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Screening of microbial population in Sabah tea kombucha pellicle for its potential as prebiotic and probiotic supplement

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Abstract. This research aimed to determine and analyze the microbial population in kombucha pellicles derived from Sabah black tea, specifically focusing on bacteria and yeast, to gain insights into their abundance, diversity, and potential as prebiotic and probiotic supplements. Despite the growing interest in kombucha in Malaysia, the specific microbial composition of the pellicle from locally sourced Sabah black teas remains underexplored. Understanding this composition could reveal its potential as a sustainable source of health-promoting microbes. It is hypothesized that Sabah tea kombucha pellicle harbours a beneficial microbial population that can be utilized as a low-cost prebiotic and probiotic supplements. The kombucha pellicle was prepared using 10 g of Sabah black tea, 1 L of sterile water containing 10% sugar (w/v), and a 10% kombucha symbiotic culture of bacteria and yeast (SCOBY). The process included boiling black tea with sugar, adding SCOBY culture, and allowing fermentation for 30 days to obtain cellulosic pellicles. After fermentation, the pellicle was separated, homogenized, and stored for further use. Then, kombucha pellicle genomic DNA was extracted and subjected to 16S and ITS metagenomic analysis to identify the bacteria and fungi population. The 16S and ITS metagenomic results showed that Sabah tea kombucha pellicle contains a potentially beneficial microbial population, mainly *Komagataeibacter*, *Zygosaccharomyces* and *Starmerella*, that may serve as a sustainable probiotic. This current study provides promising evidence for using Sabah tea kombucha pellicle as a low-cost prebiotic and probiotic supplement. This will indirectly help advertise and commercialize Sabah tea as one of the local products in Sabah.

Keywords: fermentation, *Komagataeibacter*, kombucha, Sabah tea, *Starmerella*, *Zygosaccharomyces*

INTRODUCTION

Gut health is the foundation of wellness. In recent years, Malaysia has witnessed a significant shift in dietary habits, with an increasing awareness of the importance of gut health and its impact on overall well-being. One notable trend that has gained prominence is the daily consumption of probiotic products among Malaysians as an initiative to improve the digestive system, immune system, and mental well-being (Mohd Ashri *et al*., 2021; Tay *et al.,* 2023). Even though both antibiotics and probiotics are reported to fight pathogenic bacteria, and reduce mortality, probiotics are efficacious as they inhibit pathogenic microorganisms' growth in the host, are safer, and are more cost-effective than antibiotics (Dominguez-Bello *et al.,* 2019; Pilla & Suchodolski, 2020; Zimmermann & Curtis, 2019). As awareness of these benefits spreads, a diverse range of probiotic-rich products has permeated the Malaysian market, including yogurt, fermented beverages, and dietary supplements. This trend is not only reflective of a growing interest in preventive healthcare but also indicative of the

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influence of global health and wellness movements shaping dietary choices.

The fermentation of sugared black tea (*Camellia sinensis*) via a symbiotic culture of bacteria and yeast (SCOBY), especially acetic acid bacteria, produces kombucha, a promising low-cost functional beverage (Villarreal-Soto *et al.,* 2018). It has been reported that kombucha possesses probiotic properties such as antimicrobial, antioxidant, antidiabetic, and anticancer (Al-Mohammadi *et al*., 2021; Ivanišová *et al.,* 2020; Pavlović *et al*., 2023; Villarreal-Soto *et al*., 2019; Zhou *et al*., 2022). Sabah tea, a renowned tea brand in Malaysia, particularly in Sabah, is one of the popular teas used to brew kombucha. During the fermentation process, kombucha pellicle, which is made up of cellulosic fiber and contains diverse microbial populations, is formed, but it is often discarded. This nourishing pellicle is a source of prebiotics with a possible probiotic bacterium, which can be used as a supplement to reduce waste. However, different kombucha contains different microbial populations depending on the fermentation periods, temperature, sugar concentration, substrate, and SCOBY used during the fermentation process (Nasir *et al.,* 2022). These factors also indirectly contribute to the unique properties and health benefits of each kombucha variety. As the result, there is a growing interest in the specific microbial composition of the kombucha pellicle from locally sourced Sabah black teas, which is currently underexplored. And to date, the potential of consuming kombucha pellicle has not been well documented. Therefore, this research focused on screening the bacteria and yeast population in Sabah tea kombucha pellicle to determine their abundance, diversity, and potential as prebiotic and probiotic supplements. The findings of this research would contribute to a cost-effective and sustainable prebiotic and probiotic that can be used in food supplements to improve overall health.

MATERIALS AND METHODS

Preparation of Sabah tea kombucha pellicle

Sabah tea (*C. sinensis*) was purchased from a local market in Kota Kinabalu, Sabah, Malaysia, while kombucha SCOBY was purchased from a local supplier. Sabah tea kombucha pellicle was prepared following the method described by Zubaidah *et al.* (2020), with slight modification. In brief, 10 g of black tea was added to 1 L of sterile water containing 10% sucrose (*w*/*v*) and boiled for 15 min. The tea was poured into a sterile container to cool to room temperature before adding 10% of SCOBY solution starter culture (*v*/*v*). Next, the container was covered with a sterile cloth and secured tightly with a rubber band to prevent contamination. After that, the culture was kept at room temperature, ranging from 23 to 27°C, to allow fermentation and pellicles to form. Kombucha was fermented for 30 days in the dark to obtain sufficient cellulosic pellicles. After 30 days, the pellicle was separated aseptically and pooled into a sterile flask containing sugared black tea to ensure homogeneity of the samples from different batches before being stored at 4 °C up to one month for further use.

Identification of probiotic microbes in Sabah tea kombucha pellicle

DNA extraction

The kombucha pellicle was extracted following the modified blending method described by Matthes *et al.* (2020). In brief, the kombucha pellicle was chopped into small pieces and homogenized in 35 mL lysis buffer (100 mM Tris-hydrochloride, 100 mM sodium ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate, 1% cetyltrimethylammonium bromide, pH 8) using an electrical blender (Philips, Malaysia). Then, the pellicle was centrifuged (Eppendorf, Hamburg, Germany) at 11,000× *g* for 20 min, resuspended for 5 min using a vortex (IKA, Staufen, Germany), and centrifuged for another round to obtain supernatant.

The kombucha pellicle genomic deoxyribonucleic acid (DNA) was extracted from the supernatant using the NucleoSpin® Microbial DNA kit (Macherey-Nagel Inc., Allentown, PA, USA), according to the manufacturer's protocols, with slight modifications. The concentration and purity of the DNA samples were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA samples were electrophoresed (Advance Co. Ltd., Tokyo, Japan) on 1% Tris-acetate-EDTA agarose

gel and viewed using a UV image analysis system (Bio-Rad Laboratories Inc., CA, USA). The DNA samples were sent to the sequencing company (Apical Scientific Sdn. Bhd., Malaysia) for 16S and ITS Metagenomic analysis.

16S and ITS metagenomic sequencing

The DNA with an A260/A280 ratio ranging from 1.6 to 2.0, extracted from kombucha pellicles, was amplified using 16S amplicon of V3-V4 region and internal transcribed spacer (ITS) region of ITS2 for MiSeq library amplicons preparation. Bacterial polymerase chain reaction (PCR) was performed using 16S forward (5'-CCTACG GGNGGCWGCAG-3') and reverse (5'- GACTA CHVGGGTATCTAATCC-3') primers, while fungal PCR was performed using ITS2 forward (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse (5'-GCTGCGTTCTTCATCGATG C-3') primers, with amplification conditions as follows: 5 min at 95 °C, then 40 cycles (30 s at 95°C, 30 s at 60°C, 20 s at 72°C) and a final extension for 5 min at 72 °C. Each PCR reaction contained 25 µL REDiant 2X PCR Master Mix (Axil Scientific Pte. Ltd., Singapore), 5 µL of each primer (10 mM), and 5 µL of template DNA ranging from 9.30 to 143.55 ng/µL. The first PCR were carried out using KOD-Multi & Epi-® (Toyobo Co., Ltd., Osaka, Japan), according to the manufacturer's protocols, with slight modifications.

Then, the amplified PCR product from the first PCR was used as a template for the second PCR. The quality check for the second PCR product was done using Agilent Bioanalyzer 2100 System (Agilent Technologies Inc., Santa Clara, CA, USA) and Helixyte GreenTM Quantifying (AAT Bioquest Inc., Pleasanton, CA, USA). Following the Illumina-recommended protocol, the libraries were normalized and pooled before being sequenced on the MiSeq platform using 300 paired-ends.

RESULTS

Development and observations on the color changes, thickness of the pellicle, and ph levels during kombucha fermentation

Based on the observations, the kombucha pellicle's color changed from pale beige to yellow and finally to dark brown, and its thickness increased noticeably throughout the fermentation process, as shown in Figure 1. The old pellicle would slowly turn yellow and dark brown due to the aging of the cellulose. As fermentation progresses, the thickness of the pellicle would increase due to the addition of the new layer formed under the old pellicle and over time, these old layers of the pellicle will sink as shown on day 21 of the fermentation. After 30 days of fermentation, Sabah tea kombucha fermentation can produce up to 100 g of pellicle. A substantial reduction in pH levels from 3.71 to 2.58 was also recorded, accompanied by the emergence of a distinct tangy aroma following a 30-day fermentation period.

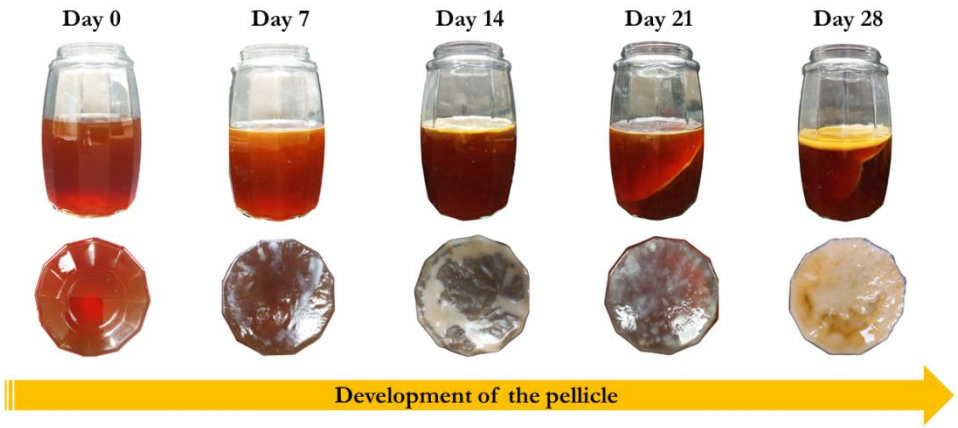

Figure 1. The development of Sabah tea kombucha pellicle in 30 days

Dominant microbial population in Sabah tea kombucha pellicle

The results of 16S metagenomic sequencing of 30 days Sabah tea kombucha pellicle showed that the pellicle is dominated by *Komagataeibacter* (99.97%), followed by *Bacillus* (0.02%) and *Bifidobacterium* (0.01%), as shown in Figure 2. The results also showed significant differences in total abundance between bacteria in Sabah tea kombucha pellicle after 30 days of fermentation. Based on the results, *Komagataeibacter* was not only a predominant bacterium but also led the bacterial succession in Sabah tea kombucha after 30 days of fermentation. *Komagataeibacter* (previously known as *Gluconacetobacter*) is a Gram-negative bacterium in the family Acetobacteraceae, order Acetobacterales, class Alphaproteobacteria, and phylum Pseudomonadota (synonym Proteobacteria).

On the other hand, the results of ITS metagenomic sequencing of 30 days Sabah tea kombucha pellicle showed that the pellicle is dominated by the species *Zygosaccharomyces bisporus* (60.76%), followed by *Starmerella davenportii* (38.66%), as shown in Figure 3. The results also showed significant differences in total abundance between fungi in Sabah tea kombucha pellicle after 30 days of fermentation. Based on the results, both *Z. bisporus* and *S. davenportii* (previously known as *Candida davenportii*) are fungi in the order Saccharomycetales, class Saccharomycetes and phylum Ascomycota. After 30 days of fermentation, it was discovered that Sabah tea kombucha pellicle had significant fungal diversity but low bacterial diversity due to the dominance of a single bacterium, as shown in Figures 2 and 3.

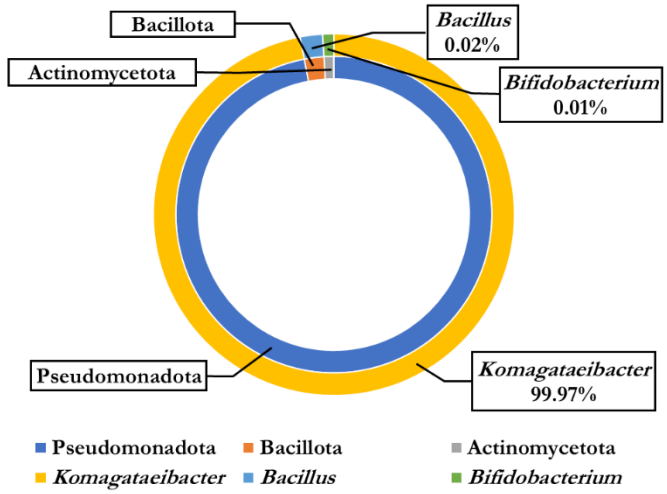

Figure 2. The dominant bacterial genus population found in Sabah tea kombucha pellicle after 30 days of fermentation

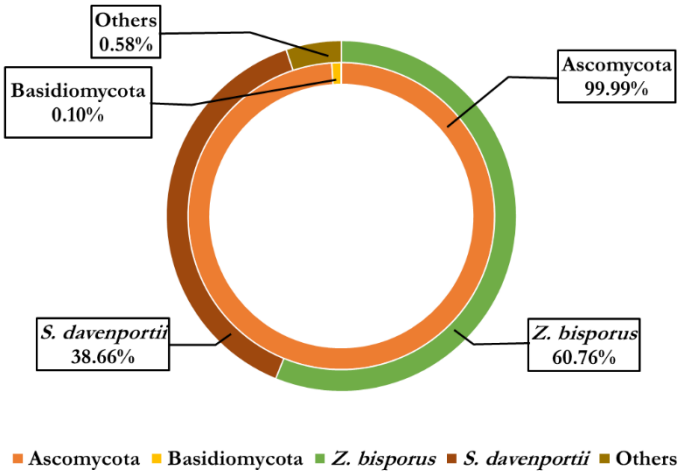

Figure 3. The dominant fungal population found in Sabah tea kombucha pellicle after 30 days of fermentation

DISCUSSION

Kombucha has been proposed as a good source of supplement due to its probiotic potential (Selvaraj & Gurumurthy, 2023) and the presence of pellicles, which can serve as a source of cellulosic fiber and prebiotics (Vargas *et al.,* 2021). Previous literature showed that kombucha harbors a wide range of microbial diversity, which may vary depending on several factors such as fermentation duration, temperature, sugar concentration, substrate, and SCOBY, with limited information reported on the microbial population derived from the pellicle (Nasir *et al.,* 2022). This diverse microbial community in SCOBY, particularly bacteria and yeast, is responsible for fermenting sweet tea into kombucha.

Based on our 16S data, the dissected kombucha pellicle showed a notable presence of *Komagataeibacter*, an obligate aerobic bacterium that possesses a high oxygen affinity for growth and this has been reported by Harrison and Curtin (2021) and Żywicka *et al.* (2021). This result is aligned with the role of the kombucha pellicle as a floating piece that allows embedded microorganisms to access oxygen (Tran *et al.*, 2020a). Furthermore, the presence of predominant *Komagataeibacter* in the kombucha pellicle can be seen from the increased thickness of the pellicle throughout the fermentation (Figure 1). This is due to the ability of *Komagataeibacter* to synthesize cellulose (Mangayil *et al.*, 2021; Vashukova *et al.*, 2022), which is the structure matrix of the kombucha pellicle, by metabolizing the nutrients in the tea. The microbes found in this study also aligned with other studies conducted on kombucha which demonstrated the presence of various *Komagataeibacter*, such as *K. xylinus*, *K. rhaeticus*, and *K. saccharivorans* in kombucha pellicle (Harrison & Curtin, 2021; Tran *et al.*, 2020a). In contrast, the low abundance of *Bacillus* and *Bifidobacterium* in our sample after 30 days of fermentation may be linked to the absence of sugar that is necessary for bacterial growth (Abd Aziz *et al.,* 2020; Parhi *et al.,* 2022). A similar finding was reported by Harrison and Curtin (2021), where the data showed a lower population of *Bacillus* at the upper layer of kombucha as compared to the lower layer after 7 days of fermentation which may be due to the limited access of sugar at the upper layer of the kombucha pellicle.

Based on a prior study conducted by Vashukova *et al.* (2022), the phyla Actinomycetota (also known as Actinobacteria), Bacillota (synonym Firmicutes), and Pseudomonadota were the most common bacteria observed in kombucha pellicle. In their finding, they found that Pseudomonadota, mainly represented by *Komagataeibacter*, a well-known acetic-acid-producing bacteria, is dominating the bacterial population in the kombucha pellicle during the first week of fermentation, and this trend continues until 90 days of the fermentation, which is consistent with our finding. Additionally, more alcohol will be converted to acetic acid as fermentation progresses, leading to the accumulation of acetic acid and acidic conditions in prolonged fermented kombucha (Tran *et al.*, 2020b). As *Komagataeibacter* can withstand an acidic condition, it has become the predominant bacteria mostly found in prolonged fermented kombucha (Hooi *et al.,* 2023; Vashukova *et al.*, 2022).

On the other hand, our ITS data showed that *Z. bisporus* and *S. davenportii*, the prominent yeasts species in kombucha, were clearly present in the dissected kombucha pellicle. These two species are resistant to weak acid preservatives such as benzoic and sorbic acid and are capable to growth in low pH conditions of kombucha (Péter, 2021; Stratford *et al.*, 2002; Tu *et al.,* 2020). The presence of yeasts in our sample are important as they are the organisms most directly responsible for ethanol fermentation, the process by which sugar is converted into ethanol and carbon dioxide (Maicas, 2020). Although *Z. bisporus* and *S. davenportii* are not cellulose-producing yeasts, however, metabolic activity of both yeasts indirectly helps sustains the growth of *Komagataeibacter* which was responsible for the cellulose production, as *Komagataeibacter* oxidizes ethanol produced during ethanol fermentation into acetic acid, which gives the kombucha its tangy flavor (Tran *et al.,* 2020b). This shows the critical role of *Komagataeibacter sp., Z. bisporus* and *S. davenportii* in Sabah tea kombucha for proper fermentation and the development of its distinctive flavor, aroma, and pellicle's texture, as highlighted by Harrison and Curtin (2021). It is also inferred that their dominance in our sample

after a lengthy fermentation period is due to their unique metabolic capabilities and adaptability to flourish in the increasingly acidic and complex environment of the kombucha brew, as reported by Yang *et al.* (2022).

Interestingly, all predominant bacteria in our Sabah tea kombucha pellicle sample have been reported as beneficial bacteria that has probiotic potential and could provide health benefits when consumed adequately (Vera-Santander *et al.*, 2023). *Bacillus* and *Bifidobacterium* are widely utilized probiotics that have been reported in both commercial and homebrewed kombucha, although their prevalence is typically lower than that of acetic acid bacteria, particularly *Komagataeibacter* (Harrison & Curtin, 2021; Vargas *et al.,* 2021; Vashukova *et al.,* 2022; Vera-Santander *et al.,* 2023). However, among all *Komagataeibacter* species mentioned above, *K. xylinus* has been acknowledged as a novel probiotic bacterium that has been reported to help lowering blood glucose and promotes weight reduction, thus helping in lowering the risk of developing obesity and diabetes indirectly (Kaashyap *et al.,* 2021; Lavasani *et al.*, 2019). In addition, *K. xylinus* is also resistant to bile and acid and has a high survival rate in harsh gastrointestinal conditions, such as low oxygen pressure, which are ideal characteristics of probiotics (Nadar Rajivgandhi *et al.,* 2021; Rasika *et al.*, 2021). Remarkably, *Starmerella* sp. found in this Sabah tea kombucha pellicle is also recognized as a novel potential probiotic yeast with ability to growth at different temperatures, has high tolerance to acid and bile salts, withstanding gastric juice, and can reduce cholesterol, as reported by Tu *et al.* (2020).

The data from this study exhibited that after 30 days of fermentation, lower microbial diversity was shown in Sabah tea kombucha pellicle compared with the one reported in the literature, which is typically fermented for 7 to 14 days. This finding agrees with Harrison and Curtin (2021), where they found that the bacterial population in kombucha pellicle was initially dominated by *Lactobacillus* (44.2%) and was later suppressed by Acetobacteraceae (90.3%), particularly *Komagataeibacter*, while *Enterococcus* and *Bacillus* populations decreased significantly in the upper layer after 7 days. In contrast, they also found that the yeast population in kombucha pellicle became more complex and variable after 7 days where *Zygosaccharomyces* and *Saccharomyces* populations decreased significantly, while population of *Starmerella* increased, followed by the emergence of some new fungi commonly involved in alcoholic fermentation such as *Lachancea* and *Issatchenkia*, in small abundant (Bellut *et al.*, 2020; Shi *et al.,* 2019). Compared to our sample, the pellicles of commercial kombucha that were fermented for a short period for human consumption were more enriched. This may be due to several factors, such as depletion in glucose concentration and accumulation of acetic acid which lead to the decrement in pH value and acidic condition as the fermentation progresses (Vashukova *et al.,* 2022). All these will result in intense competition for microbial growth., and the acidic condition of kombucha would favor the growth of acid-tolerant bacteria (acetic acid and lactic acid bacteria) such as *Komagataeibacter* and *Bacillus* (Hooi *et al.,* 2023) and yeasts such as *Zygosaccharomyces* and *Starmerella*.

Besides, kombucha pellicle can be a good source of prebiotic as it is made up of cellulosic fiber that can serve as substrate for the proliferation and flourishing of probiotics in the gastrointestinal tract, which is necessary for improving the overall health. Vargas *et al.* (2021) reported that the cellulosic fiber of kombucha might promote the development and survival of probiotics as it contains polysaccharides and is not easily hydrolyzed, which makes it suitable to serve as a prebiotic supplement (Khan *et al.,* 2022). To date, there is no information on the utilization of Sabah tea kombucha pellicle has been reported. The findings from this study offer valuable insights into the potential application of Sabah tea kombucha pellicles as a groundbreaking and sustainable prebiotic and probiotic supplement. This innovative supplement can aid digestion and enhance nutrient absorption, leading to better utilization of dietary components, including proteins, carbohydrates, and minerals, thereby contributing to overall health and well-being.

CONCLUSION

Sabah tea kombucha pellicle has been shown to contain potentially beneficial microbes, mainly *Komagataeibacter sp., Z. bisporus* and *S. davenportii*,

that may offer probiotic benefits. These findings may open new opportunities for the development of Sabah tea kombucha pellicle as a low-cost and sustainable supplement with prebiotic and probiotic potential for improving gut health and overall well-being in both humans and animals.

To fully grasp the potential of kombucha pellicle as a prebiotic and probiotic supplement for both humans and animals' health, future research should focus on its nutritional content and conduct comprehensive clinical trials to validate the health benefits and safety of these microbes in both humans and animals. It will also be important to explore how its fibers affect gut health and how these microbes interact with gut microbiota. This research will help us understand how effective and safe kombucha pellicle can be as a supplement.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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