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Metabolite Profiling of Kombucha Fermented Beverage with Substrate Variation

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ABSTRACT
Kombucha is a fermented beverage of tea and sugar with symbiotic culture of bacteria and yeast (SCOBY/symbiotic culture of bacteria and yeast). Changes in chemical composition which are functional constituents and flavor components of kombucha beverages can occur during kombucha fermentation. Substrate is one of the factors determining the characteristics of kombucha. Generally, the substrate used in making kombucha comes from *Camellia sinensis* tea leaves. Modification of substrates for kombucha fermentation with herbs can increase the various potentials of kombucha. The aim of this study was to analyze untargeted metabolomics for metabolite profiling of kombucha fermented from different substrates (clove leaves, guava leaves, mint leaves, and morel berry leaves). The research methods used were controlled fermentation for fourteen days, and metabolomic analysis using Gas Chromatography-Mass Spectrometry. The results showed that different substrates affected the metabolite content profile of fermented kombucha. The metabolite profile of Gas Chromatography-Mass Spectrometry results identified from clove kombucha as many as 26 metabolites, from guava kombucha 13 metabolites, from mint leaf kombucha 24 metabolites, and morel berry leaf kombucha with 21 metabolites. There is a metabolite identified from each kombucha with these substrate variations, namely 5-Hydroxymethylfurfural.
Key words: metabolomics; *Syzyygium aromaticum; Psidium guajava; Mentha piperita; Physalis angulata*

INTRODUCTION

Kombucha is a traditional-fermented beverage that is rich in probiotics and bioactive factors. Kombucha is generally produced from tea plus sugar fermented with a culture of bacteria and yeast (SCOBY/symbiotic culture of bacteria and yeast). The microbial association of kombucha is a complex and dynamic microbial ecosystem, and its composition changes along with the fermentation process. With the dynamic changes of the microbial community, changes in the chemical composition (including functional constituents and flavor components) of kombucha tea may occur during kombucha fermentation. Substrate is one of the factors determining the characteristics of kombucha. Generally, the substrate used in making kombucha comes from *Camellia sinensis* tea leaves. However, kombucha can also be produced from the fermentation process of other substrates, such as various types of herbal plants that are commonly consumed by the community or from fruit extracts (Bishop et al., 2022, Zubaidah et al., 2018, Mardiana et al., 2022). Kombucha produced from the fermentation process of other substrates, such as various types of herbal plants, will have an impact on its biological activity.

Several factors influence kombucha fermentation, one of which is the substrate. However, kombucha can also be produced from the fermentation process of other substrates (other than variants of tea-black tea, green tea) such as various types of herbal plants commonly consumed by the public or from fruit extracts (raspberries, goji berries, red grapes, cactus pears, blackthorn, dates, coconut, melon, watermelon, and sour cherries). Other substrates that can be used for kombucha preparation include milk, coffee, whey, wine, vinegar, Jerusalem artichoke, black carrot juice concentrate, and oak wood) (Bishop et al., 2022, Yang, 2023, Chakravorty et al., 2016, Kuzu et al., 2023). Different varieties (black, green, or oolong) of tea, along with the plants used in fermentation, changes in concentration and fermentable substrate, fermentation time, and SCOBY composition could all play a role in compositional differences between different kombuchas as well as impact their biological activity (Kuzu et al., 2023).

Traditional kombucha is made using black, green, or oolong tea as the base. Kombucha can be made with other substrates such as Jerusalem artichoke tuber extract, wine, milk, fruit juices, and plant infusions, each substrate bringing nutritional and health benefits based on the chosen medium (Bishop et al., 2022) Various studies have been conducted related to kombucha, namely the utilization of plants that are used as herbal teas, namely lemon balm, rooibos, yarrow, wheatgrass, guava leaves, cinnamon, cardamom, Shirazi thyme, African mustard, *Lippia citriodora* leaves, *Eucalyptus camaldulensis*, *Timus vulgaris* L. *Litsea glaucescens*, *Rosmarinus officinalis, Foeniculum vulgare,* and *Mentha piperita*. In addition, the variety of kombucha fermentation substrates are the bark of the faloak plant (*Sterculia quadrifa* R. Br.), bay leaves (*Syzygium polyanthum* (Wight) Walp.), cocoa leaves (*Theobroma cacao,* betel leaves, coffee leaves, soursop leaves, and bay leaves (Neffe-Skocinska et al., 2017, Suhardini and Zubaidah, 2016).

Modification of the substrate for kombucha fermentation with herbs can improve the various potentials of kombucha. Clove leaves (*Syzygium aromaticum* L. Merr. & L.M. Perry), guava leaves (*Psidium guajava* L.), mint leaves (*Mentha piperita* L.), and morel berry leaves (*Physalis angulata* L.) were known to contain bioactive compounds that have pharmacological activities that are good for health. Based on this, modifying the kombucha fermentation substrate with various types of herbs (clove leaves, guava leaves, mint leaves, morel berry leaves) as a substrate can increase the benefits of kombucha. However, there were no reports of metabolomic profiles related to kombucha from these herbs. Each substrate brings different nutritional and health benefits based on the chosen medium. The selection of different fermentation substrates also results in different substances in traditional kombucha. Very little information is available regarding the dynamic changes to flavor constituents of kombucha tea during the fermentation process with substrates of clove leaves, guava leaves, mint leaves, morel berry leaves. The aim of this study was to analyze the metabolite profile of kombucha fermented from different substrates.

METHODS

Kombucha fermentation

The samples to be used in making kombucha were clove leaves, guava leaves, mint leaves, and morel berry leaves. All the leaves were dried until dry (without sun exposure). After drying, each leaf was weighed as much as eight grams and filled into a tea bag. Next, for each type of leaf, one litre of fermentation was done. One litre of boiling water was added to 0.8% (b/v) of each leaf separately. After 20 minutes, 10% (b/v) sugar was added and then cooled (to room temperature). After that, each jar was added with SCOBY and a 100 ml kombucha starter. Then kombucha fermentation was carried out for fourteen days at room temperature (Kolompoy et al., 2024).

GC-MS Analysis

Kombucha samples were prepared for metabolomics analysis performed by GC-MS Agilent 8890 GC System, with HP-5MS column. The sample used was a kombucha solution that had been filtered using the Extrelut tool. The filtering results were then injected 0.2 to 1 μl using methanol solvent into the injector and inserted into the GC-MS instrument. Sample preparation is done by dissolving three grams of sample with methanol and then injected into the injector. The injector temperature was 290ºC with a split ratio of 50:1, the carrier gas was helium with a flow rate of 1 ml/min. The column temperature was programmed at 40ºC, rising 15ºC/min until 290ºC then held for 10 minutes. The detector used was MSD with a solvent delay of 2.6 minutes. Data were analyzed using MassHunter Workstation Quantitative Analysis software (version 10.0.707.0).

RESULTS AND DISCUSSION

Kombucha Fermentation

In this study, kombucha was made from various substrates of cloves leaf tea (KC), guava leaf (KJB), mint leaf (KDM), and morel berry leaf (KDC), using 0.8% (w/v) tea leaves and 10% (w/v) sugar, as well as 10% (v/v) starter culture and SCOBY. The fermentation process was carried out for seven days, but for metabolomic analysis, kombucha was harvested on the 7th day of fermentation. The color of the kombucha was associated with the color of the substrate tea used (**Figure 1**). Guava leaf kombucha (KJB) was more intense/dark compared to the other kombuchas (**Figure 1**).

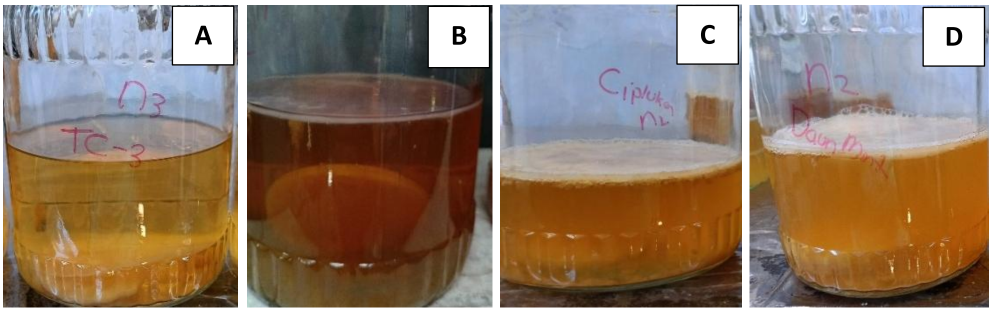


Figure 1. Kombucha (A) clove leaves (KC), (B) guava leaves (KJB), (C) morel berry leaves (KDC), and (D) mint leaves (KDM), after seven days of fermentation.

During the fermentation process, a membrane thickens over time, known as pellicle or biofilm, or commonly referred to as a thin layer of SCOBY (**Figure 1**). After 7-10 days of fermentation, biofilm begins to appear on the kombucha, but in cloves leaf kombucha (KC), the layer that forms showed very slow growth compared to kombucha from other substrates (**Figure 1A**). The layer on the surface of cloves leaf kombucha starts to thicken after 10 days of fermentation.

In KJB, KDC, and KDM kombucha with a fermentation of 10 days, the pellicle structure is thick and flat, indicating that this treatment has the thickest surface layer. Additionally, we found that different tea substrates in the production of this kombucha showed uneven SCOBY growth and formed an uneven pellicle based on thickness measurements (**Figure 2**). The pellicle structure in KC kombucha is the kombucha with the slowest SCOBY growth, making it appear the thinnest. The end of the fermentation process is marked by the sour smell produced by kombucha, and there are no signs of contamination in the kombucha.

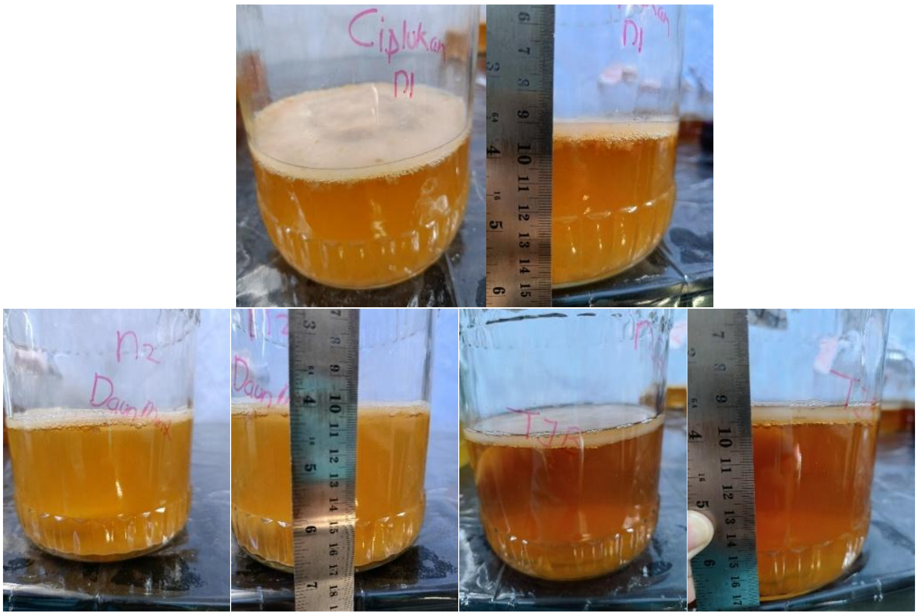


Figure 2. Growth of the SCOBY pellicle on the surface of kombucha after ten days of fermentation.

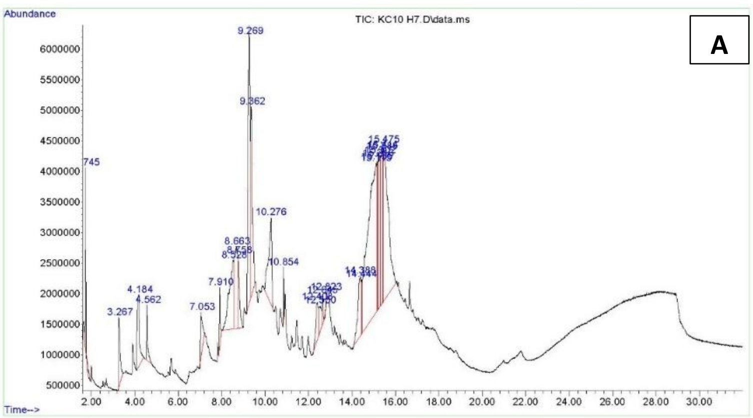
During the fermentation period of kombucha production lasting 7–12 days, a thin layer of SCOBY grows on the surface of the liquid. This layer consists of a mass of cells where bacteria and yeast adhere. During the fermentation process, the yeast and bacteria added from the kombucha starter and pellicle form the SCOBY, which is the initial sign of fermentation. SCOBY plays a role in hydrolyzing sucrose. Sucrose is hydrolyzed by the yeast colonies present in SCOBY, breaking down sucrose into fructose, which is then used by the yeast in the formation of ethanol. The ethanol produced by the yeast is then used by bacterial colonies, such as AAB (Acetic Acid Bacteria) and LAB (Lactic Acid Bacteria), to oxidize ethanol into acetic acid and lactic acid (Hsieh et al., 2021). The acid products from AAB and LAB cause kombucha to experience a decrease in pH and produce an acidic character. Kombucha will appear cloudy due to suspended solids (colloids), but if filtered, it will look clearer. Particles present in a suspension can consist of microorganisms or large molecules with sizes ranging from 1 to 1000 nm (Bishop et al., 2021).

GC-MS Metabolite Profile

In GC-MS analysis, larger peak areas are usually associated with compounds that have more stable physical and chemical properties and can be better identified through mass spectrometry. Therefore, the highest area on the GC-MS chromatogram can be used as an indicator to determine the most dominant compound in a sample and as a reference for further analysis (Mukminin et al., 2022). The GC-MS chromatography results of cloves leaf kombucha showed 26 base peak chromatograms, guava leaf kombucha with 13 base peaks, mint leaf kombucha with 24 base peaks, while morel berry kombucha had 21 base peaks (**Figure 3**). All those areas were then identified qualitatively through the method of comparing the obtained spectrum data with the spectrum data in the data bank. The results of untargeted metabolomic analysis using GC-MS can be seen in **Table 1**. The compounds produced in kombucha with different tea substrates also differ from each other.

Based on the GC-MS results obtained, cloves leaf kombucha contains 26 compounds, with 3 compounds dominating with the highest area (%) being 3-Deoxy-d-mannoic lactone (22.80%), 5-Mercaptotetrazole (13.77%), and 5-Hydroxymethylfurfural (9.95%). In mint leaf kombucha, the three compounds with the highest area are 3-Deoxy-d-mannonic acid (15.21%), 5-Hydroxymethylfurfural (13.71%), and caffeine (10.88%). The three compounds that dominate the guava leaf kombucha are 5-Hydroxymethylfurfural (35.47%), Stevioside (9.80%), and 1,2,3-Propanetriol, triacetate (7.01%). Meanwhile, in morel berry kombucha, it is dominated by caffeine compounds (17.19%), 2R, 3S-9-[1,3,4-Trihydroxy-2-butoxymethylguanine] (13.25%), and 2-Hydroxy-2-cyclopenten-1-one (9.72%).

In the content of cloves leaf kombucha, the three highest compounds are 3-Deoxy-d-mannoic lactone (22.8%); 5-Mercaptotetrazole (13.77%); 5-Hydroxymethylfurfural (9.95%). The three compounds are known to have antioxidant potential. The antioxidant activity of clove kombucha is not only from these three compounds but also from other compounds contained in clove kombucha according to GC-MS analysis results, namely Acetic acid; Diglycolic acid; 4H-Pyran-4-one,2,3-dihydro-3,5-di hydroxy-6-methyl; Propanal, 2,3-dihydroxy-, (S)-; Erythritol; 1,2,3-Propanetriol, 1-acetate; Eugenol; Butanoic acid,3-oxo-, propyl ester; D-erythro-Pentose, 2-deoxy-; Gluco-octanoic acid lactone; 5-hydroxy-2-methyl-1,3-dioxane; 1,4-Anhydro-d-galactitol; 1,4-Anhydro-d-galactitol; and Oxiranecarboxylate.



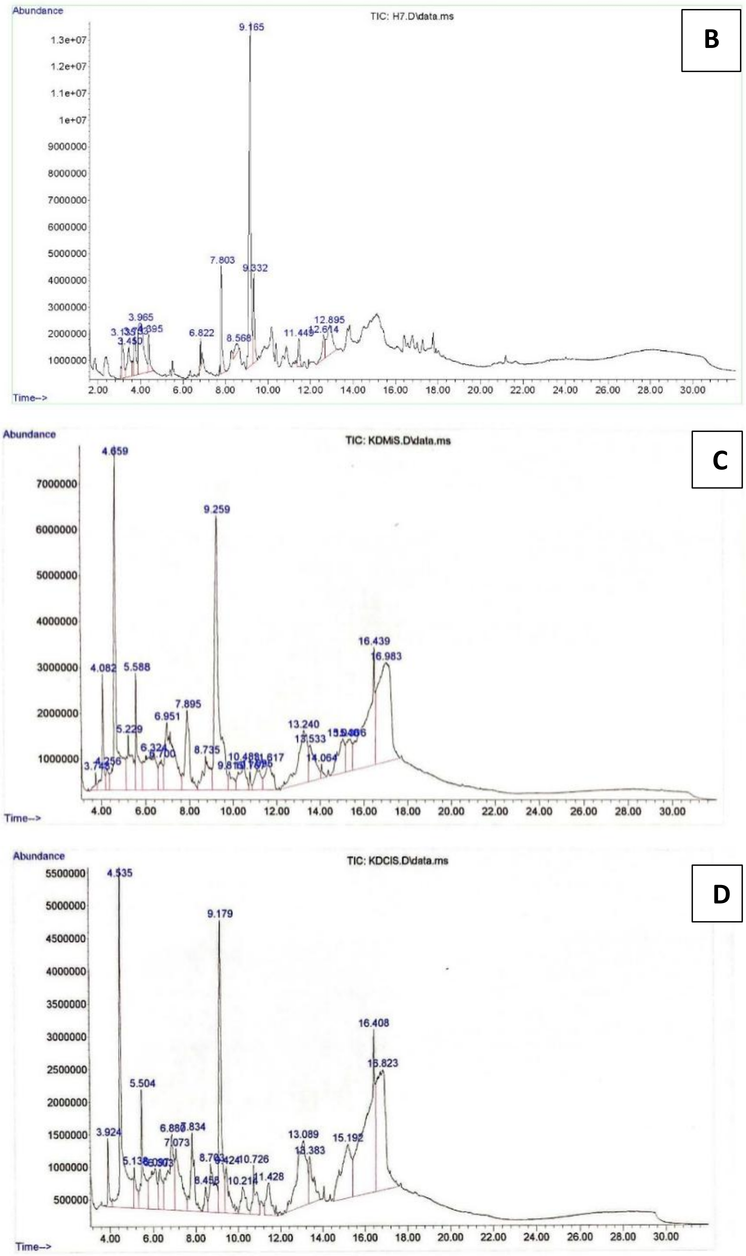


Figure 3. GC-MS Chromatogram of kombucha of cloves leaf (A), kombucha of guava leaf (B), kombucha of mint leaf (C), and kombucha morel berry leaf (D)

Table 1. GC-MS Results of Kombucha

Compounds	Area (%)	*Compounds*	Area (%)
Kombucha Cengkeh (99,32%)		*Kombucha Daun Mint (99,99%)*	
3-Deoxy-d-mannoic lactone	22,80	3-Deoxy-d-mannonic acid	15,21
5-Mercaptotetrazole	13,77	**5-Hydroxymethylfurfural**	13,71
5-Hydroxymethylfurfural	9,95	Caffeine	10,88
1,2,3-Propanetriol, 1-acetate	5,75	2-Hydroxy-2-cyclopenten-1-one	9,89
Propanal, 2,3-dihydroxy-, (S)-	5,18	Adenosine,4'-de(hydroxymethyl)-4'-[N-ethylaminoformyl]-	7,05
4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-	4,87	D-erythro-Pentose,2-deoxy-	5,97
Erythritol	4,72	1-Hexan-4,4-D2-ol, acetate	4,71
Ethyl trans-3-methyl-2-oxiranecarboxylate	4,19	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	4,24
1,4-Anhydro-d-galactitol	4,13	(3E)-3-(dimethylhydrazinylidene)propanoic acid methyl ester	3,20
Gluco-octonic acid lactone	3,35	2H-Thiazolo[3,2-a]pyrimidine-3,5-7(6H)-trione	2,88
N-Methyl-D3-aziridine	2,96	2H-Pyran-2,6(3H-dione	2,80
1,4-Anhydro-d-galactitol	2,83	Butanoic acid, ethyl ester	2,72
Propanal, 2,3-dihydroxy-, (S)-	1,92	(pyro)-catechol	2,65
cis-5-hydroxy-2-methyl-1,3-dioxane	1,89	1,2,3-Benzenetriol	2,30
Diglycolic acid	1,75	d-Mannitol,1,4-anhydro-	2,16
Propanediamide, 2-amino-	1,65	Acetamide, N-(aminocarbonyl)-	2,08
Pyrimidine-4,6-diol, 5-methyl-	1,42	1-Aminopentan-2-ol	2,06
5-Mercaptotetrazole	1,33	1,3-Dioxane,4,6-dimethyl-,cis-	1,51
Acetic acid	1,15	Isosorbide dinitrate	1,40
Butanoic acid, 3-oxo-, propyl este	1,05	Triacontyl acetate	1,00
4,5-Dihydro-2-methylimidazole-4-on	0,87	Dimethoxane	0,70
4H-Pyran-4-one, 2,3-dihydro-3,5-di hydroxy-6-methyl-	0,75	2-Methylpyrazole-3,4-diamine	0,30
3-Deoxy-d-mannoic lactone	0,66	Cyclic-isopropylidene cyclopropane-1,1-dicarboxylate	0,30
1-(2-Methyl -1,2,4-triazol-3-yl)ethanone	0,53	N-[3-(Cyano-dimethyl-methyl)-1-methyl-cyclopentyl]-acetamide	0,27

Phenol, 2-methoxy-3-(2-propenyl)-	0,37		
D-erythro-Pentose, 2-deoxy-	0,14		
Kombucha Daun Jambu Biji (88,99%)		***Kombucha Daun Ciplukan (100%)***	
5-Hydroxymethylfurfural	35,47	Caffeine	17,19
Stevioside	9,80	2R, 3S-9-[1,3,4-Trihydroxy-2-butoxymethylguanine]	13,25
1,2,3-Propanetriol, triacetate	7,01	2-Hydroxy-2-cyclopenten-1-one	9,72
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	6,45	**5-Hydroxymethylfurfural**	8,37
2-Furanmethanol	5,20	Guanosine	7,82
Dihydro-2(3H)-thiophenone	4,96	2-t-Butyl-4-methyl-5-oxo-[1,2]dioxalone-4-carboxylic acid	6,65
1,3-Dihydroxyacetone	3,50	Adenosine,4'-de(hydroxymethyl)-4'-[N-ethylaminoformyl]-	5,08
2-Hydroxy-2-cyclopenten-1	3,50	1,3-dioxolan-2-one	4,27
2-Furancarboxaldehyde	3,96	3-Hydroxy-2H-pyran-2-one	3,33
4-Amino-1,6-dihydro-1-methyl-6-oxo pyrimidine	3,34	Butyl alcohol-2D1	3,29
Ethanamine, N,N-diethyl	2,66	(3E)-3-(dimethylhydrazinylidene)propanoic acid methyl ester	3,29
Acetylmannosamine	2,04	Undecane, 6,6-dideutero-5-methyl-	3,09
1,3,5-Triazine-2,4,6-triamine	1,10	Cytidine	2,45
		(endo)-4,5,7,7-Tetramethyl-6,8-dioxabicyclo[3.2.1]octane	2,27
		1,2,3-Benzenetriol	1,96
		3-Amino-2-oxazolidinone	1,79
		3-Methyl-5-hyrazinopyrazine	1,76
		Acetic acid n-octadecyl ester	1,52
		Pentyl acetate (2,2-dideuterated)	1,26
		Propanoic acid, 2-oxo-, methyl ester	0,95
		2(3H)-Furanone, dihydro-4-hydroxy-	0,7

The profile of metabolites produced by kombucha with different substrates showed varying contents in each kombucha. However, there is one compound that is always produced by kombucha regardless of the substrate, namely 5-Hydroxymethylfurfural. In cloves leaf kombucha, the 5-Hydroxymethylfurfural content is 9.95%, in mint leaf kombucha it is 13.71%, in morel berry kombucha it is 8.37%; while in guava leaf kombucha, it has the highest content at 35.47%.

The compound 3-Deoxy-d-mannoic lactone, which has the highest area in clove kombucha, includes compounds with antibacterial and antioxidant activities. Mannoic lactone is among the compounds that impart a distinctive taste and aroma (Anjarsari et al., 2020). The compound 5-Mercaptotetrazole is a tetrazole derivative with a sulfur atom bound to the tetrazole ring, and its chemical structure indicates that this compound is capable of capturing free radicals. Research conducted by Zhao et al. (2013) shows that 5-Hydroxymethylfurfural (5-HMF) has antioxidant strength that can help prevent the occurrence of free radicals (ROS) and slow down the oxidation process.

As with cloves kombucha, the antioxidant activity of guava kombucha is also produced by other compounds according to the GC-MS results, namely 2-Furancarboxaldehyde; 2-Furanmethanol; Dihydro-2(3H)-thiophenone; 1,3-Dihydroxyacetone; 2-Hydroxy-2-cyclopenten-1; 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl; and Acetylmannosamine (Tabel 2). The stevioside compound (9.80%) contained in guava kombucha is a sweet compound that contains steviol glycoside diterpenes, which are the main components responsible for the sweetness in *Stevia rebaudiana*. Stevia contains antioxidant compounds that can prevent the spread of genes causing DNA damage, such as free radicals and superoxide (Tavarini dan Angelini, 2013).

Based on the metabolite profile analysis after the seventh day of fermentation in all kombucha samples, one compound was found in all kombucha with different substrates; 5-Hydroxymethylfurfural. These compounds can be further studied as characteristic compounds of kombucha. Research using the GC-MS method conducted by Isdar et al. (2023), using langsat kombucha, produced seven types of metabolite compounds, two of which are 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl and 5-Hydroxymethylfurfural.

The difference in the metabolite profile between the tea medium that served as the substrate and the kombucha after 7 days of fermentation is due to the fact that during the kombucha fermentation process, several precursor compounds play an important role in the synthesis of the resulting bioactive compounds. Compounds such as acetic acid, gluconic acid, glucuronic acid, folic acid, malonic acid, oxalic acid, and other acids produced during kombucha fermentation can also be considered precursors in the synthesis of kombucha bioactive compounds (Khamidah dan Antarlina, 2020). In addition, there is also glucose that acts as a precursor compound. Glucose that is converted into various types of acids, vitamins, and alcohol by bacteria during the kombucha fermentation process is also referred to as secondary metabolite fermentation. In the kombucha fermentation process, various microorganisms including lactic acid bacteria (LAB), acetic acid bacteria (AAB), and yeast work together in symbiosis to metabolize glucose and produce various metabolic products. In the synthesis of bioactive compounds in kombucha, these compounds act as raw materials that are transformed by microorganisms. Fermentation involves a complex interaction between yeast and bacteria, leading to the production of various compounds that contribute to the changes in metabolite compounds in kombucha.

During the fermentation process, the compounds contained in kombucha will change. The longer the fermentation, the different the resulting metabolites will be. This is due to the high content of bioactive compounds in kombucha, such as gluconate, glucuronate, oxalate, acetate, phenolate, and folate. The metabolites can vary depending on the tea base and the fermentation time (Nasution dan Nasution, 2022). The substrate used also greatly influences the metabolite profile contained in the sample. Kombucha with substrates of clove leaves, guava leaves, mint leaves, and morel berry leaves used in this study has different chemical compositions that can affect the metabolite compounds produced in kombucha.

CONCLUSION

Kombuchas with substrates of clove leaves, guava leaves, mint leaves, and morel berry leaves used in this study has different chemical compositions that can affect the metabolite compounds produced in kombucha. The metabolite profile of GC-MS results identified from clove kombucha as many as 26 metabolites, from guava kombucha 13 metabolites, from mint leaf kombucha 24 metabolites, and morel berry leaf kombucha with 21 metabolites. There is a metabolite identified from each kombucha with these substrate variations, namely 5-Hydroxymethylfurfural.

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