Elucidation of aerobic bacteria diversity in neera water from coconut and nipa tree

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Abstract. Neera water is among Malaysian favorite local beverage, there were a lot of studies on its composition and its nutrional value, however the microbial diversity inside the water has been understudy. Two type of neera water were collected and cultured on nutrient agar (NA) media. Single colonies of bacteria formed were selected and re-isolated on NA agar to obtain a pure culture and incubated at 37⁰C±1⁰C. The isolates were proceeded to further identification by morphological, biochemical and molecular analysis. 23 aerobic bacteria was isolated and identified in this research, 14 isolates were obtained from nipa neera and nine isolates were obtained from coconut neera. Those bacteria were identified as Serratia sp, Staphylococcus sp, Streptococcus sp, Bacillus sp, Microbacterium sp, Enterobacter cloacae, Klebsiella aerogenes, Bacillus cereus and Pantoea anthophila respectively. Most of the identified bacteria were first reported to be present in neera sap which show a good fundamentals knowledge for others researcher. The bacteria present in nipa neera water and coconut neera water were also found to be different.

1. Introduction
Nipa palm tree (Nypa fruticans) and coconut tree (Cocus nucifera L.) is one of the most widely distributed and useful trees [14]-[17] which usually found in Asia. Nipa palm can be found mostly in South, Ocenia and Southest Asia [17]. One of the benefits of coconut and nipa palm tree is producing sap. The sap was known as Neera. Neera water is one of Malaysian favourite beverage. There is a lot of studies and claimed on its nutritional value. According to Jose et al. [8] neera and neera product is a healthy option for diet. It is diabetic-friendly due to its low Glycemic Index (GI) and also is rich in minerals, vitamins, and glucamic acid [3]. According to Jose et al. [8] there are various advantages of consuming Neera beverage as stated here; Neera can help improve the human immune system because it contains glutamic acid, an amino acid that can be used by the human body to make protein. Because of its high vitamin and mineral content,

Neera beverage can increase the structure resistant and allow the immune system to combat various diseases. Neera also has a high level of vitamin K and iron, when consuming Neera, the iron level in the human body can also be raised which can prevent anaemia from occurring. Neera can also improve respiratory health as it has anti-bacteria and anti-viral properties. Liver cancer also can be cure by
regularly consuming *neera*, because *neera* can dispose of acetaldehyde which is a poisonous metabolic result that can lead to liver problems. In addition, for other types of cancer, phytochemicals, and antioxidant properties in *neera* drink can help in prevention of other types of cancer. High amount of calcium in *neera* also give benefit to human’s bone and an prevent from bone disease such as osteoporosis. *Neera* has anti-bacterial properties as mention before and has antioxidant properties. These properties help in reducing in skin related problem. Consuming *neera* can protect human’s skin from oxidation damage that usually caused by free radicals. Furthermore, consuming *neera* also can increase eye’s vision. *Neera* is beneficial for eyes health because of the presence of vitamin C. For diabetic patient, *neera* is one of the ideal beverages because it has lower glycemic index. Lastly, drink *neera* can help in reducing blood pressure because it is rich in potassium. All these advantages show high potential of *Neera* as beneficial food for human health.

The major volatiles element in fresh *neera* are aliphatic ketones, aromatic hydrocarbons, ester, alcohols, and a heterocyclic compound. Fatty acids such palmitoleic acid and palmitic acid also found in *Neera*. Besides that, acid and aliphatic hydrocarbons also one of the dominant components in coconut and nipah sap. The next major element in of the coconut sap is ethyl lactate. The overall flavour of the fresh *neera* may be contribute by the the four components of alcohol which are phenyl ethyl alcohol, 1-hexanol, two other sesquiterpene alcohols – nerolidol and farnesol-along with four ketones, namely, 3-hydroxy-2-pentanone, 2-hydroxy-3-pentanone, tetradecanone and hexadecanone. 0.07% of the ethyl alcohol can be found in fresh *neera* [2].

*Neera* from different plants have different taste and smells, besides the chemical composition, microbial composition was said to play a role in this property [15]. *Neera* sap extract, collected by tapping the spadix of coconut or nipah palm. This sap extract is consumed on the same day, max 3-4 hour after harvested, within the limited time frame, if not consumed the *Neera* will became fermented and were called Toddy. To maintain the *Neera* water, it must be preserved in the freezer. The richness of carbon souces of *Neera* water also make it perishable to microbes, conception of raw *Neera* without processing may cause various foodborne illnesses.

There not much study were conducted on *Neera* microbial diversity, recently Widyaningrum et al. [18] isolate 11 yeast from *neera* sivalan from Rembang Central Java to be used for bioethanol production as *Neera* is one of beverage that easily fermented. In 2019, Somashekararaih et al. [16] screen for Probiotic bacteria from coconut tree in India and found that 40 isolate exhibit promising probiotic properties and are favorable for use in functional fermented foods as preservatives. The studies also showed seven isolates proved to have significant antibacterial activity against all the enteric pathogens. This indicates that there is huge potential for underexplored microbes in *Neera* to be use in various industries.

2. Materials and Methods

2.1 Sampling

*Neera* water samples for nipa *neera* and coconut *neera*, respectively, were collected from Kedah and Kelantan. The samples were kept at 0 °C to prevent any chemical and physical changes. The sample was diluted by transferring 1 mL of sample to 9 mL of sterile distilled water up to 3 (10^0, 10^1, 10^2) dilution. Using a hockey stick, 100 μL of each diluted sample was streaked onto a Nutrient Agar (NA) plate. Plates were incubated at 37°C for 24-48 hours for growth monitoring. Any growth of bacteria was observed and recorded. Each of the different of a single colony were selected and purified by re-streaking on the NA plate. The re-streak plates were also incubated at 37°C for 24-48 hours.

2.2 Morphological Identification

Gram staining of each isolates was performed using a standard procedure and confirmed using the KOH lysis method [7]. Shapes of the bacteria were observed under light microscope with 100X magnification and morphology of the bacteria were observed by naked eyes.
2.3 Biochemical Analyses
Catalase activity, oxidase activity, protease activity, Indole assay, Simmon’s Citrate assay, Methyl Red (MR) assay, Urea assay and the hydrolysis of casein, starch, were determined as described by Cowan and Steel [4].

2.4 Genetic Analyses
Genomic DNA was extracted using a NucleoSpin Microbial DNA kit (Macherey-Nagel) according to the manufacturer’s instructions. The 16S rRNA gene of selected bacteria was amplified by PCR using a forward primer (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′) and a reverse primer (1525R: 5′-AAGGAGGTGATCCAGCC-3′), as described by Lane [10]. The quality of the PCR product was quantified using NanoDrop (Thermo Fisher Scientific). Gene sequencing was performed by CCB USM.

3. Results and Discussion
A total of 14 bacteria colonies were isolated from nipa neera and 9 bacteria colonies from coconut neera. The isolates were labelled as NB2, NB3, NB5, NB9, NB11, NB13, NB15, NB16, NB17, NB18, NB19, NB20, NB21 and NB22 for nipa neera and CB1, CB2, CB4, CB5, CB6, CB7, CB8, CB9 and CB10 for coconut neera. From the result, 13 out of 14 bacteria isolated from nipa neera is a Gram-positive bacteria (Figure 1). Meanwhile, 7 out of 9 bacteria isolated from coconut neera are Gram-negative (figure 2). Eight biochemical tests and 16s rRNA analyses were performed to characterize and identified the isolated bacteria such as Catalase, Indole, Simmon’s Citrate, Methyl Red (MR), Urea Agar Base (UAB) Oxidase and Triple Sugar Iron (TSI) Test. The observation on the biochemical test is shown in table 4.1. From the morphology, biochemical and molecular analyses, 16 bacteria were able to be identified and 8 it still unknown culture. The diversity of bacteria obtain from each neera beverage were shown in Figure 3.
Figure 1. Gram reaction and shapes of isolates NB2 (A), NB3 (B), NB5 (C), NB9 (D), NB11 (E), NB13 (F), NB15 (G), NB16 (H), NB17 (I), NB18 (J), NB19 (K), NB20 (L), NB21 (M) and NB22 (N) isolated from Nipa neera under 100x magnification.
Figure 2. Gram reaction and shapes of isolates CB1 (A), CB2 (B), CB4 (C), CB5 (D), CB6 (E), CB7 (F), CB8 (G), CB9 (H) and CB10 (I) isolated from coconut neera under 100x magnification.

Table 1. Morphological and biochemical analysis of bacteria isolated from nipa and and coconut neera beverages.

| Sample     | Bacteria           | Shapes      | Morphological and Biochemical Test |
|------------|--------------------|-------------|-----------------------------------|
|            |                    | Gram Reaction | Catalase | Indole | Citrate | MR | Urea | Oxidase | TSI | Slant | Butt | Gas | H2S |
| Nipa neera | NB2 Cocco-bacillus | +            | +        | -      | +      | -  | +    | Alkaline | Alkaline | - | - |
| NB3 Bacillus | -                 | +            | -        | -      | +      | +  | +    | Acid     | Acid     | + | - |
| NB5 Bacillus | -                 | -            | +        | -      | +      | -  | -    | Acid     | Acid     | - | - |
| NB9 Short Bacillus | -          | +            | -        | +      | -      | +  | -    | Alkaline | Acid     | - | - |
| NB11 Short Bacillus | +          | +            | -        | +      | +      | +  | -    | Alkaline | Acid     | - | - |
| NB13 Cocco-bacillus | +          | +            | -        | +      | -      | +  | +    | Alkaline | Acid     | - | - |
| NB15 Bacillus | +                 | +            | -        | -      | +      | +  | -    | Acid     | Acid     | + | - |
| NB16 Coccos | +                 | +            | -        | +      | -      | +  | +    | Acid     | Acid     | + | - |
| NB17 Cocco-bacillus | +          | +            | -        | +      | -      | +  | -    | Acid     | Acid     | + | - |
| NB18 Bacillus | -                 | +            | -        | -      | +      | -  | -    | Acid     | Acid     | - | - |
| NB19 Coccos | +                 | +            | -        | +      | +      | +  | -    | Alkaline | Alkaline | - | - |
| NB20 Bacillus | +                 | -            | -        | +      | -      | -  | -    | Acid     | Alkaline | + | - |
| NB21 Bacillus | +                 | -            | +        | -      | -      | +  | +    | Alkaline | Alkaline | + | - |
| NB22 Bacillus | +                 | -            | -        | -      | +      | +  | +    | Alkaline | Alkaline | - | - |
From the identification, it shown that Bacillus sp. and Streptococcus sp. are the dominant genera found in both neera beverage. Both genera have been reported to be present in palm and coconut trees. Research by Kajs et al. [9], Morales-Lizcano et al. [12] and Law et al. [11] shows the present of Bacillus sp. in neera beverage originate from the palm tree, meanwhile Atputharajah et al. [1] also reported Bacillus sp. as predominant genera present in toddy. In 2020, research by Dinoto et al. [5] found a Streptococcus mitis isolated from African oil palm neera (Elaeis guineensis).
In this research, the bacteria diversity is different between Nipa neera and Coconut neera beverages, 3 bacteria genera that can only be found in Nipa neera are Serratia sp. Staphylococcus sp. and Mycobacterium sp. A few studies also reported the presents of Staphylococcus sp in neera sap such as the present of Staphylococcus roseus from fermented neera of Asian palmyra palm (Borassus flabellifer) [13]. However, the present of Serratia sp. and Microbacterium sp has never been reported before to be present in any neera beverage. Both genera were well known as environment microbes for palm tree [6]. In Coconut neera, 4 bacteria which are Enterobacter cloacae, Klebsiella aerogenes, Bacillus cereus and Pantoea anthophila were found to be different from bacteria isolated from Nipa neera. The present of all these bacteria in neera beverage from any source has never been reported before, we believe that the environmental conditions also influence the diversity of bacteria found in the neera sap of both plants. For the unknown culture, we are unable to obtain any species or genera due to insufficient biochemical and bioinformatics data, other possibility is that it might be a new species of bacteria.

4. Conclusion
By knowing the microbial diversity of bacteria inside Neera water that are consumed by a lot of Malaysian people, it will improve our health by eliminating food that may contain risk to human health and at same time might give a new insight for potential good bacteria that can be explored to enhance human health. In this study we also found a new fundamentals knowledge on new bacteria that are present in both nipa and coconut neera that could help researcher to understand the diversity of microbial inside both beverages.

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