EISSN 2602-473X

AQUATIC SCIENCES AND ENGINEERING

Aquat Sci Eng 2024; 39(4): 248-254 • DOI: https://doi.org/10.26650/ASE20241542227

Research Article

Effects of Bacterial-Fungal Coinfection on Biochemical Parameters and Organ Structure in African Catfish (*Clarias gariepinus*)

Cigdem Urku[1], Atanas Bozhkov[2], Dimitrinka Zarpyanova[3], Alexander Atanasoff[4]

Cite this article as: Urku, C., Bozhkov, A., Zarpyanova, D., & Atanasoff, A. (2024). Effects of Bacterial-Fungal Coinfection on Biochemical Parameters and Organ Structure in African Catfish (*Clarias gariepinus*). *Aquatic Sciences and Engineering, 39(4)*, 248-254. DOI: https://doi.org/10.26650/ASE20241542227

ABSTRACT

Five infected African catfish (605±45.98 g) from the Experimental Aquaculture Base at Trakia University exhibited abnormal behaviours, including sluggish movements, lethargy, skin haemorrhages, and fin bleeding. Samples from the lesions were cultured on Sabouraud Dextrose Agar (SDA), and the fungus was identified as *Rhizopus* sp. based on colony morphology and microscopy. Bacteriological samples from the kidney, spleen, liver, and blood were cultured on Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA), revealing the presence of *Yersinia ruckeri, Aeromonas hydrophila*, and *Pseudomonas aeruginosa* based on their physiological, morphological, and biochemical traits. Significant differences ($p<0.01$) were observed in the serum protein profiles, including total protein, albumin, globulin, and glucose levels. Histological examination showed pathological changes in the liver, kidneys, and gills, as well as haemorrhages and polymorphonuclear leukocyte infiltration in the skin lesions, with intense muscle necrosis. These biochemical and histopathological findings are valuable for monitoring health and diagnosing fungal diseases in African catfish.

Keywords: African catfish, biochemical parameters, pathogen bacteria, *Rhizopus* sp.

ORCID IDs of the author:
C.U. 0000-0003-0381-9321;
A.B. 0000-0001-8371-2940;
D.Z. 0000-0002-0430-1690;
A.A. 0000-0003-3460-9589

[1]Istanbul University, Faculty of Aquatic Science, Department of Aquaculture and Fish Diseases, Onalti Mart Sehitleri, Istanbul, Türkiye

[2]Trakia University, Faculty of Agriculture, Department of Aquaculture, Students campus, Stara Zagora, Bulgaria

[3]Trakia University, Faculty of Veterinary Medicine, Department of Biochemistry, Students Campus, Stara Zagora, Bulgaria

[4]Trakia University, Faculty of Veterinary Medicine, Department of Animal Husbandry, Students Campus, Stara Zagora, Bulgaria

Submitted:
02.09.2024

Revision Requested:
30.09.2024

Last Revision Received:
01.10.2024

Accepted:
02.10.2024

Online Published:
07.10.2024

Correspondence:
Cigdem Urku
E-mail:
curku@istanbul.edu.tr

INTRODUCTION

The African catfish (*Clarias gariepinus*) is an omnivorous freshwater fish and is widely introduced in Africa, Asia and Europe. It was introduced for fish farming and aquaculture from Africa to Europe several times in the early 1970s. For the first time in Bulgaria was introduced in 2007 at the Ovcharitza Reservoir for aquaculture (Bozhkov et al., 2023). It is a preferred aquaculture fish species because of its rapid growth rate, ability to survive in unsuitable environments, and resistance to diseases and stress (Ikpegbu, Ezeasor, Nlebedum & Nnadozie, 2013). Fungal diseases are the most recurrent type of disease problem, which cause low productivity of fish and serious economic losses in aquaculture (Bangyeekhun & Sylvie, 2011). Fungal infections occur in all life stages of fish and are mainly caused by immune suppression. *Rhizopus stolonifer* is one of the most common and fastest-growing species in the *Zygomycota* phylum and is commonly known as black bread mould, a worldwide distributed species, which may cause disease, especially under favourable predisposing conditions, resulting in unacceptable mortality (Bautista-Baños, Bosquez-Molina & Barrera-Necha, 2014). *Rhizopus* sp. was isolated and identified from different fish species (Rashmi & Chandan, 2015). Bulging eyes, loss of colour, and ulcers (cysts) in internal organs have been reported in catfish infected by *Rhizopus stolonifer*. In addition, the pathogen can cause damage to multiple body systems, such as the liver, kidney, and brain. Over 70% of these infections are polymicrobial, as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aeromonas hydrophila* and *Escherichia coli* are the most com-

monly co-isolated bacterial species (Kumar & Prakash, 2020). Despite significant interest in African catfish aquaculture, knowledge about fungal pathogens and mixed infections in farmed fish remains limited. This study reports the first case of *Rhizopus* sp. and opportunistic bacterial infections in Bulgaria. This paper details the biochemical changes and histological features of internal organs, offering important baseline information for diagnosing and treating disease agents in cultivated African catfish (*Clarias gariepinus*).

MATERIALS AND METHODS

Fish sampling and clinical examination

Five African catfish (605±45.98g) from the Experimental Aquaculture Base, Trakia University in Stara Zagora, Bulgaria - 42°23'32.39" N 25°34'10.19" E were investigated. The medical history of the diseased fish (anamnesis vitae and anamnesis morbi) was taken, and the clinical findings of haemorrhage and white lesions on the whole body surface (Figure 1b, c) and haemorrhagic fin (Figure 1d) were observed in the physical examination. The rearing conditions were as follows: photo-period 8 h L: 16 h D with low light intensity (40 lux) in RAS with low water exchange. Fish are fed twice daily (3% body weight) with commercial pellets (Aqua Wels Swim, Garant-Tiernahrung GmbH, Austria).

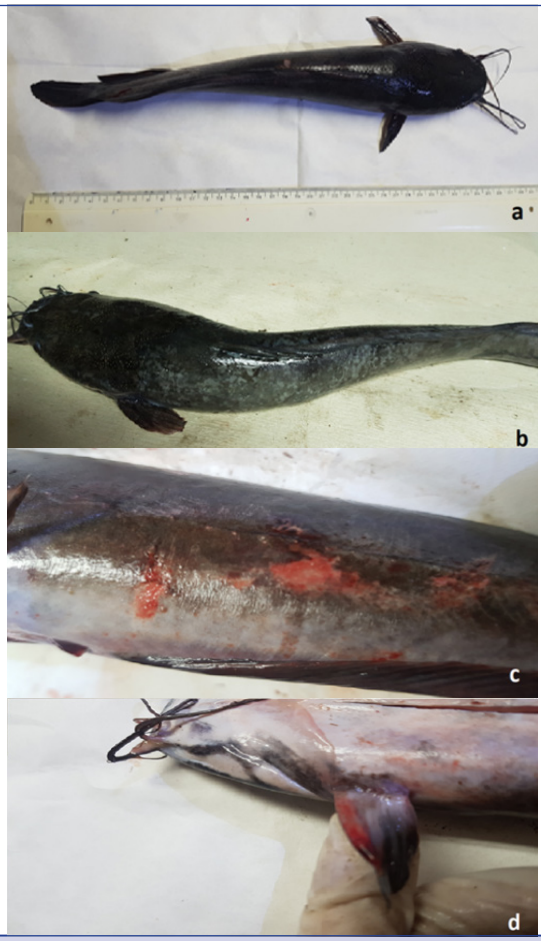

Figure 1. Control fish (a), white (b), and haemorrhagic lesions (c) on the skin, haemorrhagic fins (d).

Hydrochemical analyses

Water samples were collected 80 cm below the water surface in aseptic containers and immediately shipped to the laboratory in a cold environment. The hydrochemical parameters (SO_4-, NO_3-N, NO_2-N, NH_3-N, PO_4- and permanganate oxidizability) were measured using a colorimeter (Hach DR/850, USA) and compatible reagents (HACH Company, Loveland, USA) according to the operating manual. The values of sulphates were 50.75±1.48 mg L^{-1}, nitrate nitrogen 6.42±1.15 mg L^{-1}, nitrite nitrogen 0.160±0.02 mg L^{-1}, nitrogen 0.515±0.08 mg L^{-1}, phosphates 1.37±0.24 mg L^{-1}, permanganate oxidizability 1.33±0.08 mg L^{-1} respectively.

Biochemical analysis

Blood for analysis (approximately 1.2 ml) was drawn from the caudal vein using an aseptic technique in plain containers for biochemical analysis (K_2EDTA). Selected serum biochemical parameters were analysed using Auto Chemistry Analyser (Mindray BS-240VET, Mindray Bio-Medical, China). Plasma cortisol was assayed using a laser fluorescence reader (i-chroma TM Reader, Boditech Med, Korea). The fibrinogen concentration was measured using the BN2 System (Siemens Healthcare Diagnostics).

Parasitical and fungal examination

The presence of external parasites was investigated by examining fresh samples from haemorrhagic and white skin lesions (Buchmann, 2007). The samples were taken from diseased fish's white lesions and inoculated onto Sabouraud Dextrose Agar (SDA) plates for fungal analysis (Bautista-Baños et al., 2014). The choice of culture media was based on our knowledge that *Aspergillus* sp. and *Mucor* sp. showed very good growth on the three culture media but *Rhizopus* sp. grew only on PDA (Potato Dextrose Agar) and SDA. Fungi recovered from the skin lesions of diseased catfish were identified according to their microscopic character and colony structure.

Bacterial identification

Bacteriological samples were taken from the liver, spleen, kidney, and blood and inoculated onto Tryptic Soy Agar (TSA) and Brain Heart Infusion Agar (BHIA). The isolated bacteria were identified using the conventional bacteriological method (Gram staining, motility, cytochrome oxidase and catalase activity, fermentative degradation of glucose (O/F), O/129 (2,4-diamino-6,7-diisopropyl pteridine) sensitivity,beta-galactosidase), and API 20E (Austin & Austin, 2016).

Histopathological examination

The changes induced by the detected disease agents in the tissues were examined using routine histological methods Culling (1963). The fish were sacrificed by concussion according to Council Regulation (EU) 1099/2009 and autopsied. The tissue samples from the skin, gill, kidney, liver, and spleen were immediately fixed in 10% buffered formalin at room temperature for 24 h, dehydrated with graded series of ethanol, treated with xylene, and processed for paraffin embedding. Paraffin blocks were sectioned (4-5 µm thickness) on a microtome Leica RM 2125 (Leica Microsystems GmbH, Austria), dewaxed and stained with haematoxylin and eosin (H&E), and the slides were examined under an Olympus BX-51 light microscope equipped with an Olympus

DP72 digital camera (Culling, 1963).

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA). The results are presented as the mean and standard deviation of the mean (Mean ± SD). Statistical significance is indicated by $p < 0.05$* and $p < 0.01$ **.

RESULTS AND DISCUSSION

External examination

During the physical examination, it was observed that the fish presented with lethargic and erratic swimming activity. It may be hypothesised that low water temperature, and the increased glucose levels found at a later stage, generally affected the behaviour of the fish. According to Barton (2002), the changes in fish behaviour and disease resistance may result directly or indirectly from the primary and secondary responses in the organism.

Affected fish demonstrated clinical signs of haemorrhage and white lesions were present on the surface of the skin and haemorrhagic fin, however clinical findings such as bulging eyes and loss of colour described by Kumar & Prakash (2020) or deeply cottony surface texture reported by Adamu, Muhammad, Ahmad, Ahmad, & Yakubu (2020) have not been observed. The described symptomatic fungal lesions were not only superficial but also penetrated the fish muscles (Figure 1). These results are in agreement with those of Haroon et al. (2014) where the posterior part, which includes all fins and body of the fish, had a significantly higher infection rate (53%) as compared to the anterior part of the fish.

The morphological features of the colony included a cottony texture with a white to grey-brown colour on the top and a pale white colour on the reverse of the plate. The microscopic photograph shows the shape and arrangement of the sporangium. The microscopic examination was used for further identification of isolates up to the genus level. The colony structure and microscopic characteristics of fungal isolates from the skin lesions of moribund catfish were very similar to *those of Rhizopus* sp. (Bautista-Baños et al., 2014; Rashmi & Chandan, 2015) (Figure 2). Based on the body of the mycelia and the types of hyphae, sporangiophores were identified *as Rhizopus stolonifer* as the cause of the infection. The results align with previous studies that identified the same fungal species in catfish (Kalan et al., 2016).

Condition and hydrochemical analysis

From the fish farm hygiene point of view, some of the prerequisites that increase the threat of fungal infection are hydrochemical parameters, water temperature, moisture of feed (above 14%), etc. The current fungal species are determined to be infectious and may spread through the contamination of feed (Ikpegbu et al., 2013). The development of fungal infection as well as mycotoxin production is environment sensitive, and has suggested the huge humidity in aquaculture to be a prerequisite for the flourishing of them in feed. To rule out the probability that the disease was due to the presence of mycotoxins with a sufficient level of certainty, a sample was sent to an approved laboratory, where they were evaluated for AFB1; DON; 3-Acetyl-DON; 15-Acetyl-DON; DON-3-glucoside; OTA; T-2/TH-2; FB1; FB2 and ZEA using liquid chromatography-tandem mass spectrometry

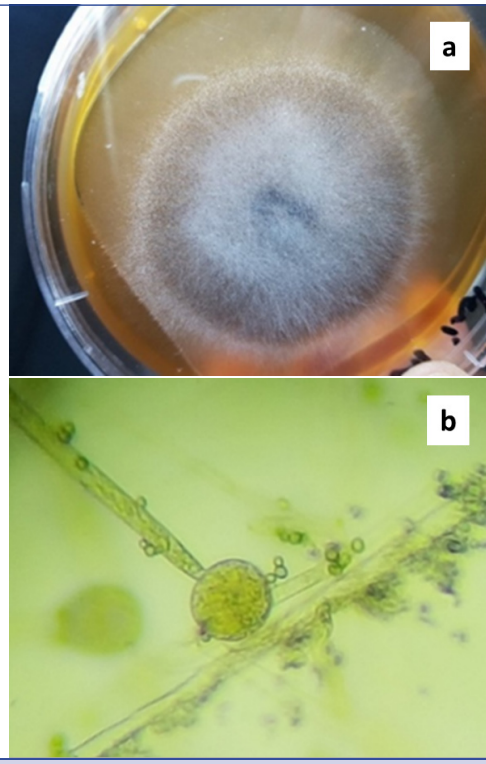

Figure 2. *Rhizopus* sp. on the SDA (a), Microscopic character of *Rhizopus* sp. with its pin head sporangium (b).

(LC-MS/MS) (TSQ Quantum Access MAX, Thermo Fisher Scientific Inc, USA). The final result from the analysis (Protocol A3694) does not involve positive data.

Poor water quality is one of the most important factors favouring the fungus growth. Kumar & Prakash (2020) reported that one of the main reasons for the development of infection in African catfish (*Clarias batrachus*) is cultivated in substandard water quality or an overstocked fish tank. In the current case, except for the water temperature (at the time of sampling was 19.3°C), the physical-chemical parameters of the water as well as the feed were according to the standards for cultivation and requirement of the species. Hence, it can be concluded that the low temperature of water was the main prerequisite for the occurrence of the fungal infection.

Internal examination

Rhizopus spp. have been considered as opportunistic pathogens and they may cause disease under favourable predisposing conditions (Refai, Laila, Amany & Shimaa, 2010). Additionally, the microbial invasion with pathogen bacteria may be facilitated by the fungal agent, *Rhizopus* sp., which causes skin and muscle damage and creates a gateway for infection. This earlier finding is in agreement with the recent findings by Adamu et al. (2020), who reported that *Rhizopus* sp. was the most frequent isolate and may easily cause secondary bacterial infection. *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* are the most commonly co-isolated bacterial species (Kumar & Prakash, 2020).

After the incubation of the bacteriological inoculations from the visceral organs, three different bacterial colonies on TSA and

BHIA were observed at 24-25 °C for 48 h. The three different types of bacteria have Gram-negative and bacillus forms; It has been determined that one bacterial species gives a negative reaction to cytochrome oxidase, unlike other bacterial species. According to the API profile results, the bacteria isolated from the catfish were identified as *Y. ruckeri* (530510057), *A. hydrophila* (724752656), and *P. aeruginosa* (220600403). There have been reports that these pathogenic bacteria were isolated from diseased catfish (Magdy, El-Hady, Ahmed, Elmeadawy & Kenwy, 2014).

Biochemical analysis

The results of the biochemical analysis are presented in Table 1.

Table 1. Blood plasma biochemical parameters of infected African catfish (Mean±SD).

Parameters	Infected	Healthy
Total Protein, g/L	33.07±0.35	41.53±2.91**
Albumin, g/L	13.83±0.23	16.89±1.61**
Globulins, g/L	19.16±0.35	24.79±3.06**
A/G ration	0.72/1	0.68/1
Glucose, mmol/L	13.09±0.23	5.19±2.25**
Triglycerides, mmol/L	2.32±0.11	2.46±2.07
Total cholesterol, mmol/L	3.44±0.37	5.45±2.08
AST, U/L	94.5±34.9	76±2.12
ALT, U/L	23.67±7.77	24.1±2.54
Alkaline phosphatase, U/L	11±2.41	27.95±16.82*
Creatinine, µmol/L	21.16±7.86	19.01±1.79
Urea, mmol/L	0.66±0.11	0.57±0.19
Uric acid, µmol\l	23.17±11.83	8.4±4.11*
Calcium, mmol/L	6.12±1.47	4.55±0.83*
Phosphorus, mmol/L	2.88±0.07	2.73±0.35

Different superscripts *(p < 0.05) and **(p < 0.01) indicate significant differences

Aquaculture conditions expose fish to several environmental and husbandry-related stressors such as salinity, hypoxia, and temperature. One of the main stressors in catfish is cold-shock stress because it cannot tolerate cold water. In African catfish, the immune system slows down in exposition to low water temperature (less than 20°C) and eventually leads to illness/death. Stressed fish will have a disturbance in homeostasis and reduced endurance, making them vulnerable to coinfection, whether caused by parasites, bacteria, fungi, or viruses. In addition, stress caused by fungal infection leading to haemostatic imbalances in fish affects the blood biochemical profile of infected fish. An indication that fish are stressed is an increase in blood glucose levels. Results of plasma glucose values revealed a significant elevation ($p<0.05$) in the diseased fish compared with the healthy fish. These results are in agreement with those of Reid et al., (2022), who explained that acute cold stress led to increased blood glucose concentrations and suppression of antioxidant enzyme activity in the kidneys, liver, and gills, whereas chronic cold stress led to decreased blood glucose, enlarged liver protein content, and elevated lipid peroxidation in the same internal organs. Regarding chronic cold stress, the last one is not accepted as a rule, as it can vary among fish species and duration of the stressor.

In the veterinary practise, some biochemical parameters are used to measure kidney function, including creatinine, urea, and uric acid. The rising concentrations of creatinine and urea in the blood are signs of kidney damage. In the present case, the urea accumulated in the plasma was relatively the same as that in other airbreathers' teleosts, and the rate of urea excretion was unchanged via the ornithine-urea cycle (OUC). These results are in agreement with those of Ip et al. (2004) who described the absence or low activities of hepatic OUC enzymes, especially ornithine transcarbamylase in the liver of the adult *C. gariepinus*. In contrast, the uric acid showed a significant increase ($p<0.05$) in the diseased fish compared to the healthy ones. We sought an explanation for these results, in renal impairment, particularly with regard to the process of excretion (Figure 3a).

In declining renal function accompanied by an excess of purine precursors, higher uric acid concentrations may be observed. Regarding the data of AST in our study, it may be suggested that activities were elevated because of compensation by providing alpha-ketoglutarate due to a positive correlation between am-

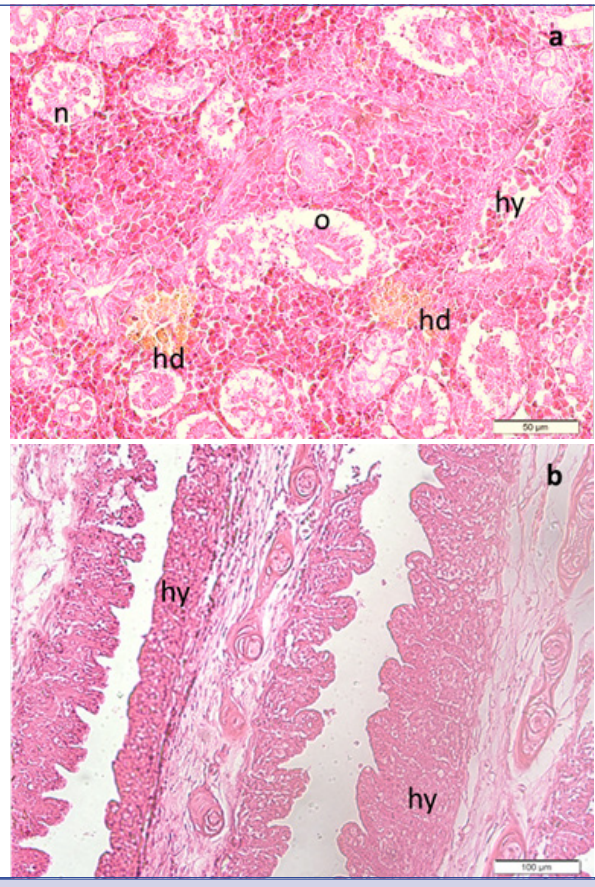

Figure 3. Necrosis of the renal tubules, hyperaemia (hy), peritubular oedema (o), and hemosiderin deposits (hd) in the kidney (a); hyperplasia (hy) between the primary gill lamellae (b) (H&E).

monia components and enzyme level. Considering that more than 80% of the nitrogen excretion in the teleost is by passive diffusion through the gills, with only a small amount excreted by the kidney, it may be reasonable to assume that the pathological changes in the gills also affect the blood plasma concentrations (Figure 3b). Separately, the hydrochemical parameters, especially NH_3 near the gill surface (present case 80 cm), are also important for ammonia excretion, but in the current case, except for the water temperature, all the measured parameters were according to the standard for the cultivation of the species. Moreover, gill hyperplasia is a pathological condition of gill tissue caused by disease, poor water quality, or gill injury. It is thought that the hyperplasia detected in this study may have been caused by secondary infection (fungi and bacteria).

The values obtained for total protein, albumin, and globulin in this current investigation were slightly lower than those for healthy catfish but within the reference value for species as reported by Secer et al. (2018). Also, the present study documents that a decrease in total protein, albumin, and globulin levels may be due to the activation of a humoral immune response against fungal invasion at cold water temperatures.

Certain enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) are used as indicators of tissue damage and liver dysfunction. In general, blood ALP levels increase in the fish during stress and parasitic infection, but the pathophysiological reason for the elevation is still unclear. In this case, the blood levels of ALP were twice as high as with an AST/ALT fluctuation that was never above the reference range. An opportunistic infection can be assumed to have caused the obstruction and cholestatic liver injury, which is demonstrated by papillary stenosis or intrahepatic or extrahepatic biliary strictures (Figure 4a, b). All of this makes it likely that *Rhizopus* sp. is the primary factor in increasing ALP.

In the current study, the hemosiderin deposits around the red pulp and discharge in the white pulp was observed in the spleen tissue. Hemosiderin, which appears brown with the H&E staining method, is the pigment found in MAs (Macrophage aggregates). Hemosiderosis is known to be caused by an increased rate of destruction of erythrocytes in the spleen. This pathological condition may be caused by alpha- and beta-haemolytic bacteria isolated from the diseased catfish in this study.

When tissue samples taken from the lesional skin were examined histopathologically, the presence of club cells in the epidermis, haemorrhage, enlargement of the stratum spongiosum of the dermis, and infiltration of polymorphic nuclear leukocytes were detected. In the current case, haemorrhage and polymorphonuclear leukocyte infiltration in the skin lesions and intense necrosis in the muscles were observed (Figure 5a, b, c, d). Most fungi are characterised to attack the external tissues and only a few species infect the internal organs of fish. In this study, the bacteria-accompanying fungal infection were the bacteria that cause septicaemia. For this reason, more lesions (haemorrhage, polymorphous nuclear leukocytes) may have occurred on the skin.

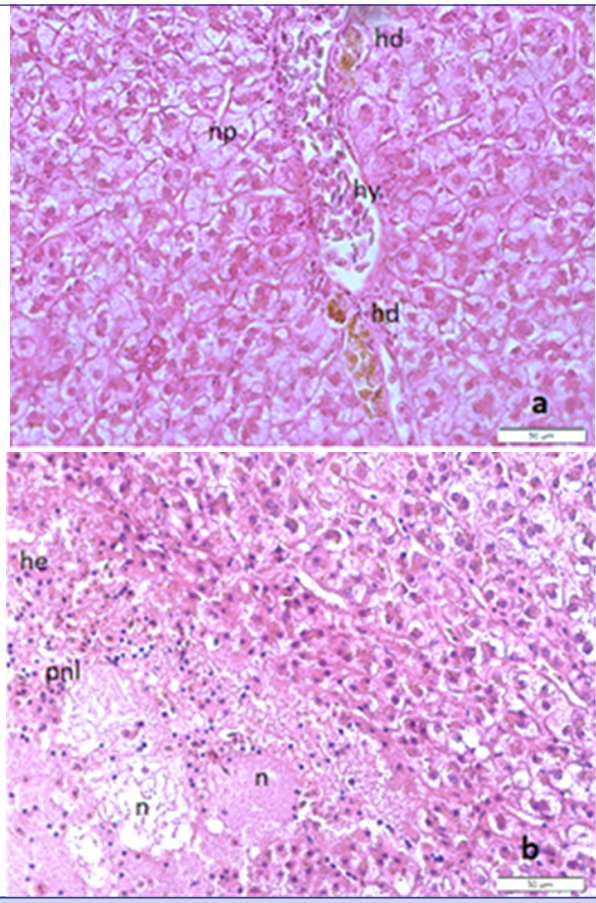

Figure 4. Deposition of hemosiderin (hd) around the blood vessels, hyperaemia (hy) and necrotic hepatocytes (np) (a), necrosis (n), polymorphous nuclear leukocytes (pnl) infiltration, haemorrhages (he) (b) (H&E).

Treatment

Currently, there is no known cure for *Rhizopus* sp. co-infection in fish. The available scientific literature, advances in research and new medicines being developed, obliges us to a certain extent to try to present starting points for achieving adequate treatment. We will first focus on external damages. The haemorrhagic fins are considered less serious damages and after sodium chloride (NaCl) or broad-spectrum antibiotic treatment (Optimal dose/3 days) frequently complete recovery and fish themselves survive. However, severe cases of fungal infection may lead to complete erosion of the fins. In addition, accompanied by a disseminated infection in the gills and eyes, they become fatal. In such a condition, medical treatment is not applicable and the patients die.

Second, certain amino acids, such as L-alanyl-L-glutamine (Recommended dosage 1-4 g/kg) and arginine (1.5-5 g/kg) supplemented by feed in high-fat diets with prebiotics for African catfish, may improve intestinal metabolism and antioxidation mechanism even at low water temperatures (Hu, Zhao, Wang, Sun & Wang, 2022).

Third, nowadays, antifungal antibiotics mainly target the cell wall or cellular membrane metabolism, and their effect is sometimes

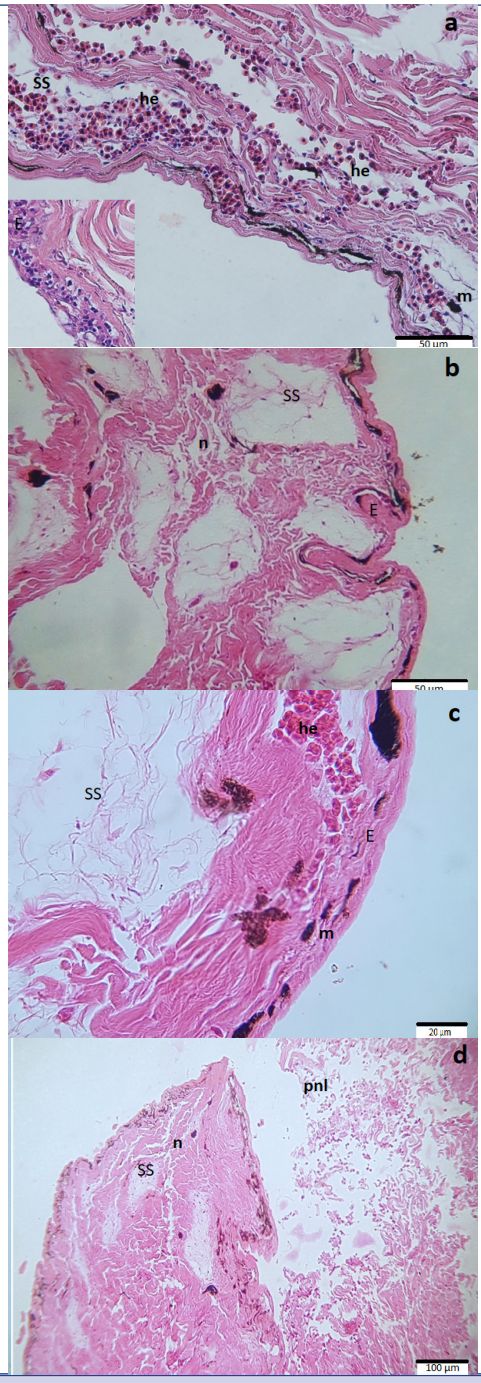

Figure 5. Club cells in the epidermis (E), haemorrhage (he) in the dermis layer (a,c), enlargement in the stratum spongiosum (SS) of the dermis (b,c), polymorphic nuclear leukocyte (pnl) infiltration in the lesioned area (d) (m: melanocyte)

controversial. In addition, the emerging antibiotic resistance represents a serious problem as well. One of the commonly applied antifungal antibiotics is itraconazole (40 mg/kg feed/7 days), which is prepared by mixing the agent with commercial feed pellets. Considering that *P. aeruginosa* inhibits the germination and therefore the virulence of *Rhizopus microsporus* by iron-chelating molecules, it would be more appropriate to administer the antibiotic at a later stage. Iron is essential for the survival and pathogenicity of *R. microsporus*; therefore, it is desirable to first apply pyoverdine (80µg ml^{-1}) (Kousser, Clark, Sherrington, Voelz & Hall, 2019).

CONCLUSIONS

This research is the first to report the pathogenic effects of *Rhizopus* sp. in conjunction with *Y. ruckeri, A. hydrophila*, and *P. aeruginosa* in RAS-farmed African catfish (*C. gariepinus*). Our findings could help develop better disease control and treatment strategies in aquaculture and prevent outbreaks in poor conditions. Additionally, fungi affecting fish can also cause serious human infections due to similarities in their epidemiology and lesions. Although the list of zoonotic fungal agents in aquaculture is limited, Mucorales fungi like *Rhizopus* sp. pose significant public health risks.

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethics Committee Approval: The authors affirm that ethical approval is unnecessary for this study.

Financial Disclosure: This study was self-sponsored.

REFERENCES

Adamu, K., Muhammad, H., Ahmad, A.S., Ahmad, M., & Yakubu, A. (2020). Diversity of bacteria and fungi associated with freshwater fishes from Mijawal River, Nasarawa, Nigeria. *Journal of Applied Sciences & Environmental Management*, *24*(6), 1085-1092.

Austin, B. & Austin, A. (2016). *Bacterial fish pathogens: Disease in farmed and wild fish*. UK: Springer. ISBN 9783319326733.

Bangyeekhun, E. & Sylvie, M. (2001). Characterization of Saprolegnia isolate from catfish. *Diseases of Aquatic Organisms*, *45*, 53-59.

Barton, B.A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*, *42*(3), 517-525.

Bautista-Baños, S., Bosquez-Molina, E. & Barrera-Necha, L. (2014). Rhizopus stolonifer. In S. Bautista-Baños (Ed.), *Postharvest decay. Control strategy* (pp. 1-44). US: Academic Press. ISBN 9780124115521.

Bozhkov, A., Nikolov, G., Urku, C., Zapryanova, D., Atanasoff, A., Secer, F.S. & Kandir, S. (2023). Effect of different light intensities on prolactin and cortisol plasma concentration in farming African catfish *(Clarias gariepinus)* in RAS with low-water exchange. *The Israeli Journal of Aqua-culture – Bamidgeh*, *75*(1834930), 1-7.

Buchmann, K. (2007). Introduction to fish parasitological methods: Classical and molecular techniques. Biofolia, Frederiksberg. ISBN 978-879-1319-39-6.

Culling, C.F.A. (1963). *Handbook of histopatological techniques*. 2nd edition, London, UK: Butterworth, ISBN 0407729011.

Haroon, F., Iqbal, Z., Pervaiz, K. & Khalid, A.N. (2014). Incidence of fungal infection of freshwater ornamental fish in Pakistan. *International Journal of Agriculture and Biology*, *16*(2), 411-415.

Hu, J., Zhao, H., Wang, G., Sun, Y. & Wang, L. (2022). Energy consumption and intestinal microbiome disorders of yellow catfish (Pelteobagrus fulvidraco) under cold stress. *Frontiers in Physiology, Aquatic Physiology*, https://doi: 10.3389/fphys.2022.985046.

Ikpegbu, E., Ezeasor, D.N., Nlebedum, N.C. & Nnadozie, O. (2013). Morphological and histochemical observations on the oesogaster of the domesticated African catfish *(Clarias gariepinus)*. *Bulgarian Journal of Veterinary Medicine*, *16*(2), 88-95.

Ip, Y.K., Zubaidah, R.M., Liew, P.C., Loong, A.M., Hiong, K.C., Wong, W.P. & Chew, S.F. (2004). African sharptooth catfish does not detoxify ammonia to urea or amino acids but actively excretes ammonia during exposure to environmental ammonia. *Physiological and Biochemical Zoology: Ecological and Evolutionary Approaches*, *77*(2), 242-254.

Kalan, L., Loesche, M., Hodkinson, P.B., Heilmann, K., Ruthel, G., Gardner, S.E. & Grice, E, (2016). Redefining the chronic-wound microbiome: Fungal communities are prevalent, dynamic, and associated with delayed healing. *mBio*, *7*(5), e01058-16.

Kousser, C., Clark, C., Sherrington, S., Voelz, K. & Hall, R.A. (2019). *Pseudomonas aeruginosa* inhibits *Rhizopus microsporus* germination through sequestration of free environmental iron. *Scientific Reports*, *9*(1), 5714.

Kumar, A. & Prakash, S. (2020). Effect of *Rh. stolonifer* on morphology and haematology of catfish. *International Peer-Reviewed Journal*, *4*(5), 6-9.

Magdy, I.H., El-Hady, M.A., Ahmed, H.A., Elmeadawy, S.A. & Kenwy, A.M. (2014). A contribution on *Pseudomonas aeruginosa* infection in African catfish (*Clarias gariepinus*). R*esearch journal of pharmaceutical, biological and chemical sciences*, *5*(5), 575-588.

Rashmi, K. & Chandan, K. (2015). Fungal infection in some economically important freshwater fishes in Gandak River near Muzaffarpur Region of Bihar. *International Journal of Life science and Pharma Research*, *5*(1), 1-11.

Refai, M.K., Laila, A.M., Amany, M.K. & Shimaa, M.K.A. (2010). The assessment of mycotic settlement of freshwater fishes in Egypt. *Journal of American Science*, *6*(11), 595-602.

Reid, C.H., Patrick, P.H., Rytwinski, T., Taylor, J.J., Willmore, W.G., Reesor, B. & Cooke, S.J. (2022). An updated review of cold shock and cold stress in fish. *Journal of Fish Biology*, *100*(5), 1102-1136.

Secer, F.S., Atanasoff, A., Zapryanova, D., Nikolov, G. & Cagiltay, F. (2018). Haematological and biochemical reference intervals for African catfish *(Clarias gariepinus)* reared in RAS. *Albanian Journal of Agricultural Sciences*, *3*, 142-145.