The microbial composition and functional roles of different kombucha products in Singapore

Suet Li Hooi[1,*], Jacky Dwiyanto[2], Kai Yee Toh[1], Gwendoline Tan[1], Chun Wie Chong[1,5], Jonathan Wei Jie Lee[1,3,4], Jeremy Lim[1,*]

[1]AMILI Pte Ltd, 89 Science Park Drive #03-09, The Rutherford, Lobby C, Singapore Science Park 1, Singapore 118261

[2]Department of Medical Microbiology, Faculty of Medicine, Universiti Malaya, 50603, Kuala Lumpur, Malaysia

[3]Division of Gastroenterology & Hepatology, National University Hospital, Singapore

[4]Yong Loo Lin School of Medicine, National University of Singapore, Singapore

[5]School of Pharmacy, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Bandar Sunway, Selangor Darul Ehsan, Malaysia

*Corresponding author

1) Suet Li Hooi

Email: suetli.hooi@amili.asia

2) Jeremy Lim

Email: jeremy.lim@amili.asia

Abstract

Kombucha is a fermented tea traditionally known for its health-enhancing properties owing to the bioactive compounds generated by acetic acid bacteria (AAB) and lactic acid bacteria (LAB). We compared the distribution of AAB and LAB and their functional pathways across nine commercial kombucha products in Singapore using shotgun metagenomic sequencing. A high prevalence of *Komagataeibacter* species including *Komagataeibacter saccharivorans* (82.93% in B), *Komagataeibacter xylinus* (93.38% in D) and *Komagataeibacter rhaeticus* (92.20% and 30.62% in G and I) was detected in AAB-dominant kombucha. LAB-dominant kombucha was largely represented by *Bacillus coagulans* (~99% in E and F) and *Lactobacillus nagelii* (~60% in H). Despite differences in bacterial composition, both LAB-and AAB-dominant kombucha harbour pathways involved in the biosynthesis of short-chain fatty acids (SCFAs), amino and organic acids and vitamin B12. "Fatty acid and beta-oxidation II (peroxisome)" and "fatty acid and beta-oxidation I" were detected in the LAB but not the AAB-dominant kombucha.

Keywords: Bioactive compounds, functional pathways, shotgun metagenomic sequencing, *Komagataeibacter*, *Bacillus coagulans*, SCFA, kombucha, microbiome, microbiota

Introduction

Kombucha is a traditionally fermented drink produced from the fermentation of sweetened green, black or oolong tea by the symbiotic culture of bacteria and yeast (SCOBY) [1,2]. This traditional beverage has been widely consumed worldwide over the last decade due to its purported health benefits. For instance, regular consumption of kombucha is believed to reduce blood pressure, aid in weight loss, improve immunity, facilitate digestion, prevent the development of metabolic and gastrointestinal diseases as well as cancer

[1,3]. These positive health effects of kombucha are mainly attributed to the unique microbial consortium and the bioactive compounds these microbes produce during the fermentation process [4].

The SCOBY in kombucha is primarily made up of yeast and a large proportion of bacteria called acetic acid bacteria (AAB). AAB are Gram-negative obligate aerobes (which strictly require oxygen to survive) belonging to the *Acetobacteraceae* family. They can grow optimally at temperatures of approximately 25 to 30 °C and a pH of 5.0 to 6.5 [5,6]. The most prevalent AAB in kombucha are the species of the genera *Komagataeibacter*, *Gluconobacter*, *Acetobacter* and *Gluconacetobacter* [6]. Common AAB species found in commercial kombucha are *Komagataeibacter xylinus*, *Komagataeibacter saccharivorans*, *Acetobacter xylinum* and *Gluconobacter oxydans* [3,7,8]. These bacteria produce acetic acid and cellulose through the fermentation process. The metabolic products, in-turn confer various health-promoting properties such as antimicrobial activities and the prevention of metabolic diseases like diabetes and obesity by decreasing intestinal glucose absorption and converting glucose into cellulose [2,9].

Lactic acid bacteria (LAB) are less frequently detected in kombucha. When present, LAB are usually found in relatively low abundances [6,7,8]. However, LAB such as *Bacillus coagulans* and *Lactobacillus* species are often added in commercial kombucha fermentation to enhance the probiotic potential of the beverage [8,10]. LAB are Gram-positive, facultative anaerobic bacteria (can survive in the presence or absence of oxygen) belonging to the *Firmicutes* phylum, which grows optimally at temperatures between 25 to 40°C and a pH of 4.0 to 6.0. LAB can be categorised into either homofermenters or heterofermenters [11,12]. *Lactobacillus*, *Lactococcus* and *Streptococcus* species are among the most common homofermenters which utilise glucose to produce lactic acid as the primary end-product of glycolysis. Meanwhile, heterofermenters such as *Leuconostoc* and *Weisella* spp. generate lactic acid, carbon dioxide and ethanol via the pentose

phosphate pathway [6,11,12]. Similar to AAB, the metabolites produced by LAB facilitate digestion, prevent infections and exert immunomodulation properties [11,12].

The different preparation and brewing processes have resulted in variable microbial composition across kombucha. In this study, we profiled the microbial composition and functional pathways of nine commercial kombucha products in Singapore using shotgun metagenomics sequencing. We further compared the microbial and functional differences between the AAB-and LAB-dominant.

Methods

Sample procurement and processing

Nine kombucha products were obtained from commercial supermarkets in Singapore. Each product was brewed according to the manufacturer's instruction. The products were stored at 4ºC, with a shelf-life of up to 12 months. Unpasteurised kombucha products were procured and sent to the laboratory for subsequent steps on the same day.

DNA extraction and shotgun metagenomic sequencing

A total of 300 to 350mL of each product was centrifuged at 6000×g. The resulting supernatant was removed, and the pellet was stored at −80°C. The DNA from the frozen pellet was extracted using QIAamp PowerFecal Pro DNA kit according to the manufacturer's protocol and quantified using Qubit 4 fluorometer. DNA sequencing was performed using Illumina NovaSeq with 2×150bp paired-end configuration and a read depth of 20 million paired-end reads. The raw sequence reads were processed using BioBakery3 workflows [13]. Read-level quality control was conducted using KneadData v0.11.0 with default settings [13]. Taxonomy annotations were performed using MetaPhlan version 3.0 which uses clade-

specific markers to identify microbial species and their relative abundances. Meanwhile, functional pathways annotations were conducted using HUMAnN version 3.0 based on the MetaCyc pathway database [13].

Data analysis

Species and pathway abundances were analysed using phyloseq R package version 1.36.0 [14]. Species and functional pathways with <10% and <0.01% relative abundances respectively were discarded using genefilter_sample and prune_taxa functions from the phyloseq R package version 1.36.0 [14]. The bar plots were generated using ggplot2 R package version 3.3.5 whereas the heatmap was generated using ComplexHeatmap R package version 2.13.1 [15,16]. The products were categorised as AAB- or LAB-dominant if the relative abundances of either AAB or LAB species reached more than 30%. Non-AAB- and LAB-dominant products (relative abundances of AAB, LAB or other species (<30%) were categorised as "others".

Results

All nine products were unpasteurised, and most (n = 5) used either SCOBY or acidifier (n = 2) as the starter culture. *Bacillus coagulans* which was added in E and F and was detected in our analysis with relative abundances of almost 100% (99.95% and 99.98% respectively). However, the species of *Lactobacillus*, *Acetobacter*, *Gluconobacter* and *Saccharomyces*, which claimed to be added in products D and G by the respective manufacturers were either below detection limit or present in low abundance (2.06% of *Saccharomyces cerevisiae* in G) **(Table 1** and **Fig 1a).**

Table 1. Commercial claims and microbiome profiles of the kombucha products (*n* = 9) included in this study.

Starter culture	Product	Type of dominant bacteria present	Probiotics added*	Number of microbial species	Number of functional pathways	Average pathways per species
Acidifier	A	Others	None	27	314	11.63
Acidifier	B	AAB	None	4	130	32.50
SCOBY	C	Others	None	96	390	4.06
SCOBY	D	AAB	Yeast and *Lactobacillus* species	4	148	37
Unknown	E	LAB	*Bacillus coagulans*	3	117	58.5
Unknown	F	LAB	*Bacillus coagulans*	2	137	45.67
SCOBY	G	AAB	Yeast of the *Saccharomyces* genus and bacteria from *Acetobacter* and *Gluconobacter* species	3	97	32.33
SCOBY	H	LAB	None	7	192	27.43
SCOBY	I	AAB	None	6	142	23.67

* As claimed by the kombucha manufacturer

Overall, 141 microbial species were detected across all nine products. Eighty-one species remained after filtering for low species abundance (<10%) **(Fig 1a)**. Product C had the highest species diversity, followed by product A and the remaining products contained less than ten species **(Table 1)**.

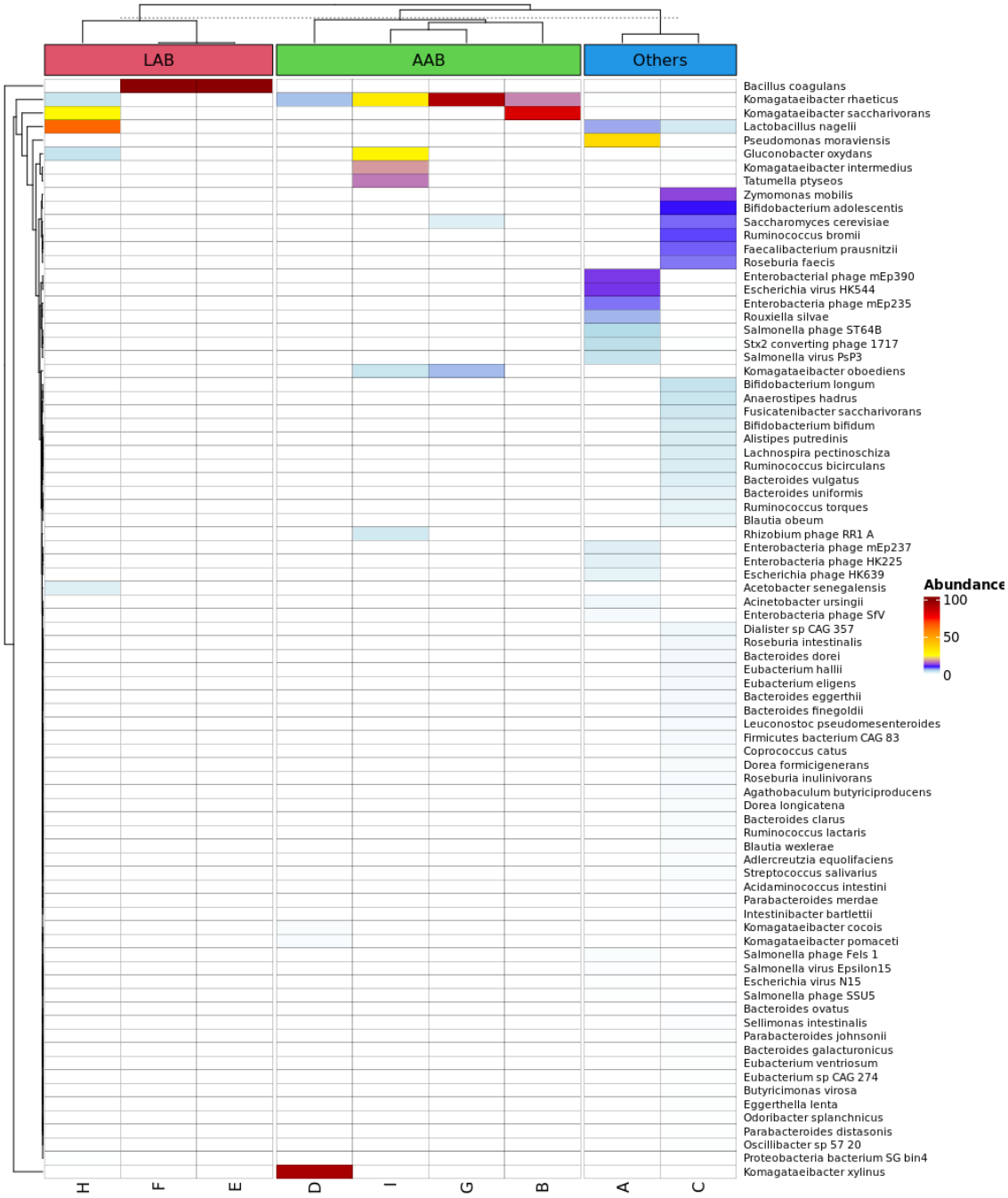

Fig 1a. Heatmap of microbial species. The heatmap of 81 microbial species detected across the nine kombucha products after filtering for species with >10% relative abundance.

The classification of species from AAB and LAB was based on the definition set by previous literature [5,11]. Four out of the nine products were predominated by AAB, notably the species from the *Komagataeibacter* genus (relative abundance > 30%). These four products, B, D, G and I were predominated by *Komagataeibacter saccharivorans* (B), *Komagataeibacter xylinus* (D) and *Komagataeibacter rhaeticus* (G and I) with relative abundances of 82.93%, 93.38%, 92.20% and 30.62%, respectively. LAB-dominant products were E, F and H, with relative abundances of *Bacillus coagulans* in E and F at 99.95% and 99.98%, respectively and *Lactobacillus nagelli* in H at 64.12% **(Fig 1a)**.

After removing functional pathways with <0.01% relative abundance, 59 out of 433 pathways remained. The top five most abundant functional pathways were the pentose phosphate pathway (non-oxidative branch), aerobic respiration I (cytochrome c), aerobic respiration II (cytochrome c) (yeast), guanosine deoxyribonucleotides de novo biosynthesis II and adenosine deoxyribonucleotides de novo biosynthesis II **(Fig 2a)**.

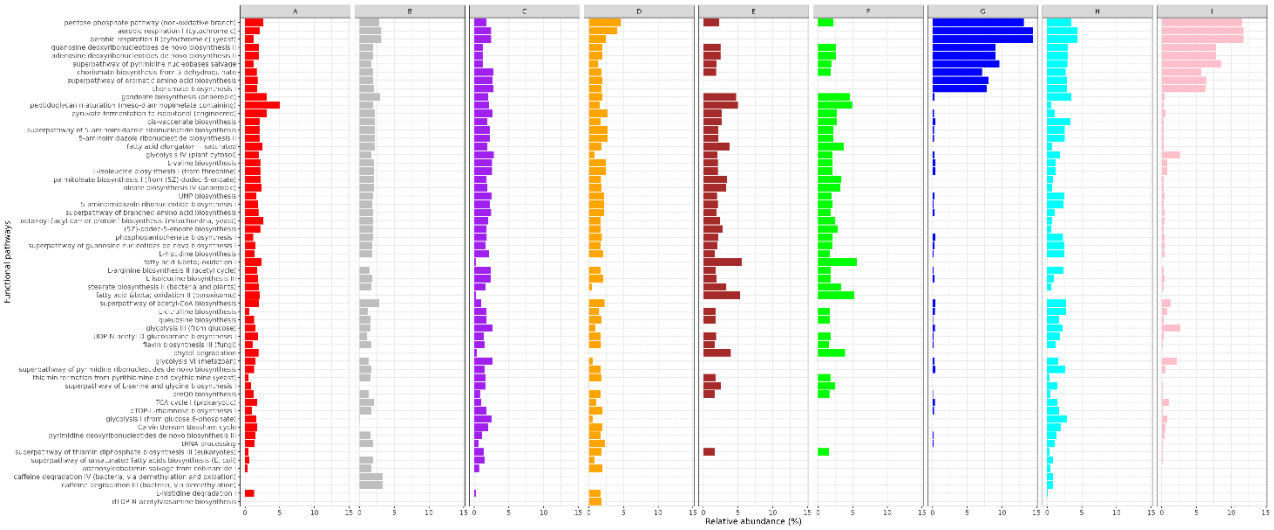

Fig 2a. Functional pathways. The top 59 functional pathways after removing those with less than 0.01% relative abundance (the relative abundances were normalised to 100% in this figure) across all the nine kombucha products.

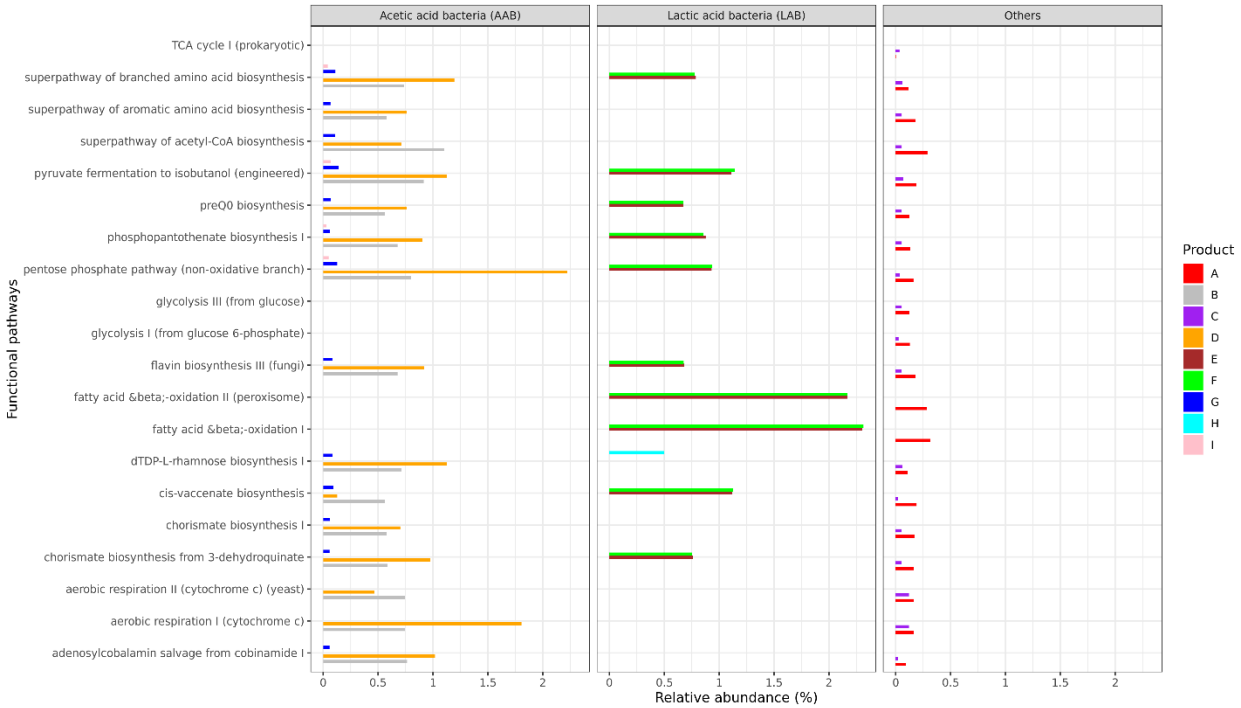

Fig 2b. Species contribution. The relative abundances of the top 20 functional pathways contributed by

three categories of microbes; AAB, LAB and others. Unclassified species contributing to the pathways across the nine products were excluded.

Of the 59 pathways, we further selected 20 that are involved in the production of beneficial metabolites. AAB was found to harbour 15 out of the 20 selected pathways (75%), while 11 pathways (55%) were detected in LAB. Notably, 2 out of 20 functional pathways (10%) were exclusively contributed by LAB and "others". These pathways were "fatty acid and beta-oxidation II (peroxisome)" and "fatty acid and beta-oxidation I". Meanwhile, 6 out of 20 (30%) were exclusively contributed by AAB and "others" **(Fig 2b)**.

Discussion

The growing popularity of kombucha in the last decade has motivated many beverage companies to introduce various new fermentation strategies, starter cultures and additional flavours, which results in unique microbial and metabolite profiles among different kombucha products. Notably, the bacterial composition in kombucha is comprised mainly of AAB and LAB. These bacteria play an essential role in producing bioactive compounds, thereby contributing to the pro-health effects of kombucha [3,6]. In this study, we analysed nine commercial kombucha products in Singapore and compared the composition of AAB and LAB and their functional potentials. Shotgun metagenomics sequencing revealed that the studied kombucha can be largely classified into three categories, namely the AAB- dominant (B, D, G and I), LAB-dominant (E, F and H) and others (A and C).

Consistent with other parallel kombucha studies, *Komagateibacter saccharivorans*, *Komagataeibacter xylinus* and *Komagataeibacter rhaeticus* are the most abundant and prevalent *Komagataeibacter* species found in the AAB-dominant products [8,17,18]. Interestingly, the high prevalence of *Komagataeibacter*

spp. was found across geographically distant locations (e.g. Australia, China, Turkey and USA), suggesting a degree of standardisation in the kombucha preparation [7-8,17-19]. The species of *Komagataeibacter* are reported to exhibit efficient glucose-metabolising activities (e.g. high glucose conversion rate to cellulose) which have a putative role in preventing the onset of metabolic diseases such as type-2 diabetes and obesity [9,18].

Our study found the three LAB-dominant products (E, F and H) were predominated by *Bacillus coagulans* (~99% relative abundances in E and F) and *Lactobacillus nagelii* (64.12% relative abundance in H). Our finding was consistent with the previous studies conducted in America which also detected the predominance of these LAB species in their tested kombucha samples [7,8]. *B. coagulans* are often added to food products not only due to their numerous probiotic characteristics but also their high tolerance to extreme environments such as high temperature and acidity. *B. coagulans* possess a wide range of probiotic attributes such as promoting intestinal digestion, modulating the immune system, preventing pathogenic bacteria colonization, and alleviating certain diseases and gastrointestinal tract (GIT) disorders such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and colorectal cancer [8,20,21]. The predominance of *B. coagulans* in these products was consistent with the claims made by the product manufacturer. Nonetheless, some probiotic species labelled by the manufacturers were not detectable, likely due to the detection limit of our sequencing approach, or the unfavourable storage conditions. *Lactobacillus nagelii*, a common *Lactobacillus* species found in kombucha predominated product H. The species of *Lactobacillus* (LAB) are known to enhance the health-promoting properties of kombucha and widely regarded as probiotics as they can produce short-chain fatty acids (SCFAs), amines, bacteriocins and vitamins involved in antimicrobial and antioxidant activities as well as gut health maintenance, among others [10,12].

To evaluate the health benefits of kombucha, we analysed the functional potential of the microbes across the nine products. The top five out of 59 functional pathways detected were related to sugar metabolism

(pentose phosphate pathway (non-oxidative branch), aerobic respiration I (cytochrome c), aerobic respiration II (cytochrome c) (yeast)) and the production of purines (guanosine deoxyribonucleotides de novo biosynthesis II and adenosine deoxyribonucleotides de novo biosynthesis II). Some of the products of these pathways such as ribose-5-phosphate sugar, nicotinamide adenine dinucleotide phosphate (NADPH), adenosine triphosphate (ATP) and purines act as essential growth factors and energy sources for the microbes to synthesise bioactive compounds in the kombucha culture [1].

We further selected 20 functional pathways including SCFAs production, biosynthesis of precursors for B vitamins, amino acids and organic acids [1,4,8]. We noted that functional pathways such as "fatty acid and beta-oxidation II (peroxisome)" and "fatty acid and beta-oxidation I" were only contributed by LAB but not AAB. These pathways metabolised fatty acids to produce acetyl-CoA in an anaerobic condition. Acetyl-CoA is a precursor for the biosynthesis of acetate, one of the common SCFAs that play an essential role in maintaining gut health, immune homeostasis and preventing disease development [22]. LAB, which are facultative anaerobes may utilize these pathways to generate SCFAs when oxygen level is depleted during kombucha fermentation, unlike AAB, which requires oxygen to grow [6,20,23-24].

In comparison, AAB contributed 20% more pathways involved in sugar metabolism, respiration, biosynthesis of amino acids and B vitamins than LAB. For instance, superpathway of aromatic amino acid biosynthesis, superpathway of acetyl-CoA biosynthesis, chorismate biosynthesis I, aerobic respiration II (cytochrome c) (yeast), aerobic respiration I (cytochrome c) and biosynthesis of adenosylcobalamin (vitamin B12) were among the pathways found in AAB but absent in LAB. Meanwhile, lower abundances of species from the "others" group contributed to the 20 selected pathways as compared to AAB and LAB. This could suggest that AAB and LAB possess greater capability in producing beneficial metabolites as compared to non-AAB and -LAB species.

Nevertheless, the exact metabolite composition in kombucha would require further targeted chemical assays. One of the limitations of our study is the small sample size, contributed mainly by the relatively limited number of kombucha brands in Singapore. Future work to assess the stability of kombucha microbiome over different production batches is warranted to understand the consistency of microbial and chemical compositions in kombucha. Clinical studies including *in vivo* tests and bioavailability assessments are indispensable to provide clinical evidence of kombucha's biological activities in humans, thereby confirming the positive health implications of this popular beverage.

Conclusion

Komagataeibacter species, namely *Komagateibacter saccharivorans*, *Komagataeibacter xylinus* and *Komagataeibacter rhaeticus* were the most prevalent and dominant bacteria present in AAB-dominant products (B, D, G and I) whereas *Bacillus coagulans* and *Lactobacillus nagelii* were the dominant species detected in LAB-dominant products (E, F and H). Although the functional pathways contributed by AAB differed from those contributed by LAB, both categories of bacteria can generate specific organic acids, amino acids, vitamin B compounds and SCFAs with health-enhancing properties in kombucha. Future work to characterise the microbial and metabolite concentrations and their consistencies in kombucha across production batches is necessary.

Funding

This study was supported by AMILI Pte. Ltd., Singapore.

Author Contributions

Conceptualization, GT, TKY and JL.; Methodology, JD, HSL, CCW, JLWJ; Writing – Original Draft Preparation, JD, HSL.; Writing – Review & Editing, GT, TKY, JL, CCW, JD, HSL. All authors have read and agreed to the published version of the manuscript.

Competing interests

SLH, KYT, GT are employees of AMILI Pte. Ltd. JWJL and JL are co-founders of AMILI Pte. Ltd. JD and CWC declare no conflict of interests.

References

1. Jayabalan, R.; Malbaša, R.V.; Lončar, E.S.; Vitas, J.S.; Sathishkumar, M. A Review on Kombucha Tea-Microbiology, Composition, Fermentation, Beneficial Effects, Toxicity, and Tea Fungus. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 538-550, doi:10.1111/1541-4337.12073.

2. de Miranda, J.F.; Ruiz, L.F.; Silva, C.B.; Uekane, T.M.; Silva, K.A.; Gonzalez, A.G.M.; Fernandes, F.F.; Lima, A.R. Kombucha: A review of substrates, regulations, composition, and biological properties. *J. Food Sci.* **2022**, *87*, 503-527, doi:10.1111/1750-3841.16029.

3. Antolak, H.; Piechota, D.; Kucharska, A. Kombucha Tea-A Double Power of Bioactive Compounds from Tea and Symbiotic Culture of Bacteria and Yeasts (SCOBY). *Antioxidants (Basel)* **2021**, *10*, doi:10.3390/antiox10101541.

4. Villarreal-Soto, S. A.; Beaufort, S.; Bouajila, J.; Souchard, J.-P.; Taillandier, P. Understanding Kombucha Tea Fermentation: A Review. *J. Food Sci.* **2018**, *83* (3), 580–588, doi:10.1111/1750-3841.14068.

5. Qiu, X.; Zhang, Y.; Hong, H. Classification of Acetic Acid Bacteria and Their Acid Resistant Mechanism. *AMB Express* **2021**, *11* (1), doi:10.1186/s13568-021-01189-6.

6. Bishop, P.; Pitts, E. R.; Budner, D.; Thompson-Witrick, K. A. Kombucha: Biochemical and Microbiological Impacts on the Chemical and Flavor Profile. *Food Chem* **2022**, 100025, doi:10.1016/j.focha.2022.100025.

7. Harrison, K.; Curtin, C. Microbial Composition of SCOBY Starter Cultures Used by Commercial Kombucha Brewers in North America. *Microorganisms* **2021**, *9* (5), 1060, doi:10.3390/microorganisms9051060.

8. Yang, J.; Lagishetty, V.; Kurnia, P.; Henning, S.M.; Ahdoot, A.I.; Jacobs, J.P. Microbial and Chemical Profiles of Commercial Kombucha Products. *Nutrients* **2022**, *14*, 670, doi:10.3390/nu14030670.

9. Lavasani, P.S.; Motevaseli, E.; Sanikhani, N.S.; Modarressi, M.H. Komagataeibacter xylinus as a novel probiotic candidate with high glucose conversion rate properties. *Heliyon* **2019**, *5*, e01571, doi:10.1016/j.heliyon.2019.e01571.

10. Nguyen, N.K.; Dong, N.T.; Nguyen, H.T.; Le, P.H. Lactic acid bacteria: promising supplements for enhancing the biological activities of kombucha. *Springerplus* **2015**, *4*, 91, doi:10.1186/s40064-015-0872-3.

11. Mokoena, M. P. Lactic Acid Bacteria and Their Bacteriocins: Classification, Biosynthesis and Applications against Uropathogens: A Mini-Review. *Molecules* **2017**, *22* (8), 1255, doi:10.3390/molecules22081255.

12. Wang, Y.; Wu, J.; Lv, M.; Shao, Z.; Hungwe, M.; Wang, J.; Bai, X.; Xie, J.; Wang, Y.; Geng, W. Metabolism Characteristics of Lactic Acid Bacteria and the Expanding Applications in Food Industry. *Front. Bioeng. Biotechnol.* **2021**, *9*, doi:10.3389/fbioe.2021.612285.

13. McIver, L.J.; Abu-Ali, G.; Franzosa, E.A.; Schwager, R.; Morgan, X.C.; Waldron, L.; Segata, N.; Huttenhower, C. bioBakery: a meta'omic analysis environment. *Bioinformatics* **2018**, *34*, 1235-1237, doi: 10.1093/bioinformatics/btx754.

14. McMurdie, P.J.; Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **2013**, *8*, e61217, doi:10.1371/journal.pone.0061217.

15. Wickham, H. ggplot2: Elegant Graphics for Data Analysis. Available online: https://ggplot2.tidyverse.org (accessed on 10 March 2022).

16. Gu, Z.; Eils, R.; Schlesner, M. Complex Heatmaps Reveal Patterns and Correlations in Multidimensional Genomic Data. *Bioinformatics (Oxford, England)* **2016**, *32* (18), 2847–2849, doi:10.1093/bioinformatics/btw313.

17. Arikan, M.; Mitchell, A.L.; Finn, R.D.; Gurel, F. Microbial composition of Kombucha determined using amplicon sequencing and shotgun metagenomics. *J. Food Sci.* **2020**, *85*, 455-464, doi:10.1111/1750-3841.14992.

18. Kaashyap, M.; Cohen, M.; Mantri, N. Microbial Diversity and Characteristics of Kombucha as Revealed by Metagenomic and Physicochemical Analysis. *Nutrients* **2021**, *13*, doi:10.3390/nu13124446.

19. Zhao, Z.; Sui, Y.; Wu, H.; Zhou, C.; Hu, X.; Zhang, J. Flavour Chemical Dynamics During Fermentation of Kombucha Tea. *Emir. J. Food Agric.* **2018**, *30*, 732-741, doi:10.9755/ejfa.2018.v30.i9.1794.

20. Su, F.; Xu, P. Genomic Analysis of Thermophilic Bacillus Coagulans Strains: Efficient Producers for Platform Bio-Chemicals. *Sci. Rep* **2014**, *4*, 3926, doi:10.1038/srep03926.

21. Cao, J.; Yu, Z.; Liu, W.; Zhao, J.; Zhang, H.; Zhai, Q.; Chen, W. Probiotic characteristics of *Bacillus coagulans* and associated implications for human health and diseases. *J. Funct. Foods* **2020**, *64*, 103643, doi:10.1016/j.jff.2019.103643.

22. Tan, J.; McKenzie, C.; Potamitis, M.; Thorburn, A. N.; Mackay, C. R.; Macia, L. The Role of Short-Chain Fatty Acids in Health and Disease. *Adv. Immunol.* **2014**, 91–119, doi:10.1016/b978-0-12-800100-4.00003-9.

23. Schönfeld, P.; Wojtczak, L. Short- and Medium-Chain Fatty Acids in Energy Metabolism: The Cellular Perspective. *J Lipid Res*. **2016**, *57* (6), 943–954, doi:10.1194/jlr.R067629.

24. PubChem. Fatty Acid Oxidation. Available online: https://pubchem.ncbi.nlm.nih.gov/pathway/PathBank:SMP0000781 (accessed on 25 May 2022).