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Transient or Permanent Inhabitants: Cultured Internal Microbiota of *Cichlidogyrus thurstonae* and *Scutogyrus longicornis* (Monogenea: Ancyrocephalidae) from *Oreochromis* sp.

(Penghuni Sementara atau Kekal: Mikrobiota Dalaman Biakan *Cichlidogyrus thurstonae* dan *Scutogyrus longicornis* (Monogenea: Ancyrocephalidae) daripada *Oreochromis* sp.)

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ABSTRACT

Monogeneans, a class of parasitic platyhelminthes, are usually found on the surface of fish. When it feeds on its host, it harbours bacteria, which can survive in its gut. Occasionally, the monogeneans may cause damages on fish tissue while feeding and may allow secondary infection. The present study aimed to isolate and identify culturable bacteria obtained within the gill monogeneans, *Cichlidogyrus thurstonae* and *Scutogyrus longicornis*, and gill surface of the tilapia fish, *Oreochromis* sp. based on 16S ribosomal RNA gene sequencing. Monogeneans were isolated from the fish gill filaments and surface disinfected using 70% ethanol before squashed aseptically on Luria Bertani (LB) agar to isolate the internal microbiota. A total of five bacteria species, namely *Burkholderia* sp., *Enterobacter hormaechei*, *Enterobacter* sp., *Ochrobactrum intermedium* and *Pantoea* sp., were found within *C. thurstonae*, whilst a total of eight bacteria species, namely *Burkholderia contaminans*, *Pantoea dispersa*, *Sphingomonas yabuuchiae*, *Rhizobium pusense*, *O. intermedium*, *Acinetobacter bereziniae*, *Escherichia hermannii*, and *Staphylococcus saprophyticus*, were found within *S. longicornis* in which *B. contaminans*, *P. dispersa,* and *S. yabuuchiae* were also found on the surface of the tilapia fish gill filaments. *Enterobacter bugandensis* and *Acinetobacter pittii* were found solely on the surface of the gill filaments. These bacteria are also found in the environment and some of them are believed to be pathogenic to fish. We suggest that monogeneans may serve as potential bacteria reservoirs, which facilitate the transmission of bacteria.

Keywords: Bacteria; identification; internal microbiota; molecular; monogenean; tilapia

ABSTRAK

Monogenea ialah salah satu kelas parasit platyhelminthes yang sering ditemui pada permukaan badan ikan. Apabila monogenea memakan tisu hos ikan, ia memperoleh bakteria yang dapat hidup di dalam usus monogenea. Kadangkala, monogenea boleh menyebabkan kecederaan pada tisu ikan semasa makan yang mungkin menyebabkan jangkitan sekunder pada ikan. Kajian ini bertujuan memencilkan dan mengenal pasti bakteria yang boleh dikultur daripada monogenea, *Cichlidogyrus thurstonae* dan *Scutogyrus longicornis* serta pada permukaan insang ikan tilapia, *Oreochromis* sp. berdasarkan jujukan gen 16S ribosomal RNA. Monogenea dipencilkan daripada filamen insang ikan dan permukaan dibilas dengan 70% etanol sebelum dilenyek secara aseptik pada agar LB untuk pemencilan mikrobiota dalaman. Sebanyak lima spesies bakteria, iaitu *Burkholderia* sp., *Enterobacter hormaechei*, *Enterobacter* sp., *Ochrobactrum intermedium* dan *Pantoea* sp., dijumpai di dalam *C. thurstonae* manakala sejumlah lapan spesies bakteria, iaitu *Burkholderia contaminans*, *Pantoea dispersa*, *Sphingomonas yabuuchiae*, *Rhizobium pusense*, *O. intermedium*, *Acinetobacter bereziniae*, *Escherichia hermannii* dan *Staphylococcus saprophyticus* dijumpai pada *S. longicornis* dengan *B. contaminans*, *P. dispersa* dan *S. yabuuchiae* juga dijumpai pada permukaan filamen insang ikan. Walau bagaimanapun, *Enterobacter bugandensis* dan *Acinetobacter pittii* hanya dijumpai pada permukaan filamen insang sahaja. Bakteria ini juga boleh didapati daripada persekitaran dan sebahagian daripada bakteria ini dipercayai bersifat patogen kepada ikan. Kami mendapati monogenea berkemungkinan berfungsi sebagai perumah yang memudahkan penularan bakteria.

Kata kunci: Bakteria; mikrobiota dalaman; molekul; monogenea; pengenalpastian; tilapia

Introduction

Monogeneans are ectoparasitic platyhelminthes, that mainly infest marine or freshwater fish. Most of them can be found on external parts of fish such as body surface, fins, and gills (Bychowsky 1957; Yamaguti 1963). Monogeneans are commonly be subdivided into monopisthocotylean and polyopisthocotylean (Sproston 1946; Yamaguti 1963). Both sub-classes have different feeding habits and attachment apparatus, which are well documented by Kearn (1999, 1994). Monopisthocotylean is the skin feeding monogenean, which mainly feeds on the epidermis and mucus of its host whereas polyopisthocotylean is the monogenean that feeds on the blood of its host (Kearn 1994, 1963). Infection of monogeneans on fish often lead to secondary infection by bacteria and fungi, and this increases fish mortality especially in high density fish farming (Ogawa 2015; Whittington 2012).

Previous studies have shown the association of microorganisms either internally or externally with monogeneans and other parasitic platyhelminthes species (Table 1). One of the earliest discoveries of potential bacteria observed associated with the monogenean, *Diclidophora merlangi* was reported by Morris and Halton (1975) based on electron microscopy. Other microorganisms such as virus-like particles, myxosporidians and microsporidians have also been found associated with monogeneans by other researchers (Table 1). From the literature surveys, information on the internal microbiota of parasitic Platyhelminthes, including monogeneans is scarce (Table 1). Sepúlveda et al. (2017) had used the whole monogenean, *Zeuxapta seriolae*, to elucidate the culturable microbiota (surface and internal) present in the monogenean, however, the monogenean used was unsterile and may contain bacterial contaminations. Recent reviews on the interaction between helminths and gut microbiota in various vertebrate hosts have showed the importance of their complex relationships in maintaining the health and homeostasis of the hosts (Cortés et al. 2019; Morley 2016). In addition, White et al. (2018) reported that the study of the internal microbiota of helminthic parasites was essential in order to understand their roles in infection and survival of parasites in the hosts. Knowledge on the bacteria community within platyhelminthe parasites is unknown and thus, such research will throw some light on their ecological roles, for instance, bacteria reservoir and functional roles on the hosts. For the first time, we aimed to isolate and identify the culturable internal bacteria of the gill monogeneans, *Cichlidogyrus thurstonae* and *Scutogyrus longicornis* of the tilapia fish, *Oreochromis* sp.

Materials and Methods

Collection of fish samples

A total of 15 tilapia (*Oreochromis* sp.) with body lengths range from 10 to 12 cm were collected by a fisherman from a fish pond located at Kampar, Perak, Malaysia. The fish were brought to the laboratory for isolation of monogeneans. The animal ethical approval (U/SERC/94/2017) was approved by UTAR Scientific and Ethical Review committee held on 13 November 2017.

Isolation and identification of monogeneans from fish gills

Gill arches from the left and right sides of the tilapia were removed and placed separately into two petri dishes containing sterile 0.85% saline water. A self-made toothpick with an attached eyelash was disinfected using 70% ethanol and used to dislodge monogeneans that were attached on the gill filaments. The dislodged monogeneans were then transferred carefully, cleaned and flushed with sterile saline using an autoclaved glass Pasteur pipette in a sterile glass cavity block. The monogenean species was identified based on the morphological characteristics of its reproductive organs and haptoral sclerites (Lim et al. 2016; Pariselle & Euzet 2009, 1995). The identified monogeneans were placed into different sterile glass cavity blocks for extraction of internal bacteria.

Isolation of internal culturable bacteria of *C. thurstonae* and *S. longicornis*

To obtain the internal bacteria of *C. thurstonae* and *S. longicornis*, individual monogenean was hold anteriorly using a pair of sterile fine forceps and dipped into 70% ethanol for about 5 s to disinfect its surface. The monogenean was then washed with sterile 0.85% saline water and placed into a drop of 50 µL sterile 0.85% saline water on a LB agar plate. The monogenean was then squashed using a sterile inoculating loop to release the internal fluid of the monogenean onto the plate under a stereomicroscope (Motic NSZ-810, China) in a laminar flow cabinet. A total of four *C. thurstonae* and six *S. longicornis* were surface-disinfected for isolation of their internal bacteria.

TABLE 1. Internal and external microorganisms associated with parasitic platyhelminthes (viz., monogeneans, trematodes, and cestodes)

Class	Species	Associated microorganism	Site of infection		Method			Reference
			External	Internal	LM	EM	ML	
Monogenean	*Diclidophora merlangi*	Bacteria		✓		✓		Morris & Halton (1975)
	Cichlidogyrus halli typicus	Bacteria		✓		✓		El-Naggar & Kearn (1989)
	Cichlidogyrus thurstonae	Bacteria		✓			✓	Present study
	Gyrodactylus avalonia	Bacteria	✓			✓		Cusack and Cone (1985)
	Gyrodactylus colemanensis	Bacteria	✓		✓	✓		Cusack et al. (1988)
	Gyrodactylus salaris	Bacteria, flagellate	✓			✓		Bakke et al. (2006)
	Gyrodactylus salmonis	Bacteria	✓			✓		Cone and Odense (1984)
	Microcotyle sp.	Virus-like particles	✓			✓		Justine and Bonami (1993)
	Pseudodiplorchis americanus	Microsporadian	✓			✓		Cable and Tinsley (1992)
		Bacteria		✓		✓		
	Pseudodactylogyrus bini	Myxosporidian	✓		✓			Aguilar et al. (2004)
	Scutogyrus longicornis	Bacteria		✓			✓	Present study
	Zeuxapta seriolae	*Bacteria	✓	✓			✓	Sepúlveda et al. (2017)
Trematode	*Clinostomum marginatum*	Bacteria	✓			✓		Aho et al. (1991)
	Culaeatrema inconstans	Bacteria	✓			✓		Lasee and Sutherland (1993)
	Megalodiscus temperatus	Bacteria	✓			✓		Morris (1973)
	Gyliauchen nahaensis	Bacteria	✓			✓		Hughes-Stamm et al. (1999)
Cestode	*Caryophyllidean* sp.	Bacteria	✓			✓		Poddubnaya and Izvekova (2005)
	Eubothrium rugosum	Bacteria	✓			✓		Izvekova (2006); Lapteva and Izvekova (2004)
	Proteocephalus cermuae	Bacteria	✓			✓		Korneva (2008); Korneva and Plotnikov (2009)
	Proteocephalus percae	Bacteria	✓			✓		Korneva and Plotnikov (2012, 2009)
	Proteocephalus torulosus	Bacteria	✓			✓		Korneva and Plotnikov (2012, 2009)
	Schistocephalus solidus	Bacteria		✓			✓	Hahn and Dheilly (2018)
	Triaenophorus nodulosus	Bacteria	✓			✓		Korneva and Plotnikov (2012, 2009); Lapteva and Izvekova (2004); Plotnikov and Korneva (2008)

LM, light microscope; EM, electron microscope; ML, molecular method; *, mixture of surface and internal bacteria)

For non-surface disinfected *C. thurstonae* and *S. longicornis*, individual monogenean was held anteriorly using a pair of sterile fine forceps, and washed by dipping it into sterile 0.85% saline water for about 5 s. The monogenean was then placed in a drop of 50 µL sterile 0.85% saline water on a LB agar plate. Similarly, the monogenean was then squashed using a sterile inoculating loop onto the agar plate. A total of three monogeneans for each species were used for non-surface disinfected *C. thurstonae* and *S. longicornis*. This step was performed to verify the effectiveness of the surface-disinfection method of the monogoeneans.

Gill filaments of *Oreochromis* sp. (approximately 5 mm in length) were also treated with the abovementioned surface-disinfection method with a 15 s dip in 70% ethanol, however, this step was replaced with a washing time of 15 s in 0.85% saline water for non-disinfected gill filaments.

The same 70% ethanol disinfection method was applied on a pair of forceps without holding a monogenean or a gill filament in order to verify the disinfection process of the monogeneans. All the prepared spread plates were kept in an incubator at 30 °C for 24 to 48 h.

PURIFICATION AND GRAM STAINING OF ISOLATED BACTERIA

Bacteria colonies with different morphologies were stained with Gram staining. Bacteria isolates were then selected, and subcultured onto Luria Bertani (LB) agar plates until pure bacteria colonies were obtained. The pure bacteria colonies isolated within the monogeneans and on the surface of the gill filaments were processed for molecular identification.

DNA EXTRACTION OF ISOLATED BACTERIA

The bacterial DNA was extracted using the heat treatment method described by Dashti et al. (2009) with some modifications. Two loopfuls of bacteria colonies were suspended in 200 µL deionized water and centrifuged at 13,000 rpm for 5 min. The resulting cell pellet was resuspended with 200 µL deionized water and centrifuged again at 13,000 rpm for 5 min. The pellet was resuspended with 200 µL deionized water and heated at 95 °C for 10 min using a heat block. The suspension was immediately placed in an ice bath for 10 min followed by a centrifugation at 13,000 rpm for 5 min. The resulting supernatant was analysed using gel electrophoresis to confirm the presence of bacterial DNA.

POLYMERASE CHAIN REACTION (PCR)

The 16S rRNA of the isolated bacteria was amplified by a 30-cycle PCR with a set of universal primer: 27F (5'-AGAGTTTGATCMTGG-3', where M is C or A) and 1492R (5'-TACCTTGTTACGACTT-3') (Lane 1991). The PCR condition was performed with an initial activation step (95 °C for 5 min), denaturation step (95 °C for 30 s), annealing step (55 °C for 30 s), extension step (72 °C for 1 min), and a final extension step (72 °C for 5 min).

MOLECULAR IDENTIFICATION OF ISOLATED BACTERIA

The PCR products were purified and sequenced using the Sanger method by Apical Scientific Sdn Bhd, Malaysia. The DNA sequencing results were then trimmed using BioEdit (version 7.0.5) and compared to the sequences in the GenBank database using the similarity search programme performed by BLAST.

RESULTS

A total of four *Cichlidogyrus thurstonae* and six *Scutogyrus longincornis* were collected and identified from two tilapia fish. Different bacterial colonies were observed on the LB agar plates containing surface-disinfected squashed monogeneans (Supplementary Figure 1), non-surface disinfected squashed monogeneans (Supplementary Figure 2) and non-disinfected gill filaments (Supplementary Figure 3). However, there was no bacterial colony observed on the LB agar plates of disinfected gill filaments (Supplementary Figure 4) and disinfected forceps without holding a monogenean or a gill filament (Supplementary Figure 5).

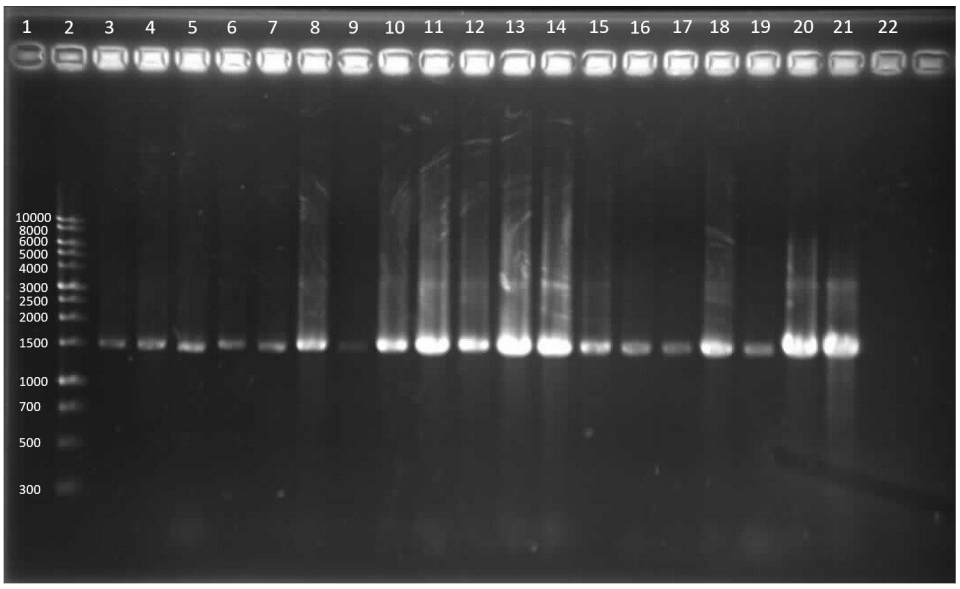

FIGURE 1. An electrophoresis gel image of the PCR products. Lane 2 was loaded with 1 kb DNA ladder. Lanes 3 to 21 were loaded with 16S rRNA amplicons of each bacteria sample. Lane 22 was loaded with a negative control

Base on the different morphology of the culturable pure bacterial colonies (isolated within the monogeneans and on the surface of gill filaments) and their Gram staining results, a total of 34 bacteria were selected, and proceeded for bacterial DNA extraction, PCR and followed by DNA sequencing. The electrophoresis gel image shown in Figure 1 confirms the expected band size of the PCR products of 1.5 kb.

After performing the similarity comparison of the DNA sequences and the GenBank database, the closest possible identified bacteria species within the monogeneans and the fish gill filaments were tabulated in Table 2. Based on the closest similarity with sequence databases using BLAST, a total of five and eight bacteria species were identified in *C. thurstonae* and *S. longicornis*, respectively (Table 2). Among these bacteria species, *Enterobacter hormaechei* was found twice out of four *C. thurstonae* specimens whilst *Burkholderia contaminans* was isolated in four out of six *S. longicornis* specimens. On the surface of *Oreochromis* gill filaments, six bacteria species were identified.

TABLE 2. Bacteria species within *Cichlidogyrus thurstonae* (n = 4) and *Scutogyrus longicornis* (n = 6), and on the surface of gill filaments (n = 2) of *Oreochromis* sp. based on gene sequence analysis using BLAST

Species	Locations			Similarity (%)	Accession number
	Gill surface	Within *C. thurstonae*	Within *S. longicornis*		
Acinetobacter pittii	✓			100	NR_117621.1
Burkholderia contaminans	✓ ✓			99	NR_104978.1
Enterobacter bugandensis	✓			99	NR_148649.1
Ochrobactrum intermedium	✓			100	NR_113812.1
Pantoea dispersa	✓			99	NR_116797.1
Pantoea dispersa	✓ ✓			100	NR_116755.1
Sphingomonas yabuuchiae	✓			100	NR_0286341
Burkholderia sp.		✓		91	AB545643.1
Enterobacter hormaechei		✓ ✓		100	NR_126208.1
Enterobacter sp.		✓		98	KF843698.1
Ochrobactrum intermedium		✓		100	NR_113812.1
Pantoea sp.		✓		97	MG602694.1
Pantoea sp.		✓		98	KF730646.1
Acinetobacter bereziniae			✓	100	NR_117625.1
Burkholderia contaminans			✓ ✓ ✓ ✓	99	NR_104978.1
Escherichia hermannii			✓	100	NR_104940.1
Ochrobactrum intermedium			✓	100	NR_113812.1
Pantoea dispersa			✓	100	NR_116797.1
Pantoea dispersa			✓	100	NR_116755.1
Rhizobium pusense			✓	99	NR_116874.1
Sphingomonas yabuuchiae			✓	99	NR_028634.1
Staphylococcus saprophyticus			✓	100	NR_074999.2

"✓" indicates the frequency of bacteria being identified from different samples

DISCUSSION

In the present study, six genera of bacteria were found on the surface of the gill filaments in which two bacteria species, viz., *A. pittii* and *E. bugandensis* were not found in any of the studied monogenean species (Table 2). The bacteria, which were found on the surface of gill filaments, are likely to have originated from the environment. For example, *A. pittii* had been reported from pond water

(Nemec et al. 2011), and the gill of the diseased blunt snout bream (Li et al. 2017). *Burkholderia contaminans* was found in the fresh or marine waters (Fang et al. 2011; Maravić et al. 2012; Olapade et al. 2005; Vanlaere et al. 2009) and in diseased tilapia (Mahboub et al. 2022). On the other hand, *B. contaminans*, *Pantoea dispersa* and *Sphingomonas* species had been isolated from soil (Hall et al. 2015; Leung et al. 1999; Selvakumar et al. 2008). For *Sphingomonas* species, it had also been isolated from the biofilm of an aquaculture recirculating system (King et al. 2004). *Ochrobactrum intermedium*, which belongs to the family Brucellaceae, has been isolated from various environments such as waste water and soil (Aujoulat et al. 2014). Interestingly, *E. bugandensis*, which was found exclusively on the gill filament of *Oreochromis* sp. in the present study, had only been isolated on the International Space Station environmental surfaces (Urbaniak et al. 2018), and in neonatal blood (Pati et al. 2018) but not in water or soil. In a study done by Pakingking et al. (2015), the authors had shown that majority of the bacteria found on the gill and intestine of *Oreochromis niloticus* also originated from the water and sediment. However, it was also believed that the bacteria community present in the fish and in the water may change in different water quality parameters (Ismail et al. 2016). The presence of bacteria found on the surface of the gill filaments of *Oreochromis* species in this study indicates that these bacteria are probably opportunistic (Mahboub et al. 2022) or associated with the gill tissues (due to their ecological niche) but this requires further investigations.

Like other monopisthocotyleans, *C. thurstonae* and *S. longicornis* probably feed on the epithelial tissues of the tilapia fish (Kearn 1963). The present study showed that four bacterial genera, namely *Burkholderia, Enterobacter, Ochrobactrum* and *Pantoea*, found on the fish gill filaments, were also present within *C. thurstonae*. For *S. longicornis*, four bacterial genera, namely *Burkholderia, Ochrobactrum, Pantoea* and *Sphingomonas,* found inside the monogeneans were also observed on the gill surface of the tilapia. These results indicate that the monogeneans may have ingested these bacteria located on the gill epithelial tissues or in the water column while the monogeneans were feeding on the gills. However, we could not determine if the ingested bacteria later will serve as the native intestinal gut microbiota of *C. thurstonae* and *S. longicornis* in the present study. In addition, the different bacterial communities found in the two monogenean species, *C. thurstonae* and *S. longicornis*, could be due to the different monogenean gut physiological condition.

There are four bacteria species, namely *Acinetobacter bereziniae, Escherichia hermannii*, *Rhizobium pusense,* and *Staphylococcus saprophyticus*, found solely within *S. longicornis* in the present study. Among these species, *E. hermannii*, *R. pusense,* and *S. saprophyticus* were isolated from fish. For example, *E. hermannii* had been isolated from tilapia (Almeida et al. 2017), *R. pusense* from brown trouts (Al-Hisnawi et al. 2015), and *S. saprophyticus* from barbs (Rahman et al. 2018). On the other hand, *A. bereziniae*, which is commonly isolated from human clinical specimens (Nemec et al. 2010), for the first time, was isolated from the natural environment in the present study. Generally, other *Acinetobacter* species had also been reported in soil (Doughari et al. 2011). In *C. thurstonae*, out of five bacteria species isolated in the present study, only one identified bacteria species, viz., *Enterobacter hormaechei*, was isolated solely from *C. thurstonae*. This bacterium also probably originated from the environment as well because it has been isolated from water (Garg et al. 2013). Some questions arose here such as if these bacteria found inside the monogeneans act as endosymbionts, and assist them in digestion as well as protection, or serve as opportunistic or transient bacteria in the guts of the monogeneans. In a recent study, the nematode, *Trichuris muris*, was shown to acquire its host intestinal microbiota to enhance their survival in the hosts (White et al. 2018). We hope that this preliminary study on the culturable bacteria within a monogenean will initiate more studies in future to investigate the bacteria community present in other monogeneans, and elucidate their possible functions in the parasites.

The internal culturable bacteria community, identified from *C. thurstonae* and *S. longicornis* in the present study, was not similar to that of the previous studies done by Cusack et al. (1988) who investigated the external bacteria community using biochemical methods as well as Sepúlveda et al. (2017) who studied the internal and surface bacteria community of the marine monogenean, *Zeuxapta seriolae* based on molecular analyses. The differences in the culturable microbiota inside different species of monogeneans are probably affected by the external and internal environments such as the chemical and physical properties of the ingested water, food, and the gut environment of the monogeneans. The selection of gut microbiota associated with the monogeneans is probably a natural selection process as similar suggestions had been proposed in an earthworm-microorganism interaction study (Thakuria et al. 2010). However, further studies are required to examine the internal and external factors that may determine the microbiota in different monogeneans.

The present study indicates that almost all identified bacteria inside the monogeneans are Gram-negative bacteria except for *S. saprophyticus*. This is similar to the observations reported by Cusack et al. (1988) and Sepúlveda et al. (2017). Previous evidence also indicated that Gram-negative bacteria are generally the pathogenic disease-causing bacteria in fish (Pękala-Safińska 2018; Sudheesh et al. 2012). If monogeneans are in favour of harbouring Gram-negative bacteria, they are likely to serve as reservoirs and transmit potential pathogenic bacteria. However, more studies on the internal microbiota of monogeneans are needed.

Conclusion

In conclusion, this first report on the culturable bacteria found within the monogeneans, *C. thurstonae* and *S. longicornis*, indicates that these bacteria are generally Gram-negative, and probably originated from the surface of the fish gill filaments. The monogeneans may serve as potential bacteria reservoirs, which may facilitate the administration of its gut bacteria into the fish while the monogeneans are feeding on the gill tissues. Further investigations on the monogeneans microbiome may provide us a better understanding on the importance of monogeneans in initiating secondary bacterial infections of fish.

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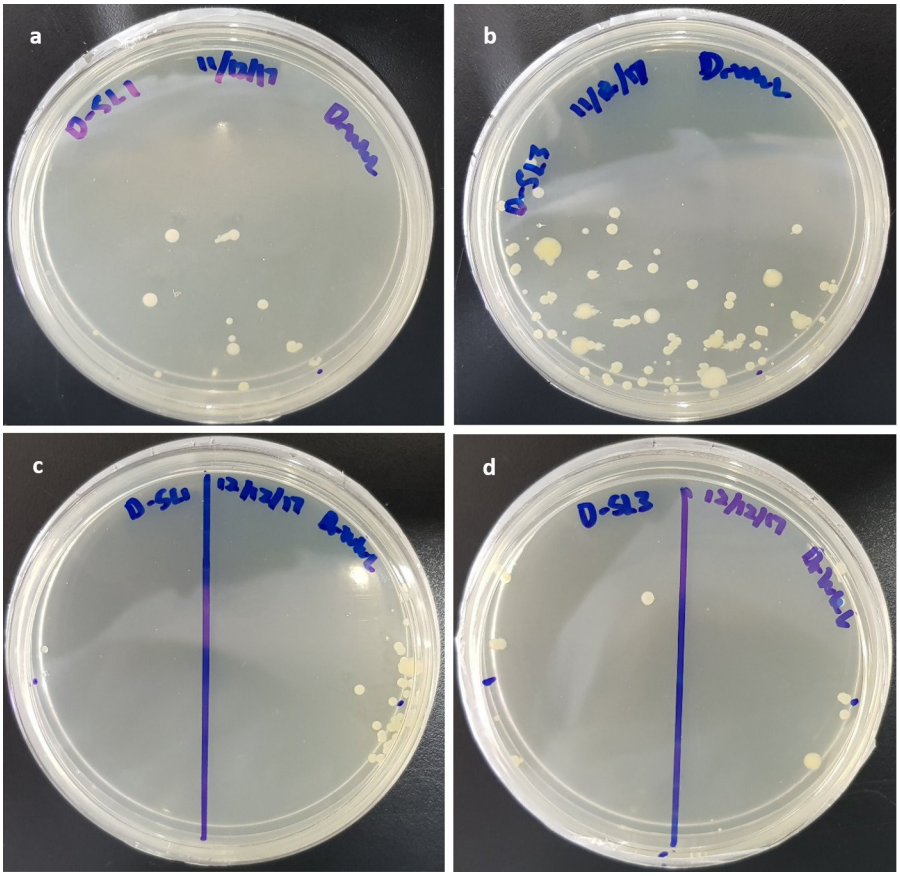
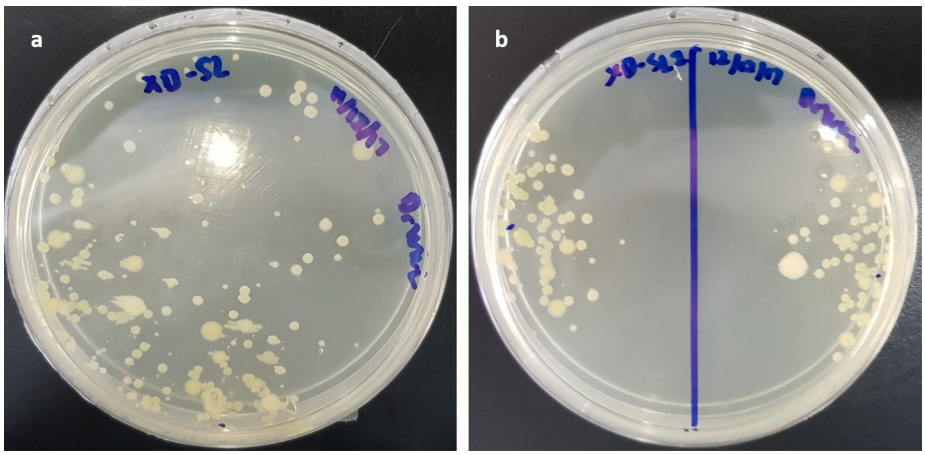
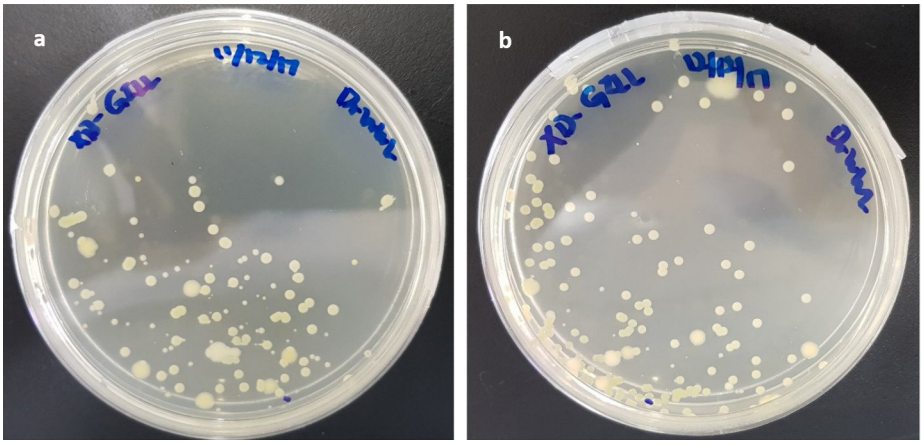


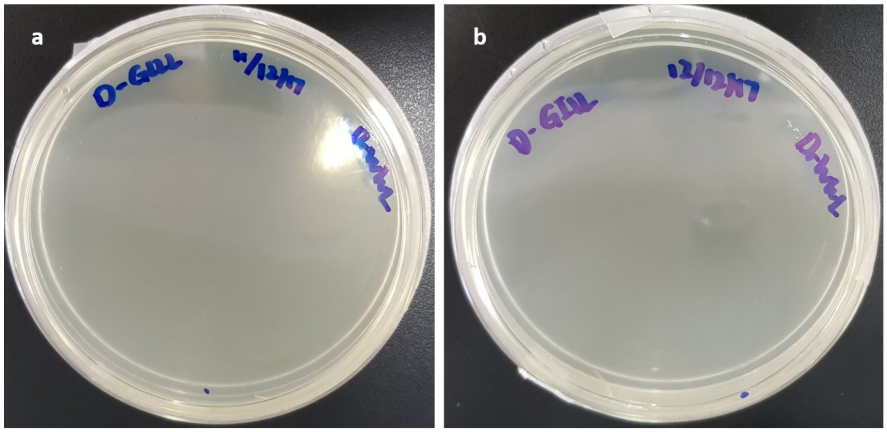
SUPPLEMENTARY FIGURE 1. LB culture plates (a) and (b), each with one surface disinfected *Scutogyrus longicornis* from the first tilapia. LB culture plates (c) and (d), each with two surface-disinfected *S. longicornis* from the second tilapia



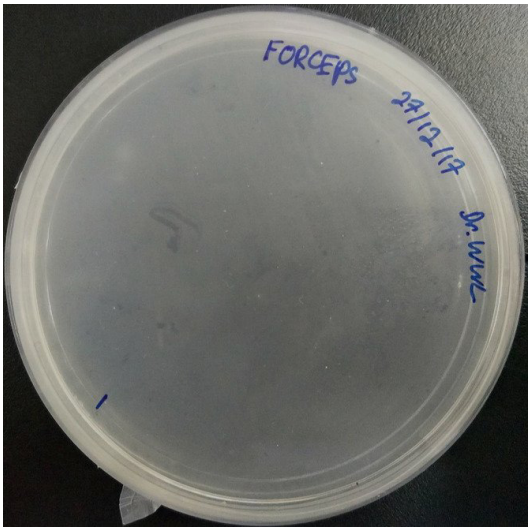
SUPPLEMENTARY FIGURE 2. LB culture plate (a) with one non-surface disinfected *Scutogyrus longicornis* from the first tilapia. LB culture plate (b) with two non-surface disinfected *S. longicornis* from the second tilapia



SUPPLEMENTARY FIGURE 3. Culture plate (a) and (b) with one non-disinfected gill filament from the first and second tilapia, respectively



SUPPLEMENTARY FIGURE 4. LB culture plates (a) and (b) with one disinfected gill filament from the first and second tilapia, respectively



SUPPLEMENTARY FIGURE 5. LB culture plate with the disinfected forceps without holding *S. longicornis*