using the neighbor-joining method, with 1000 bootstrap repetitions [34].

Antimicrobial susceptibility testing

The antibiotic sensitivity of pure bacterial strains was identified through the disk diffusion approach. The bacterial isolates were inoculated into Tryptic Soy broth (Himedia, India) supplied with 2% NaCl and placed overnight at 25 °C. After adjusting each suspension to a 0.5 McFarland standard turbidity, 100 µl was streaked onto Muller-Hinton agar plates (Himedia, India) supplied with 2% NaCl. To prepare the antibiograms, commercial antibiotic discs were used: Florfenicol (FFC, 30 µg), Erythromycin (E, 15 µg), Ampicillin (AMP, 10 µg), Amoxicillin (AMX 25 µg), Doxycycline (DO 30 µg), Streptomycin (S, 10 µg), Sulphamethoxazole – Trimethoprim (SXT, 1.25 µg, and 23.75 µg, Ciprofloxacin (CIP, 5 µg), Novobiocin (NV 30 µg), and Oxytetracycline (OTC 30 µg). After placing the antibiotic disks onto the plates, they were incubated for 24 hours at 25 °C. Each disk's surrounding inhibition zone diameter was measured and noted. In accordance with CLSI standards, the results were categorized as susceptible (S), intermediately resistant (I), or resistant (R) [35].

Histopathological examination

Samples from the liver, spleen, kidneys, and ovaries were taken, preserved in 10% neutral buffered formalin, cleaned, dried, and embedded in paraffin. The paraffin-embedded blocks were cut at a thickness of 5 microns and stained with Hematoxylin and Eosin [36]. The stained sections were examined by a light microscope (Olympus BX50, Japan).

Treatment strategy

Brood stocks in the affected tanks were treated with florfenicol powder (50%) (Aquaflor®). Florfenicol was selected based on the results of antibiotic sensitivity testing. The dosage of florfenicol was 30 mg/kg body weight for 00 successive days at a feeding rate of 3% of the fish body weight [3]. The drug was coated onto the surface of the pellets using molasses. Twenty-four hours after the last medication, kidney samples were collected for microbiological analysis. Tank water disinfection strategy was also adopted concurrently with the oral antibiotic therapy at a dosage of 1.5 mL /m^3 Hydrogen peroxide® (50 %) for 2 hrs in tank water [37].

Immune-stimulatory strategy

An immune-stimulatory strategy has been competently used to enhance broodstocks immune response:

Oral probiotic: Sanolife PRO-F® powder (a combination of *Bacillus subtilis* 3.25 × 10^9 CFU/g, *Bacillus licheniformis* 3.50 × 10^9 CFU/g, and *Bacillus pumilus* 3.25 × 10^9 CFU/g; with a total number of 1.0 × 10^{10} CFU/g) was directly mixed with the diet, using sunflower oil (20 ml/kg diet), at a dosage of 1 g / kg. Broodstocks were fed this diet on daily for 2 weeks, with a feeding ratio of 3% of body weight [38].

Vitamin C: Vitamin C 100 % powder has been added to broodstock diets daily at a dosage of 1.5 g/ Kg diet [39].

The following minerals have been added daily to diets: Organic selenium at a dosage of 0.5g/Kg diet and organic zinc at a dosage of 0.25 g/Kg diet [40].

Daily, β-glucan was added to broodstock diets at a dosage of 1.5 g/Kg and nucleotides was added to diets at a dosage of 1.5 g/Kg diet [41].

Results

Clinical signs and post-mortem lesions

The prominent clinical findings of chronic moribund Gilthead seabream broodstocks were anorexia and lethargy. Externally, broodstocks showed generalized skin darkening, hemorrhages at the base of fins together with pale gills. Internally, the spleen, liver, and kidney were remarkably infiltrated with several whitish various sized nodules, ascetic fluid in the abdominal cavity, and engorged gall bladder. In addition, an unpleasant odor originated from the abdominal cavity of necropsied broodstocks.

Water Analysis

The average recorded water values for un-ionized ammonia (0.7 – 0.9 mg/L), water temperature (20 - 23°C), water pH (9 – 9.5), dissolved oxygen (3.5 – 4.5 mg/L), and salinity (30-32 g/L).

Phenotypic and Biochemical Characteristics Photobacteriumdamselae subsp. piscicida strains

were gram-negative, non-motile, coccobacilli rod-shaped with characteristic bipolar staining, cytochrome oxidase and catalase positive, and sensitive to vibriostatic agents (O/129, 150 µg). The strains produced small pale non-separated colonies on BHIA agar 2% NaCl, non-hemolytic activity on sheep blood agar 2% NaCl, did not grow on TCBS agar, and produced unique API 20E code (2005004). No other pathogenic bacteria were detected in the current case. The phenotypic and biochemical

characteristics of the retrieved strains (Table 1).

16S rRNA gene sequencing

The assembled sequences of two *Photobacterium damselae subsp. Piscicida* strains have been submitted to GenBank under the accession numbers: PP952735 and PP952301. Depending on sequence analysis, the current sequences were confirmed to be *P. damselae subsp. Piscicida.* The GenBank accession nos. (PP952735 and PP952301) showed 100 % to 99.9% identity with the accession numbers of *P. damselae subsp. Piscicida* (OR484840, OR785475, ON146435, MN186608, MW063536, ON376819 and ON322861) (Fig.1).

Antimicrobial susceptibility testing

The tested *Photobacterium damselae* subsp. *piscicida* strains were sensitive to Florfenicol (FFC 30µg), Sulphamethoxazole – trimethoprim (SXT,

1.25 µg, and 23.75 µg, Oxytetracycline (OT30µg), Ciprofloxacin (CIP5µg) and Doxycycline (DO 30µg). Conversely, these strains were resistant to Eythromycin (E 15 µg), Ampicillin (AMP 10 µg), Novobiocin (NV 30 µg), Streptomycin (S, 10 µg), and Amoxicillin (AMX 25 µg) (Table 2).

Histopathological findings

Histopathological examination of ovaries showed the presence of multiple granulomas embedded in ovarian tissue, these granulomas are characterized by central area of necrosis and surrounded by thin fibrous connective tissue capsule, these granulomas were infiltrated by mononuclear inflammatory cells (Figure 2 a & b). Concerning kidneys, there was activation of melanomacrophage centers (Figure 2 c), degeneration and necrosis of tubular lining epithelium (Figure 2 d), focal areas of mononuclear inflammatory cells aggregation (Figure 2 e) with the presence of multiple granulomas with central necrosis containing bacterial colonies and fine connective tissue capsule Figure 2 f & g). Spleen revealed activation of melanomacrophage centers and congestion of splenic blood vessels (Fig. 3 a), also there were variably sized granulomas embedded in splenic tissue (Figure 3 b & c). Pancreas showed activation of melanomacrophage centers (Fig. 3 d), hepatocytes revealed focal areas of steatosis (Fig. 3 e) and also activation of melanomacrophage centers (Fig.3 f), granulomas were embedded in hepatic parenchyma with areas of necrosis (Fig. 3 g).

Therapeutic trials

The application of florfenicol powder has been successfully used to stop brood stocks mortalities after medication. Tank water disinfection strategy using H2O2 has augmented the treatment plan andensured a swift recovery among infected brood stocks. The health and survival of treated brood stocks were enhanced, as shown by normal swimming behaviour and improved feed intake. To confirm such efficacy, kidney samples from the affected tanks were subjected to microbiological analysis. No colonial growths were detected on the cultured plates.

Discussion

Broodstock is the first building stone in the marine production cycle. Therefore, marine hatchery managers exert too much effort to ensure better health, fertility, and fecundity for their broodstock populations. A successful marine production cycle initially relies on a competent biosecurity strategy, efficient infectious disease preventive strategy, rigorous water quality management, rich nutrition plan, and genetic improvement strategy [42]. The first generation of Gilthead seabream broodstocks in native marine hatcheries are mainly collected from open lakes or seawater. In Egyptian marine hatcheries, around 20-30 % of the hatchery-reared old gilthead seabream broodstocks are annually replaced by comparable populations from the open water to avoid genetic bad traits' segregation as a simple means for genetic improvement. However, this old /only available genetic improvement strategy has a critical negative impact on marine hatchery biosecurity plan implementation [43]. Even though wild gilthead seabream broodstocks might look apparently healthy at the time of collection, yet, they might harbor some latent viral, bacterial, or any potential infectious agents [44].

Photobacteriosis caused by *Photobacterium damselae subsp. Piscicida* is one of the most critical latent bacterial infections affecting marine fish, contributing to frequent mortality episodes [3, 10]. It has been incriminated in massive mortalities among cultured seabream (*Sparus aurata*), grey mullets (*Mugil cephalus*), and seabass (*Dicentrarchus labrax*) in several Egyptian localities [10, 45].

In marine aquaculture, the disease usually occurs when the water temperature rises above 20°C, the salinity is between 20 ppt and 30 ppt, the dissolved oxygen concentration is low and the water quality is poor [46]. It is well known that, poor water quality such as low dissolved oxygen, elevated salinity, and temperature are major infection triggers in marine aquaculture**.** However, at the hatchery level, the major trigger of infection is the dependence on trash fish (sardine, mackerel, and squid) as the sole food source prior or during the spawning period. Such a risky feeding strategy together with broodstock age progress could pose a suitable epidemiological trigger for photobacterium, vibrio, and other bacterial threats [3, 10].

In the current study, *P. damselae subsp. Piscicida* was revealed as the direct etiology behind the disease eruptions and mortalities among Gilthead seabream broodstocks. The identification of the recovered isolates was ultimately determined by morphological, biochemical, and molecular screening matching the standard criteria of this pathogen according to [3, 17]. All extracted molecular data within the genetic study and phylogram confirmed the final identity of the isolated *Photobacterium* strains to be *P. damselae subsp. Piscicida* [47].

The clinical signs and gross lesions reported in the current research were concordant with those previously recognized by Essam *et al.* [17] who observed typical whitish granulomatous-like deposits in the spleen and kidney, engorged gall bladder and empty gastrointestinal tract, and with Eissa *et al.* [3] who observed pale liver with multiple whitish nodules in liver and kidney. In addition, an unpleasant odor originated from the abdomen of necropsied fish.

Several literatures have confirmed that progress in the age of a marine fish plays a critical role in defining the form of photobacteriosis [48, 49]. In the current study, Gilthead seabream broodstocks were between 5-7 years old which is an older age enough to allow a subacute or chronic form of photobacteriosis to develop. This conclusion runs in accordance with Noya *et al.* [48] findings, which documented that gilthead seabream's susceptibility to fish photobacteriosis varies depending on their age. They suggested that fish weighing less than 5 g are vulnerable to the illness, but that fish weighing more than 50 g develop resistance to it. Furthermore, they concluded that larger seabream's neutrophils and macrophages were found to have effective phagocytic activities, whereas smaller fish showed no evidence of these host cell types' function. This is due to the functionality of macrophages and neutrophils in larger seabream, which can professionally phagocytize and destroy the bacteria [48, 49].

In the current study, the development of multiple granulomas containing macrophage-laden bacteria together with focal areas of necrosis in the anterior kidney, spleen, and liver of the older Gilthead seabream broodstocks highly supports the above hypothesis [50]. The focal necrotic areas that appeared within the kidney, spleen, and liver could have developed earlier in age because of the virulent nature of the *P. damselae subspp piscicida* strain. However, the application of cellular immune system modulators such as B glucan, nucleotides, and some organic trace minerals such as selenium and zinc in broodstock diets could have stimulated neutrophils and macrophage activity with consequent enhancement of cytokines secretion with special reference to TNFα [51]. These cell-mediated rich environment with the trigger of TNFα could have resulted in subsiding the disease progress through killing the bacteria in macrophages, necrosis of central tissue, and encircling the whole reaction by connective tissue capsule while broodstock is surviving the infection through years of cohabitation with such nasty pathogen [50 - 52].

In our case, the latent infection has been responsible for recurrent mortalities in hatchery-reared old broodstocks mostly at the end of the spawning cycle. The successive spawning seasons for an old broodstock could have resulted in an abrupt breakdown in mucosal immunity and its associated lymphoid tissues in the intestine (GALT), skin (SALT), and Gills (GILT) [50, 53, 54]. This could be attributed to the deleterious continuous change in intestinal / gill milieu and microbiome associated with noxious chemicals produced by *P. damselae subspp piscida* [50, 54].

Gilthead seabream is a benthopelagic coastal species, that inhabiting seagrass beds, rocky and sandy bottoms. A possible explanation for the presence of the pathogen in gill tissues is the possible lodging of the bacteria through gills by water circulation through the gas exchange process and its swift passing into blood stream from the branchial blood circulation. Moreover, the pathogen invades the intestinal mucosal/submucosal tissues arriving the blood circulation with consequent iron sequestering action where bacteria are capable of iron acquisition from blood with possible increase in bacterial virulence with the development of anemia manifested by pale gills during the subacute and chronic stages [55, 56].

To practically intervene with photobacterium outbreaks, antibiotics have been used as timely therapeutic strategy. However, *Photobacteria* have developed wide resistance to many of these antibiotics [3, 12]. Therefore, adequate preventive antibiotic alternatives should be adopted in aquaculture production to manage infectious diseases including Photobacterium, and sustain fish health and immunity [53].

In the present study, *P. damselae subsp. piscicida* strains were sensitive to florophenicol, oxytetracycline, ciprofloxacin, doxycycline and sulphamethoxazole / trimethoprim and moderately resistant to ampicillin, amoxicillin, erythromycin, streptomycin and novobiocin. These susceptibility patterns were agreed with Varvarigos *et al.* [57]**.** According to the antibiotic sensitivity profiles of *P. damselae subsp. Piscicida* florfenicol was selected for treatment of chronically infected broodstocks in

diets at a dosage of 30 mg / Kg body weight for 10 successive days as a systemic therapeutic strategy where mortalities have been stopped at the end of the treatment period [3, 58].

Sanolife PRO-F®, a commercial probiotic containing *Bacillus subtilis, B. licheniformis,* and *Bacillus pumilus* strains, was synergistically added in diets of broodstocks as a supportive immunostimulant mean of disease control. The probiotic has an inhibitory effect on the growth of virulent strains of *P. damselae* subsp *piscicida* which in turn has a very positive effect on intestinal milieu and microbiome [50, 53]. *The Bacillus* strains compete with bacterial pathogens over nutrients and attachment sites [59]. They are responsible for production of inhibitory compounds, enhancement of enzymatic activities, and improvement of water quality [60]. Further, the oral probiotic has cellular immunostimulatory effects at the intestinal mucosal level with indirect enhancement of phagocytic activity, lysozyme activity, respiratory burst, and GALT activation [50, 61].

β-glucans can be also included in the diet to prevent or minimize broodstock mortalities due to photobacteriosis by boosting immunity and enhancing resistance to diseases [62]. In the current research, the dietary supplementation of β-glucans at 1.5 g /kg diet for 2 weeks synergized with other immuno-stimulants in the reduction of broodstock mortalities. This assumption is supported by certain immunological studies which indicate its boosting effects on innate immune reactions and resistance to pathogenic bacteria at least 2 weeks following oral administration [63]. In aquaculture, vitamin C is also well recognized for its ability to modulate immunity and has a positive effect on the prevention and control of diseases [39]. It enhances immune protection against infections by promoting the innate and adaptive immune responses. Several studies have verified the positive effects of Vitamin C on immunological parameters such as respiratory burst, serum protein level, phagocytic, lysosomal activity, and complement activity [39, 62]. Ultimately, we have used Vitamin C at a dosage of 1.5 g / Kg diet as an integral component of the immunostimulatory protocol used throughout the study

Conclusion

The addition of curcumin at a lower concentration (0.5 mmol) to freezing extender media has been demonstrated to improve the post-thaw quality of rabbit sperm. This enhancement positively affects various parameters, such as progressive motility, viability, plasma membrane integrity, acrosome integrity, and sperm DNA fragmentation. The protective mechanism at play involves the preservation of adequate levels of antioxidant-related transcripts and the antifreeze-related transcript HSP90, which together reduce the accumulation of reactive oxygen species (ROS) and improve mitochondrial function, This ultimately results in improved post-thaw viability and motility of cryopreserved spermatozoa, which is essential for the advancement of assisted reproductive technologies (ART) in rabbits and other livestock species, thereby supporting the conservation of genetic resources in the face of challenges such as climate change and declining fertility rates in farm animals.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This study was run in accordance with the guidelines instated by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University, Egypt (Vet CU 18042024940).

TABLE 1 Phenotypic and biochemical characteristics of retrieved isolates

Characteristics	*Photobacterium damselae* subsp. *piscicida*
Gram- staining	Gram-negative coccobacilli
Bipolar staining	Bipolar bacilli
Brain heart infusion agar with 2% NaCl	Small pale non separated colonies
Sheep blood agar with 2% NaCl	Small grayish non-hemolytic colonies
Growth on TCBS	No growth
Motility	Non-motile
O/129 sensitivity (150 mg)	Sensitive
Cytochrome oxidase	+
Catalase	+
O/F test	Fermentative without gas production.
B–Galactosidase production (OPNG)	-
Arginine dihydrolase production (ADH)	+
Lysine decarboxylase production (LDC)	-
Ornithine decarboxylase production (ODC)	-
Citrate utilization (CIT)	-
H2S production (H2S)	-
Urease production (URE)	-
Tryptophane deaminase production (TDA)	-
Indole production (IND)	-
Acetoin production (VP)	+
Gelatinase production (CEL)	-
Acid from glucose (GLU)	+
Acid from mannitol (MAN)	-
Acid from inositol (INO)	-
Acid from Sorbitol (SOR)	-
Acid from rhamnose (RHA)	-
Acid from sucrose (SAC)	-
Acid from melibiose (MEL)	-
Acid from amygdalin (AMY)	-
Acid from arabinose (ARA)	-
H2S production (H2S)	-
Urease production (URE)	-
Tryptophane deaminase production (TDA)	-
Indole production (IND)	-
Acetoin production (VP)	+
Gelatinase production (CEL)	-
Acid from glucose (GLU)	+
Acid from mannitol (MAN)	-

Notes: +, positive; −, negative.

TABLE 2. Antibiogram of the retrieved bacterial isolates

Antimicrobialagent	Code (concentration)	*Photobacterium damselae subsp. piscicida*
Ampicillin	AMP (10 μg)	R
Amoxicillin	AMX (25 μg)	R
Erythromycin	E (15 μg)	S
Novobiocin	NV (30 μg)	S
Trimethoprim–Sulphamethoxazole	SXT (1.25 μg + 23.75 μg).	S
Oxytetracycline	OTC (30 μg)	S
Doxycycline	DO (30 μg)	S
Florfenicol	FFC (30 μg)	S
Ciprofloxacin	CIP (5 μg)	S
Streptomycin	S (10 μg)	R

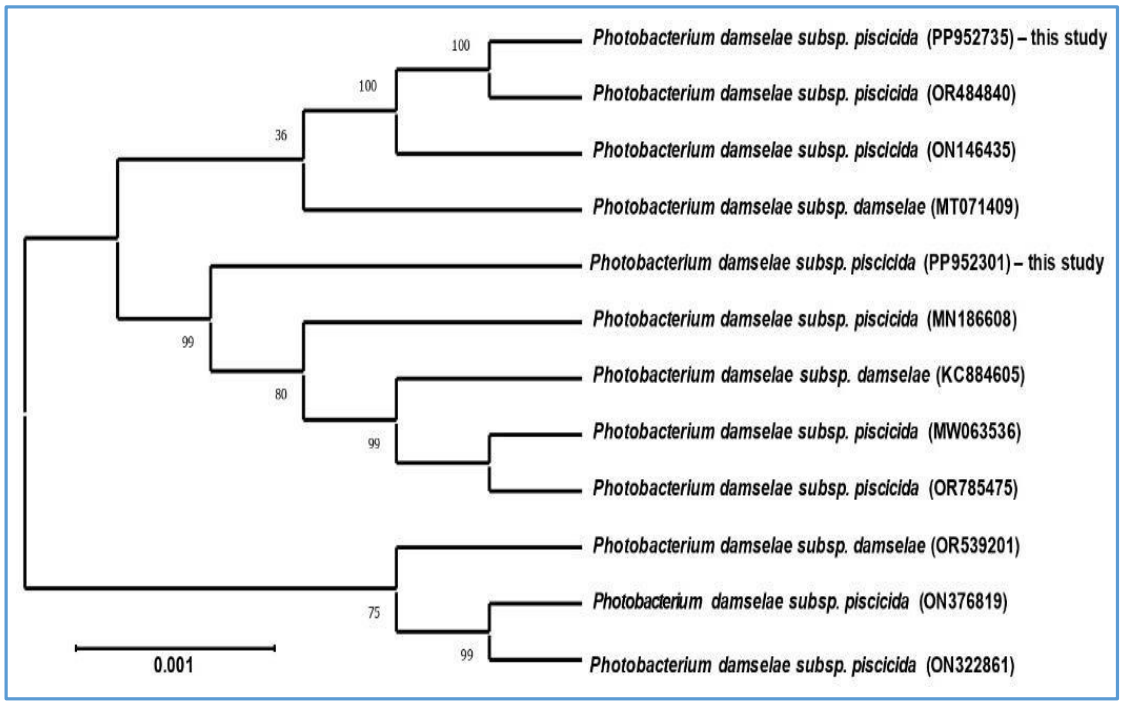

Fig.1. Phylogenetic tree of *Photobacterium damselae subsp. piscicida* isolated from Gilthead seabream broodstocks based on 16S rRNA sequences.

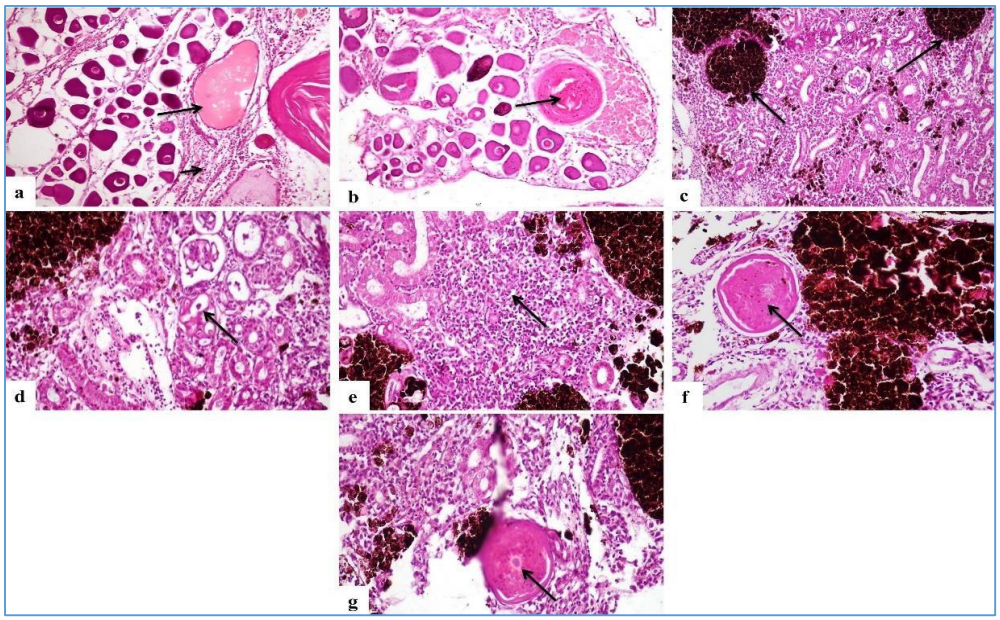

Fig.2. **Representative photomicrograph showing, (a) and (b) multiple granulomes in ovaries**, granulomes characterized by central area of necrosis containing bacterial colonies and surrounded by thin fibrous connective tissue capsule (long arrow) and also infiltrated with mononuclear inflammatory cells (short arrow) (H&EX200). (c) kidneys showing activation of melanomacrophage centers (arrows) (H&EX100), (d) degeneration and necrosis of tubular lining epithelium (arrow) (H&EX200), (e) focal area of mononuclear inflammatory cells aggregation (arrow) (H&EX200) and (f and g) multiple granulomes with central area of necrosis containing bacterial colonies and encircled with fine connective tissue capsule (arrow) (H&EX200).

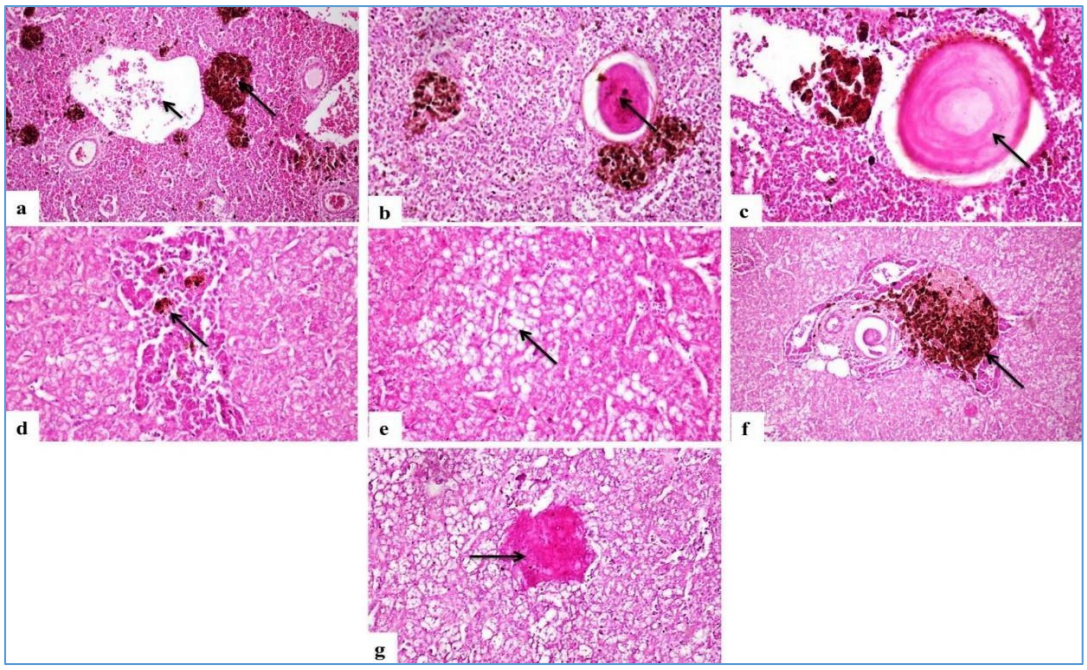

Fig. 3. Representative photomicrograph showing, (a) Spleena, ctivation of melanomacrophage centers (arrow) (H&EX400), (e) activation of melanomacrophage centers (long arrow) andhepatocytes showing focal steatosis (arrow) (H&EX400), (f) liver congestion of splenic blood vessels (short arrow) (H&EX100), (b)showing activation of melanomacrophage centers (arrow) spleen, granulome embedded in splenic tissue (arrow)(H&EX100), (g) granulome are embedded in hepatic parenchyma (H&EX200). (c) Higher magnification showing characteristics ofwith areas of necrosis (arrow) (H&EX400).

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عدوى بكتريا *Photobacterium damselae subsp. Piscicida* السبب المستمر للوفيات في أمهات أسماك الدنيس: الأدلة الجزيئية ولباثولوجيه واستراتيجية المكافحة

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الملخص

تقوم معظم المفرخات البحرية المصرية بتجميع أمهات الدنيس من المياه المفتوحة في مثلث الديبا أو دمياط حيث من الممكن أن تحتوي بعض هذه الأمهات على عدوى مخبأة مثل *Photobacterium damselae subsp. piscicida* في الدراسة الحالية، تم فحص إجمالي ٢١ أم ميتة/محتضرة عمرها ٧-٥ سنوات ظاهريًا وتم إخضاعها للفحص الميكروبيولوجي والهستوباثولوجى. كانت الأمهات المحتضرة خاملة وفاقدة للشهية. خارجياً، كانت الأمهات تعاني من اسوداد في الجلد وأنزفه عند قاعدة الزعانف بالإضافة إلى شحوب الخياشيم. داخليًا، كان هناك عقيدات بيضاء مختلفة الحجم في الطحال والكبد والكلى بشكل ملحوظ، رائحة كريهة تنشأ من تجويف بطن الأمهات. كشف الباثولوجي عن وجود أورام حبيبية مع ارتشاح الخلايا اللمفاوية والالتهابية فى أنسجة الكلى والطحال والكبد الأمامية جنبًا إلى جنب مع التنشيط الشديد لمركز الميكروفاج الميلاني.(MMC) وكانت الخلايا المحملة بالبكتيريا ملحوظة أيضًا من خلال الأنسجة المصبوغة. تم عزل *Photobacterium damselae subsp piscicida* من الأعضاء الداخلية للأسماك المحتضرة والميتة. تم التشخيص المبدأى على أساس صبغة ثنائي القطب، وAPI 20 E، واختبار الحساسية للمضادات الحيوية. تم تأكيد التشخيص باستخدام التسلسل الجزئي ل جين16S rRNA. اعتمدت استراتيجية المكافحة في البداية على العلاج بالمضادات الحيوية عن طريق الفم (فلورفينيكول) لمكافحة العدوى ومطهر للمياه (مياه الأكسجين) لتقليل الحمل البكتيري، يليه برنامج معزز للمناعة باستخدام إضافات الأعلاف مثل البروبيوتيك، والبيتاجلوكان، النيوكليوتيدات، فيتامين C، الزنك العضوي والسيلينيوم. ثم متابعة أحواض الأمهات لمدة شهر واحد بعد بدء إستراتيجية المكافحة ولم يلاحظ وجود حالات نفوق بين الأمهات المربأة.

الكلمات الدالة: الدنيس، الأمهات، المفرخ، *Photobacterium damselae subsp. piscicida*، الأمراض المزمنة، استراتيجية المكافحة.