Bacterial contamination from selected catfish pond farm and its susceptibility to antimicrobial agents

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ABSTRACT

Aims: The aquaculture industry has been identified as one of the sources for the emergence of antimicrobial resistant bacteria. This study aimed to determine the level of bacterial contamination from selected catfish pond farming at Jasin, Melaka and its susceptibility to antimicrobial agents. The correlation between the sampling points' Water Quality Index and the level of bacterial contamination was also determined.

Methodology and results: Sampling was carried out at six sampling points of the river as well as selected catfish pond farming. Microbial counts were assessed using the membrane filtration and isolates were further identified by using the polymerase chain reaction (PCR) method. Antimicrobial susceptibility tests were carried out using the disk diffusion method. Results showed that the total bacterial count obtained was not significantly different ($P>0.05$) between sampling points with an average of 4.47 ± 0.41 log CFU/mL. A total of 27 different bacterial species were identified where 63% were identified as Gram-negative bacteria. A medium negative correlation (r=-6.16, $P<0.01$) was found between the sampling points' Water Quality Index and the level of bacterial contamination. Among bacterial species identified, 18 species are pathogenic strains.

Conclusion, significance and impact of study: Antibiotic susceptibility test indicated that only 4 isolates showed resistance to antimicrobials tested and the study shows the potential cross contamination between fishponds and Kesang River, Melaka. Thus, public health concerns are tangible, and prevention of the cross contamination is crucial for the farm.

Keywords: Antibiotic resistant, aquaculture, bacterial contamination, wastewater, water quality index,

INTRODUCTION

River is one of the major sources of water supply in Malaysia which supplies approximately 98% of the water to the people (Chan, 2012). River water quality care is essential and should be maintained to ensure the quality of water supplied to consumers is safe (Basheer *et al.*, 2017). The deterioration of river water quality can cause various diseases to humans and aquatic life (Fulazzaky *et al.*, 2010). In Malaysia, water pollution can be caused by effluent discharge released from the industrial development process. This uncontrolled development will lead to an increase in the disposal rate of waste material into the river thus causing disruption to the aquatic system and ultimately causing the water quality to decline. According to a report released by the Department of Environment Malaysia (2016), the main causes of river water quality deterioration in Malaysia are domestic waste, industrial, animal husbandry and agricultural-based industries.

Fishery sector has played an important role as a main source of protein to Malaysia's population for decades. The total fishery production of the country has amounted to 1.7 million tonnes in 2017. This value is contributed by close to 1.5 million tonnes from captured fishes and 0.2 million tonnes from aquaculture fishes (Kuthoose *et al.*, 2021). However, increasing the population increases the demands for fishes. This situation urged fishermen to venture into the aquaculture sector to meet the country's demands. Aquaculture industry in Malaysia is mainly dominated by the growing of aquatic plants, such as seaweed. Among finfish, the most reared species is African catfish followed by red tilapia, sea bass, river catfish and red snapper (Starkl *et al.*, 2018).

Aquaculture industry is said to cause negative impacts to the environment (Dauda *et al.*, 2018). Previous studies

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stated that the aquaculture industry causes destruction of natural ecosystems, acidification of soils, water pollution, ecological impacts and changes of landscape (Hua, 2015). Most of the impacts are caused by untreated effluent discharge into the river (Basheer *et al.*, 2017). Aquaculture industry is a rapidly emerging industry in Malaysia and contributes to the multimillion product value in the local economy. However, the sector is not highly monitored to protect the environment (Zarkasi and Nazari, 2018). Lack of knowledge on proper rearing process and treatment of effluent also gives negative effects. Crowded ponds and unsuitable feeds given to the fish could lead to biological contamination (Dauda *et al.*, 2018). The main biological concerns to humans when consuming aquatic foods are pathogens and biotoxins (Atkinson *et al.*, 2011). These pathogens when present in aquatic food in excess could cause illnesses (Helms *et al.*, 2002).

Healthy aquatic ecosystems are supported by good physicochemical properties and biological diversity (Kathleen *et al.*, 2016). Various existing bacteria come not only from domestic wastewater or human and animal waste, in fact bacteria from aquaculture or animal husbandry industries are also present from the food sources provided to fish in farming ponds. More than 140 species of invasive bacteria have been identified in lakes and rivers. All these bacterial species contribute negatively to human health and the country's economic impact in various forms, especially environmental degradation (Karim *et al.*, 2016).

Antibiotics have been widely used in fish farming either for therapeutic, prophylactic, or other purposes. The antibiotics are being misused to increase growth as well as feed efficiency in the animals (Kathleen *et al.*, 2016). Due to misuse and abuse of antibiotic drugs, it has led to the development of antibiotic resistance across the globe. According to previous research, there are more than 30 types of antibiotics found in wastewater, soil and drinking water samples taken from several places of aquatic environment (Kemper, 2008).

Therefore, this study aims to determine the level of bacterial contamination of catfish pond farming at Jasin, Melaka and their source of water from Kesang River that correlates with the Water Quality Index of the targeted location to characterize the bacterial species obtained and its susceptibility towards selected antibiotics.

MATERIALS AND METHODS

Study area and sampling stations

The study was conducted in Jasin, Melaka which is in the west coast of Peninsular Malaysia between the latitude of 2°11'53.88" and longitude of 102°30'14.76". The schematic map of the sampling location is as shown in Figure 1. Water sources for the fish farm are coming from Kesang River. The river is about 35 km in length, running through Jasin Town as a border between the southern states of Melaka and the state of Johor. It supplies 55 million L of raw water daily to the state. The river passes through a big palm oil plantation and a few industrial development activities are located at the sides of it. Sampling activities were carried out four times in between February until June at six sampling stations which represent the upstream of the river (Station 1), early stage of catfish pond farming (Station 2), middle stage of catfish pond farming (Station 3), final stage of catfish pond farming (Station 4), effluent of the catfish pond farming (Station 5) and the downstream of the river (Station 6) (Figure 1). Four samples per station were collected for each sampling time. The water samples were transported to the laboratory in an ice-packed container stored for microbiological and physicochemical analysis. All samples were analysed within 24 h after the sampling.

Water quality index analysis

Samples were analysed by following the methods outlined in the Interim National Water Quality Standards (INWQS) (Leong *et al.*, 2018). Six parameters were measured which are dissolved oxygen (DO), biochemical oxygen (demand (BOD), chemical oxygen demand (COD), pH, ammoniacal nitrogen (AN) and total suspended solid
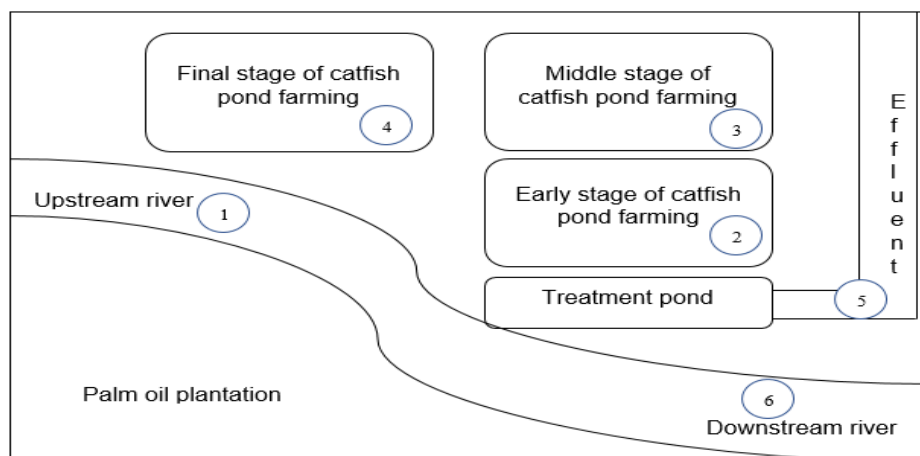
Figure 1: Sampling stations at fish farms and Kesang River.

(TSS) (Ma'arof and Hua, 2015). Briefly, the BOD values were determined based on the DO difference before and after the samples were stored in an incubator at 20 °C for 5 days. *In situ* data measurements were recorded for DO and pH values using DO meter and pH meter respectively. The COD concentration was analysed by using Reactor Digestion method while AN concentration was analysed using Nessler method (Leong *et al.*, 2018). Standard WQI classification and method were applied for each parameter (Ma'arof and Hua, 2015). The overall WQI of the water was calculated using the following formula (Department of Environment Malaysia, 2016):

$$WQI = (0.22 \times SIDO) + (0.19 \times SIBOD) + (0.16 \times SICOD) + (0.15 \times SIAN) + (0.16 \times SISS) + (0.12 \times SIpH)$$

Note: WQI = Water quality index; SIDO = Dissolved oxygen sub-index; SIBOD = Biological oxygen demand sub-index; SICOD = Chemical oxygen demand sub-index; SIAN = Ammoniacal nitrogen sub-index; SISS = Suspended solids sub-index; SIpH = pH value sub-index

Bacteriological analysis

A total of 100 mL of water samples were taken using microbiological sample bottles and stored in containers containing ice to be analysed in laboratories. Samples were diluted 6 times of the dilution using 0.85% normal saline in 1:9 ratio. Diluted water samples were then filtered using a 0.45 µm membrane filter and were placed on nutrient agar (NA) plates and incubated at 37 °C for 24 h for bacterial colony growth. The amount of bacterial colony is then calculated and recorded in the CFU/mL as the number of colonies per plate multiplied by the dilution factor and divided with 100. Bacterial colonies then are subculture to other NA plates to obtain single colonies. Gram staining was performed for each single colony for differentiating Gram-positive or Gram-negative bacterial groups. Then, morphological observation of the colony and cell was performed to identify the type of bacterial colony that grows on the NA plates after being incubated for 24 h. Macroscopic identification is based on identification information according to the Determinative Bergey's Bacteria Manual (Williams, 2000; Zarkasi *et al.*, 2018). Microscopic identification and bacterial identification were conducted, and bacterial colonies were identified by shape, diameter, height, surface, opacity and pigmentation (Moyes *et al.*, 2009; Boyanova, 2017; Zarkasi *et al.*, 2019; Kuthoose *et al.*, 2021). Each identified colony is given a different ID.

DNA extraction

The DNA extraction method used in this research is the boiling method (Lai *et al.*, 2011; Suhaimi *et al.*, 2019). Bacterial colony from nutrient agar was suspended in 100 µL sterilised distilled water and vortex to homogenize the sample. The sample was then suspended in a 70 °C water bath for 10 min. After 10 min, the sample was immediately cooled in an ice bath for 5 min. The sample

was then centrifuged at 20,000× *g* for 3 min. The supernatant obtained was transferred into new sterilised tubes and stored in -20 °C until further used (Abdullahi *et al.*, 2021).

Polymerase chain reaction (PCR) and 16S rRNA gene analysis for pure colonies

Supernatant extracted in the boiling method was used in the PCR reaction (Halim *et al.*, 2020). The 50 µL reaction assay contained 25 µL master mix, 2 µL 27F primer (5' TAC GGY TAC CTT GTT ACG ACT T 3'), 2 µL 1492R primer (5' CCA GCA GCC GCG GTA ATA CG 3'), 16 µL sterilised water and 5 µL extracted DNA. Thermocycling was performed using a C1000 Thermal Cycle (Bio-Rad, California, US). The thermal cycler condition consists of initial denaturation at 95 °C for 10 min, 94 °C for 4 min, annealing at 45 °C for 2 min and extension at 72 °C for 7 min and soaked at 15 °C (Ahmad *et al.*, 2021). 35 cycles of PCR amplification were performed. The purified amplicons were then sequenced using the Big Dye direct cycle sequencing kit on an ABI 3730 automated sequencer. To define the microbial taxa, individual rRNA gene sequences were compared to those available in the BLAST database (http://blast.ncbi.nlm.nih.gov/) (Zarkasi *et al.*, 2019).

Antibiotic susceptibility test

Twelve isolated bacteria were selected from the total isolates obtained based on their frequency of presence (more than 4 times) among all the sampling points (Table 1). The selected isolated bacteria were assessed for their susceptibility to different antibiotics utilizing the disc diffusion method according to method described by Clinical and Laboratory Standard Institutes (CLSI, 2012) on Mueller Hinton agar (Table 2). A total of six antibiotics that were commonly found in the aquaculture products and environment (Chen *et al.*, 2020) were chosen that includes ciprofloxacin, sulfamethoxazole, oxytetracyline, chloramphenicol, erythromycin and penicillin G. All Gram-negative strains were not applicable to be tested with antibiotic penicillin G and erythromycin as these antibiotics only work on Gram-positive bacteria (Mokhtar *et al.*, 2023). Briefly, fresh bacterial culture in Mueller Hinton broth was measured for its optical density (0.08 to 0.13 nm) at 625 nm wavelength. The bacterial culture was then swabbed on Mueller Hinton agar (MHA) using sterile cotton swabs. The antibiotic discs (Oxoid, UK) were evenly embedded onto the inoculated agar incubated at 37 °C for 18 h (Mokhtar *et al.*, 2023).

Statistical analysis

One-way analysis of variance (one way-ANOVA) followed by post hoc Tukey was used to compare the Water Quality Index (WQI) and the total bacterial count among the sampling stations (Abdullahi *et al.*, 2020). All descriptive and statistical analysis were conducted using EXCEL 2016 and IBM SPSS statistical software. In

Table 1: List of bacterial isolates tested for antibiotic susceptibility testing.

No.	Strain ID	Strain species
1.	14H	*Escherichia fergusonii*
2.	27U	*Fictibacillus rigui*
3.	28B	*Bacillus cereus*
4.	32L	*Bacillus wiedmannii*
5.	32N	*Citrobacter murliniae*
6.	34B	*Enterobacter hormaechei* subsp. *xiangfangensis*
7.	61H	*Chryseobacterium cucumeris*
8.	64D	*Citrobacter freundii*
9.	65C	*Klebsiella pneumoniae*
10.	65E	*Bacillus aryabhattai*

Table 2: List of antibiotics used in this study. Zone of inhibition for tested antibiotics.

Name of antibiotics	Disc concentration	Inhibition zone standards*		
		Sensitive (mm)	Intermediate (mm)	Resistant (mm)
Penicillin (P10)	10 unit	≥ 29	-	≤ 28
Erythromycin (E15	15 μg	≥ 23	14-17	≤ 13
Chloramphenicol (C30)	30 μg	≥ 18	13-17	≤ 12
Ciprofloxacin (CIP5)	5 μg	≥ 21	16-20	≤ 15
Oxytetracycline (OT30)	30 μg	≥ 19	15-18	≤ 14
Sulfamethoxazole-trimethoprim (SXT25)	25 μg	≥ 16	11-15	≤ 10

*Based on Clinical and Laboratory Standard Institutes (2012).

addition, the Pearson correlation test was used to determine the correlation between the average value of WQI and the total bacterial count for each sampling point. All statistical analyses were considered as significant if $P<0.05$.

RESULTS

Water quality index

The DO concentration values varied from 10.86 ± 4.62% to 70.79 ± 13.95% among all the stations. The lowest and the highest value of DO concentration is at Station 4 and Station 1, respectively. Based on this figure, there is no average DO percentage value per station that achieved the standard value set by the Department of Environment (DOE) of 80%. pH values range from 6.55 ± 0.68% to 7.32 ± 0.36% which averagely all stations are in Class 1 and 2. The lowest and the highest value of pH is at Station 6 and Station 4, respectively. Each station indicates the average value of the pH recorded is within minimum and maximum standard value of 5 to 9 set by the DOE. The average level of BOD concentration ranged from 6.08 ± 4.62 mg/L to 60.08 ± 26.68 mg/L in the water which shows that all stations are in Class 4 and Class 5. The lowest value was recorded at Station 1 while the highest was at Station 3. The average value of BOD Station 1 and 6 is equivalent to the standard values set by DOE while Station 2, 3, 4 and 5 exceed the prescribed standard values of 6 mg/L. The average COD level shown that it is range from 21.17 ± 16.33 mg/L to 769.17 ± 540.64 mg/L. Station 1 is in Class 2, Station 6 is in Class 3 while the others are in Class 5. The highest value of

COD level was recorded at Station 2 while the lowest was at Station 1. Each stations average COD concentration value was recorded exceeds the standardized value of the DOE of 10 mg/L. The average AN level ranged from 0.36 ± 0.1 mg/L to 93.91 ± 25.65 mg/L. The highest value of AN level was recorded at Station 4 while the lowest was at Station 1. Station 1 and 6 are in Class 3 while the others are in Class 5. The AN level for all stations exceeds the standard value (0.3 mg/L) of INWQS. The TSS average level ranges from 33.66 ± 6.38 mg/L to 980.33 ± 131.51 mg/L. The highest value of TSS level was recorded at Station 4 while the lowest was at Station 1. Station 1 and 6 are in Class 2 while the others are in Class 5. The TSS level for all stations does not exceed the standard value (1000 mg/L) of INWQS. Table 3 shows the results of all parameters tested for WQI determination of all stations while Table 4 shows the Water Quality Index for all stations.

Bacterial contamination level and its correlation with WQI

One-way ANOVA tests were used to compare the Water Quality Index (WQI) between water samples taken from different sampling points. Based on the statistical tests performed, the mean value of WQI is significantly different ($P<0.05$) between each sampling point. Next, the Tukey post hoc test is used to determine the difference in average value of WQI recorded for each sampling point. Based on the test, four sampling points, Station 2, 3, 4 and 5 were significantly different ($P<0.05$) with two other sampling points, Station 1 and 6 (Figure 2). Furthermore, one-way ANOVA tests were used to compare bacterial

Table 3: Values of Water Quality Index parameters.

Stations	Values of 6 Water Quality Index parameters					
	DO (%)	pH	BOD (mg/L)	COD (mg/L)	AN (mg/L)	TSS (mg/L)
1	70.79 ± 13.95	6.60 ± 0.85	6.08 ± 2.84	21.17 ± 6.34	0.36 ± 0.11	33.67 ± 15.35
2	23.45 ± 8.15	6.92 ± 0.25	50.75 ± 11.27	769.17 ± 301.78	18.48 ± 7.68	655.83 ± 295.46
3	30.32 ± 13.87	6.71 ± 0.53	60.08 ± 16.69	692.50 ± 238.07	15.83 ± 7.51	688.17 ± 219.74
4	10.86 ± 4.62	7.32 ± 0.36	48.67 ± 8.76	436.67 ± 217.89	93.92 ± 25.65	980.33 ± 270.86
5	28.43 ± 11.61	7.28 ± 0.41	35.17 ± 14.37	498.89 ± 220.81	13.02 ± 5.97	524.17 ± 278.64
6	62.06 ± 15.34	6.55 ± 0.68	6.20 ± 2.91	41.83 ± 21.61	0.76 ± 0.06	42.83 ± 15.26

Table 4: Water Quality Index according to all sampling stations.

Stations	Water Quality Index	Class	Water Quality Classification (Department of Environment Malaysia, 2016)
1	78	2	Clean
2	13	5	Polluted
3	14	5	Polluted
4	10	5	Polluted
5	19	5	Polluted
6	69	3	Slightly polluted

contamination levels between water samples taken from different sampling points. There was no significant difference of total viable count of bacterial contamination between the sampling points of selected catfish pond farming in Jasin, Melaka (*P*>0.05) with an average reading of 4.47 ± 0.41 log CFU/mL (Figure 2). In addition, the Pearson correlation test has been used to determine the degree of relationship between the mean value of the Water Quality Index (IKA) and the average value of Total Bacterial Count at each sampling point. There was a significant medium negative correlation between Water Quality Index and total viable count of bacterial contamination at each sampling point (r=-0.616, *P*<0.01).

Distribution of bacterial species across sampling locations and pathogenicity

The major dominant bacterial species isolated from this study were pathogenic bacteria to human (52%) followed by non-pathogenic bacteria (29%) and pathogens to human and animal (15%) and majority of the bacterial species belongs to Gram-negative bacteria (63%) (Figure 3). Pathogenicity classification of the isolated strains was reported based on published literature (Table 5). Down to the species level, the most frequent bacterial species isolated in this study were *Escherichia fergusonii*, *Klebsiella pneumoniae*, *Fictibacillus rigui*, *Citrobacter murliniae* and *Enterobacter hormaechei* (Figure 4). The results show consistency among sampling stations indicating its population dynamic.

Antibiotic susceptibility test

The results of susceptibility testing of selected isolated bacteria against selected antibiotics are summarized in Table 6. Based on the results, only four isolated bacterial strains (14H, 32L, 65E and 28B) were found to be resistant towards some of the antibiotics tested. Strain 14H and 32L each showed resistance towards two types of antibiotics with both strains showing resistance towards oxytetracycline. None of the strains tested showed resistance towards erythromycin. However, three Gram-positive
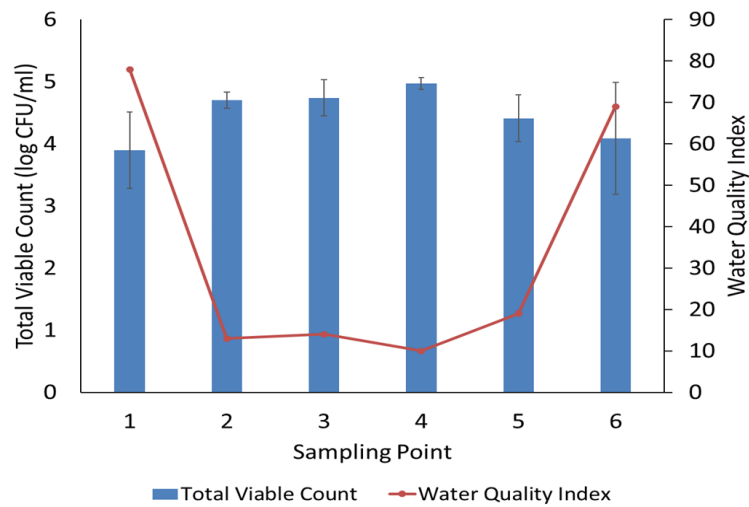
Figure 2: Total viable count and Water Quality Index per sampling point.
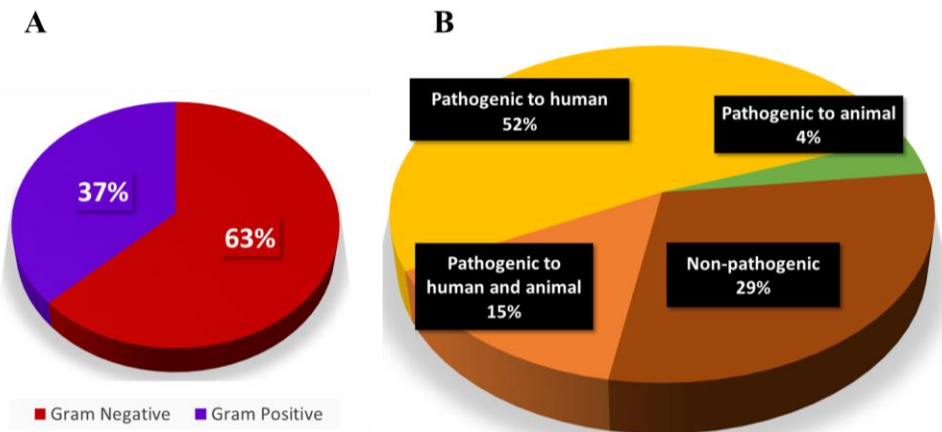
Figure 3: Bacterial isolates distribution based on (A) Gram-stain and (B) pathogenicity types.

strains showed resistance towards penicillin G.

DISCUSSION

The results showed that the water in the catfish pond farming averagely is in contaminated condition. This is because all sampling points taken from the pond farming are in Class 5 based on the classification according to the Interim National Water Quality Standard (INWQS) (Department of Environment Malaysia, 2016). Based on INWQS, the Water Quality Index (WQI) in Class 5 is not suitable for any activity. This shows that catfish that are reared in the pond have been breeding in contaminated pond water and should not be sold or eaten. Classification of water quality for fish farming according to INWQS should be in Class 3. Water quality Class 3 is suitable for fish farming for fishes that can tolerate environmental conditions (Department of Environment Malaysia, 2016).

The use of contaminated fish farming as food can lead to health implications, such as diarrhoea, cholera, typhoid

and abdominal inflammation (Njoku *et al.*, 2015). Whereas WQI for sampling points taken from the upstream and downstream rivers are generally in a slightly contaminated condition, which is Class 3. This river requires extensive treatment to improve the degree of its cleanliness and suitability as raw and drinking water. The cleaning process of these two sampling points shall also be made to reduce the decomposition materials contained therein. This process should be carried out to reduce the BOD values of both sampling points and thus increase the value of upstream DO and downstream of the river.

WQI of Kesang River shows the decline of WQI from 2014 (Class 2) to 2016 (Class 3). The decline was due to the release of wastewater from the catfish pond farming directly into the Kesang River without any prior treatment (Azzlan *et al.*, 2016) Based on the observation during the sampling process, there was a pond for wastewater treatment. However, due to the overgrowth of bamboo trees used to treat wastewater, the treatment
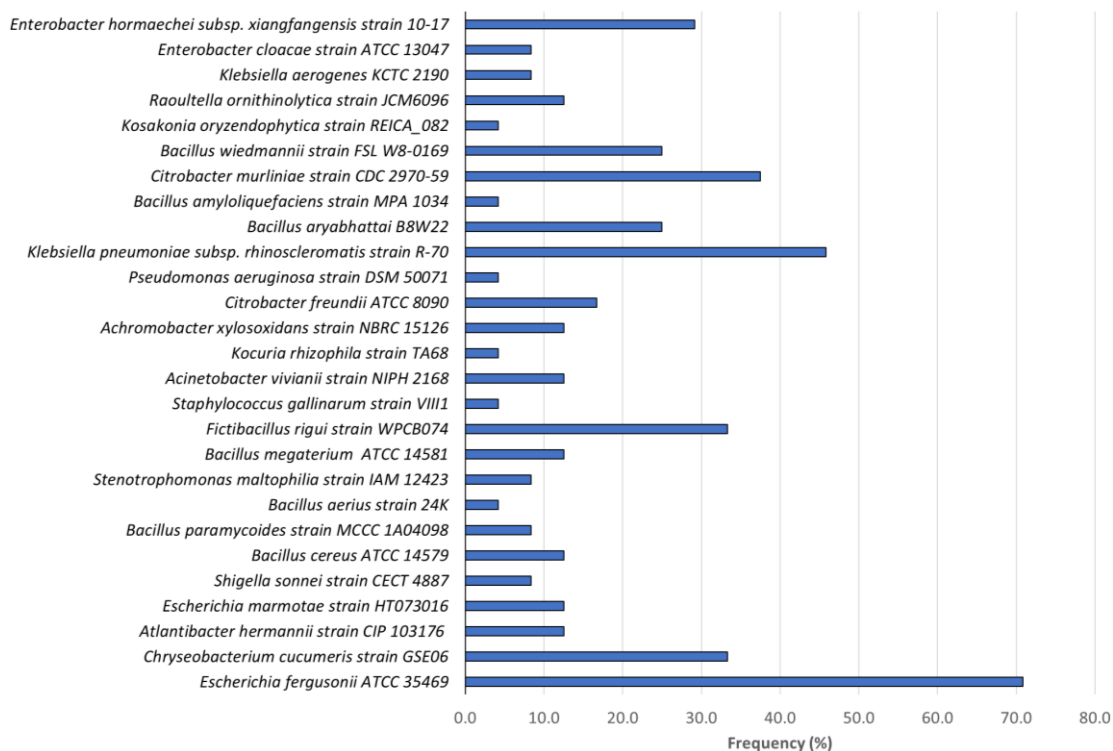
Figure 4: Percentage occurrence of isolated bacterial strains for all sampling sites.

Table 5: List of bacterial species isolated and its pathogenicity.

ID	Bacterial species	Pathogenicity	Reference
61G	*Bacillus megaterium*	Non-pathogenic	Luo *et al.* (2021)
27U	*Fictibacillus rigui*	Non-pathogenic	Wu *et al.* (2020)
28B	*Bacillus cereus*	Human pathogen	Tuipulotu *et al.* (2021)
33U	*Staphylococcus gallinarum*	Animal pathogen	Sorour *et al.* (2023)
32L	*Bacillus wiedmannii*	Human pathogen	Miller *et al.* (2016)
65H	*Bacillus amyloliquefaciens*	Non-pathogenic	Rong *et al.* (2021)
33O	*Kocuria rhizophila*	Human pathogen	Zhang *et al.* (2023)
65E	*Bacillus aryabhattai*	Non-pathogenic	Tepaamorndech *et al.* (2019)
32X	*Bacillus aerius*	Non-pathogenic	Meidong *et al.* (2018)
32G	*Bacillus paramycoides*	Non-pathogenic	Yousuf *et al.* (2023)
14H	*Escherichia fergusonii*	Animal pathogen	Kandhan *et al.* (2023)
34B	*Enterobacter hormaechei*	Human pathogen	Yeh *et al.* (2022)
23G	*Raoultella ornithinolytica*	Human pathogen	Hajjar *et al.* (2020)
14B	*Stenotrophomonas maltophilia*	Human pathogen	Trifonova and Strateva (2019)
23F	*Klebsiella aerogenes*	Human pathogen	Passarelli-Araujo *et al.* (2019)
33E	*Acinetobacter vivianii*	Human pathogen	Hristova *et al.* (2023)
64D	*Citrobacter freundii*	Animal pathogen	Behera *et al.* (2022)
64F	*Pseudomonas aeruginosa*	Human and animal pathogen	Azam and Khan (2019)
31K	*Shigella sonnei*	Human pathogen	Shad and Shad (2021)
65C	*Klebsiella pneumoniae*	Human and animal pathogen	Marques *et al.* (2019)
11F	*Enterobacter cloacae*	Human pathogen	Mustafa *et al.* (2020)
61H	*Chryseobacterium cucumeris*	Non-pathogenic	Sherpa *et al.* (2021)
32N	*Citrobacter murliniae*	Human pathogen	Behera *et al.* (2022)
29N	*Kosakonia oryzendophytica*	Non-pathogenic	Sherpa *et al.* (2021)
66A	*Escherichia marmotae*	Human pathogen	Sivertsen *et al.* (2022)
64K	*Atlantibacter hermannii*	Human pathogen	Hristova *et al.* (2023)
29C	*Achromobacter xylosoxidans*	Human pathogen	Pickrum *et al.* (2020)

Table 6: Susceptibility testing of selected isolated bacteria against selected antibiotics.

Antibiotic	Zone diameter (mm)						
	Standard*	S. aureus[1]	E. coli[1]	14H	32L	65E	28B
Ciprofloxacin (CIP5)	≤15	29	31	14	24	27	28
Sulfamethoxazole (SXT25)	≤16	31	26	30	19	31	20
Oxytetracycline (OT30)	≤14	42	31	0	13	26	18
Chloramphenicol (C30)	≤12	29	27	27	35	27	19
Erythromycin (E15)	≤13	24	NT	NT	25	25	22
Penicillin G (P10)	≤28	42	NT	NT	26	26	0

Note: *Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2012). Value denotes the range of inhibition zone that indicates resistance towards antibiotic tested. **Red box indicates resistance towards tested antibiotics, [1]- Control strains, NT- not tested.

pond is no longer used. In addition, the treatment pool is seen to be in a dry condition although there is wastewater at Station 5 (effluent) which is flowing normally. Based on Table 4, it is notable that there is a general trend in which the decline of WQI from Station 1 to Station 5 and WQI is rebounded at Station 6. The decline of WQI is due to the various factors affecting the water quality. Among them are decreasing concentration of dissolved oxygen (DO) concentration, increasing concentration of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS) and high concentrations of nitrogen ammonia (AN) at Station 2-5. However, the increase in WQI at the sampling point 6 (downstream) is due to the dilution factor of river water along the distance of 2 km from the sampling point 5 (effluent) to the sampling point 6.

The decomposition of waste from aquaculture activity is a factor of low DO concentration and high concentrations of AN are due to high organic content (Suratman *et al.*, 2015). The dissolved oxygen (DO) value averagely is less than 5 mg/L from each sampling point. Generally, DO concentration below 5 mg/L can affect the function and survival of aquatic organisms. The death of aquatic organisms can occur when the DO concentration is lower than 3 mg/L (Njoku *et al.*, 2015). Observation during the sampling process found many carcasses floating on the surface of the fishponds. Fish deaths may be due to the lack of oxygen concentration in the pond. The decline in DO concentration in catfish pond farming may also be caused by the high organic content accumulated due to unconverted pond water and not being released as wastewater over three months.

The reading of pH value recorded from all sampling points are within the minimum and maximum standard values set by INWQS. The pH value required and appropriate for aquaculture and river activities is 6.5 to 9.5 (Njoku *et al.*, 2015). Measurement of pH values helps to determine whether the water is providing a suitable environment for fish, although most catfish can tolerate extreme environmental conditions. The Melaka Water Regulatory Agency found that the pH value of Sungai Kesang was acidic (Azzlan *et al.*, 2016). River water is found to be acidic due to the decay of organic matter that releases hydrogen ions into the environment. Additionally, soil and drought factors that occur in January to April 2014 also affect the level of acidity of Kesang River.

However, throughout the sampling process, a dry season of 32 °C only occurred in May. However, the pH value obtained in this study is similar to Njoku *et al.* (2015). which investigates the microbiological profile and physical properties of fishponds in the Niger Delta region of Nigeria.

The results showed that BOD and COD concentrations were high at Station 2 to Station 5 and low at Station 1 and Station 6. The high concentrations of BOD and COD, coincided with low DO concentrations, high pH values and high concentrations indicate the effects of organic pollution on the sampling point (Suratman *et al.*, 2015). BOD and COD concentrations each show the amount of dissolved oxygen required to decompose organic matter by microorganisms and chemicals. High organic and chemical pollutants in the aquatic system result in increased BOD and COD concentrations. Pollution of organic matter occurs as a result of waste material such as animal faces used as a pond-making fertilizer or produced by fish faeces (Njoku *et al.*, 2015). Overall, the ammoniacal nitrogen concentration obtained from the results exceeds the standard value set by INWQS. Ammoniacal nitrogen is a measurement of ammonia content, a toxic and organic pollutant contained in a body of water. Through the ammonification process, bacteria convert nitrogen to plants that decompose into ammonia (Purwono *et al.*, 2017; Lau *et al.*, 2019). The highest concentration of AN is recorded at Station 4. Station 4 is a fish breeding pond to be disposed of. The fish in the pond can breed up to their maximum size until it reaches its death, and the carcasses are left as food to other surviving fish. Decomposition of fish carcasses causes high pollution of organic matter in the pond. The presence of ammonia in the pool is a result of high protein (chicken) collection, fish waste and microbial decomposition of organic matter (Njoku *et al.*, 2015).

Total suspended solids (TSS) are usually due to the introduction of external factors brought by the flow of rainwater which causes an increase in the concentration of this parameter (Rossi *et al.*, 2005). TSS concentrations are also influenced by various anthropogenic factors such as industry, aquaculture and agricultural activities (Atkinson *et al.*, 2011). Although the TSS concentration does not exceed the value of the INQWS standard, the TSS concentration is high at Station 2 to Station 5

compared to Station 1 and Station 6. This is because Station 1 and Station 6 are streams flowing from upstream to downstream with only a small amount of sediment yielded. Whereas Station 2, 3, 4 and 5 are stationary fishpond farming without any flow. Stationary water stream will cause the inorganic particles to be dissolved in water and become sediments (Ma'arof and Hua, 2015).

On average, the total bacterial count is high at each sampling point. Total bacterial counts indicate the concentration of bacteria that exist in the water body. The river water quality has been reported to have a negative correlation with the intensity level of anthropogenic activity (Leong *et al.*, 2018). Various human activities such as industrialization, agriculture and aquaculture activities have a significant influence on the presence of bacteria in the body of water. This is because of the release of wastewater generated from activities carried out into the river without any treatment. In addition, decomposition of fish and water residues into nutrients for plants and microorganisms. This leads to an increase of bacteria growth (Njoku *et al.*, 2015). The results of total bacterial count for each sampling point are exceeding the standards set by the World Health Organization (WHO) of 100 CFU/mL or 2 log CFU/mL. This indicates that each sampling point is contaminated and is not safe to use without treatment.

The results from the bacterial strain identification showed that Gram negative bacteria were dominant to the overall bacterial strain obtained. Previous studies by Njoku *et al.* (2015) also found that Gram negative bacteria were the dominant bacterial strains of fishponds studied around Nigeria. Gram negative bacteria have a slender formulation that forms a layer of biofilms in the outer surface of the bacteria. This biofilm helps bacteria survive heat temperatures from the environment in the ponds (Sahalan *et al.*, 2018). The high Gram-negative bactericidal resistance to extreme environments makes the bacteria more difficult to eliminate using antibiotics. This makes Gram negative bacteria as a bacterium more harmful to human health and aquatic animals (Hussin *et al.*, 2018).

The assessment of antibiotic resistance among bacteria from aquaculture to antimicrobial agents is important to be constantly updated. This is to know the degree of antibiotic resistance and to monitor the emergence of new antibiotics that are resistant to bacteria. Kathleen *et al.* (2016) said that there was a report on the detection of antibiotic resistance to bacterial isolation from aquaculture that could affect human health. This will cause difficulties for medical practitioners to provide effective treatment of antibiotic resistant antibiotics as the choice of antibiotic use is limited (Friedman *et al.*, 2015). Therefore, antibiotic resistance to bacterial strains should be carefully monitored. According to Rodloff *et al.* (2008), the term susceptible, intermediate and resistance has been adopted worldwide according to the new standard ISO 20776-1 guideline. Based on these standards, susceptible terms are sensitive bacterial strains, which are susceptible to antibiotics and indicate

susceptibility when *in vitro* inhibited by antibiotic concentrations that give rise to therapeutic effects (Kalia, 2014). Intermediate terms mean that bacteria exposed to antibiotics show an *in vitro* inhibition zone by antibiotic concentrations but are associated with uncertain therapeutic effects. The term inhibition means *in vitro* antibiotics against antibiotics by concentrations that indicate failure to therapeutic effects.

Four out of a total of ten bacterial isolates that have been selected for antibiotic susceptibility test showed resistance towards selected antibiotics tested. Penicillin was found to have the highest number of strains that showed resistance. Some published studies conducted on aquaculture product and environment samples in Malaysia (Jalal *et al.*, 2010; Sahilah *et al.*, 2014; Kathleen *et al.*, 2016; Mohamad *et al.*, 2019) also noted a high percentage of resistance to penicillin against tested bacteria. Antibiotics of penicillin have been widely used in clinical areas (Lu *et al.*, 2014) and are reported as resistance profiles commonly found in food and environment products (Sallam *et al.*, 2014).

In addition, two strains showed resistance towards oxytetracycline. Studies by Mog *et al.* (2020), Preena *et al.* (2020) and Nadella *et al.* (2021) also found similar observations where bacterial isolates obtained in their studies showed highest resistance towards oxytetracycline. Oxytetracycline residues were reported to be commonly found in aquatic products. In addition, the usage of this particular antibiotic is commonly authorized in most of the aquaculture countries (Lulijiwa *et al.*, 2019; Chen *et al.*, 2020) which may be the contributing factor to the high reports of resistance.

No resistance was found against chloramphenicol by the tested bacterial strains in this study. Similarly, studies conducted previously find that a high percentage of bacterial susceptibility is against this antibiotic (Sahilah *et al.*, 2014; Kathleen *et al.*, 2016). Since 1983, the use of chloramphenicol antibiotics in aquaculture has been banned in several countries including Malaysia, Indonesia, Korea and Japan. This is because countries in South Asia alone have used fish as a food item of 33.6 kg per person within a year. Chloramphenicol adversely affects human health even with low doses. Among the adverse effects is anaemia and cardiovascular function failure (Yoo *et al.*, 2003). The ban on the use of antibiotics helps to alleviate the problem of human health and reduce the percentage of antibiotic resistance to bacterial strain.

Growth of microorganisms in water is highly influenced by environmental factors. Therefore, the parameter analysis used to determine the Water Quality Index is closely related to the total amount of bacteria (Leong *et al.*, 2018). This is evidenced by the results of this study which records that the highest total bacterial count is at the sampling point 4. This causes the sampling point 4 to have the lowest WQI value compared to other sampling points. The value of WQI in this sampling point is low due to the low DO concentration factor with high concentration of BOD, COD, TSS and AN (Al-Badaii *et al.*, 2013). There is no significant difference between the

total bacterial counts for each sampling point. However, a trend can be seen where there is an increase in the amount of bacterial count from the sampling point 1-4 and the reduction from the sampling point 4 to 6. This matter can be associated with high DO concentration values for sampling points 1 and 6 and low DO concentration for sampling points 2 to 5. The high DO concentration indicates the lack of organic matter contained in the sampling points 1 and 6 compared to the other sampling points.

High pollution of the catfish ponds studied can provide a variety of adverse implications. Livestock and wastewater released from the pond can be a carrier of microbial and human-toxic pollution (Adebayo-Tayo *et al.*, 2012). In addition, environmental degradation can also occur. Organic pollutants and wastes released into aquatic ecosystems can cause chemical pollution, water quality decline, death and destruction of aquatic habitats (van Schothorst *et al.*, 2009). From the aspect of food safety, van Schothorst *et al.* (2009) determined that fish that are reared in the pond are often given the impression of the community that it is healthier and nutritious. However, the fish that are reared in the pond have a high concentration of toxic substances compared to the wild fish in the ocean. Pollution in poultry also has a bad effect on antibiotic drugs. The misuse of antibiotics to maintain the growth of livestock can be carcinogenic and cause antibiotic resistance to human consumption (Pall *et al.*, 2013). This will cause the authorities to act with more attention to the aquaculture industry such as tightening of the law and conducting regular monitoring at the breeding site for more often.

Continuous usage of antibiotics as growth promoters and infection treatment without proper monitoring will lead to persistence of antibiotics in the aquaculture environment. Presence of antibiotic residues from un-ingested feed containing antibiotics and aquatic faeces would result in prolonged periods of selective pressure in the environment, leading to the increase of antibiotic resistance in microorganisms. The acquired resistance could remain in the microbial community and induce antibiotic resistant genes transfer mechanisms which further worsen the issue (Preena *et al.*, 2020).

Implementation of proper regulations and enforcement of antibiotic usage, increasing the global capacity in reducing aquatic diseases, prudent use of antimicrobials as well as keeping the aquaculture environment clean to reduce the risk of diseases are essential strategies to combat this global threat (Preena *et al.*, 2020). Development of alternatives to antibiotics such as vaccines, usage of probiotics and functional feed additives as well as changes to aquaculture practices should be explored to be used as preventive measures, thus faltering the progress of the emergence of antibiotic resistance.

CONCLUSION

Generally, the catfish pond farming at Jasin, Melaka has a polluted water quality due to release of untreated wastewater that could cause significant effect ($P<0.05$). High pollution of organic matter leads to decrease of pH and DO concentration while increasing the concentration of BOD, COD, AN and TSS. High bacterial contamination also causes the catfish to breed in contaminated water that could make them act as carriers of microbial contamination and disease to humans. Four isolated bacterial strains were detected with resistance towards few antibiotics that may source from antibiotics used in the pond farming process. The correlation between WQI and total bacterial counts showed that increases in bacterial contamination levels among the stations led to destruction of WQI of water bodies. Thus, the wastewater of catfish pond farming and the river will need extensive treatment and regular monitoring for it to be suitable for its uses and not give a negative impact towards human health, aquatic systems, and the environment.

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