Jurnal Riset Veteriner Indonesia
Journal of the Indonesian Veterinary Research
P-ISSN: 2614-0187, E-ISSN:2615-2835
Volume 5 No. 2 (July 2021), pp. 53-65
journal.unhas.ac.id/index.php/jrvi/

The identification of gram positive bacteria and their effects on kidney histopathology of Amazon Sailfin Catfish *(Pterygoplichthys pardalis)* in Lapompakka and Sidenreng Lakes, Wajo.

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Abstract

Pterygoplichthys spp or commonly referred as Pleco is originated from Amazon River in South American Continent. The aim of this study is to isolate and identify the pathogenic gram-positive bacteria in pleco's kidney (Pterygoplichthys pardalis) and to observe the pleco's kidney histopathology colonized by gram-positive pathogenic bacteria in Sidenreng and Lapompakka Lakes. The samples employed in this experiment includes 10 plecos with each 5 samples represented each lake. The isolation and identification of positive gram bacteria were performed through bacterial culture produced from pleco's kidney swab test on blood agar using gram staining followed by biochemical testing with Vitek 2 compact system. From the results, there were 4 gram-positive bacteria successfully isolated, one of them was Enterococcus faecalis which is the suspected pathogen. Specimen preparation for kidney histopathology was carried out by 10% neutral buffered formalin (NBF) fixation, graded ethanol series dehydration, paraffin wax embedding, 4 μm sectioning, Hematoxylin and Eosin Staining and after that, the specimen was observed. Data analysis employed qualitative descriptive method. From the observation, damages such as necrosis, inflammatory cell infiltration, hemorrhage, and hypertrophy were identified from the pleco's kidney. Kidney damages were most probably caused by the infection by gram-positive pathogenic bacteria Enterococcus faecalis.

***Keywords** : Sidenreng Lake, Lapompakka Lake, Kidney, Histopathology, Plecos.*

Introduction

Sidenreng and Lapompakka Lakes are located near Tempe Lakes. These three lakes were surrounded by three regions of Wajo, Soppeng, and Sidrap. The three lakes are interconnected and located in the central part of South Sulawesi (Husnah *et al.*, 2008). Sidenreng Lake holds a great potential as an ecosystem in South Sulawesi, especially in Sidenreng Rappang (Sidrap). A different species of fish lived in both Sidenreng and Lapompakka lakes begin to indicate signs of extinction (Andy Omar, 2010). One possible cause of such sudden change was the annual

population explosion of invasive species such as the Plecos or Amazon Sailfin Catfish (*Pterygoplichthys pardalis*). The presence of this invasive species threatens the endemic fish population.

The local community in Sidenreng and Lapompakka Lakes, so far, did not fully utilize plecos as a food source considering the difficult processing because of their armoured-like scutes (Dewi, 2019). Most of the locals would only use plecos as tank cleaners because they are well known for their scavenging behaviour and they feed on aquarium food scraps and algae (Istanti, 2005). On the other hand, there are some other regions that attempts to utilize plecos as food sources for a variety of food products such as fish balls, nuggets, pleco fish meal, gellatin, and Otak-otak (Southeast Asian Fish Cake) with good nutritional content and attractive colouring (Chaidir, 2001; Mahdia, 2002; Erawaty, 2001; Tiyansmainar, 2001; Hermanto *et al.*, 2014).

For healthy and safe food processing involving pleco's meat, there are a number of standardized requirements and procedures. One of the important regulations is contamination-free from pathogens that may harm the consumers (Kwantes dan Isaac, 1975 as cited in Manurung dan Susantie, 2017). Therefore, investigation on pathogenic contamination of plecos in SIdenreng and Lapompakka Lakes is considered necessary.

A histological examination is needed to confirm the presence of infectious pathogens and to detect the abnormal pathological changes of infected fish at the tissue scale (Asniatih *et al.*, 2013). One biological tissue with great potential as an indicator for such examination is the kidney (Sukenda *et al.*, 2008). The kidney is an excretory organ in all vertebrates and it plays an important role in sustaining homeostasis by filtering the metabolic waste such as ammonia (Safratilofa, 2017).

Therefore, a histopathological examination on the kidney to confirm bacteria identification is very important. Such examination may be carried out by the identification of gram-positive bacteria and their effects on kidney histopathology of pleco (*Pterygoplichthys pardalis*) from Sidenreng and Lapompakka Lakes.

Materials and Methods

This study employed a primary data descriptive analysis. Research site is located in Sidenreng Lake, Wette'e District, Panca Lautang Sub-District, Sidrap and Lapompakka Lake in Tanasitolo District, Wajo. The research was conducted for 2 (months) from February to March 2021. Data source in this descriptive study was collected from laboratory observation performed by Municipal Health Service Laboratory in Makassar and The Laboratory of Veterinary Clinic of Hasanuddin University to observe the histopathological damages on pleco's kidney. Sample collection employed a random sampling technique at one stage from each lake. The data in this study is primary data. The method of data collection includes direct observation, review of literature, and documentation.

Results and Discussion

Sample Collection

From each lake, 5 samples were collected randomly without any specific criteria. There were 10 samples in total. The plecos were trapped using traditional fishing tool or *Jebba* fish trap. After the samples were collected, a necropsy was carried out to dissect the kidney from the other organs. The swab method was used to collect the contaminating bacteria in kidney using

sterilized cotton and the bacteria were cultured on Brain hear Infusion Broth (BHIB). The dissected whole kidneys were preserved in 10% formalin for histopathological examination. Each swab sample on BHIB was transferred to the Municipal Health Service laboratory using a cool box during the travel for pathogen identification. The kidney sample organ on 10% formalin was delivered to The Laboratory of Veterinary Clinic, Hasanuddin University for kidney histopathological examination on plecos.

Identification of Gram-Positive Bacteria

The identification of gram-positive bacteria was performed by biochemical testing with *VITEK Densi CHEK Plus*. Before conducting biochemical testing, isolation of the samples was carried out on Blood agar culture media to acquire bacteria isolates with gram-positive characteristics.

From 10 samples of plecos (*Pterygoplichthys pardalis*), 5 from Sidenreng Lake and the remaining 5 from Lapompakka Lake were identified to be contaminated by 7 strains of bacterial isolates. From 7 bacterial isolates, 4 isolates were identified from Sidenreng lake plecos and 3 other isolates were identified from Lapompakka lake. Such result was obtained after the testing of isolate selection found in plecos' kidney (*Pterygoplichthys pardalis*) and overall, there were 7 pure isolates identified with gram-positive characteristics.

Bacterial Colony Morphology and Potential Gram-Positive Bacteria Cell in Plecos' Kidney (Pterygoplichthys pardalis)

The characteristics of potential gram-positive isolates obtained from pleco's kidney (*Pterygoplichthys pardalis*) isolation can be observed from the colony morphology consisting of colour, size, hemolysis, and shapes as well as the morphology of bacterial cells on blood agar medium. The morphological characteristics of 7 isolated colonies are presented in Tables 1 and 2

Table 1. Morphological Characteristics of Kidney Bacterial Isolate Colonies from Sidenreng Lake (SL) Plecos

No.	Samples	Colony	colour	Size	Shapes	Hemolysis	Gram Staining	Bacterial cell morphology
1.	DSG1	a	Grey-white	Large	circular and flat colony shape	β-Hemolysis	+	Bacili
2.	DSG2	a	Grey-white	Large	circular and flat colony shape	β-Hemolysis	+	Bacili
3.	DSG3	a	Grey-white	Large	circular and flat colony shape	β-Hemolysis	+	Coccus
4.	DSG4	b	creamy colour	Medium	Bulat, cembung	α-Hemolysis	+	Coccus

Table 2. Morphological Characteristics of The Isolate Colony in The Kidney of Lapompakka Lake Plecos

No	Samples	Colony	Colour	Size	Shapes	Hemolysis	Gram Staining	Bacterial cell morphology
1.	DLG1	b	creamy colour	Small	Bulat, cembung	γ-Hemolysis	+	Coccus
2.	DLG3	B	creamy colour	Medium	Bulat cembung	α-Hemolysis	+	Coccus
3.	DLG5	B	creamy colour	Medium	Bulat, cembung	α-Hemolysis	+	Coccus

Characterization and Identification of Gram-Positive Bacteria in Pleco

Bacterial colony isolated on agar blood media was tested using biochemical testing with VITEK 2 compact system. Vitek 2 compact system is a highly automatic system to perform bacterial identification and antimicrobial susceptibility testing according to the principles of advanced colourimetry and turbidimetry.(Prihatini *et al.*, 2018) In this study, GP card was used for identifying gram-positive aerobic cocci and non-spore-forming bacilli, while BCL card was used for identifying gram-positive spore-forming bacilli (Biomerieux,2013). Vitek 2 compact system identification sheet includes a data validation level that provides percent probability results (Biomerieux, 2013, Pincus, 2014).

Table 3. Identification results by Vitek 2 compact system on Sidenreng Lake pleco kidneys.

Samples	Colony	Gram Staining	Bacterial	Confidance
Sidenreng Lake, Sample 1	DS G1a	Bacilli, gram-positive	*Bacillus sp*	*Low discrimination*
Sidenreng Lake, sample 2	DS G2a	Bacilli gram-positive	*Bacillus cereus/thuringiensis/mycoides*	85%
Sidenreng Lake , sample 3	DS G3a	Coccus, gram-positive	*Staphylococcus gallinarum*	99%
Sidenreng Lake, sample 4	DS G4b	Coccus. Gram-positive	*Enterococcus faecalis*	98%

Table 4. Identification results by Vitek 2 compact system on Lapompakka Lake pleco kidneys.

Sample	Colony	Gram Staining	Bacterial	Confidance
Lapompakka Lake, sample 1	DLG1b	Coccus, gram-positive	*Staphylococcus equorum*	95%
Lapompakka Lake, sample 3	DLG3b	Coccus, gram-positive	*Enterecoccus faecalis*	98%
Lapompakka Lake, sample 5	DLG5b	Coccus, gram-positive	*Enterecoccus faecalis*	99%

Low discrimination on the DSG1a isolate validation level, identification only reaches the limit of the bacteria genus of Bacillus in accordance with the explanation by Biomerieux (2013), and Pincus (2014). To identify and discriminate further species of bacteria, biochemical testing is required because there were possibilities of 2 or 3 species (taxa) with a similar pattern and therefore, it was considered as low discrimination. Similarly, DSG2a isolates or *Bacillus sp* has the identification accurate validation of 85% (acceptable identification).

a. *Bacillus sp*

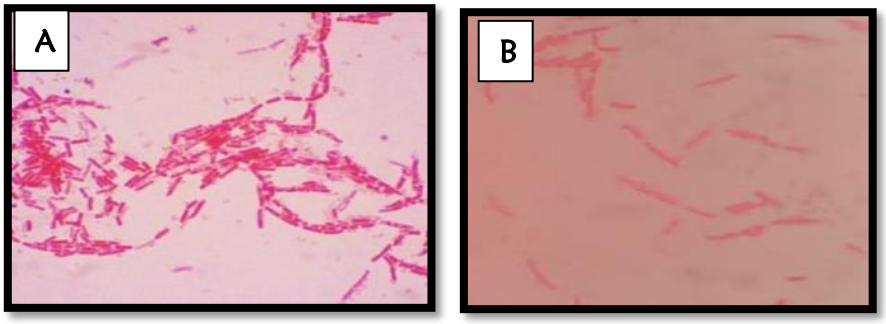


Figure 1. The Appearance of Bacteria Colony *Bacillus sp* under Microscope (*Olympus corpuration*, Japan), at 100x magnification; A : DSG1a Isolate, B: DSG2a Isolate

In DSG1a and DSG2a isolates, Bacillus bacteria were isolated. However to identify dominant bacteria species in Plecos. Further research is necessary. On DSG2a isolates, *Bacillus cereus*, *Bacillus muicoides*, and *Bacillus thuringiensis* were isolated at 85% validation level (confidence)

Based on the observation of *Bacillus sp* colony morphology on blood agar media in Table 1, it was identified that this colony has a creamy colour, circular and flat colony shape, β-Hemolysis trait and rod shape (bacilli) under microscope. This is in accordance with the study performed by Feliatra *et al.,* (2004) stating that the bacteria colony colour of *Bacillus sp* is creamy and the shape is circular. Bacteria cell shape was rod. In addition to the finding on Similar *Bacillus cereus* colony morphology and its characteristics on blood agar media was also found in the study performed by Fatmasari (2015) that the colony appearance of Bacillus cereus is creamy with β -Hemolysis trait. *Bacillus sp* are mostly used as probiotics in aquaculture because of its ability in producing enzymes and other anti-microbial components, bacteriocin that could inhibit pathogenic bacteria according to Umoro (2016)

b. *Enterecoccus faecalis*

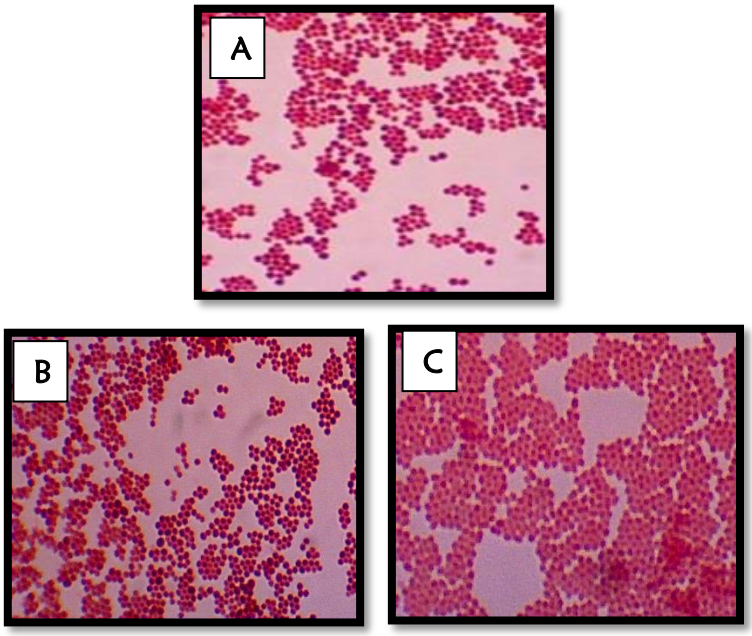


Figure 2. Colony appearance of *b. Enterecoccus faecalis* under a microscope (*Olympus corpuration*, Japan) at 100x magnification; A: DLG3b Isolate, B: DLG3b Isolate, C: Isolate DSG4b

Isolates DSG4b, DLG3b, and DLG5b were identified to be *Enterecoccus faecalis* (Table 1 and 2). These bacteria were the only bacteria found in the samples from Sidenreng and Lapompakka Lake. The morphology characteristics of *E.faecalis* were a creamy colour, spherical and convex and spherical. Staining results indicated the pairs of cocci and α-Hemolysis traits. This is in accordance with the study performed by Evans *et al.,* (2002) stating that *Enterococcus faecalis* is a gram-positive coccus with a diameter of 0,5 – 1 μ and may colonize in pair, chain, or single. According to Arumugam *et al* (2017), fish contaminated or infected by positive bacteria *E. faecalis* may demonstrate clinical symptoms of fatigue, abdominal ascites, organ colour change, spleen necrosis and hemorrhage in the kidney. *E. faecalis* is also commonly described as fish or human pathogen (Eley, 1992).

c. *Staphylococcus equorum*

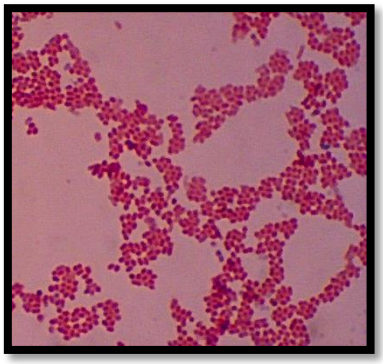


Figure 3. Colony of *Staphylococcus equorum* under microscope (*Olympus corpuration*, Japan), at 100x magnification;

In the DLG1b isolate from Lapompakka lake samples, *Staphylococcus equorum* (Table 2) identified with the colony morphology of creamy colour, convex and spherical shape, γ-Hemolytic trait, and coccus cell shape is also a gram-positive bacteria. This is in accordance with the study performed by Febriyana, (2017) indicating that *Staphylococcus equorum* is a non-spore-forming bacteria. The colony commonly known for its white, creamy and sometimes orangish yellow colour. *Staphylococcus equorum* falls under the category of Coagulase-negative Staphylococci and is frequently isolated from fermented food products or food processing environments. *S. Equorum* played an important role in the formation of aromatic compounds during the fermented food maturation or ripening process, primarily in cheese and sausages (Irlinger *et al.,* 2012).

Staphylococcus equorum is a part of normal microbiotes found in human and animal skin or mucous membrane as well as spread in different places including in the soil, air, water, or food materials (Coton *et al.,* 2010). *S.equorum* falls under the category of Coagulase-negative Staphylococci which is initially non-pathogenic. However, due to its opportunistic traits, such bacteria may cause harm and demonstrate pathogenic traits, especially when the host immune system is weak or there is a supportive environment for growth (Lee *et al.,* 2018).

d. Staphylococcus gallinarum

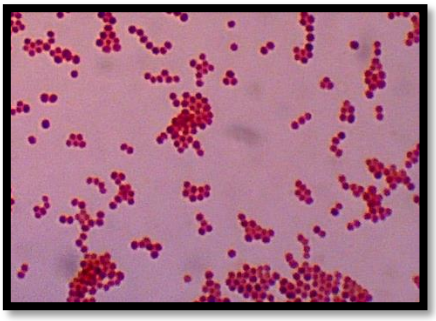
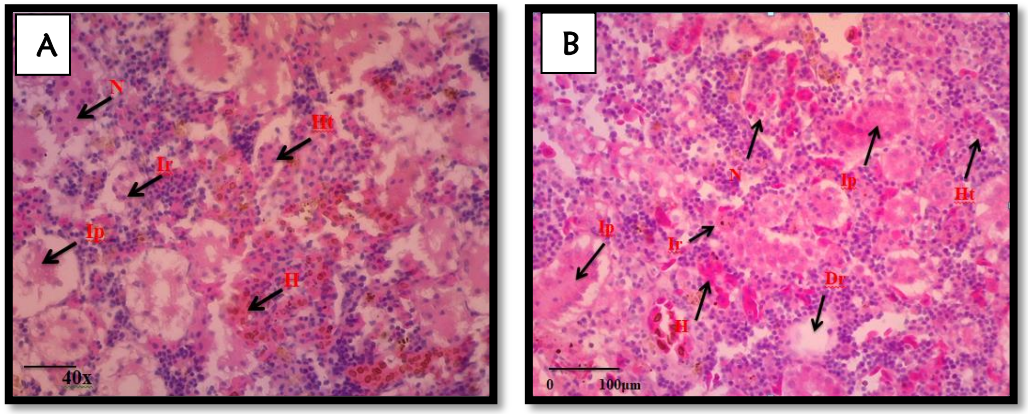


Figure 4. Colony appearance of *Staphylococcus qallinarum* under microscope (*Olympus corpuration*, Japan), at 100x magnification;

Isolate DSG3a from Sidenreng Lake samples (Table 1) were *Staphylococcus gallinarum* The colony morphology of *Staphylococcus gallinarum* observed in blood agar has grey-white colour, flat, spherical and large shape, β-Hemolytic, cell shape of coccus. This is in line with the explanation in Jawetz, Melnick & Adelberg's Medical Microbiology by Geo *et al.*, (2008) stating that *S. gallinarum* has a non-motile coccus shape, non-spore-forming trait, gram-positive. The colony is flat, cloudy, dry, and yellow or has no pigment. Infection capacity of *S. gallinarum* is categorized as low and the bacteria have low effect on human (Yu *et al.*, 2008) Similar to *S. equorum, S. gallinarum* falls under the category of Coagulase-negative Staphylococci which is non-pathogenic but opportunistic (Lee *et al.*, 2018).

Pleco's (*Pterygoplichthys pardalis*) Kidney Histopathology

Based on the results of biochemical testing using VITEK 2 Compact System in this study, it was identified that from 7 isolates, 4 bacteria were detected to be gram-positive. Pleco's (*Pterygoplichthys pardalis*) Kidney Histopathology is presented as follows:



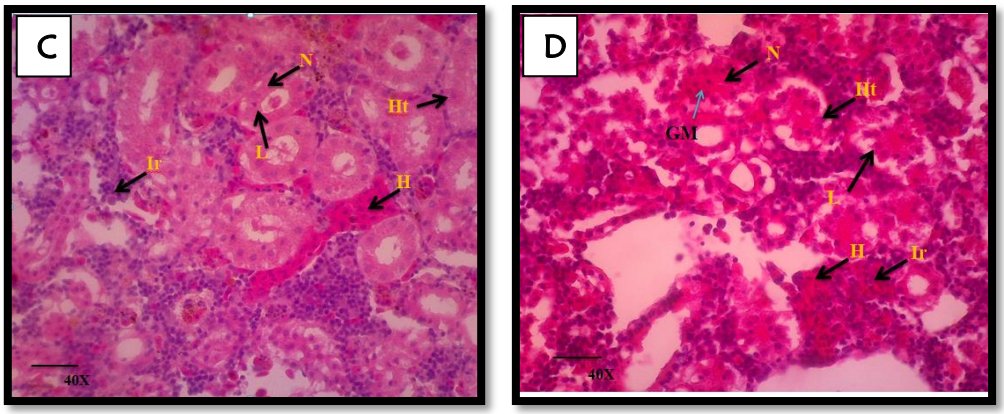


Figure 5. Pleco's (*Pterygoplichthys pardalis*) Kidney Histopathology identified as gram-positive

Description: A. DSG1a Isolate (*Bacillus sp*), B. DSG3a Isolate (*Staphylococcus gallinarum*), C. DLG3b Isolate (*Enterococcus faecalis*), D. DLG1b Isolate (*Staphylococcus equorum*); Ht: Hypertrophy; H: Hemorrhage; N: necrosis; Ir: Inflammatory Cell Infiltration; Ip: Protein Infiltration; Dr: Renal Dilatation. (Magnification at 40x10; Scale Bar = 10 μm).

Histopathological changes generally found in Sidenreng and Lapompakka lake Plecos encompassed cell hypertrophy, necrosis, hemorrhage, and infiltration. Such kidney histopathological changes in plecos from Sidenreng and Lapompakka Lakes demonstrated the most serious degree of damages according to the kidney histological damage categories introduced by Camargo and Martinez (2007). Previously, pathogen presence in both of the lakes were known and the most dominant pathogen from those lakes was *Enterococcus faecalis*. This was in line with the previous study performed by Ritnoga (2016) that contaminated Carps by *E. faecalis* also indicated similar clinical symptoms such as lesions, ulcers, and excess mucus on the fish body.

According to (Figure 5), pleco's kidneys indicated some tubular and glomerular damages such as tubular and glomerular hypertrophy which eventually triggered cell lysis, necrosis, and scar tissue. Damages may cause different impacts either functionally or morphologically. Morphologically, glomerular damages may be represented by necrosis, proliferation of membrane cell and leukocyte infiltration. The functional glomerular damage is indicated by the protein escapes, reduced blood flow perfusion, and other macromolecules excess in glomerular filtrate. Initial symptoms of necrosis was the presence of lysis and hyperthrophy (Wahyuni *et al.,* 2020). Such cases has an impact on the kidney function and metabolism. Glomerular hypertrophy occurs because of the toxic compound inhibition. Despite its low concentration, persistent contamination may cause hypertrophy reaction (Takashima and Hibiya, 1995).

The longer the kidney exposed to toxic compounds, the more kidney tissue suffers from necrosis. Necrosis may also happened to trauma, biological agents (virus, bacteria, fungi, and parasites), chemical agents or blood supply disruption in particular organs (Plumb, 2018). Cell death may be indicated by the cellular loss which appears like fenestrae in the glomerulus. (Cahyaningrum *et al.,* 2015). Hemorrhage may occur due to trauma, blood vessel rupture, porosity increase stimulated by bacterial or viral infection and toxic compound contamination. Leukocyte infiltration was also apparent from the Pleco's kidney most probably because of the fish immune reaction to pathogen presence.

Enterococcus faecalis was also identified from DSG4b, DLG3b and DLG5b isolates. According to Arumugam *et al,m* (2017) *E.faecalis* is pathogen to fish and human. Histopathological damages on the observed Pelco's kidneys in Table 6 indicated that samples infected by *E. faecalis* suffered from kidney haemorrhage. Such findings were also confirmed by Arumugam *et al*

(2017) that the infected fish by *E. faecalis* showed clinical symptoms including kidney haemorrhage. Haemorrhage occurred because of the blood vessel rupture or increasing porosity triggered by the pathogen. According to Sumatri, (2013) virulence factors playing important roles in the pathogenesis of *Enterococcus faecalis* consisted of a number of components. Such components encompassed *Aggregation Substance* (AS), *cytolysin, surface adhesins, Lipoteichoic Acid* (LTA), *sex pheromones*, *Extraceluller Superoxide Production* (ESP), *hyaluronidase*, and *gelatinase lytic enzyme* and AS-48. *E. faecalis* pathogenic capacity may also be seen from Tables 1 and 2 confirming their α-Hemolytic trait. This implied that the bacteria may lyse blood cells completely. A study performed by Semedo *et al.,* (2003) found that *E. faecalis* may sometimes be β-Hemolytic or α-Hemolytic.

Serious damages based on pleco's kidney histopathology also occurred in pleco's kidney infected by gram-positive *Bacillus sp* in DSG1a and DSG2a isolates where damages such as necrosis, hemorrhage, inflammatory cell infiltration and hypertrophy as well as β-Hemolytic trait were identified (Table 1). However, the specific Bacillus bacteria species that caused such damages were not known and therefore, biochemical testing was required for further identification. From DSG2a isolates, the identified bacteria encompassed *Bacillus cereus, Bacillus thuringiensis* and *Bacillus mycoides.* According to Latifah *et al.,* (2014) kidney damages also occurred in Gourami infected by popeye eye disease-related bacteria. The bacteria causing such disease were *Bacillus sp*, especially *Bacillus mycoides*, and they may cause damages such as necrosis, cell congestion, and kidney cell degeneration. According to the study by Setyowati *et al.* (2007), necrosis may be identified by the loss of tissue structure. After that, kidney tissue cells suffer from cell damage causing necrosis. The damages most probably occurred because of the growing colony of bacteria in kidneys and it is highly associated with the toxic bacteria infection in popeye disease.

As for the DSG3a (*Stapylococcus gallinarum*) and DLG1b (*Staphylococcus equorum*) Isolates, both species of bacteria are categorized as Coagulase-negative Staphylococci which is non-pathogenic, but opportunistic. Such bacteria may cause harm and demonstrate pathogenic traits, especially when the host immune system is weak or there is a supportive environment for growth (Lee *et al.,* 2018). The damages found from the isolates including hypertrophy, hemorrhage, necrosis, inflammatory cell infiltration, and renal dilatation. The damages were caused by the disruption in immune system and stimulating the opportunistic bacteria to become actively pathogenic. This was in accordance with Hardi *et al.,* (2011) clarifying that the microbial behavioral changes and organ morphology in the body may happen because of the immune system disruption affected by bacterial infection or environmental factor. Austin and Austin (2008) stated that behavioral changes in the infected fish that can be observed including disorientation, fatigue, popeye and instability.

According to Dong *et al.,* (2018) the observed changes from the kidney histopathology of infected fish by either gram-negative or positive bacteria cannot be distinguised since both types of bacteria causes identical damages such as necrosis, inflammatory cell infiltration, degeneration, hydrolysis, haemorrhage, and fat degeneration. Kidney damages, according to Dong *et al.,* (2018) were also found in almost all isolates of gram-positive bacteria. In this study, the samples of Plecos (*P. pardalis*) also indicated similar damages such as necrosis, inflammatory cell infiltration, haemorrhage, and cell hypertrophy. A similar finding confirmed by Latifa *et al.,* (2014) occurred in Gourami's (*Osphronemus gouramy*) kidney triggered by gram-negative bacteria and caused symptoms such as congestion, necrosis, haemorrhage, and cell degeneration. In line with those studies, Heimesaat *et al.,* (2021) confirmed that the gram-negative bacteria may cause damages such as cell death, and tissue damage in fish liver, kidney, and intestine. According to Takashima and Hibiya (1995) damages in the kidney may also occur

due to chronic exposure to toxic compounds. The longer the exposure, the more severe the damages it causes in the kidney and the more cell suffers from necrosis.

Conclusion and Recommendation

There were 4 different bacteria identified as gram-positive bacteria in plecos (*Pterygoplichtthys pardalis*). The bacteria encompassed *Bacillus sp, Staphylococcus gallinarum*, *Enterococcus faecalis*, dan *Staphylococcus equorum*. Histopathological changes that frequently happened in the kidney samples from plecos infected by pathogen were necrosis, inflammatory cell infiltration, hemorrhage, and hypertrophy.

As a recommendation for further research, research on water quality from both lakes are also necessary to identify their toxicity level. Therefore, there will be more information available concerning the consumption feasibility of water biota from Sidenreng and Lapompakka Lakes in Wajo. The local community should concern with the lake cleanliness as a aquatic habitat for fish in order to anticipate harmful microbial contamination for the local community themselves.

Acknowledgement

The authors would like to express their gratitude to the Municipal Health Service of Makassar, the Agricultural and Livestock Service of Sidrap and Wajo as well as other parties involved in supporting this research. The authors also would like to express their gratitude to all lecturers from the Faculty of Veterinary Medicine that always encouraged and assisted the authors until the completion of this research. The authors have no conflicts of interest to declare in relation to the involved parties in this research.

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