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Determination of bacteria in *Corbicula fluminea* tissue using 16S rRNA gene sequences

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***Corbicula fluminea* is a highly consumed traditional snack by the locals in Kelantan, Malaysia. However, the consumption is suspected to be a cause of diarrhoeal disease that originated from bacterial contamination in *C. fluminea* tissue. Poor handling and unhygienic processing were hypothesised to contribute largely to the bacterial contamination. Hence, this study aims to compare the bacterial community in *C. fluminea* tissue at each processing stage to determine the root of contamination source in *C. fluminea* tissue. Polymerase chain reaction was performed to amplify the hypervariable regions V3–V4 of the 16S rRNA gene from bacterial DNA isolated from *C. fluminea* tissue. The communities at each processing stage were sequenced on the Illumina Miseq platform and then analysed using the Divisive Amplicon Denoising Algorithm 2 pipeline. The sequencing regions were examined using the Silva v132 reference database. A total of 4 195 108 raw reads were obtained from six bacterial DNA samples of *C. fluminea*, and the assembled reads were assigned to 888 amplicon sequence variants. Proteobacteria (87.8%), Firmicutes (8%) and Bacteroidetes (3.1%) were the most dominant groups at the phylum level, while *Aeromonas* (47%), *Klebsiella* (15.7%) and *Enterobacter* (10.1%) were predominant at the genus level. The presence of these pathogenic bacteria in the *C. fluminea*, especially in the smoking and selling stages indicate unhygienic handling by human during preparation and selling stages and this could pose a health risk to consumers.**

1. Introduction

Corbicula fluminea, which is also known as *etok* or *etak* in Kelantan, Malaysia, is a freshwater clam that originated from Asia. It is treated as a popular snack among the Kelantanese when watching television, studying and chatting – a consumption habit that has been passed on from one generation to the next. *C. fluminea* has been greatly harvested to fulfil the demand of the locals (Aweng, 2012). Recently, this clam, which is mostly sold in wet markets and the street, has become one of the most important sources of income for the locals in Kelantan (Aweng and Kutty, 2018).

C. fluminea is able to survive in freshwater rivers, including brackish and estuarine waters (Zhivoglyadova and Revkov, 2018). The clam can be found in various kind of substrates, particularly soft clay, fine sand and coarse sand substrates (Kramer-Wilt, 2008). Because it is sensitive to even slight changes in microclimate, its population density and distribution were affected by temperature; salinity; hypoxia; eutrophication; bacteria, viruses and parasites; substrate size; predators; and genetic changes (Deng *et al*., 2005; Foster *et al*., 2017; Aweng *et al*., 2018; Castañeda *et al*., 2018). A high level of contamination inhibits the growth and even increases the mortality of *C. fluminea* (Cataldo *et al*., 2001; Shoults *et al*., 2009).

C. fluminea is well recognised for its nutritive value and pharmacological function (Centre for Agriculture and Bioscience International, 2018; Chijimatsu *et al*., 2011; Liao *et al*., 2013). However, three processing stages (the fresh stage, the smoked stage and the selling stage) of *C. fluminea* in Kelantan are suspected of contributing to bacterial contamination. The fresh stage involves the natural environment inhabited by *C. fluminea* in most of the rivers in Malaysia including the Kelantan River, Perak River and Terengganu River. Some of the rivers are polluted by the industrial wastewater and municipal discharges (Ab Razak *et al*., 2015). This clam has potential in biosorption of bacteria in water columns and sediments into their soft tissue. In this study, fresh *C. fluminea* sold in Pasir Mas and Tumpat, Kelantan, is imported from Perak, Malaysia. In the smoked stage, fresh *C. fluminea* undergoes a smoking process to remove harmful microorganisms and enhance their flavour. In smoking process preparation, the locals marinate the clam with ingredients such as sugar, salt, blended lemongrass, shallots, monosodium glutamate, ginger and garlic for 2 h. Then, the clam is smoked on a bamboo platform on top of the firewood for 15 to 30 min. In the selling stage, the sellers transfer the smoked *C. fluminea* into their selling sites that are located on the street with low-cost seating facilities. The unhygienic condition in smoking processing sites and selling sites increases the risk of foodborne bacterial contamination and

diseases. Lately, local newspapers have reported that *C. fluminea* consumption caused diarrhoea outbreak in Kelantan.

Several studies on the microbiological safety and quality of bivalves, especially oyster, have been carried out (Froelich and Noble, 2016; King *et al*., 2012; Travers *et al*., 2015). However, data on the prevalence of pathogenic bacteria in *C. fluminea* are limited. The bacterial community in *C. fluminea* tissue is yet to be elucidated. To our knowledge, no studies on the changes of the bacterial community for each processing stage of *C. fluminea* have been conducted. Hence, the objective of this study is to assess the bacterial community using metagenomics analysis on 16S rRNA of bacterial DNA in *C. fluminea* tissue at each processing stage in Kelantan. The bacterial community was obtained from the soft tissue surface of *C. fluminea* in order to evaluate the changes of the bacterial community at each processing stage. Besides, bacteria obtained from the soft tissue surface are able to reduce the bioprocess of bacteria in *C. fluminea* (Gomes *et al*., 2018). The 16S rRNA gene sequencing was used due to its high accuracy in identifying the bacterial species (Srinivasan *et al*., 2015).

2. Materials and methods

2.1 Sample collection

Fresh, smoked and sold *C. fluminea* samples were purchased from the six major *C. fluminea* sellers located within Pasir Mas (06°02′59″N, 102°10′09″E) and Tumpat (06°08′48″N, 102°13′06″E), Kelantan, in March and June 2018. The smoked samples were purchased promptly after the completion of the smoke processing, while the selling samples were purchased after they were exposed for 3 h on the street-side vendor's kiosk. Approximately 100 g of *C. fluminea* samples was collected for each stage. Samples were collected in zip-lock bags, preserved below 4°C during the transportation to the laboratory and tested once arrived in the laboratory.

2.2 Temperature of the smoking process

Temperatures of the smoking platform were identified using a cooking thermometer during the smoking process.

2.3 Bacterial DNA extraction

Fifteen pieces of *C. fluminea* were randomly chosen. The shell of *C. fluminea* was opened using sterilised forceps and a scalpel blade. After the shell was opened, the clam was held firmly using forceps, and the soft tissue of the clam was flushed with sterilised water. The water was collected using a sterilised glass petri dish (150 × 20 mm). These steps were repeated for all 15 clams for each processing stage. The flushed bacteria in the collected water were subjected to DNA extraction in triplicates following the modified cetyltrimethylammonium bromide protocol (Dee *et al*., 2020). The extracted bacterial genomic DNA samples were pooled together and stored at −20°C.

2.4 PCR amplification and sequencing

The hypervariable regions V3–V4 of the 16S rRNA gene from the bacterial DNA of *C. fluminea* were amplified using universal primer sets 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) (Fadrosh *et al*., 2014). Polymerase chain reaction (PCR) was performed in triplicates using 10 µl reaction with 1.6 µl PCR buffer, 0.75 µl magnesium chloride ($MgCl_2$; 25 mM), 0.25 µl deoxynucleoside triphosphate (25 mM), 0.5 µl primers (10 mM), 1 µL DNA template (150 ng/µl) and 0.3 µl DNA taq polymerase (Promega, USA).

The amplification program was set as 98°C for 30 s (initial denaturation), 30 cycles of 98°C for 15 s (denaturing), 58°C for 15 s (annealing) and 72°C for 15 s (extension) and then kept at 72°C for 1 min (final extension). The amplified PCR products were examined using 1.2% gel electrophoresis and purified using the Qiaquick® Gel Extraction Kit (Qiagen, Germany) (Abraham *et al*., 2017).

The DNA was then quantified using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific). The purified products were pooled together for each processing stage and sent to the commercial laboratory in dry ice condition. The library preparation and high-throughput sequencing were performed based on the protocol from Illumina Miseq (2 × 300 bp) in 100k tags. The steps from bacterial DNA extraction to PCR amplification and sequencing are summarised in Figure 1.

2.5 Bioinformatics analysis

The raw sequences were assessed using FastQC v0.11.3 and pre-processed for quality control such as removing the adapters, artefacts and low-quality bases (<Qv20). Reads longer than 480 bp were removed based on the estimated hypervariable region size of 460 bp. The pre-processed reads were imported into Quantitative Insights into Microbial Ecology (Qiime 2 v2018.8) (Bolyen *et al*., 2018). After that, a Divisive Amplicon Denoising Algorithm 2 pipeline was used to process the input reads into exact amplicon sequence variants (ASVs) (Callahan *et al*., 2016). Then, a classifier was adopted for classifying the ASVs into their taxonomic groups based on the reference database Silva v132 (Quast *et al*., 2012). The mitochondria, chloroplast, archaea and unassigned taxa were filtered and removed.

The diversity analysis was performed using Qiime 2 v2018.8. The taxonomic composition and alpha diversity (Chao1 and Shannon indices and Faith's phylogenetic diversity [PD]) were determined (Faith, 1992; Sarma and Das, 2004). Also, the taxonomic beta diversity (unweighted and weighted Unifrac indices) was calculated as the diversity of the bacterial community between the fresh samples, smoked samples and selling samples. The rarefaction curves and bar chart of species composition using Chao1 indices were constructed by Qiime 2. Besides, the principal coordinate analysis (PCoA) plots and heatmap were produced from the unweighted and weighted Unifrac indices between the fresh stage, smoked stage and selling stage. The analysis of shared and unique ASVs at the processing stages was performed using Venn diagrams.

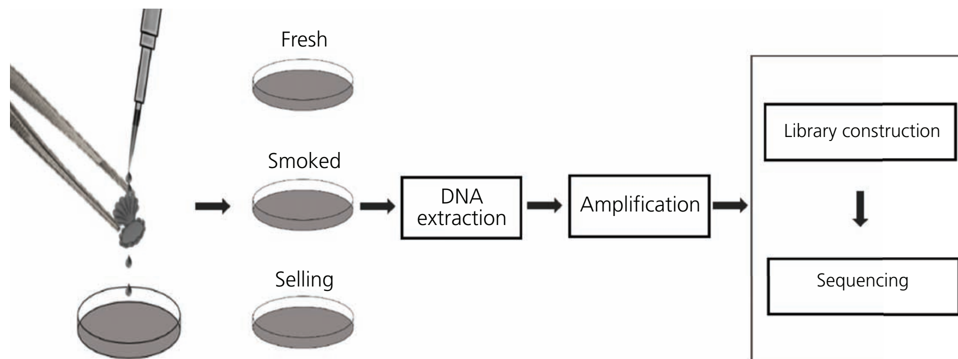

Figure 1. Overview of experimental design. A schematic diagram that shows how *C. fluminea* was processed for the high-throughput sequencing on the 16S rDNA gene

3. Results

3.1 ASVs of the bacterial community

The total throughput for the six samples was 4 195 108 raw reads obtained from the V3 to V4 hypervariable regions of the 16S rRNA gene. By pre-processing, denoising and removing chimeras, 194 417 to 263 443 sequences were collected from each sample, resulting in a total of 1 439 520 sequences from all samples. All sequences were then classified into 888 ASVs based on the reference in the Silva v132 database (Quast *et al*., 2013). The taxonomy numbers of ASVs for all samples, namely Fresh A, Smoked A, Selling A, Fresh B, Smoked B and Selling B, are tabulated in Table 1. The ASV numbers of the bacterial community from the phylum level to the genus level are presented in Table 2.

3.2 Alpha diversity of bacterial community composition

The alpha diversity represents the mean number of bacteria within a single sample. The Chao1 index accounts for bacterial richness, while the Shannon index accounts for both bacterial abundance and richness. Faith's PD is expressed as the minimum length of phylogenetic branches required to span the taxa of the phylogenetic tree. Larger PD values indicated greater expected feature diversity (Faith and Baker, 2007). The results show that the fresh samples have the largest alpha diversity indices, followed by the smoked samples and the selling samples (Table 3).

Table 1. The description and ASVs found in *C. fluminea* tissue for each sample

Samples	Sampling month	Sample description	ASVs
Fresh A	March	Fresh *C. fluminea* in the first sampling	331
Smoked A	March	Smoked *C. fluminea* in the first sampling	205
Selling A	March	Selling *C. fluminea* in the first sampling	158
Fresh B	June	Fresh *C. fluminea* in the second sampling	420
Smoked B	June	Smoked *C. fluminea* in the second sampling	418
Selling B	June	Selling *C. fluminea* in the second sampling	336

Table 2. The ASVs of the bacterial community in *C. fluminea* from the phylum to genus levels

Samples	Phylum	Class	Order	Family	Genus
Fresh A	14	19	49	74	116
Smoked A	15	20	44	57	83
Selling A	17	23	40	55	76
Fresh B	19	33	64	94	147
Smoked B	19	28	60	95	149
Selling B	17	30	66	104	146

The new discovered ASVs decreased at 30 000 reads, and the rarefaction curve approached a saturation plateau (Figure 2). This indicated that the sequencing depth was sufficient to estimate most bacterial diversity in *C. fluminea* (Schöler *et al*., 2017). The alpha diversity of Fresh B is higher than that of Fresh A, and this could be attributed to the increased rainfall in their habitat in June. This is supported by Wang *et al*. (2019) as they found that the alpha index was significantly higher in the wet environment than in the dry environment.

All sequences were identified into 25 phyla, ranging from 14 to 19 phyla per sample. Phyla with a relative abundance of >0.1% are presented in the bar graph of species distribution (Figure 3).

Table 3. Alpha diversity indices of the bacterial community in *C. fluminea* tissue at different processing stages

Samples	Chao1	Shannon	Faith PD
Fresh A	331.233	4.724	18.147
Smoked A	205.000	4.314	12.828
Selling A	158.267	3.319	13.211
Fresh B	420.133	5.491	23.691
Smoked B	418.025	5.456	22.099
Selling B	336.000	4.220	22.415

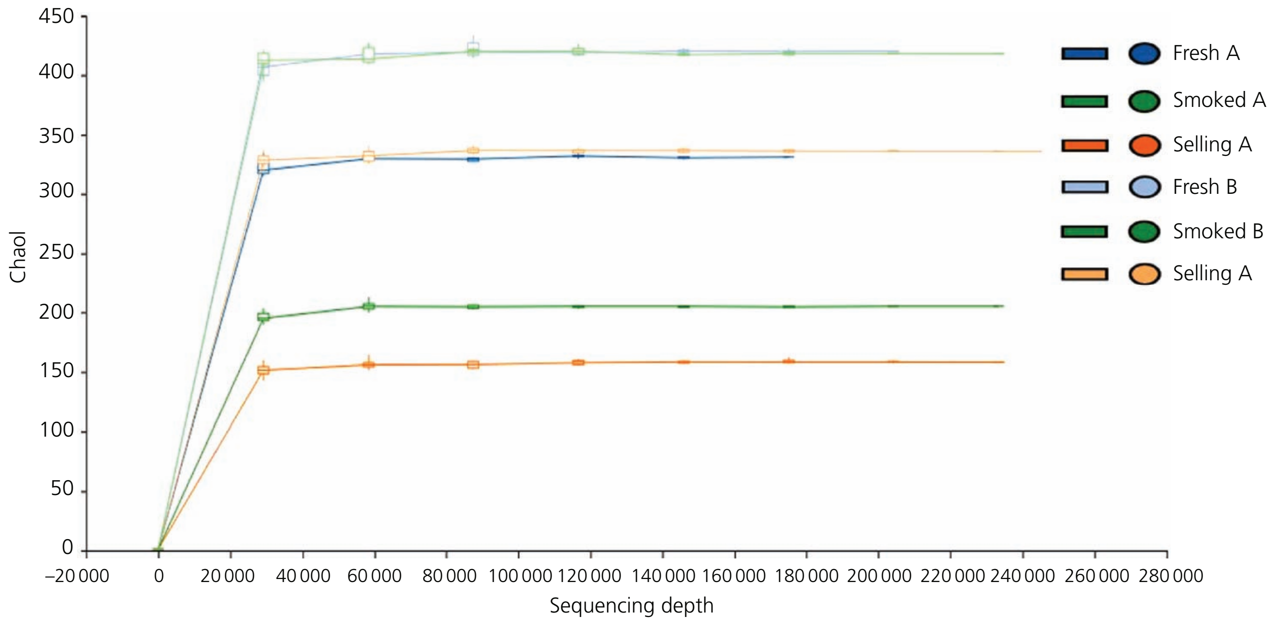

Figure 2. Alpha rarefaction curve based on the Chao1 index

16S rRNA gene sequences recovered from the *C. fluminea* samples fell into three main phyla, namely Proteobacteria (87.7%), Firmicutes (8.0%) and Bacteroidetes (3.1%), which contributed to 98.8% of the total sequences.

At the genus level, the sequence results for all the samples were assigned to 287 genera, ranging from 76 to 149 genera per sample, which showed a large bacterial community across all samples of *C. fluminea*. The relative abundances of the bacterial community in the *C. fluminea* samples at the genus level (i.e. >0.1%) were constructed (Figure 4). The samples were dominated by nine major genera, where the highest number of genera was detected in Smoked B (149) and the lowest number of genera was found in Selling A (76). Meanwhile, the top three genera that are present in all the samples are *Aeromonas*, *Klebsiella* and *Enterobacter* with values of 47, 15.7 and 10.1%, respectively, amounting to

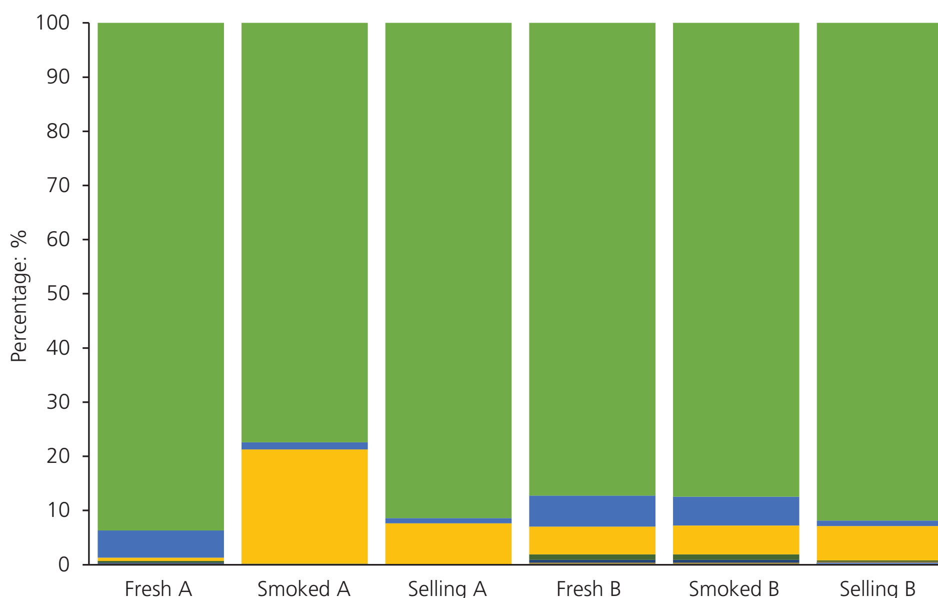

Figure 3. Taxonomy bar plot at the phyla level with a relative abundance of >0.1%

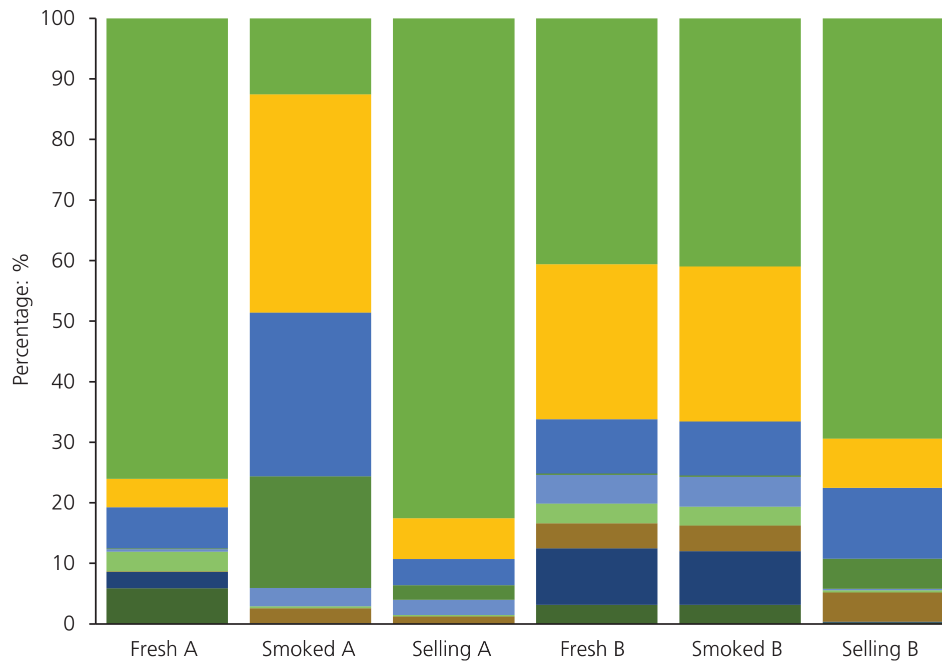

Figure 4. Taxonomy bar plot at the genus level with a relative abundance of >0.1%

72.8% of the total bacterial community. This is consistent with previous studies by Wangkahad *et al*. (2015), who found similar bacteria genera in the river water, and Dey *et al*. (2018), who reported the presence of these bacteria in street foods.

The relative abundance of *Aeromonas* spp. was 68.3% in Fresh A, which was reduced sharply to 11.6% in Smoked A. After being sold for 3 h on the street, *Aeromonas* spp. increased sharply to 78.5% in Selling A. For the second sampling, the relative abundance of *Aeromonas* spp. in Fresh B (33.5%) maintained in Smoked B (34.1%), but it sharply increased in Selling B (57.4%). This showed that the selling stage always has a higher relative abundance of *Aeromonas* spp.

In the first sampling, both *Enterobacter* spp. and *Klebsiella* spp. were high in Smoked A (25.0% and 33.3%, respectively) compared to Fresh A (6.1% and 4.1%, respectively) and Selling A (4.2% and 6.4%, respectively). This trend also could be observed in the relative abundance of *Escherichia-Shigella* spp., *Lactococcus* spp. and *Bacillus* spp., which are much prevalent in Smoked A. The relative abundances for *Enterobacter* spp. and *Klebsiella* spp. were reduced in the selling stage due to the increasing relative abundance of the *Aeromonas* spp. contamination.

3.3 Shared and unique bacteria community

A comparison of the numbers of shared and unique ASVs is presented using Venn diagrams in Figure 5. The number of shared ASVs for Fresh A, Smoked A and Selling A was 79, accounting for 16.7% of the total number of shared and unique ASVs. The unique ASVs varied from 42 to 215, where Fresh A was found to have the highest number and Smoked A and Selling A the reduced numbers with values of 215, 76 and 42, respectively. In the second sampling, the number of shared ASVs for Fresh B, Smoked B and Selling B was 158, accounting for 23.1% of the total number of shared and unique ASVs. The unique ASVs varied from 101 to 147, where Fresh B has the lowest number and Smoked B and Selling B have the slightly increased numbers with values of 101, 104 and 147, respectively. The high number of shared ASVs found in this study indicated that a high number of bacteria from the fresh stage remained in the smoked stage and selling stage. In each of the samples, it was also found that a high number of unique ASVs indicated that some bacteria originated from the smoked stage and selling stage, especially in Selling B.

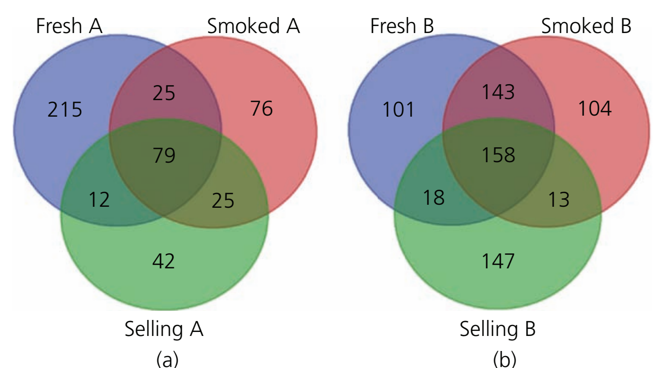

Figure 5. Venn diagram. The numbers of shared and unique ASVs were compared at each processing stages in 2 months

The higher number of shared ASVs in the second sampling compared with that in the first sampling was believed to be due to the bacteria complex in the habitat, unsanitary conditions of the smoking platform and improper handling practices of the sellers at the selling sites in the second sampling.

3.4 Beta diversity of bacterial community composition

The heatmap analysis associated with bacterial community similarity at the genus level, which shows bacterial richness and PD between each sample, is presented in Figure 6. Fresh B and Smoked B showed the highest similarity, while the other samples are less similar to each other.

PCoA represented the dissimilarities of bacterial community composition from *C. fluminea* using unweighted Unifrac and weighted Unifrac distance (Figures 7(a) and 7(b), respectively). The PCoA plots show the clustering of individual samples with other samples, but there was considerable variation detected using both metrics of beta diversity. In Figure 7(a), Smoked A and Selling A form a cluster, but they are apart in Figure 7(b). In contrast, Fresh B and Smoked B formed a closed cluster in Figure 7(b).

4. Discussions

In both Fresh A and Fresh B, *Aeromonas* was found as the predominant bacterial genus that is present in the *C. fluminea* samples. *Aeromonas* has high relative abundances in fresh samples because this genus highly populates the aquatic environment such as freshwater and sediment, which is the habitat of *C. fluminea* (Janda and Abbott, 2010). The bacteria were bio-absorbed into the *C .fluminea* soft tissue during pedal feeding and filter feeding (Chen *et al*., 2013).

In the first sampling, the *Aeromonas* spp. reduction in Smoked A from Fresh A was due to its heat sensitivity. This is supported by Nishikawa *et al*. (1993), who found that *Aeromonas* spp. is relatively easy to eliminate at 55°C for 2 min, while other bacteria can survive up to 55°C for 15 min. For the second sampling, the relative abundance of *Aeromonas* spp. in Fresh B (33.5%) is maintained in Smoked B (34.1%) due to the low temperature of the applied heat. The temperature of the smoking process was measured, and it was found that the maximum temperature is 53°C in the second sampling. In the selling stage, the increased relative abundances of *Aeromonas* spp. in both sampling were consistent with previous studies by Gupta *et al*. (2013) and Nyenje *et al*. (2012), who found the presence of *Aeromonas* spp. in vendor foods. Unhygienic handling of cooked clams, lack of handwashing practices among sellers and the poor sewage disposal system increase the *Aeromonas* spp. contamination (Igbinosa *et al*., 2012). Based on field observation, the sellers purchase raw fish while they were selling *C. fluminea*. This increases bacterial cross-contamination from raw fish into the soft tissue of *C. fluminea*. This is supported by Abd-El-Malek (2017), who found *Aeromonas* spp. in raw fish, which was 40% in wild and 36% in cultured Nile tilapia. The bacteria transmission from raw fish into *C. fluminea* is possible if the *C. fluminea* sellers directly handle the *C. fluminea* after fish purchase. *Aeromonas* spp. belongs to a rod-shaped group, stained as Gram negative and facultative anaerobic bacteria (Percival and Williams, 2014). *Aeromonas* spp. is classified as an enteropathogen, which is usually found in contaminated drinking water and causes gastrointestinal diseases and diarrhoea in children and travellers (Igbinosa *et al*., 2012; Drancourt, 2017). Therefore, *Aeromonas* spp. is the main factor that is associated with the diarheal outbreak in Kelantan.

In the first sampling, *Enterobacter* spp., *Klebsiella* spp., *Escherichia-Shigella* spp., *Lactococcus* spp. and *Bacillus* spp. were prevalent in Smoked A. Poor sanitation of the smoking platform, which was not washed, contributed to this trend. Besides that, the smoking platform was not covered and was exposed to poultry waste. This finding is supported by Periasamy *et al*. (2013) and Abebe *et al*. (2017), as they found *Enterobacter* spp., *Shigella* spp. and *Klebsiella* spp. in wastage of poultry.

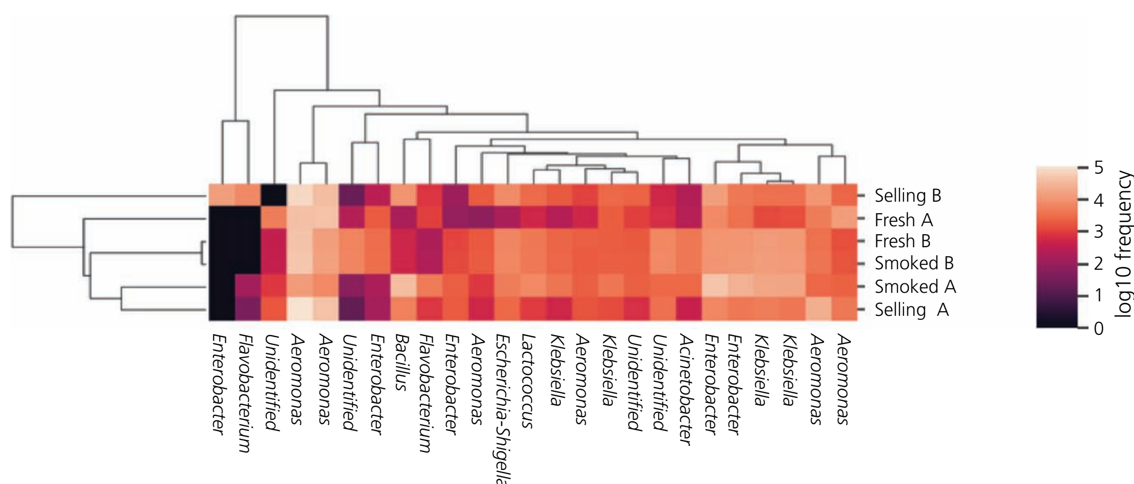

Figure 6. Heatmap. The relative abundance between the top 25 shared ASVs of *C. fluminea* were compared at each processing stage

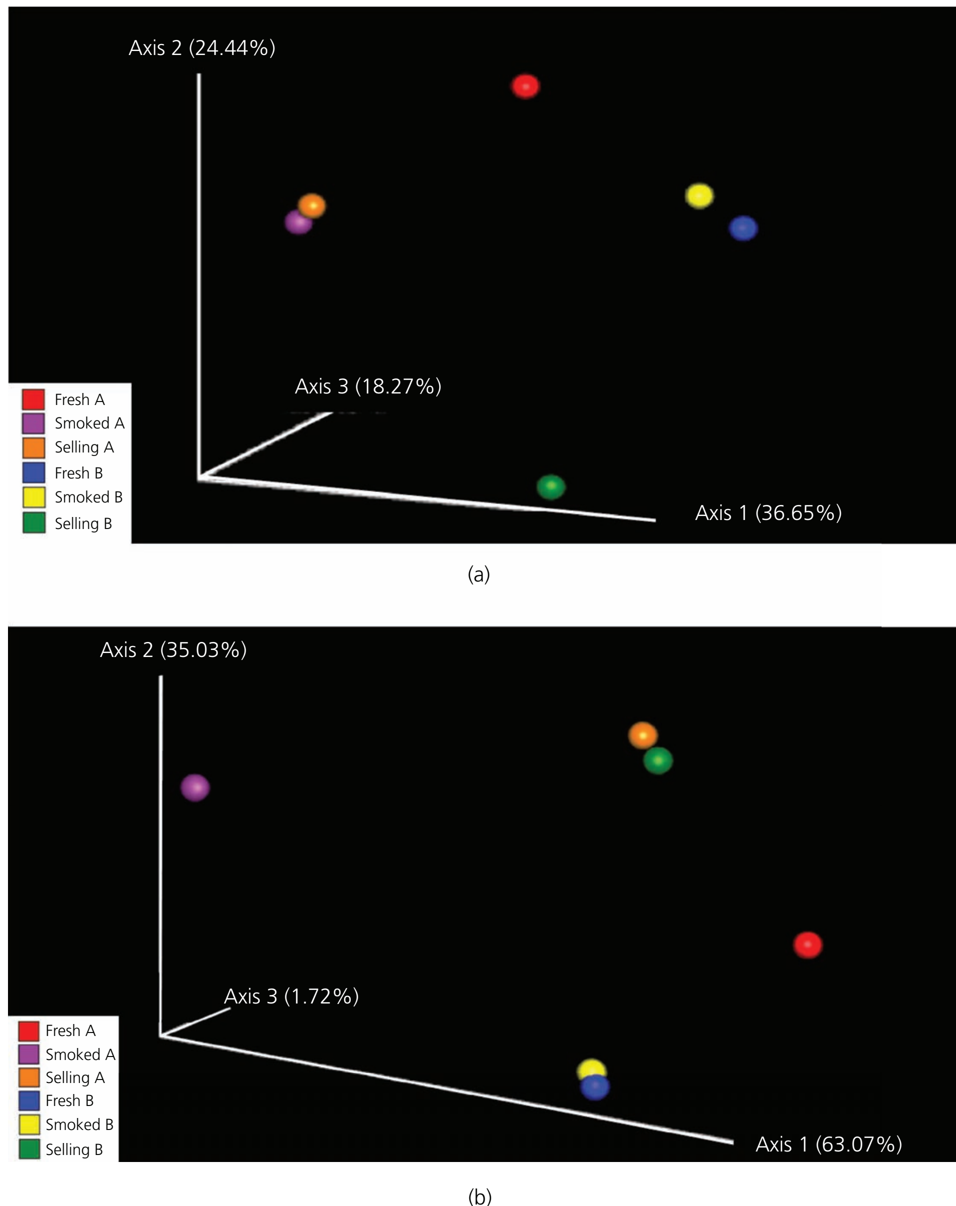

Figure 7. Three-dimensional principal coordinate analysis plot: (a) unweighted Unifrac distance and (b) weighted Unifrac distance of these six samples

In the selling stage, the presence of *Klebsiella* spp., *Enterobacter* spp. and *Bacillus* spp. is consistent with the results reported by Das *et al*. (2012), as they found the presence of similar bacteria in ready-to-eat food on the street. The unhygienic conditions of utensils used during the selling of *C. fluminea* such as the steel containers (‘*cupak*’ or ‘*leng*’ in the local dialect) used for pouring the clam into the newspaper wrap and the handling of *C. fluminea* with bare hands contaminate the clam. In India, Tambekar *et al*. (2009) found similar bacteria present in street-vended fruit juices due to the poor water quality used in juice dilution, poorly hygienic utensils and inadequate handwashing practices when peeling fruits.

In the second sampling, it was surprising to note that the bacterial communities in Fresh B and Smoked B were almost similar, suggesting that the bacteria in Fresh B remained unharmed even after the smoking process. The low temperature (53°C) in the smoking process was concluded insufficient to eliminate the bacteria in smoked *C. fluminea*. This result is supported by Nyenje *et al*. (2012), who stated that the food should be cooked at least 70°C for 2 min. The low temperature was unable to eliminate the bacteria in the Smoked B sample. This hypothesis was proven to be correct as indicated by alpha diversity indices that showed similar values for Fresh B and Smoked B. The PCoA plot of weighted Unifrac and heatmap also showed close

clustering and high similarity in terms of bacterial species, abundance and PD between Fresh B and Smoked B.

Next, most bacterial genera that were high in Fresh B and Smoked B reduced sharply in Selling B, except *Aeromonas*. The reduction is believed to be due to the lower moisture content in *C. fluminea* after being exposed on an open surface for more than 3 h on the street-side kiosks, as low moisture slows down bacterial growth (Amit *et al*., 2017). The moisture reduction in the selling stage is mainly caused by the weather, humidity and wind in that particular day. Based on our previous study, there is significant reduction of moisture content in the soft tissue of smoked and selling *C. fluminea*, as compared with that in fresh *C. fluminea* (Aweng *et al*., 2020). The alpha diversity indices were reduced in the selling stage, which indicated that bacterial abundance and richness decrease with decreasing moisture content.

The dynamics of the bacterial community in *C. fluminea* tissue are highly influenced by the temperature during the smoking process and handling of clams and utensils used during the selling process in that particular day. In general, the pathogenic bacteria found in *C. fluminea* potentially could pose health risks to consumers. Previous studies were done to determine bacteria in the food using conventional methods such as Gram staining, morphology and biochemical tests (Khan, 2018; Noor, 2016). However, these methods consume time and require a large number of samples to produce accurate results; hence, the pipeline used in the current study to investigate the bacterial community can be applied in other ready-to-eat foods in order to further evaluate the bacterial community from the surrounding factors. The application of 16S rRNA sequencing is efficient to determine the bacterial community and reduce bias from the traditional culture-based bacteria study (Cao *et al*., 2017). Besides that, the ASV method used in the current study was able to distinguish sequence variants to as little as one nucleotide and combine the benefits for subsequent analysis of closed-reference and de novo operational taxonomic units (OTUs), which reduced the error from the OTU assignation (Callahan *et al*., 2017). The comparison of the bacterial community from the *C. fluminea* processing stages enabled the researchers to gain insight into the bacteria shift in each processing site, which are in the river, smoking platform and selling area on the street.

5. Conclusion

This study was devoted to investigate the bacterial contamination of *C. fluminea* at different processing stages. Proteobacteria, Firmicutes and Bacteroidetes were found as the most dominant groups at the phylum level, while *Aeromonas*, *Echinobacter* and *Klebsiella* were predominant at the genus level. The high relative abundance of pathogenic bacteria in *C. fluminea*, especially in the selling stage, raised public concern. Bacteria contamination in the selling stage must be monitored frequently by officials as it is directly related to human consumption. The consumption of *C. fluminea* could pose health risks to consumers if the problem on contaminants (bacteria and heavy metal) in clam is not eliminated. This research would serve as a baseline study for further investigation of hygienic cooking methods, handling and packaging of *C. fluminea*, which need to be done to reduce bacterial contaminants in *C. fluminea* at each processing stage.

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