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Metagenomic profiles of core and signature bacteria in the guts of white shrimp, *Litopenaeus vannamei*, with different growth rates

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ABSTRACT

A large number of studies have previously revealed evidence suggesting a strong correlation between gut bacteria and the growth performance of aquatic animals. However, specific research reporting bacterial communities inhabiting white shrimps with different growth rates are still very limited. Thus, the present study aimed at investigating the structure and composition of core and signature bacteria in the gut of shrimps at different growth rates. A total of 60 healthy white shrimps, *Litopenaeus vannamei*, with different growth rates (slow vs fast) were collected from three shrimp ponds as replica. Bacterial 16S rRNA genes associated with these shrimp guts were extracted, amplified and sequenced using next-generation sequencing (NGS) analysis to determine the structure and composition of the bacterial community within and between groups. The result showed that 35 genera of 181 detected genera (19.34%) were considered to be the core microbiome in the gut of white shrimps regarding their prevalence in all samples including Illumatobacter, Ruegeria, Candidatus Bacillopora, Rose-ovarius, Silicimonas, Algoriphagus, Haloferula, Dinoroesobacter, Vibrio, Lactobacillus, Bdellovibrio, Shimia, and Rubiginitalea. In addition, there was a strong association between diversity and species richness of gut bacteria and the growth of white shrimps. The species richness and the Shannon index representing bacterial diversity were significantly lower in the fast-growing shrimps ($p < 0.05$), reinforcing the close relationship between gut bacteria and their host growth. Further analysis using linear discriminant analysis effect size (LEfSe) indicated that nine bacterial species were significantly higher in the fast-growing shrimps (Group 1) than the slow-growing shrimps (Group 2). The nine species were identified as *Coprococcus comes* (OTU58), *Oscillibater* sp. ER4 (OTU74), *Acidaminicoccus intestini* (OTU210) and *Bacteroidetes ovatus* (OTU671), *Oscilibacter* sp. (OTU274), *Peptococcus* sp. (OTU218), *Clostridium phoceensis* (OTU313), *Legionella* sp. (OTU682), and unidentified Clostridiales (OTU186). These results might suggest that the nine bacterial species are bacterial signatures for the high growth shrimps. However, due to the novelty of the shrimp gut bacteria, further studies are still required to understand their specific roles and contribution to the growth of white shrimps.

1. Introduction

White shrimp, *Litopenaeus vannamei*, is one of the highest economic-value products in the world including Indonesia. The shrimp has been commercially cultivated in Indonesia since 20 to replace tiger prawn, *Penaeus monodon*, which was destroyed by viral diseases such as the white spot syndrome virus (WSSV) and odon baculovirus (MBV) (Budhiman et al., 2005). A few years later, shrimp farming has increased dramatically and become the main cultivable species of crustacean in onesian aquaculture industries. Currently, Indonesia has become the fourth largest shrimp exporting country after India, Ecuador and Argentina (Henriksson et al., 2019). Recent data indicated that that total Indonesian shrimp production in 2018 was 147 thousand metric tons valued at more than 1.3 billion $ USD and make shrimp become Indonesia's leading export for fisheries commodities (Wati, 2018). With its high potency area for shrimp production which has not been used yet, the Indonesian government even set a target to be the largest shrimp exporting country in future. Therefore, various strategies have been

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developed to increase yield in shrimp farming industries including manipulation of gut bacteria through probiotic supplementation etc.

A large number of studies have previously claimed evidence suggesting that there was a close interaction between gut bacteria and the growth performance of cultured animals. A study by Fan et al. (2019a), for instance, revealed that the structure, species richness and evenness of gut microbiota was highly associated with the shrimp body weight. Similarly, Hasyimi et al. (2020) reported strong correlation between gut microbiota and feed conversion ratio as well as the specific growth rate of white shrimp. However, these studies used different diets to feed the shrimp samples. These variables have been previously reported to contribute significantly to any discrepancy of gut microbiota (Gao et al., 2019; Huynh et al., 2019; Sharawy et al., 2020). In addition, different rearing conditions, life stages, and host physiological conditions might also contribute to the difference in bacterial communities inhabiting gut tracts (Amin, 2010; Giatsis et al., 2015; Rajeev et al., 2021). With such an experimental design, we are not sure whether the difference in animal growth was caused directly by the difference in microbial composition or could be the diet which had a direct effect on the growth of culture animals, and later shaped different microbial compositions in their guts. As a consequence, the general conclusion derived from such experiments such as genus/species marker in the high growth shrimps which becomes the main finding of such study could be bias. Therefore, more specific studies are required to investigate bacterial markers in bigger bodyweight or high growth rates. However, few published data on the relationship between shrimp growth and gut microbes are available and the knowledge of the shrimp gut bacteria at different growth is still limited. Furthermore, the effect of the gut bacteria on shrimp growth performance is still uncertain and lacks support information.

The present study investigated the difference in structure and composition of the bacterial community associated with the gut of shrimp with different growth rates by taking samples from the same ponds. We presumed that other factors such as diets and environmental conditions were the same as the two samples groups shared the same diet and other rearing conditions. As replicates, we also took samples from two other ponds therefore we had a total of three replicates. This experimental design will have a better understanding of the relationship between shrimp groups. An Illumina-based next-generation sequencing (NGS) method was used to analyze the diversity and composition of the gut bacteria and seek the association among gut bacterial structure, and bodyweight of shrimp. The NGS technology may contribute to the improvement of aquaculture sustainability of Pacific white shrimp (Rodriguez-Anaya et al., 2018). An in-depth understanding of the bacterial ecology in the shrimp gut can later help to improve aquaculture productivity.

2. Material and methods

2.1. Pond culture system

Three intensive shrimp ponds (800m^2 HDPE pond; 1.2 m water depth; and 247 ind/m^2 stocking density) located at Bangkalan, Madura Island, East Java, Indonesia (7.021874° S, 112.741575° E) were selected for the present study (Fig. 1). All ponds were stocked with the same shrimp larvae (F1 larvae from a certified shrimp hatchery in Indonesia), the same feeding rate (4%bw/day) and the same feed type (commercial shrimp pellet with ~33% crude protein content). In addition water

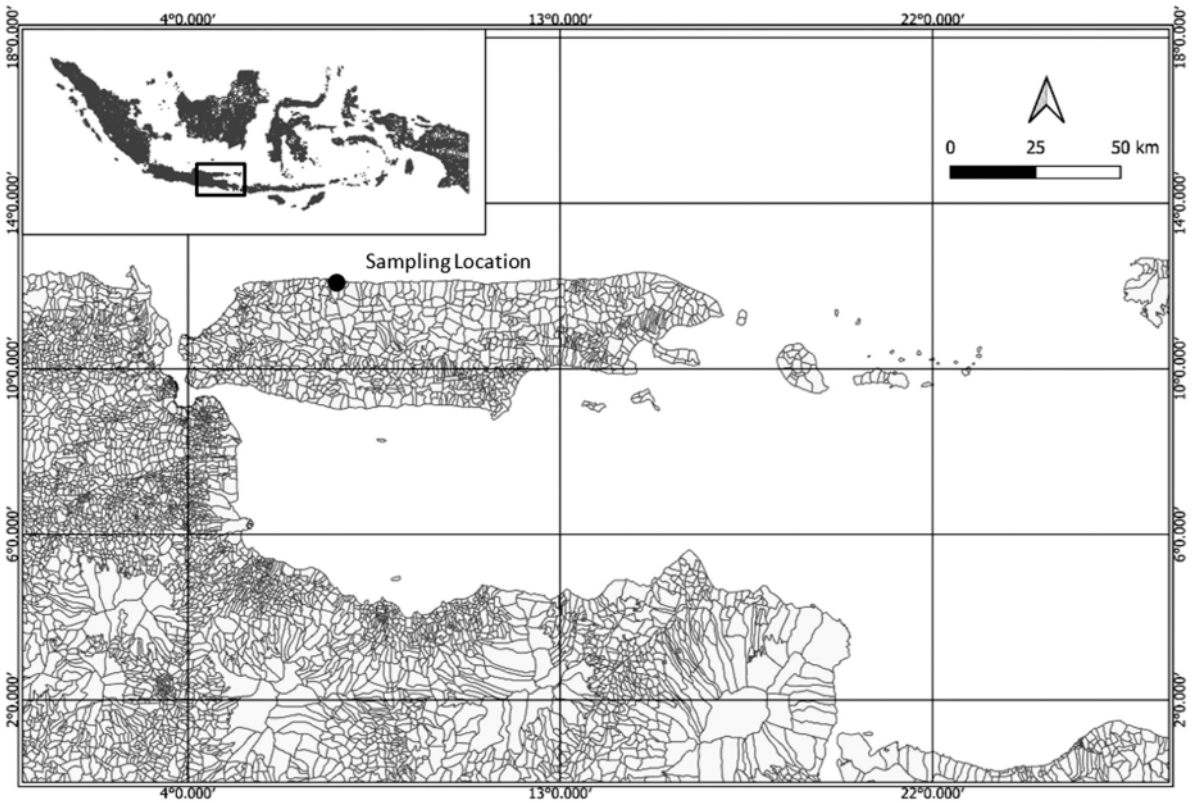

Fig. 1. Geographical location of intensive white shrimp ponds at which the shrimp samples were collected (Madura Island, East Java, Indonesia).

quality parameters among the three ponds were quite similar including pH (7.77–8.00), salinity (30.67–33 psu), temperature (28.33–28.67 °C), phosphate (0.13–0.47 mg/L), nitrite (0.25–0.28 mg/L), and dissolved oxygen (4.13–4.53 mg/L). Furthermore, no diseases had been reported during the farming period. In the beginning, 30 healthy shrimp larvae used in the culture period were randomly sampled on day 0 of culture (DOC 0) and measured its weight and length to get an average of initial shrimp size.

2.2. Gut sampling

A total of 60 healthy white shrimps at DOC 47 were collected from the three intensive shrimp ponds (@20 shrimps). The 20 shrimps from each pond consisted of 10 small shrimps and 10 big shrimps (~2 times bigger than the size of small shrimps). The average body weight and length of shrimp samples from each pond were presented in Table 1. The shrimp samples were collected using a scope net from each pond at three sites (@3–4 shrimps) according to a protocol of Rozario and Devarajan (2020) to increase sampling precision. Prior to the sampling, all shrimps were fasted for 12 h to reduce or empty the intestinal tract at the sampling time (Gao et al., 2019). In addition, all shrimps sampled during this study were in healthy condition and showed no clinically pathological signs or abnormal conditions including clean and bright skin colour, active swimming/movement, complete organ, reed gills, transparent hepatopancreas, no deformities, and free from ectoparasites (Huang et al., 2018). All shrimps were then placed in an icebox and transported to Microbiology Laboratory, Faculty of Fisheries and Marine Universitas Airlangga in ~1 h.Afterwards, shrimp guts were sampled in a laminar flow hood as previously described by Amin (2010) with some modifications. In Brief, shrimps were firstly rinsed individually with distilled water, followed by 70% ethanol, and washed with sterile distilled water. Thereafter, the shrimps were dissected aseptically in a laminar hood and the gut was stored in a 1.8 ml sterile microtube individually which was previously filled with RNA later (RNAlater: SIGMA) for bacterial DNA preservation. The gut samples were then stored in a freezer (−20 °C) until further analysis.

2.3. DNA extraction

Bacterial DNA in the shrimp guts was extracted using QIAamp DNA Microbiome Kit (Qiagen, Germany), according to the manufacturer's instructions. The concentration and quality of the extracted DNA were assessed using a DNA-RNA protein Quantification Spectrophotometer (N-913A MaestroNano Pro). The sterile microtubes containing the extracted DNA were then stored at -20 °C until further use.

Table 1
The initial weight, final weight, weight gain and the specific growth rate of white shrimp, *Litopenaeus vannamei*.

Parameters	Group 1			Group 2		
	P1	P2	P3	P1	P2	P3
IW (g)	0.083 ± 0.001	0.088 ± 0.001	0.088 ± 0.001	0.083 ± 0.001	0.088 ± 0.001	0.088 ± 0.001
FW (g)	6.80 ± 0.42^{b}	6.71 ± 0.27^{b}	6.59 ± 0.49^{b}	3.66 ± 0.46 ^{a}	3.96 ± 0.37^{a}	3.70 ± 0.45^{a}
WG (g)	6.80 ± 0.42^{b}	6.71 ± 0.27^{b}	6.59 ± 0.49^{b}	3.66 ± 0.46 ^{a}	3.96 ± 0.37^{a}	3.70 ± 0.45^{a}
SGR (%bw. day^{-1})	209.30 ± 13.04^{b}	204.96 ± 4.12^{b}	203.03 ± 7.41^{b}	143.67 ± 13.04^{a}	152.02 ± 9.40^{a}	148.86 ± 13.23^{a}

IW: Initial weight, FW: Final weight, WG: Weight gain, Group 1: shrimps with high growth rate, Group 2: shrimps with low growth rate, SGR: specific growth rate. P1: Shrimp pond 1, P2: Shrimp pond 2, and P3 is shrimp pond 3. Values are average with a standard deviation of 10 white shrimps. Different manuscripts represent significant differences in values at $p < 0.05$.

2.4. Amplification of 16S rRNA gene

The amplification of 16S rRNA genes and microbial community analysis was performed in Novogene Bioinformatical Information Technology Co. (Singapore). The hypervariable V3-V4 region of the bacteria 16S rRNA genes was amplified using a set of barcoded fusion reverse primers and the same forward primer. 16S rRNA genes of distinct 16SV3-V4 regions were amplified using a pair of specific primers (515F: CCTAYGGGRBGCASCAG, and 806R: GGACTACNNGGGTATCTAAT) with barcodes. The PCR amplification was carried out in a 50-μL reaction containing Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and 10 ng template DNA. The PCR amplification was carried out under the following conditions: initial denaturation at 98 °C for 2 min, followed by 35 cycles of 94 °C for 30s, 55 °C for 30s, and 72 °C for 90s and final extension at 72 °C for 10 min. Then, quantification and quality of PCR products were assessed by mixing the same volume of 1× loading buffer (contained SYB green) with the PCR products, followed by running the mixture in electrophoresis on 2% agarose gel. Samples with a bright main strip between 400 bp–450 bp were selected for purification with Qiagen Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Then, the PCR products were adjusted to the same concentration and subsequently sent to the Genome Sequencing Company (Novogene, China) for microbial analysis. Paired-end libraries were generated with NEBNext® UltraTM DNA Library Prep Kit for Illumina and quantified via Qubit and Q-PCR and subsequently analysed by the Illumina HiSeq.

2.5. Data treatment and bioinformatic analysis

The obtained paired-end reads were assigned to each sample group based on their unique barcodes, followed by cutting off the barcode and primer sequences. Thereafter, paired-end reads were merged using FLASH V1.2.7 to produce raw tags (Magoc and Salzberg, 2011). Afterwards, the raw tags were filtered out under specific conditions to produce high-quality clean tags according to the Qiime V1.7.0 (Caporaso et al., 2010). The clean tags were compared with the reference database (SILVA database) using the UCHIME algorithm and produced the Effective Tags. All the effective tag sequences were then analysed using Uparse software v7.0.1090, and sequences with ≥97% similarity were assigned to the same operational taxonomical unit (OTU) for annotation. The annotation of each OTU into taxonomical classification was performed as previously described by Fan et al. (2019b). OTUs abundance information was firstly normalized using a standard sequence number corresponding to the sample with the least sequences. Subsequent analyses of alpha diversity and beta diversity were all performed based on this output normalized data. In the end, linear discriminant analysis effect size (LEfSe) analysis was performed to find the taxonomic biomarkers between sample groups. In addition, SPSS ver 23 software was used to compare sample groups, OTU richness, Observed species and Shannon index.

3. Results

The present study viewed the structure, diversity and uniqueness of bacterial communities associated with the guts of shrimps with different growth rates. Two sample groups of shrimps compared in the present study consisted of fast-growing shrimps (Group 1) and slow-growing shrimps (group 2). Starting from the same initial age (PL12), weight (~17 mg), receiving the same diets and sharing the same rearing environment, the white shrimps grew significantly different ($F = 86.67$, df5, 54 $p < 0.001$). The average initial weight, final weight, weight gain and specific growth rate of each sample group collected from three ponds were presented in Table 1. The shrimp guts were then dissected out from each group sample were then used for further bacterial community studies.

3.1. Raw read sequences and selected sequences for taxonomic designation

A total of 841,201 raw sequences were obtained from the six pooled samples (3 samples from Group 1 and 3 samples from Group 2) on Illumina paired-end platform. After quality filtering, 629,212 high-quality sequencings were selected and used for constructing the operational taxonomic units (OTUs). The summarizations of sequence numbers obtained in each step of data processing were presented in Table 2.

Of 629,212 high-quality sequences, 610,916 sequences (97.1%) were clustered into 902 operational taxonomic units (OTUs) with an identity threshold of 97%. While 18,296 sequences (2.9%) were classified as unique sequences consisting of 10,099 sequences from Group 1, and 8197 sequences from Group 2. Unique was defined as the number of sequences with a frequency of 1 and only occurs in one sample. In general, the average OTU richness across all samples was 551 OTUs, ranging from 535 to 608 in the gut of Group 1, and from 453 to 640 in the gut of Group 1 (Fig. 2).

3.2. Structure of core shrimp gut bacteria

To display the microbial community structure and diversity in the samples, taxonomic annotation corresponding to the OTUs was constructed. The result showed that 94.14% of the total sequences, across all the samples, were assigned into 14 phyla which were Proteobacteria, Actinobacteria, Bacteriodetes, Tenericutes, Firmicutes, Chloroflexi, Verrucomicrobia, Cyanobacteria, Chlamydiae, Planctomycetes, Fusobacteria, Hydrogenedentes, Deniococcus and Parcubacteria. While about 5.86% of the OTUs were placed in unclassified bacteria. Among these phyla, 8 taxa were considered predominant and core phyla as their abundances were more than 1% of the total sequences. The most abundant phylum was Proteobacteria occupying 53.99% of the total sequences, followed by Actinobacteria (23.67%), Bacteroidetes (4.97%), Tenericutes (3.62%), Firmicutes (2.97%), Chloroflexi (1.39%), and Verrucomicrobia (1.34%) (Fig. 3).

While at the genus level, a total of 181 genera could be detected across all shrimp groups. Of these, the top 100 genera were selected to construct the evolutionary tree using the aligned representative sequences. The relative abundance of each genus was displayed along with the genus in Fig. 4a. In addition, 35 of these 181 genera (19%) were considered as core gut bacteria of white shrimps as their prevalence >10 sequences across all samples (Fig. 4b). The top 13 most abundant genera were Illumatobacter occupying 21.5% of the total sequences followed by Ruegeria (7.1%), Candidatus Bacillopora (3.61%) Roseovarius (2.86%), Silicimonas (1.67%), Algoriphagus (1.48), and Haloferula (1.22%). While six other genera appeared to be (<1.0%), including Dinoroesobacter, Vibrio, Lactobacillus, Bdellovibrio, Shimia, and Robiginitalea.

3.3. Diversity and structure of bacteria within and between groups

The diversity of the bacterial community and specific taxa within the sample groups were performed by analyzing the single sample (Alpha diversity) which can reflect the richness and diversity of microbial communities within each sample. The result showed that OTUs richness and observed species in Group 1 (fast-growing shrimps) were significantly lower than that of Group 2 (slow-growing shrimps) ($p < 0.01$). Similarly, the Shannon index which represents species richness and evenness were also significantly lower in the high growth shrimp compared to the low growth shrimp ($t = 12.43$, df 5, $p < 0.01$) (Fig. 5). As the growth of white shrimp was highly associated with the animals' gut bacteria, we further detected the correlation between gut bacteria and the bodyweight of shrimp.

The beta diversity of the bacterial communities associated with the gut of white shrimps between groups was investigated through a PCoA shown in Fig. 5d. The first two components explain a total of 55.77% of the variation (PC1, 25.25%; PC2, 30.52%). The figure also showed that group 2 (slow-growing shrimps) are narrower dispersion compared to group 1, indicating more differences in the intra-group of fast-growing shrimps.

3.4. Bacterial signature of fast-growing shrimps

Bacterial compositions of the shrimp guts in both groups were shown in Fig. 6. The dominant phyla in Group 1 (relative abundance >5%) were Proteobacteria (53.95%) and Actinobacteria (26.78%), which were counted for over ~87% of the total gut bacteria. Meanwhile, the guts of slow-growing shrimps (Group 2) were dominated by Proteobacteria (53.83%), Actinobacteria (20.47%), Bacteriodetes 6.02%), and Tenericutes (5.23%), which accounted for 86% of the total gut bacteria. These results showed in general that Proteobacteria and Actinobacteria were the most abundant phyla in all shrimp gut regarding the growth rate. However other phyla including Bacteriodetes and Tenericutes appeared to be lower in the guts of fast-growing shrimps. Furthermore, Family Vibrionacea was detected to be higher in the slow-growing shrimps than the fast-growing shrimps, 1.31% and 0.22% respectively.

At the genus level, a total of 181 genera were identified from all shrimp guts. The most dominant genera (>5%) in the fast-growing shrimps (Group1) were Ilumatobacter (24.25%), followed by Ruegeria (6.21%). Similar trends were obtained from the guts of slow-growing shrimps (Group 2) where Ilumatobacter (18.44%) and Ruegeria (7.91%) became the most dominant genera. However, the differences were that Vibrio and Candidatus Bacilloplasma were more abundant in the guts of slow-growing shrimps, Fig. 7. Relative abundance of Vibrio was 1.3% in the slow-growing shrimps and only 0.22% in the gut of fast-growing shrimps. Similarly, Candidatus Bacilloplasma was more abundant in Group 2 than Group 1, 5.21% and 1.92% respectively.

Further analysis using linear discriminant analysis effect size (LEfSe) indicated that 49 OTUs were picked out with the Linear Discriminant Analysis (LDA) score > 2 (Fig. 8). Nine of the 49 OTUs were significantly higher in Group 1 (high-growth shrimps) than Group 2 (low-growth

Table 2
Raw and qualified sequence reads following Illumina-sequencing of the V3-V4 segment of the 16S rRNA gene.

Sample ID	Raw PE(#)	Raw Tags(#)	Clean Tags(#)	Effective Tags(#)	Base (nt)	Avg Leng(nt)	Q20	Q30
S.1 K	138,509	133,195	131,599	114,913	46,777,239	407	98.46	94.98
S.2 K	148,614	142,217	140,249	102,917	42,025,885	408	98.31	94.48
S.3 K	137,167	131,244	129,461	94,523	39,031,351	413	98.29	94.42
S·1B	131,879	127,098	125,572	112,775	45,882,666	407	98.44	94.87
S·2B	147,256	140,300	138,502	101,593	41,474,226	408	98.37	94.65
S·3B	137,776	132,252	130,062	102,491	41,986,494	410	98.29	94.44

Note: **Raw PE** represents the original PE reads after sequencing; **Raw Tags** represents tags merged from PE reads; **Clean Tags** represents tags after filtering; **Effective Tags** represents tags after filtering Chimera and can be finally used for subsequent analysis; **Base** is the number of bases of the Effective Tags; **AvgLeng** represents the average length of Effective Tags; **Q20** and **Q30** are the percentages of bases whose quality value in Effective Tags is greater than 20 (sequencing error rate is less than 1%) and 30 (sequencing error rate is less than 0.1%).

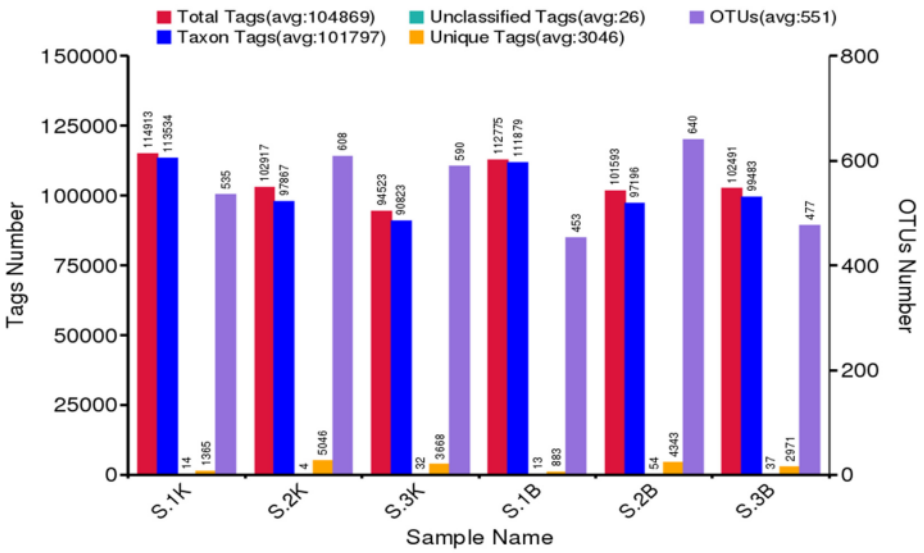

Fig. 2. Summarization of the tags and OTUs number from each sample (a pooled of 10 shrimp guts collected from 3 different ponds). S.1 K, S.2 K and S.3 K are gut bacteria of slow-growing shrimps. While, S·1B, S2B and S3B are gut bacteria of fast-growing shrimps. The Y1-axis titled "Tags Number" means the number of tags; Total tags"(Red bars) means the number of effective tags; Taxon Tags" (Blue bars) means the number of annotated tags; Unclassified Tags" (Green bars) means the number of unannotated tags; Unique Tags" (Orange bars) means the number of tags with a frequency of 1 and only occurs in one sample. The Y2-axis titled "OTUs Numbers" means the number of OTUs displayed as "OTUs" (Purple bars) in the above picture to identify the numbers of OTUs in different samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

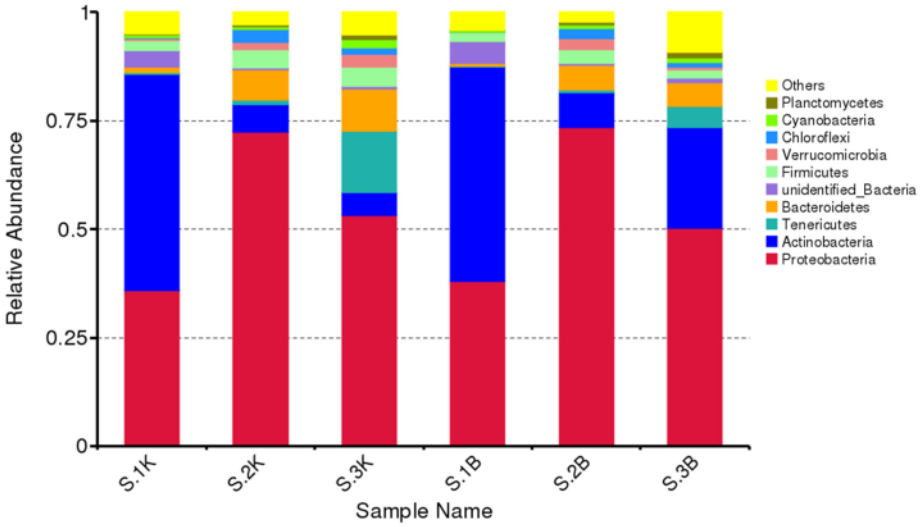

Fig. 3. Total proportional distributions of dominant phyla in guts of white shrimps at different growth rates. S.1 K, S.2 K and S.3 K are gut bacteria of slow-growing shrimps. While, S·1B, S2B and S3B are gut bacteria of fast-growing shrimps.

shrimps) which were OTU58, OTU74, OTU210 and OTU671, OTU186, OTU274, OTU218, OTU313 and OTU682. Seven OTUs belonged to Firmicutes (OTU58, OTU74, OTU210, OTU186, OTU274, OTU218, OTU313) followed by one Bacteroidetes (OTU671) and also one Proteobacteria (OTU682). The seven Firmicutes were identified as *Coprococcus comes* (OTU58), *Oscilibater* sp. ER4 (OTU74), *Acidaminicoccus intestini* (OTU210), unidentified Clostridiales (OTU186), *Oscilibacter* sp. (OTU274), *Peptococcus* sp. (OTU218), and *Clostridium phoceensis* (OTU313). While the Bacteriodetes was identified as *Bacteroidetes ovatus* (OTU671), and the other Proteobacteria was identified as *Legionella* sp. (OTU682). Meanwhile, 26 OTUs were identified higher in the low growth shrimps. The most 5 abundant OTUs were identified as *Facalibacterium* sp. (OTU 85), *Blantia* spp. (OTU61), *Dorea longicantena* (OTU48), *Prevotella* sp. (OTU145), and *Ruminococcus bromii* (OTU82).

4. Discussion

The growth of cultured animals including white shrimps,

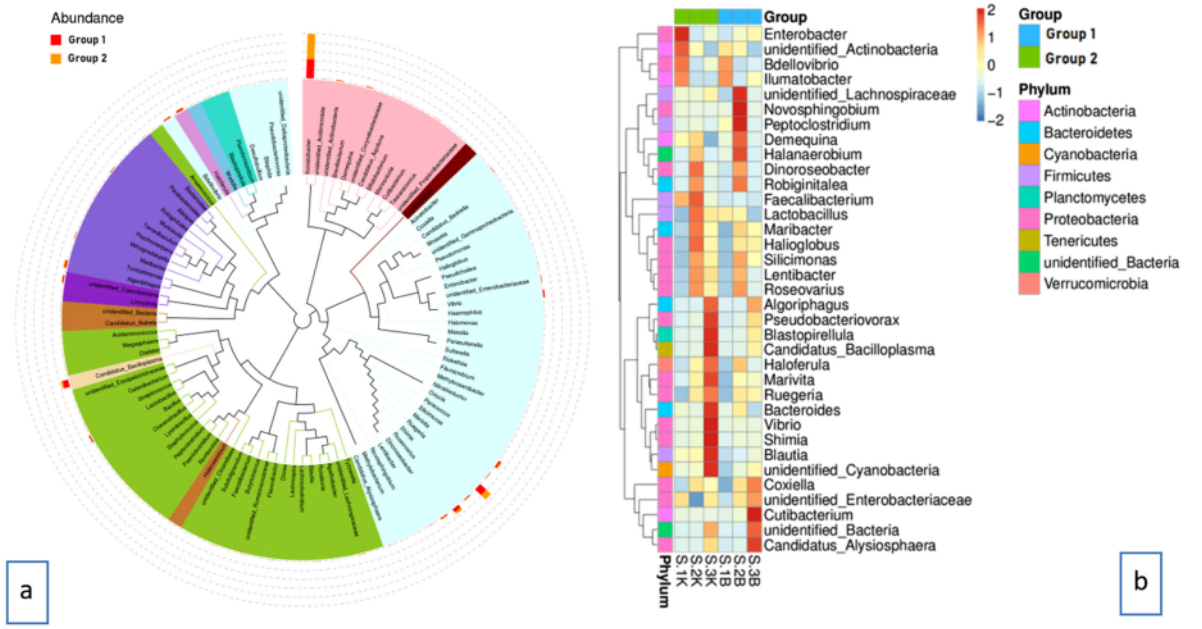

Fig. 4. Prevalence of core bacterial genera in the gut of white shrimps, *L. vannamei*. S·1B, S·2B and S·3B are Group 1 or shrimps with a high growth rate. S.1 K, S.2 K and S.3 K are Group 2 or shrimps with a slow-growth rate. a). The 100 most abundant genera associated with guts of slow and fast-growing shrimps cultured in intensive ponds. b) core bacterial genera in the gut of white shrimp cultured in intensive ponds.

L. vannamei are determined in general by host-related factors (Kooloth Valappil et al., 2021; Uengwetwanit et al., 2021) and environment-related factors (Mustafa Kamal et al., 2018). The host-related factors are those factors that come from internal organisms such as the presence and expressions of growth-promoting genes, hormone productions etc. While environmental-related factors include water quality, diets and also microbial community associated with the gut tract of the cultured animals. Among the environmental-related factors, the gut microbiota has gained considerable interest from aquatic microbiologists due to their significant contributions to the growth of their hosts via various mechanisms including digestive enzyme production for diet digestion (Amin, 2018), excreting vitamins (Chen et al., 2017) and producing short-chain fatty acids (SCFAs) (Hoseinifar et al., 2017). Many studies viewed a strong correlation between gut bacteria and the growth performances of their animal hosts. However, studies investigating the specific correlation between shrimp growth and gut bacteria are still very limited. Thus, the present study reported the structure and diversity of core gut bacteria in white shrimp shared the same environmental condition such as ponds and diet but having different growth rates. In addition, the bacterial signatures of high growth shrimps were also viewed. We found that structure and composition of gut bacteria can be linked with white shrimp growth, which may highlight the significant contribution of gut bacteria to shrimp growth. To the authors' knowledge, this is the first study to report bacterial signatures in the gut of high growth shrimps cultured in commercial ponds.

4.1. Core bacteria in the guts of white shrimp

At the phyla level, we identified at least 15 phyla across all gut samples. The most 7 abundant phyla were Proteobacteria occupying 53.99% of the total sequences followed by Actinobacteria (23.67%), Bacteroidetes (4.97%), Tenericutes (3.62%), Firmicutes (2.97%), Chloroflexi (1.39%), and Verrucomicrobia (1.34%). The domination of proteobacteria in shrimp guts was also previously reported by several authors. Gao et al. (2019) reported on white shrimp culture in China that Proteobacteria occupied 70% of gut bacteria at the age of 30 days, and even higher at the age of 60 days (95.5%). Similarly, He et al. (2020) and Zhang et al. (2019) reported the domination of Proteobacteria in the intestine of ~9.8 g white shrimps, which was accounted for 44.12% and 38.94% respectively. Zoqratt et al. (2018) reviewed the composition of gut bacteria in white shrimps cultured in commercial ponds of Vietnam and Malaysia and viewed that 50–85% of total bacterial communities were Proteobacteria, followed by Actinobacteria, Bacteriodetes and Fusobacteria. Furthermore, Hasyimi et al. (2020) found Proteobacteria to be the most abundant in the intestine of white shrimp cultured in Indonesia, followed by Bacteroides and Firmicutes in the second and third place respectively. Fan et al. (2019a) has also documented the domination of Proteobacteria in the gut tract of white shrimp cultured in China even though a little bit lower (40.83%), followed by Bacteriodetes (19.96%), Verrucomicrobia (8.26%), Firmicutes (6.17%) and Actinobacteria (1.59%). In addition, Huynh et al. (2019) found that intestines of white shrimps were dominated by Proteobacteria regardless of their culture systems (86.6% in indoor ponds, and 51.8% in outdoor ponds. Fan et al. (2019b) also reported that the gut tract of white shrimp was dominated by Proteobacteria regardless of their culture environment (fresh water and marine water). Proteobacteria appears to be always the top abundances, while other phyla such as Actinobacteria, Firmicutes, Bacteriodetes or Verrucomicrobia seemed to vary according to the culturing system and geographic locations. These results can be strong evidence that Proteobacteria are part of the core bacterial phylum and the most abundant in the gut of white shrimp regardless of their life stages, culture system and geographic locations.

In terms of the genus, the bacterial community associated with the gut of shrimp appeared to vary and seems to be influenced by many factors including age or life stages, diets, culture system and locations (Fan et al., 2019a; Fan et al., 2019b; Gao et al., 2019; Huynh et al., 2019; Hasyimi et al., 2020). The present results revealed 181 genera across all shrimp guts, but only 35 genera (19.34%) were considered to be the core microbiome of white shrimps regarding their prevalence in all samples. Among the 35, the most abundant genera were Illumatobacter (21.5%),

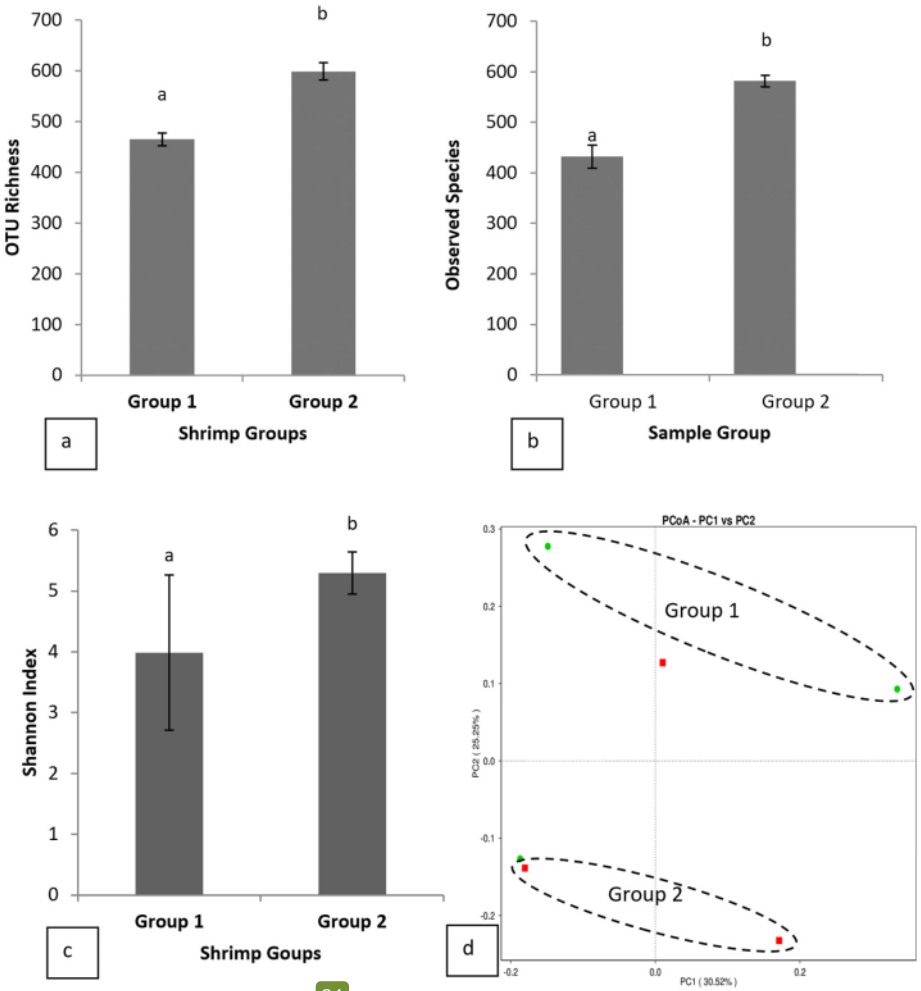

Fig. 5. Comparison of OTUs richeness (a), observed species (b), Shannon index (c), and Principal Coordinates Analysis (PCoA) (d) based on Unweighted Unifrac distance of shrimps at different growth rates of the bacterial community associated with the guts of fast-growing shrimps (Group 1) and slow-growing shrimps (Group 2). Different superscripts represent the average values were significantly different, $p < 0.05$.

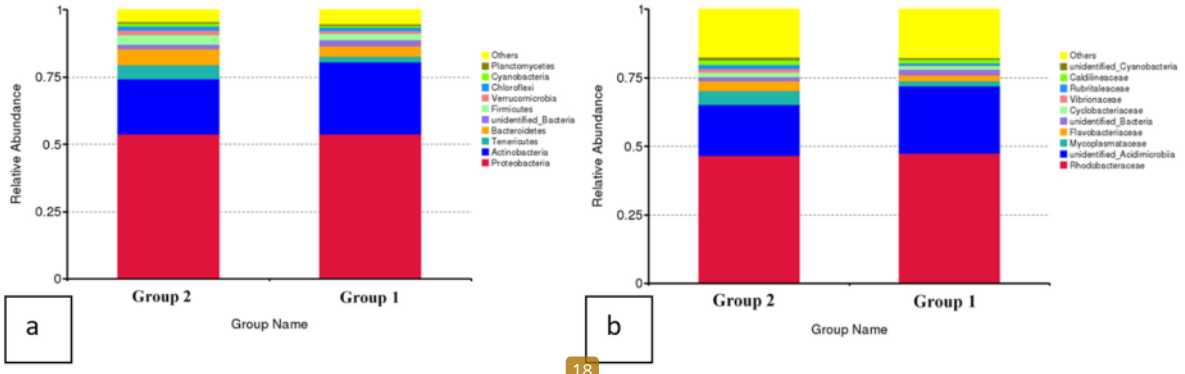

Fig. 6. Taxa relative abundance in phylum obtained from two sample groups. Relative abundance of the top 10 bacterial communities in the gut of *L. vannamei*. (a) phylum level. (b) Family level. Two samples groups were shown. The unclassified sequences or sequences that could not be classified into any known groups were assigned as 'Others'. Group 2 is slow-growing shrimps and Group 1is high growth shrimps.

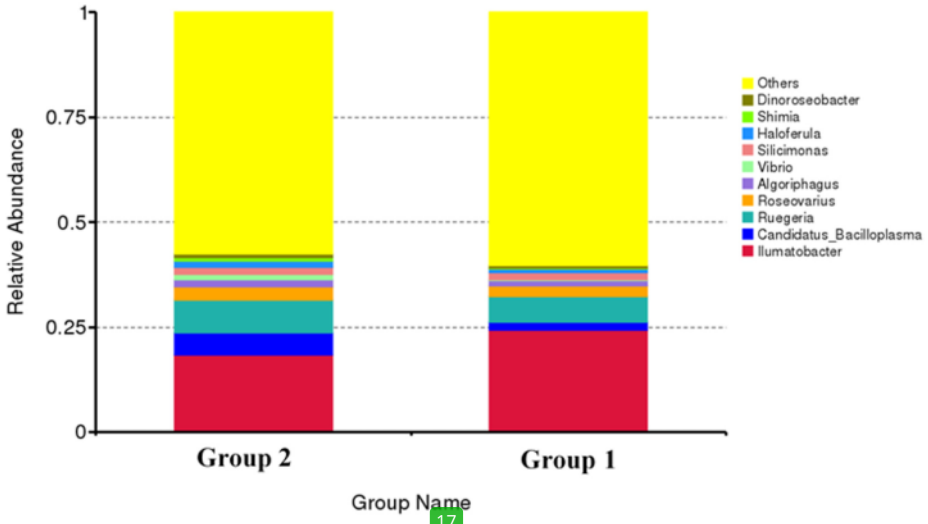

Fig. 7. Taxa relative abundance in genera obtained from two sample groups. Relative abundance of the top 10 bacterial communities in the gut of *L. vannamei*. Two samples groups were shown. The unclassified sequences or sequences that could not be classified into any known groups were assigned as 'Others'. Group 2 is slow-growing shrimps and Group 1 is fast-growing shrimps.

Ruegeria (7.1%), Candidatus Bacillopora (3.61%) Roseovarius (2.86%), Silicimonas (1.67%), Algoriphagus (1.48), and Haloferula (1.22%). Menawhile, Dinoroesobacter, Vibrio, Lactobacillus, Bdellovibrio, Shimia, and Robiginitalea (<1.0%). The present results were quite different from what had been reported by Gao et al. (2019) where the most abundant genera were Vibrio (31%), Photobacterium (23.9%) and Candidatus Bacillophora (7.6%). Different dominant genera were also previously reported from shrimp cultured in Indonesia by Hasyimi et al. (2020) in which the most 5 abundant genera were Nocardiodes, Pelotunomonas, Spongiimonas, Desulfopila and Bryobacter. Another study performed by Huynh et al. (2019) showed that the most abundant genera were Ruegeria, Desulfovibrio, Shewanella, Vibrio and Nitrospira. All these data strongly suggest that the proportion of gut bacteria in terms of the genera is highly variable. However interesting finding in the present study is that few genera such as Ruegeria, Candidatus Bacillopora, Lactobacillus were also commonly reported as members of dominant genera in white shrimps.

4.2. Bacterial signatures in the gut of high growth shrimps

Bacterial diversity represented by the Shannon index and species richness of bacteria detected from the gut shrimps were significantly lower in the gut of high growth shrimp (Group 1) compared to that of the slow growth shrimp (Group 2). These results may suggest that less diversity and species richness in the intestinal tracts are better especially relating to the growth rate. A similar conclusion was proposed by Daniels et al. (2013) revealed there was an increase in the species richness, evenness and Shannon index in shrimp guts with a high growth rate. However, the present study result is also contradictory to what has been previously reported by Fan et al. (2019a) in which white shrimp with higher growth tended to associate with a higher Shannon index. Meanwhile, Huynh et al. (2019) concluded that there was no significant correlation between the Shannon index and the growth of white shrimps as the average values of the Shannon index between high growth shrimp and slow growth shrimps were not significantly different. These data may indicate that there are other factors besides species richness and diversity of gut bacteria to signify the growth of white shrimps including the dominant genus or species level.

Many papers reported that the growth of cultured animals is frequently associated with certain bacterial species in their intestinal tract (Daniels et al., 2013; Fan et al., 2019a; Huynh et al., 2019; Amin et al., 2020). Thus, the present study investigates if there are any differences in more specific level of bacteria such as genus or species by using a linear discriminant analysis effect size (LEfSe). The results showed that at least 9 bacterial species were significantly higher in fast-growing shrimps (Group 1) which were *Coprococcus comes* (OTU58), *Oscillibater* sp. ER4 (OTU74), *Acidaminicoccus intestini* (OTU210) and *Bacteroidetes ovatus* (OTU671), *Oscilibacter* sp. (OTU274), *Peptococcus* sp. (OTU218), *Clostridium phoceensis* (OTU313), *Legionella* sp. (OTU682), and unidentified Clostridiales (OTU186). Eight of the nine unique species belonged to Firmicutes and the other species belong to Bacteriodetes. Firmicutes is the third dominant phyla in the gut of high growth shrimps (Group 1) but less dominant in slow growth shrimp (occupied by Tenericutes). As many aquaculture probiotics belong to Firmicutes (Wang et al., 2019), these members of Firmicutes might also play a significant role in the growth of white shrimps, though further investigation is still required.

Among the nine bacterial signature species of high growth shrimp, only a few species have been described in several published papers. For instance, a study by Tran et al. (2020) reported that *Coprococcus comes* was described as a fermentative bacteria in white shrimp which involved in the production of short-chain fatty acids (SCFAs), through the fermentation of carbohydrates. SCFAs as butyric acid, acetic acid and propionic acid have been described as very important compounds used in energy homeostasis, metabolism and maintenance of gut health (Chen et al., 2020). Thus, the SCFAs have been frequently used as feed additives as a way to improve growth performances, feed digestibility and immune system of many aquatic animals including white shrimps (Silva et al., 2016) and common carp, *Cyprinus carpio* (Liu et al., 2014). The bacterial species (*C. comes*) was also reported as a part of commensal microorganisms in humans and also have been reported to produce butyric acids (Anand et al., 2016). These results may suggest that *Coproccus comes* (OTU58) contribute to the white shrimp growth through its capacity to produce SCFAs, therefore should be further investigated for white shrimp probionts.

Another unique species is *Bacteroidetes ovatus* (OTU671). This species has also been reported to be a beneficial member of Bactiodetes due to possessing a xyloglucan-polysaccharide utilization loci gene which

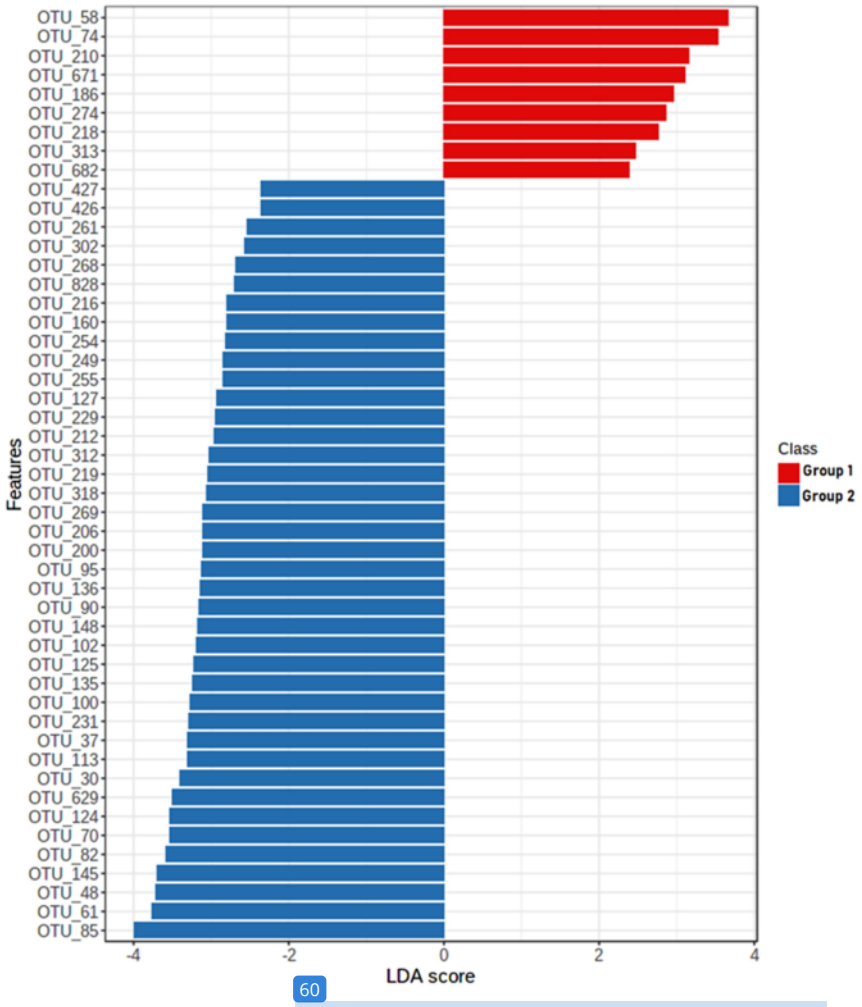

Fig. 8. Phylotype of gut bacteria corresponding to growth rate identified using Linear Discriminant Analysis Effect Size (LEfSe) analysis. The histogram is the linear discriminant analysis (LDA) score for featuring OTUs abundance between the 2 Groups. Group1 is fast-growing shrimps and Group 2 is slow-growing shrimps.

provides the ability to utilize polysaccharides (Tang et al., 2017). Another study by Larsbrink et al. (2014) had also confirmed the presence of a complex gene locus capable of hydrolysing xyloglucan to component monosaccharides for further metabolism in *B. ovatus*. Xyloglucans are a ubiquitous material constructing plant cell wall polysaccharides such as micro or macroalgae and convert for beneficial nutrition (Larsbrink et al., 2014). Similarly, Rogowski et al. (2015) identified that *B. ovatus* detected from the human gut has a capacity to synthesize enzymes for degrading complex polysaccharides such as glycan in an extensive array of both plant and animal-based diets and other complex carbohydrates. Furthermore, Tamura et al. (2017) describe *Bacteroidetes ovatus* as a member of gut Bacteroidetes and possess the ability to utilize mixed linkage beta glucans, major health-promoting cereal polysaccharides in humans. To the author knowledge, this is is the first study to report the presence of *Bacteriodetes ovatus* from the gut of white shrimps. Acknowledging the capability of the bacterium, it may suggest that this bacterium is a potential probiont for white shrimps therefore it should be further investigated.

Another unique species identified to be dominant in the gut of high growth shrimps was *Peptococcus* sp. This bacterium appears to be quite rare since authors could find only one published paper up until now. The paper which was reported by Shahina et al. (2012) described *Peptococcus* sp. isolated from shrimp as an anaerobic bacterium, and having the capacity to synthesize protease. While the rest of the unique species including *Oscillibater* sp. ER4 (OTU74), *Acidaminicoccus intestini* (OTU210) *Oscilibacter* sp. (OTU218), *Clostridium phoceensis* (OTU313), and *Legionella* sp. (OTU682) are not common to be isolated or detected, therefore information on these bacteria are very limited.

The finding of nine unique bacterial species can be considered as the main signature of gut bacteria in high growth shrimps. All these bacteria are novel species and were firstly reported to be detected in the gut of white shrimps. The present results may contribute to the development of novel probiotic strains or prebiotic compounds to increase the growth of white shrimp. The development of probiotics can be done through isolating these unique bacteria and confirming their metabolic activity in vitro and in vivo (Amin et al., 2017; Amin, 2018). The advantage of this approach preserves the isolate and just reculture them whenever are needed. However, the main challenge for this approach was the difficulty in getting the isolates. As many experimental microbiologists estimate that the total bacteria which can be cultured in the laboratory with the currently available culture media is less than 2% (Wade, 2002). This may suggest that we need to develop a new culture media in order

to grow such bacteria. While the other approach is by stimulating the growth of these bacteria by providing suitable nutrient compounds, generally known as prebiotic (Fuandila et al., 2020; Hasyimi et al., 2020). With this approach, we do not have to isolate or culture the targeted bacteria but only screen some prebiotic compounds to find the best compounds which can stimulate the growth and domination of bacterial targets in the gut of white shrimp. With all advantages and disadvantages, it is highly recommended to use a combination of both approaches in order to increase the chances of getting better results.

5. Conclusion

We identified at least 35 genera (19.34%) belonged to 15 phyla which were considered to be the core microbiome of white shrimps regarding their prevalence in all samples. The most 14 abundance were Illumatobacter, Ruegeria, Candidatus Bacillopora, Roseovarius, Silicimonas, Algoriphagus, Haloferula, Dinoroesobacter, Vibrio, Lactobacillus, Bdellovibrio, Shimia, and Robiginitalea. In addition, a strong association between diversity and species richness of gut bacteria and the growth of white shrimp, reinforcing the close relationship between gut bacteria and host growth. The reason might be the number of nine bacterial species significantly higher in high growth shrimps (Group 1): *C. comes* (OTU58), *Oscillibater* sp. ER4 (OTU74), *Acidaminicoccus intestini* (OTU210) and *Bacteroidetes ovatus* (OTU671), *Oscilibacter* sp. (OTU218), *Peptococcus* sp. (OTU218), *Clostridium phoceensis* (OTU313), *Legionella* sp. (OTU682), and unidentified Clostridiales (OTU186). However, due to the novelty of the shrimp gut bacteria, further detection and culture of these gut bacteria will be necessary to understand their specific roles in the shrimp gut in terms of growth.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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