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Gut and skin microbial profiling of healthy and dropsy diseased Malaysian Mahseer Empurau (*Tor tambroides*) following exposure to antimicrobial agents

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ABSTRACT

Aims: The study was aimed to identify bacterial pathogens from dropsy diseased *Tor tambroides*, compare the isolated bacteria from dropsy and healthy Empurau and assess their sensitivity towards various antibiotics.
Methodology and results: To identify the possible causative agents of dropsy in diseased fish, we conducted pure culture of bacteria using spread plate techniques, Gram's staining, KOH string test, 16S rRNA gene sequencing and antibiotics susceptibility test. Plate and microscopic observation revealed that most bacterial strains from healthy and dropsy diseased Empurau are Gram-negative, KOH reaction-positive and rod-shaped. The characterization of 16S rDNA sequence revealed that most of the bacteria were *Pseudomonas* spp., with a higher number identified in dropsy-affected Empurau than healthy ones. It is postulated that *P. fluorescens*, *Aeromonas* spp., *Citrobacter freundii* and *P. congelans* are the major causative agents of dropsy disease in Empurau fishes, but further investigations are required to strengthen this claim. Antibiotic susceptibility tests uncovered two multi-drug resistant bacterial strains (resistant to at least three antibiotics) from the gut and skin of healthy Empurau fish, respectively. Interestingly, all dropsy diseased Empurau gut isolates were susceptible to Morepenem and Tetracyclin, whereas all dropsy diseased Empurau skin isolates were susceptible to Chloramphenicol.
Conclusion, significance and impact of study: The data on the possible causative agents of dropsy disease in Empurau fishes will be beneficial in managing fish bacterial diseases. The local aquaculture yield can be further improved through future research and elucidation on the biochemical characterization and disease management of bred Empurau fish.

Keywords: Dropsy disease, Empurau (*Tor tambroides*), microbial profiling, multi-drug-resistant bacteria

INTRODUCTION

Aquaculture is experiencing a notable upward trajectory owing to the heightened commercialization of aquatic products, driven by the imperative to address food security concerns resulting from the burgeoning global population (Wright *et al*., 2023; Lim, 2024). Moreover, the aquaculture sector's significance in fostering development is underscored by data from the Food and Agriculture Organization (FAO, 2022), which indicates that approximately 58.5 million individuals are employed in the primary sector, and it is estimated that the livelihoods of approximately 600 million people depend at least partially on fisheries and aquaculture. Notwithstanding its pivotal role, the expansion of aquaculture activities has been accompanied by an elevated risk of severe disease occurrences. Fish diseases occur more frequently in aquaculture than in wild fisheries as the aquaculture populations are reared in close proximity at high densities, resulting in a high contact ratio, enabling pathogens to spread quickly and easily, leading to disease occurrences and outbreaks (Irshath *et al*., 2023). Among different infectious organisms, bacteria pathogens account for the majority of diseases in fish farming (Sudheesh *et al*., 2012).

Infectious abdominal dropsy (IAD), often known as dropsy disease, is a prevalent bacterial infection extensively documented by various researchers. IAD has been reported in several economically significant aquaculture fish species, including Indian major carps (*Catla catla*, *Labeo rohita*, *Cirrhinus mrigala*), rainbow trout (*Onchorhynchus mykiss*), crucian carp (*Cyprinus carassius*), silver carp (*Hypojtjalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), common carp

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(*Cyprinus carpio*), goldfish (*Carassius auratus*) and grass carp (*Ctenopharyngodon idella*) (Lau *et al*., 2022). Clinical manifestations of IAD include hemorrhagic lesions in the skin and fin, abdominal distention, exophthalmia and imbalance. Different etiological agents for IAD have been reported, such as *A. hydrophila* (Edun, 2007; Dash and Payyappilli, 2016) and *P. fluorescens* (Swain *et al*., 2008) or in some cases, mixed infections involving these two pathogens (Aly and Ismail, 2016), as well as *A. veronni* (Sreedharan *et al*., 2011).

Tor tambrodies, locally known as Malaysian mahseer or Empurau, is a highly profitable riverine fish in Southeast Asia and is a valuable aquaculture species. The burgeoning interest in cultivating this particular species is underpinned by its profound cultural and socioeconomic significance, serving as a valuable resource for food, ornamental purposes and recreational fishing (Lau *et al*., 2021a; 2021b; Lim *et al*., 2021). Given the alarming decline in the natural population and distribution of *T. tambroides*, there has been a notable surge in attention directed toward artificial propagation. This surge is driven by the dual objectives of enhancing aquaculture production and conserving this species (Asaduzzaman *et al*., 2016). Notably, Ingram *et al.* (2005) have reported that the commercial value of *T. tambroides* in the fish market can reach as high as RM 400/kg. In Sarawak, recent market prices have fluctuated within the range of RM 800 to RM 1,000/kg (Bernama, 2021). This remarkable price escalation underscores the exponential growth in the economic worth of this species over the course of the year.

The present study aims to perform microbial profiling of the gut and skin microbiota in both healthy and dropsy diseased Empurau. This investigation seeks to furnish specific insights into the indigenous microflora of healthy Empurau and to identify the pathogenic agents responsible for dropsy in this species. From the profile generated, the diseased Empurau contains the same dropsy-causing pathogenic strains and variety as seen in other fishes. Furthermore, this profile enables a comparative assessment of how dropsy affect the microbial composition of healthy Empurau. These data hold significant implications for future research, facilitating the development of preventative measures and treatments to mitigate the proliferation of causative agents within aquaculture environments. Such interventions have the potential to substantially reduce mortality rates and mitigate yield losses within the Empurau aquaculture farming industry.

MATERIALS AND METHODS

Preparation of agar media

In this study, nutrient agar, Aeromonas isolation agar, MacConkey agar and eosin methylene blue (EMB) agar media were used. The agar was prepared according to instructions from the manufacturer. The media was allowed to cool and pour into sterile disposable Petri dishes and allowed to solidify.

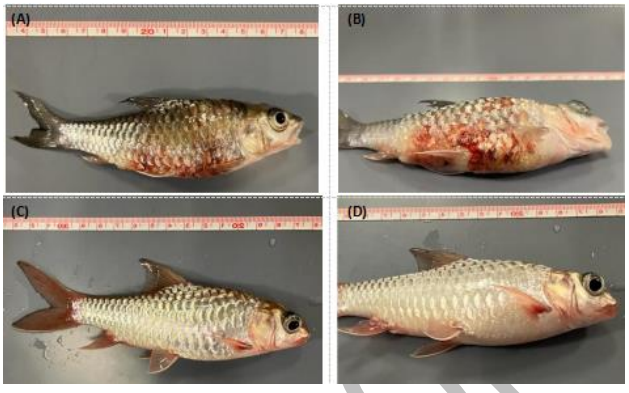

Figure 1: Morphological observation of (A) dropsy diseased Empurau with common symptoms of dropsy, (B) swollen and haemorrhagic lesion on the lower abdominal area of dropsy fish, (C) healthy Empurau with no signs of dropsy and (D) healthy lower abdominal area of Empurau with no signs of dropsy.

Fish dissection and sample collection

Capture-bred *T. tambroides* with an average length of 15 ± 0.6 cm (three showing common clinical symptoms of IAD (Figure 1) and three apparently healthy fish obtained from a local aquaculture farm were included in this study. All organisms were sacrificed according to the protocol approved by UNIMAS Animal Ethics Committee with permit number of UNIMAS/AEC/T/F07/023. The fish was euthanized using an overdose of MS-222 (Tricaine methanesulfonate, Argent Chemical Laboratories, Redmond, WA). The sections of the skin and gut of the fish were especially removed by means of a sterile scalpel and pair of scissors and kept in 50 mL falcon tube containing 10 mL of PBS solution. Homogenization was carried out to obtain a uniform distribution of cells in the stock solution. The stock solutions were stored at 4 °C until further use.

Enumeration and isolation of bacteria

Serial 10-fold dilutions (up to 10^{-6}) of each homogenized sample were made in 10 mM PBS (pH 7.2). A 100 µL of the final two dilutions (10^{-5} and 10^{-6}) were plated on solidified freshly prepared agar plates and spread using a sterile glass rod. The plates were incubated at 30 °C for 16 h. After incubation, the plates were read by considering and selecting those plates which had between 30-300 colonies. Representative bacterial colonies based on the difference in shape, size and colour from each plate were selected for pure isolation. It was subcultured on the agar plates under the same growth conditions. Pure bacterial colonies were selected for identification and were preserved at -80 °C in 40% glycerol.

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Gram staining and microscopy

Gram staining was done to distinguish the Gram properties of bacteria. Each isolate was stained by the Gram stain method following a standard staining protocol. Briefly, a loopful of the isolated bacterial colonies was emulsified in sterile distilled water, and a thin preparation was made on a glass slide. The smear was air-dried completely and was then heat-fixed by passing the slide over a flame three times. The fixed smear was then completely covered with crystal violet solution for 30 sec, after which it was washed off with flowing water. The smear was then covered with Gram iodine for 1 min and washed off with flowing water. The smear was decolourized with ethanol until the purple colour just ceased to flow away from the smear and then washed off with flowing water. Finally, safranin solution was applied to the smear for 30 sec, followed by rinsing off with flowing water. The slide was air-dried before viewing under the microscope. The slides were examined using oil immersion objectives (×100) light microscope.

Direct colony PCR and gene sequencing

Sequencing the 16S rRNA gene has been extensively used in the understanding of bacterial evolution and phylogeny, and it is regarded as the "gold standard" in bacterial identification. Sequencing of the amplicon was performed using the universal bacterial primers 21F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GTATTACCGCGGCTGCTG-3′) that targeted the V3-V4 regions of the 16S rRNA gene. Selected bacterial colonies were picked with a sterile toothpick from the agar plates and dabbed into a 0.2 mL PCR tube containing 10 µL of distilled water. The PCR tube with the picked colony was boiled at 95 °C for 10 min and used as a template for colony PCR. The following solution was then added into the PCR tube: 4.0 µL of 5× Flexi buffer, 2.0 µL of 25 µM $MgCl_2$, 0.4 µL of 10 mM dNTP, 1.0 µL of each primer 27F/519R (10 µM), 0.2 µL of Taq DNA polymerase (1.25 U) and Milli-Q water up to 20 µL. The PCR program consisted of 95 °C for 3 min; 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min; and a final extension at 72 °C for 5 min. The amplified PCR products were analysed by electrophoresis in a 2.0% agarose gel stained with ethidium bromide. The gels were visualized under UV light using UV transilluminator. PCR products were purified using a FavorPrep™ GEL/PCR Purification Kit according to the manufacturer's instructions. The DNA samples were sent for sequencing and the data obtained were analysed using the Basic Local Alignment Search Tool (BLAST) with the database from the National Centre of Biotechnology Information (NCBI). Besides, multiple sequence alignment, model test (to select the best model for the phylogenetic tree) and maximum likelihood phylogenetic tree were conducted by using MEGA X 10.2.6 software with Tamura-Nei model + G and 1000 bootstrap replications (Kumar *et al*., 2018).

Antibiotic susceptibility testing of selected bacteria

Antibiotic susceptibility testing was carried out following Kirby-Bauer disc diffusion method on Mueller Hinton agar (HIMEDIA, India) as per Clinical and Laboratory Standard Institute (CLSI) recommendations. A total of six bacterial pathogens (*A. hydrophila*, *A. veronii*, *E. tarda*, *P. mirabilis*, *S. flexneri* and *S. maltophilia*) were tested due to their high prevalence in the study area, and they were considered to represent the isolated Gram-negative bacteria, a group to which major bacterial fish pathogens belong. Seven commercially available antibiotic disks (Oxoid Ltd, Basingstoke, UK) were used in the following concentrations: tetracycline (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), clindamycin (2 µg), levofloxacin (5 µg) and meropenem (10 µg). Inhibition zones for various isolates were measured and interpreted as sensitive or resistant according to standard interpretative zone diameters suggested in CLSI guidelines. In this study, if the isolates were resistant to at least three classes of antimicrobial agents, they were regarded as multi-drug resistance (MDR).

RESULTS AND DISCUSSION

Morphological observation of bacterial strains from both healthy and dropsy diseased Empurau

Microbial profiling is a powerful technique that applies metagenomics study to determine the specific community of microorganisms. In this study, we conducted gut and skin microbial profiling on both dropsy diseased Empurau and healthy Empurau fishes. A total of 23 isolates and 22 isolates were harvested from the purified plates of gut and skin samples from healthy and dropsy diseased Empurau fishes, respectively (Figure 2). The morphologies and shapes of the colony formed were observed and recorded. Gram staining and KOH reaction were also performed on these colonies (Tables 1 and 2). Various colours were observed across all colonies isolated from both healthy and dropsy diseased Empurau, namely green, grey, yellow, pink, black, orange, white and blue, via plate observation. Almost all of them are Gram-negative, except for NHK1 (bacteria strain 1 isolated from healthy Empurau fish skin and cultured on nutrient agar). The predominance of Gram-negative bacteria in both healthy and diseased fish can be attributed to several factors related to the aquatic environment and the biology of these bacterial groups. Gram-negative bacteria are generally more abundant in aquatic environments, including freshwater and marine systems than Gram-positive bacteria. Gram-negative bacteria have developed mechanisms to adapt to challenging environmental conditions commonly encountered in aquatic systems. They are often more resilient to changes in salinity, pH, temperature and other environmental factors, which enables them to maintain a competitive edge (Anwar and Choi, 2014). This abundance in the environment

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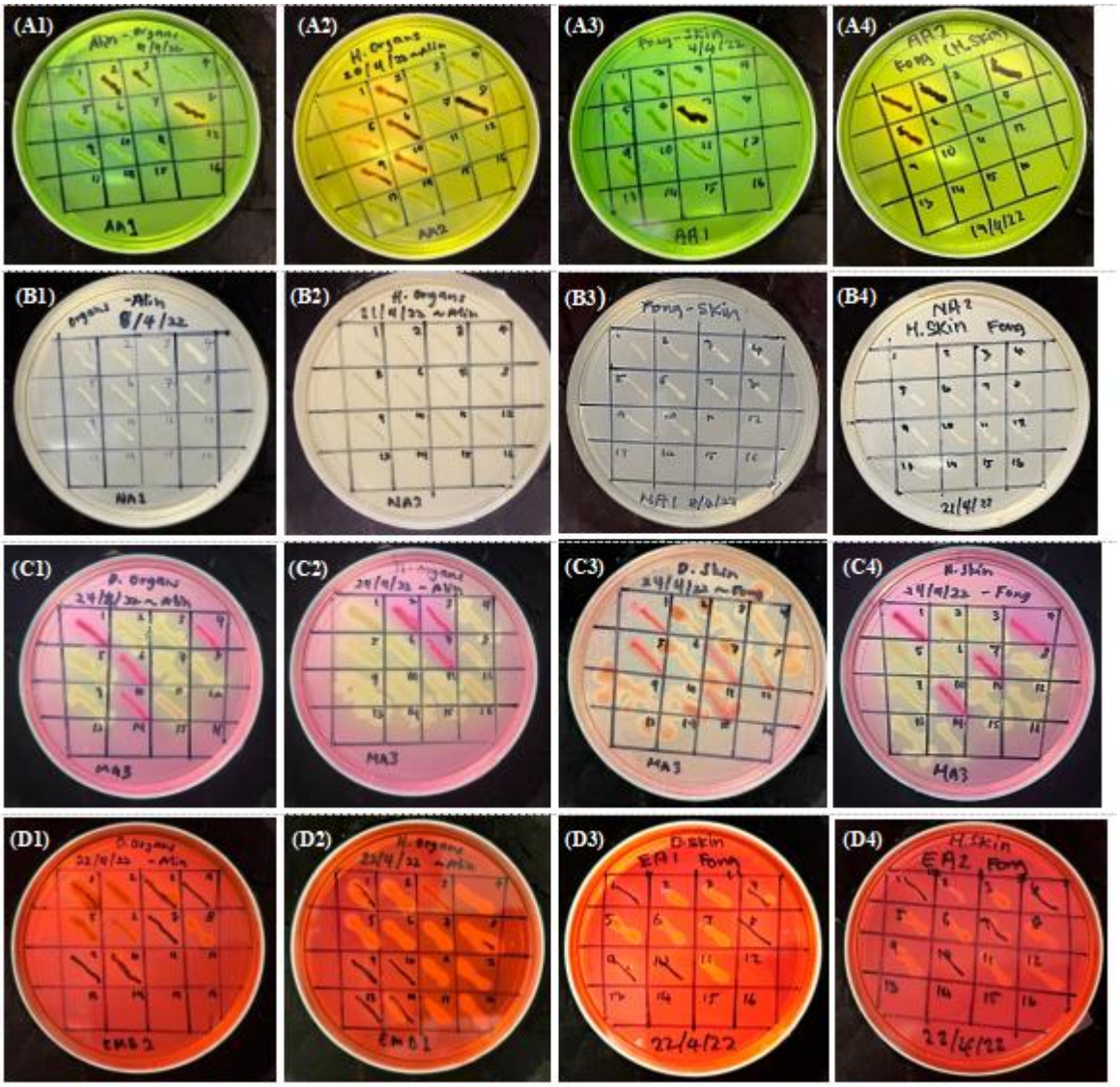

Figure 2: Purified colonies on different agar plates that were divided into 16 sections. A1, B1, C1 and D1 are streaked gut colonies of dropsy diseased Empurau on Aeromonas agar, Nutrient agar, MacConkey agar and EMB agar, respectively. A2, B2, C2 and D2 are streaked gut colonies of healthy Empurau on Aeromonas agar, Nutrient agar, MacConkey agar and EMB agar, respectively. A3, B3, C3 and D3 are streaked skin colonies of dropsy diseased Empurau on Aeromonas agar, Nutrient agar, MacConkey agar and EMB agar, respectively. A4, B4, C4 and D4 are streaked skin colonies of healthy Empurau on Aeromonas agar, Nutrient agar, MacConkey agar and EMB agar, respectively.

increases the likelihood of fish encountering and harbouring Gram-negative bacteria. The majority of the isolates are KOH reaction positive, with the exception of NDG2, NHG1 and NHK1. All of them were cultured on nutrient agar, isolated from the gut of dropsy diseased, healthy Empurau guts and healthy Empurau skin, respectively. Interestingly, only NDG2, NHG1 and NHK1 (all the aforementioned strains) were observed to be cocci in shape, whereas the others were observed to be rod-shaped under microscopic observation.

Bacterial 16S rDNA profiling for healthy and dropsy diseased Empurau

The 16S rDNA amplification was done via colony PCR to amplify a DNA fragment of around 460 bp. All 16S rRNA gene fragments (V3-V4 region, ~460 bp) from 23 gut isolates and 22 skin isolates were successfully amplified with a band observed between ladder indicators of 450 bp to 550 bp (Figure 3). These PCR amplicons were sent for sequencing and then subsequently used in BLAST

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Table 1: Plate and microscopic observations, 16S rDNA-based sequence identity and average inhibition diameter of gut isolates.

Gut	Isolate	Observation				16S rDNA-based sequence identity (%)	Average diameter of inhibition (mm)				
		Plate observation	Microscopic observation				Ampicillin	Tetracycline	Chloramphenicol	Clindamycin	Meropenem
		Colony's morphology	Gram stain	KOH reaction	Shape						
Healthy	AHG1	Yellow fluorescent, round	-	+	Rod	95	0.0 (R)	15.3 (S)	26.3 (S)	0.0 (R)	28.0 (S)
	AHG2	Pale green, round	-	+	Rod	99	-	-	-	-	-
	AHG3	Dark green with a dark centre, round	-	+	Rod	99	-	-	-	-	-
	NHG1	Opaque dark yellow, round	-	-	Cocci	93	0.0 (R)	15.7 (S)	10.7 (R)	0.0 (R)	32.0 (S)
	NHG2	Translucent pale yellow, round	-	+	Rod	99	-	-	-	-	-
	NHG3	Translucent, pale yellow (almost white), irregular	-	+	Rod	99	9.7 (R)	16.3 (S)	14.3 (I)	0.0 (R)	31.0 (S)
	MHG1	Yellow fluorescent, round	-	+	Rod	82	-	-	-	-	-
	MHG2	Bright pink, round	-	+	Rod	99	12.3 (R)	15.7 (S)	22.0 (S)	0.0 (R)	27.3 (S)
	MHG3	Translucent pale pink, irregular	-	+	Rod	99	-	-	-	-	-
	MHG4	Yellow, round	-	+	Rod	99	-	-	-	-	-
	EHG1	Translucent white, round	-	+	Rod	99	14.3 (I)	18.7 (S)	13.7 (I)	0.0 (R)	28.3 (S)
	EHG2	Translucent yellow, round	-	+	Rod	99	-	-	-	-	-
	EHG3	Opaque dark green, round	-	+	Rod	99	15.3 (I)	17.7 (S)	22.0 (S)	0.0 (R)	25.0 (S)
Dropsy	ADG1	Translucent bluish-grey, round	-	+	Rod	99	-	-	-	-	-
	ADG2	Pale green, round	-	+	Rod	99	0.0 (R)	33.0 (S)	18.3 (S)	0.0 (R)	26.7 (S)
	ADG3	Dark green with a dark centre, round	-	+	Rod	99	16.3 (I)	21.7 (S)	22.0 (S)	0.0 (R)	26.0 (S)
	NDG1	Translucent pale yellow, round	-	+	Rod	99	-	-	-	-	-
	NDG2	Opaque dark yellow, round	-	-	Cocci	99	-	-	-	-	-
	MDG1	Bright pink, round	-	+	Rod	99	-	-	-	-	-
	MDG2	Yellow, round	-	+	Rod	99	8.7 (R)	15.7 (S)	13.3 (I)	0.0 (R)	26.0 (S)
	MDG3	Translucent pale pink, irregular	-	+	Rod	99	0.0 (R)	16.7 (S)	15.3 (I)	0.0 (R)	30.3 (S)
	EDG1	Translucent white, round	-	+	Rod	99	-	-	-	-	-
	EDG2	Opaque dark green, round	-	+	Rod	99	-	-	-	-	-

G, gut; H, healthy; D, dropsy; A, Aeromonas agar; N, Nutrient agar; M, MacConkey agar; E, EMB agar; S, susceptible; I, intermediate; R, resistant.

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Table 2: Plate and microscopic observations, 16S rDNA-based sequence identity and average inhibition diameter of skin isolates.

Skin	Isolate	Observation				16S rDNA-based sequence identity (%)	Average diameter of inhibition (mm)				
		Plate observation	Microscopic observation				Ampicillin	Tetracycline	Chloramphenicol	Levofloxacin	Gentamicin
		Colony's morphology	Gram stain	KOH reaction	Shape						
Healthy	AHK1	Black opaque with yellow translucent, round	-	+	Rod	99	-	-	-	-	-
	AHK2	Black opaque with orange translucent, round	-	+	Rod	99	-	-	-	-	-
	AHK3	Greenish translucent, round	-	+	Rod	99	-	-	-	-	-
	AHK4	Greenish opaque, round	-	+	Rod	99	29.3 (S)	15.0 (R)	16.3 (R)	0.0 (R)	12.7 (R)
	NHK1	Opaque dark yellow, round	+	-	Rod	95	23.3 (S)	18.0 (S)	18.3 (S)	21.0 (S)	25.0 (S)
	NHK2	Pale yellow (almost white), round	-	+	Cocci	99	-	-	-	-	-
	MHK1	Pink opaque, round	-	+	Rod	99	25.7 (S)	15.0 (R)	18.3 (I)	14.3 (I)	23.0 (S)
	MHK2	White opaque, round	-	+	Rod	99	-	-	-	-	-
	MHK3	White opaque, irregular	-	+	Rod	99	-	-	-	-	-
	EHK1	Black, round	-	+	Rod	98	-	-	-	-	-
	EHK2	White translucent, round	-	+	Rod	99	29.3 (S)	16.0 (R)	20.0 (S)	0.0 (R)	17.0 (I)
	EHK3	White opaque, round	-	+	Rod	99	-	-	-	-	-
Dropsy	ADK1	Greenish opaque, round	-	+	Rod	100	33.7 (S)	23.3 (S)	36.0 (S)	0.0 (R)	13.7 (I)
	ADK2	Black opaque with yellow translucent, round	-	+	Rod	99	20.7 (S)	13.3 (R)	22.7 (S)	15.7 (I)	20.3 (S)
	ADK3	Greenish translucent, round	-	+	Rod	100	24.0 (S)	18.5 (S)	25.5 (S)	0.0 (R)	12.7 (I)
	NDK1	Pale yellow (almost white), round	-	+	Rod	99	27.3 (S)	18.0 (S)	28.7 (S)	0.0 (R)	12.7 (I)
	NDK2	Opaque dark yellow, round	-	+	Rod	78	24.0 (S)	18.3 (R)	30.0 (S)	30.3 (S)	26.0 (S)
	MDK1	Pink opaque, round	-	+	Rod	99	-	-	-	-	-
	MDK2	White opaque, round	-	+	Rod	99	-	-	-	-	-
	MDK3	Pink translucent, round	-	+	Rod	99	-	-	-	-	-
	EDK1	White, irregular	-	+	Rod	99	28.0 (S)	17.0 (I)	28.7 (S)	7.0 (R)	14.5 (I)
	EDK2	Black, round	-	+	Rod	99	-	-	-	-	-

K, skin; H, healthy; D, dropsy; A, Aeromonas agar; N, Nutrient agar; M, MacConkey agar; E, EMB agar; S, susceptible; I, intermediate; R, resistant.

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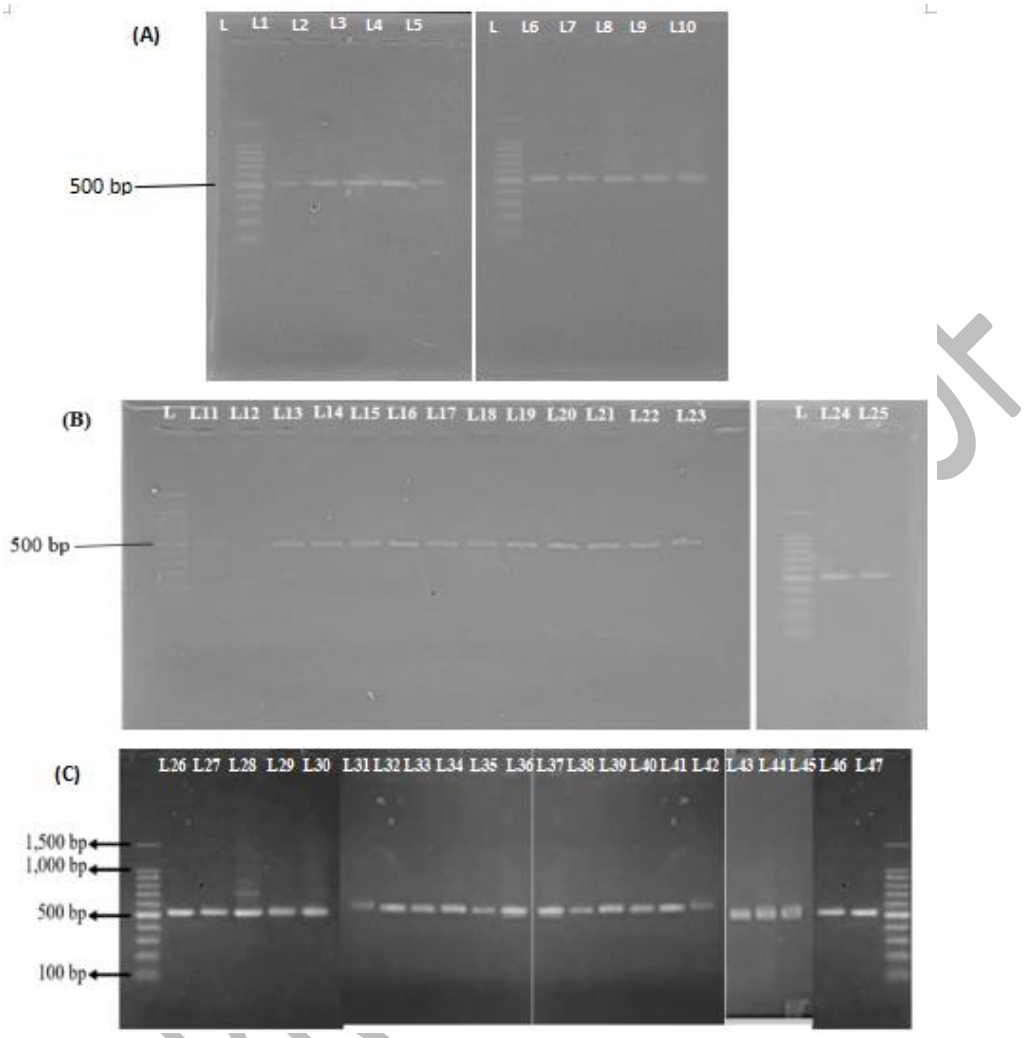

Figure 3: Electrophoresis results from colony PCR of the isolates.(A) L, ladder; L1, ADG1; L2, ADG2; L3, ADG3; L4, NDG1; L5, NDG2; L6, MDG1; L7, MDG2; L8, MDG3; L9, EDG1; L10, EDG2. (B) L, ladder; L11, AHG1; L12, AHG2; L13, AHG3; L14, NHG1; L15, NHG2; L16, NHG3; L17, MHG1; L18, MHG2; L19, MHG3; L20, MHG4; L21, EHG1; L22, EHG2; L23, EHG3; L24, AHG1; L25, AHG2. (C) L26, ADK1; L27, ADK2; L28, ADK3; L29, NDK1; L30, NDK2; L31, EHK1; L32, EHK2; L33, EHK3; L34, MHK1; L35, MHK2; L36, MHK3; L37, EDK1; L38, EDK2; L39, MDK1; L40, MDK2; L41, MDK3; L42, NHK1; L43, NHK2; L44, AHK1; L45, AHK2; L46, AHK3; L47, AHK4.

queries against the public GenBank database for match (Table 1). Around 53.8% (7 out of 13) of the healthy Empurau gut isolates were identified as *Pseudomonas* spp., whereas 60% (6 out of 10) of the dropsy diseased Empurau gut isolates belong to the *Pseudomonas* genus. On the other hand, about 58.3% (7 out of 12) of healthy Empurau skin isolates are grouped under the genus *Pseudomonas,* while 70% (7 out of 10) of dropsy diseased Empurau were discovered to be *Pseudomonas* spp. Other bacterial strains found are *Aeromonas* spp., *Staphylococcus* spp., *Citrobacter* spp., *Aeromonadale*s bacterium, uncultured gamma proteobacterium and uncultured bacterium clones. One interesting finding is that there is more *Pseudomonas* spp. identified in dropsy diseased Empurau than their healthy counterparts. This finding correlates with Lau *et al.* (2022), who found that *Pseudomonas* spp. was one of the major bacteria genera abundantly found in diseased Empurau fishes. It is postulated that *Pseudomonas fluorescens*, *Aeromonas* spp., *Citrobacter freundii* and *Pseudomonas congelans* are the major causative agents of dropsy disease in Empurau fishes, but further investigations are required to strengthen this claim.

Diseased fish gut and skin showed more diverse microbial flora as compared to healthy fishes

The microbial diversity of Empurau healthy and dropsy diseased gut and skin was illustrated via pie charts in Figure 4. *Pseudomonas azotoformans* was most found in

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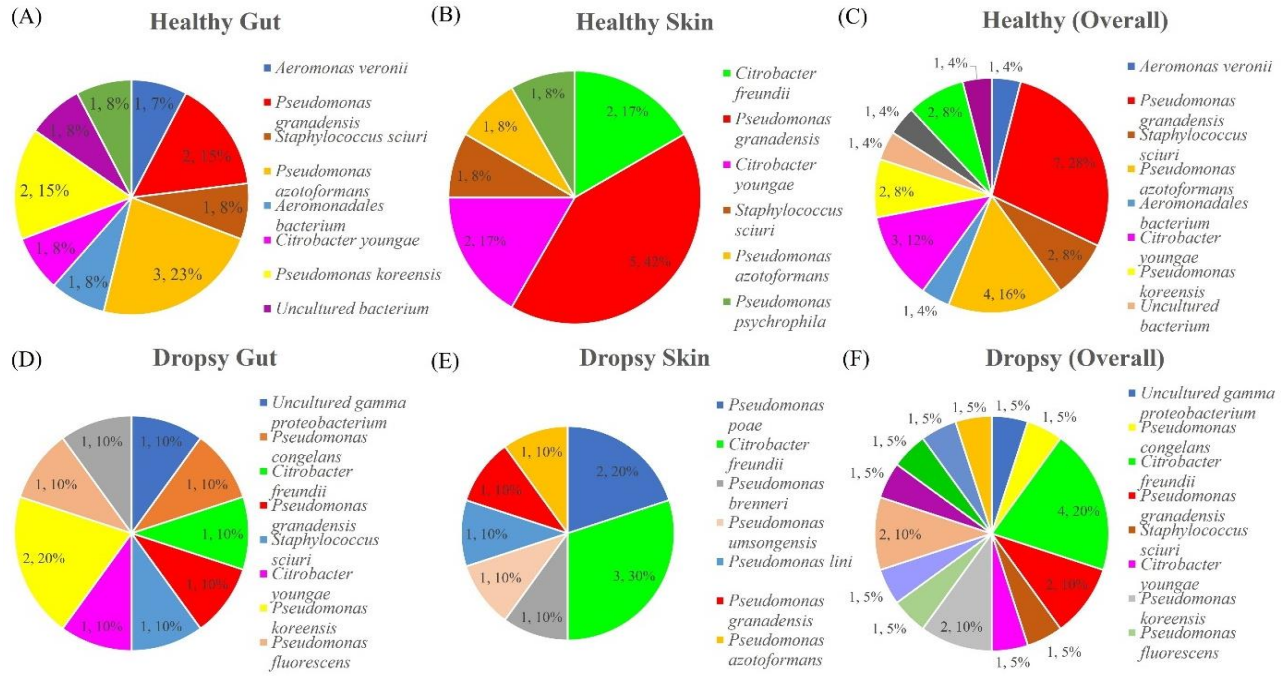

Figure 4: Microbial diversity and composition of (A) healthy gut and (B) skin, (C) healthy overall healthy, (D) dropsy diseased gut and (E) skin as well as (F) overall dropsy diseased samples.

healthy gut whereas *P. koreensis* was most found in dropsy diseased gut. Interestingly, *Pseudomonas koreensis* can only be found in the gut and not in the skin. *Pseudomonas granadensis* was most abundant in healthy skin, whereas *C. freundii* was abundantly found in dropsy diseased skin. The microbial diversity of Empurau dropsy diseased gut and skin is higher than that of their healthy counterparts. Overall, 14 types of bacteria are found in the dropsy diseased gut and skin compared to the 11 types found in the healthy gut and skin. Overall, *Pseudomonas granadensis* was found abundantly in healthy gut and skin (28%), whereas *C. freundii* was found mostly in dropsy diseased gut and skin (20%). There are several bacteria that were only found in dropsy diseased samples, namely *C. freundii* (except for AHK1), *P. koreensis* (except for EHG2), *P. poae*, *P. lini*, *P. congelans*, *P. brenneri* and *P. umsongensis*. *Pseudomonas azotoformans* was found in dropsy diseased skin, healthy skin and healthy gut, but not in dropsy diseased gut. *P. poae* was once classified as *P. fluorescens* whereby *P. fluorescens* was documented to have a close association with dropsy disease in fishes (Swain *et al*., 2008). The pathogenicity of other *Pseudomonas* spp. towards dropsy disease has not been established yet (Austin and Austin, 2016). *Citrobacter freundii* was found to be the causative agent for clinical symptoms such as eye bleeding, inflammation, and exopthalmia as well as internal organs necrotic changes in eels, carps and tilapia, whereby these symptoms were similarly observed in dropsy diseased fishes (Gallani *et al*., 2016).

Pseudomonas granadensis from healthy fish gut

The maximum likelihood phylogenetic tree was constructed to evaluate the evolutionary relationship across all gut and skin isolates (Figure 5). Cluster 1 encompasses mostly *Pseudomonas* spp. and one uncultured gamma proteobacterium. Cluster 2 consists of mainly *Citrobacter* spp. and *P. freundii*. Cluster 3 is resided by *P. lini* alone. Cluster 4 houses *Pseudomonas* spp., *Citrobacter* spp., *S. sciuri* and *A. veronii*. Cluster 5 consists of bacteria from both healthy and dropsy diseased skin and gut samples. Overall, there is no distinctive cluster that can differentiate healthy and dropsy diseased bacteria strains into their individual clusters. Interestingly, *Pseudomonas granadensis* from the healthy gut is located in different clusters from dropsy diseased and healthy skin as well as dropsy diseased gut.

Multi-drug resistant bacteria strains isolated from the gut and skin of healthy Empurau fish

Antibiotics susceptibility test was conducted on randomly selected 10 gut and 10 skin isolates from both healthy and dropsy diseased Empurau fishes. Interestingly, all 20 isolates tested depicted resistance to at least one of the antibiotics tested, the resistance level was determined based on the zone of inhibition as described by the CLSI guidelines: resistance, intermediate and susceptible (Tables 1 and 2). Seven types of antibiotics discs namely, Ampicillin (10 µg), Tetracycline (30 µg), Chloramphenicol (30 µg), Clindamycin (2 µg), Morepenem (10 µg),

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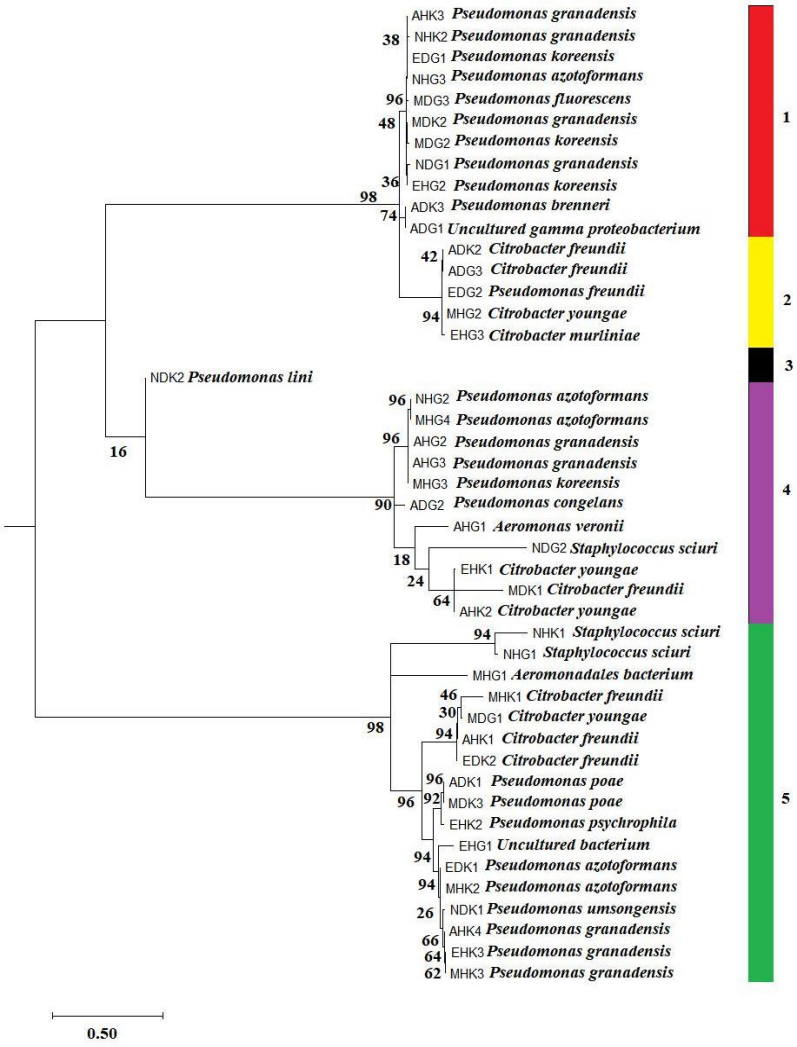

Figure 5: Maximum likelihood phylogenetic tree constructed based on Tamura Nei model with 1000 bootstrap replications, divided into 5 main clusters (coloured and labelled 1 to 5). The scale bar refers to a phylogenetic distance of 0.05 nucleotide substitutions per site. Numbers on the branches indicate the bootstrap percentage after 1000 replications in constructing the tree.

Levofloxacin (5 µg) and Gentamicin (10 µg) were used to test the sensitivity of the isolated bacteria from the healthy and dropsy diseased Empurau fish gut and skin samples. The average inhibition zone for all isolates ranged from 0.0 mm to 33 mm.

All 10 healthy and dropsy diseased Empurau gut isolates were found to be susceptible towards Morepenem and Tetracyclin but were resistant to Clindamycin (Table 1). There are 66.7% (4 out of 6) isolates from healthy Empurau gut samples that were found to be resistant to two antibiotics, namely, Ampicillin and Clindamycin while 75% (3 out of 4) of dropsy diseased Empurau isolates from the gut exhibited resistance to these two antibiotics as well. Only one gut isolate was found to be resistant to three antibiotics (Ampicillin, Chloramphenicol and Clindamycin), namely, NHG1 isolated from healthy Empurau gut cultured on nutrient agar.

All 10 healthy and dropsy diseased Empurau skin isolates were found to be susceptible to Ampicillin (Table 2). In fact, only AHK2 (*P. granadensis*) is resistant to four antibiotics, namely Tetracycline, Chloramphenicol, Levofloxacin and Gentamicin. With two out of 20 isolates that are MDR, this suggests that multi-drug resistance is relatively uncommon among these isolates. This can be viewed as a positive finding, as MDR bacteria can be challenging to treat and manage. While the current prevalence of MDR isolates is low, it is important to track changes over time to detect emerging resistance patterns and respond accordingly. This is crucial for preserving the effectiveness of antibiotics and ensuring that bacterial infections can continue to be treated effectively in the

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future (Algammal *et al*., 2020). Notably, all dropsy diseased Empurau skin isolates were susceptible to Chloramphenicol. This information indicates that Chloramphenicol may be one of the most effective agents in curbing the growth of dropsy-causing bacterial strains in farmed Empurau fishes.

CONCLUSION

In conclusion, antibiotic screening, morphological characterization and molecular identification have been done to profile the isolates obtained from both healthy and dropsy diseased Empurau fishes. Plate and microscopic observation revealed that most bacterial strains from healthy and dropsy diseased Empurau are Gram-negative, KOH reaction-positive and rod-shaped. 16S rDNA profiling indicated that most of these strains belonged to *Pseudomonas* spp., with a higher prevalence in dropsy-diseased Empurau. It is postulated that *Pseudomonas fluorescens*, *Aeromonas* spp., *C. freundii* and *Pseudomonas congelans* are the possible causative agents of dropsy disease in Empurau fishes, but further investigations are required to strengthen this claim. Antibiotics susceptibility test unearthed two multi-drug-resistant bacteria strains (each from the gut and skin of healthy Empurau fish, respectively). Intriguingly, all dropsy-diseased Empurau gut isolates were susceptible to Meropenem and Tetracycline, while skin isolates were susceptible to Chloramphenicol. These findings suggest the potential efficacy of these antibiotics in combating dropsy-causing bacterial strains in the skin and gut of farmed Empurau fishes, warranting further exploration. Data generated in this study can aid key stakeholders in Empurau farming to effectively prevent dropsy disease in fish and minimize harvest and financial losses.

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