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COMPARATIVE ANALYSIS OF VOLATILE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF KEFIR PRODUCED BY THAI BLACK JASMINE RICE

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Abstract: Volatile compounds and antioxidant activity of kefir produced from Thai black jasmine rice (Hawm nil) were investigated and compared. Thai black jasmine rice milk was prepared by ultrasonication for 24 hours, and then subjected to nine different inoculum percentages of kefir grains and fermentation temperature conditions. Volatile compounds were detected by gas chromatography-mass spectrometry (GC-MS) analysis, while antioxidant activity was determined by DPPH free radical scavenging and ferric reducing/ antioxidant power (FRAP) assays. Metabolic pathway analysis was also conducted. Results indicated that Hawm nil rice milk kefir contained 27 volatile compounds, including four organic acids, four fatty acids, six alcohols, four esters, four ethers, three amines, one ketone and one amide. The GC-MS profile of volatile compounds differed significantly between fermentation conditions; however, antioxidant capacity of Hawm nil rice milk kefir remained constant. Metabolism of pyruvate, sulfur and glycerolipid were identified as pathways affected by fermentation. Results indicated that volatile compounds in fermented Hawm nil rice milk contributed to its antioxidant property. Varying temperature and inoculum conditions were crucial factors that affected the release of these volatile compounds. Rice milk kefir showed potential as supplementary food that was effective in ameliorating multiple human diseases and could serve as a valuable tool in promoting sustainability of human health.

Keywords: Rice milk kefir, sustainability, antioxidant activity, volatile compounds, ultrasonication technique.

Introduction

Kefir is a sour, frothy, slightly alcoholic drink formed by the fermentation of lactose and sugar in various types of milk by kefir grains (Chandan, 2006). It is a probiotic drink with numerous health benefits, and studies have shown that it has antimicrobial and anti-inflammatory activity, besides promoting wound healing and gut health. Kefir can be made from the milk of cows, goats and sheep, with cow milk being widely used. Besides animal milk, milk from non-dairy sources may also be used, such as coconut, soy and rice, Kefir is typically a homemade product, but the drink has also been sold commercially (Farnworth, 2005).

Deeseenthum *et al.* (2017) recommended rice milk kefir as an anti-inflammatory drink for people at risk of chronic disease, while Chunchom *et al.* (2016, 2017) reported on its high non-toxic antioxidant activity, which may be beneficial to health. A total of 47 volatile compounds have been identified in rice kefir fermented by lactic acid bacteria (LAB) (Lee *et al.*, 2019), while 60 compounds are detected in the fermentation of grain substrates (wheat, oats, malt and barley) using the same bacteria, including linoleic acid, oleic acid, 5-hydroxymethylfurfural and acetic acid (Salmeron *et al.*, 2015).

Recently, scientific interest in kefir has increased because it contains unique volatile compounds. Rice milk kefir may add value to

products because of its high antioxidant content and health benefits**.** Rice milk kefir produced under different conditions may form diverse volatile compounds that possess disparate properties. Therefore, this study determines and compares the volatile compounds and antioxidant activity of kefir produced from the milk of Thai black jasmine rice (Hawm Nil) under various fermenting conditions.

Materials and Methods

Rice Material

Whole grains of the Hawm Nil Thai coloured rice variety (dark purple or black rice), also known as Thai black jasmine, were sourced from Kalasin Province, Thailand (Figure 1) at coordinates 16.4385° N, 103.5061° E.

Kefir Grain Activation

Homemade kefir grains were purchased from Nonthaburi Province, Thailand. The starter grains were grown in pasteurized milk **(**Dutch mill**)**, incubated at room temperature for 24 hours, and then kept refrigerated at 4 °C until required for use.

Rice Milk and Kefir Preparation

Thai black jasmine rice grains were soaked in distilled water for 24 hours before the mixture was blended at a ratio of 1**:**5 w/v **(**black rice**:** water**).** The prepared rice milk was subjected to ultrasonication according to the technique described by Deeseenthum *et al.* (2018**).** It was ultrasonicated for five minutes using a Sonics Vibra Cell Ultrasonicator **(**20 KHz**)** at low intensity with tip diameter of 25 mm, 500 ml to 1,000 ml volume and 70 % amplitude. Following this, the rice milk was pasteurised at 75 °C for 15 mins and filtered using cheese cloth**.** Activated kefir grains were inoculated into the rice milk and incubated for 24 hours at 25 ± °C at static condition. A 50 ml sample of the rice milk was collected immediately after inoculation (zero hour).

Experimental Design

This study used a central composite rotatable design (CCRD) with two factors to generate the experimental set up as presented in Table 1**.**

Production of Rice Milk Kefir

Nine experimental conditions were used in this study. Fermentation temperature and inoculation

Figure 1: A map of Thailand indicating Kalasin Province, where the Hawn Nil Thai black jasmine rice used to make rice milk in this study is sourced

Table 1: Experimental design of rice milk kefir production with code values and actual values

Experiment No.	Process variables	
	Inoculation rate (%)	Incubation temperature (°C)
1	2.5 (-1.414)	27.5 (0)
2	3 (1)	25.0 (-1)
3	3 (1)	30.0(+1)
4	4 (0)	23.9 (-1.414)
5	4 (0)	25.0 (0)
6	4 (0)	27.5 (0)
7	4 (0)	30.0 (+1.414)
8	5 (+1)	25.0 (-1)
9	5.0 (+1.414)	27.5 (0)

Note: Code values are shown in parenthesis

rates were found to be important factors for kefir biomass production (Gao *et al.*, 2012), and also significant for antioxidant activity and the production of bioactive compounds (Maleki *et al.*, 2015.). Inoculation rate was varied between 2.5 and 5.0 % w/v, while fermentation temperature was varied between 25 °C and 30 °C. After 24 hours, another 50 ml sample was collected from each fermentation experiment for biochemical analysis.

Volatile Compound Determination

Volatile compounds were analyzed and detected by gas chromatography-mass spectrometry (GC-MS) using a Shimadzu GC-MS QP2010NC instrument with a CP wax 52 CB high-polarity polyethylene glycol (PEG) phase column at the following settings — oven temperature: 50.0 °C, injection temperature: 230.0 °C, injection mode: split, injection volume: 20 µl, flow control mode: linear velocity, pressure: 53.6 kPa, total flow: 14.0 ml/min, column flow: 1.00 ml/min, linear velocity: 36.3 cm/sec, purge flow: 3.0 ml/min, split ratio: 10.0, high pressure injection: off, carrier gas saver: off, splitter hold: off, rate of oven temp: 10, temperature: 50 °C to 220 °C and hold time: five to 10 minutes.

DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) Free Radical Scavenging Assay

A stock solution of DPPH was prepared at a concentration of 10 µg/ml. One milligram of ***2, 2-diphenyl-1-picrylhydrazyl*** (DPPH) ***hydrate*** was first dissolved in 10 ml of methanol before being topped up to 100 ml. The solution was then covered with aluminium foil and stored at -20° C. To dilute the DPPH to a concentration of 0.1 µg/ml, 1 ml of stock solution was pipetted and added to 100 ml of methanol. Antioxidant activity of rice milk kefir was evaluated by free radical scavenging of DPPH radicals according to Akowvah *et al.* (2005*)*. Briefly, 100 µl of DPPH solution was added to 50 µl of sample with methanol as the negative control. The solution was mixed well and incubated for 30 min in the dark at room temperature. A microplate reader was used to measure absorbance at 517 nm. A DPPH standard was prepared at concentrations of 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg/ml, and their absorbance values were also measured at 517 nm using a microplate reader.

Ferric Reducing/Antioxidant Power (FRAP) Assay

FRAP assay was performed according to Benzie and Strain (1996) with slight modifications. The FRAP reagent was used as follows: 20 µl of sample was added to 1.50 µl of FRAP

reagent. The mixture was mixed thoroughly and incubated in the dark for 30 minutes. Absorbance was measured at 595 nm using a microplate reader. A standard concentration was prepared at 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 µg/ml, and the standard curve ($r^2 = 0.9995$) for FRAP was plotted with absorbance readings at 595 nm. A calibration curve was drawn with $FeSO_4.7H_2O$ concentration on the X axis and OD on the Y axis**.** Values obtained were expressed in µg/ml of ferrous equivalent Fe **(**II**)** per µg of sample**.**

Metabolic Pathway and Data Analyses

Metabolic pathway analysis was conducted using MetaboAnalyst 4**.**0 **(**Chong *et al.*, 2019**)**. All experiments were conducted in duplicates, with the mean and standard deviation reported. Data were subjected to statistical analysis using one-way ANOVA. Mean difference was analysed using Duncan's new multiple range test ($p<0.05$).

Results and Discussion

Effect of Process Variables on Volatile Compounds

Volatile compounds in fermented Thai black jasmine rice milk inoculated by kefir grains from Nonthaburi Province are listed in Table 2. The GC-MS profile results from the nine experiments detected the presence of four acids, four fatty acids, six alcohols, four esters, four ethers, three amines, and one ketone and amide each**.** Concentrations of inoculum and fermentation temperatures were significant factors in kefir biomass production and volatile compound extraction (Gao *et al.*, 2012). Volatile compounds such as aldehydes, ketones, acids, esters, alcohols and aromatic compounds were previously identified and reported by Dan *et al.* (2017). Profiles of volatile flavour compounds differed significantly depending on the ratio of initial proportions of *Lactobacillus delbrueckii* subsp. *bulgaricus* IMAU20401 to *Streptococcus. thermophilus* ND03 (Dan *et al.*, 2017).

Thus, inoculation percentage and proportion played important roles and could alter the profiles of volatile compounds. A total of 19 volatile compounds were found in cow milk kefir as five benzene derivatives, six acids, four ketones, two alcohols, one ester and one aldehyde (Guler *et al.*, 2016). Increasing the inoculation rate of kefir grains was found to be directly proportional to the increase in levels of volatile compounds, such as acetaldehyde, ethanol, ethyl acetate, benzene derivatives, free fatty acids C4, C6, and total free amino acids, especially proline, tyrosine, cysteine and valine (Guler *et al.*, 2016).

It was crucial to understand the processes that lead to the production of the listed metabolites. In our study, milk kefir was the inoculum and rice milk was the substrate, using a combination of aerobic and anaerobic fermentation. Ramos, et *al*. (2000) suggested that *Bacillus subtilis* produced lactate, acetate, acetoin, ethanol, succinate and 2, 3-butanediol using glucose and pyruvate as substrates during anaerobic metabolism. This concurred with our results in the detection of lactic acid, acetic acid, acetonin, ethanol and 2,3-butanediol. Chemical composition and biological activity of some volatile compounds identified from Thai black jasmine rice milk kefir are listed in Table 2.

Organic Acids

Four organic acids were detected, namely acetic acid, propionic acid, lactic acid and L**-**lactic acid (Table 2). The GC**-**MS profile showed the presence of acetic acid at all experimental conditions, while other acids were only detected at certain parameters**.** As a result of hydrolysis of butterfat (fatty acids), biochemical metabolic processes, or bacterial metabolism, organic acids could be present in dairy products. Lactate was a common end-product of bacterial fermentation (Guzel-Seydim *et al.*, 2000).

Corona *et al*. (2016) determined that the amount of lactic acid formed in red rice milk was influenced by the carbohydrate content. Most carbohydrates in red rice milk existed as starch, which was a glucose polysaccharide that would be broken down into monosaccharides. Increased amounts of kefir as a starter of

fermentation would increase polysaccharide degradation, resulting in a higher content of lactic acid as the breakdown product of glucose compounds. *Lactobacillus bulgaricus* was a homo-fermentative LAB that could break down glucose into pyruvic acid, and then convert it into lactic acid through the Embden-Meyerhof pathway. Luang-In and Deeseenthum (2016) found an abundance of *Bacillus amyloliquefaciens* in Thai milk kefir. This bacterium produced lactate dehydrogenase to form lactic acid, acetate kinase to produce acetic acid and alcohol dehydrogenase to produce alcohol.

Acetic acid is an organic substance that was widely used in the food and medical industries (Gomes *et al.,* 2018**;** Evans *et al.*, 2019)**.** In the former, acetic acid was used as a food preservative, additive and as vinegar in salads**.** Persistent retention of acetic acid inside tumour cells had been associated with complete tumour necrosis in patients with hepatocellular carcinoma (Huo *et al.*, 2004).

Table 2: Volatile compounds identified in rice milk kefir fermented under different conditions as detected by GC-MS

Compound	Peak area relations of each experiment								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Organic acids									
Acetic acid	8.28	2.06	7.07	9.37	9.48	6.71	6.35	1.51	5.74
Propionic acid	0.60	-	-	-	1.08	-	-	-	-
Lactic acid	-	-	-	35.50	-	-	-	-	-
L-Lactic acid	-	-	-	-	-	-	36.35	-	26.98
Fatty acids									
Octadecanoic acid	-	7.20	-	-	-	-	-	-	-
Docosanoic acid	-	-	-	-	-	-	-	0.91	-
Hexadecanoic acid	-	-	-	-	-	-	-	-	8.44
Carboxylic acid	-	5.41	-	-	-	-	-	-	-
Alcohols									
Ethanol	2.36	1.12	1.71	2.24	3.81	2.98	1.66	0.80	3.32
Cyclobutanol	49.68	-	-	40.98	-	-	-	-	-
2,3-Butanediol	2.11	4.07	3.56	2.93	3.83	3.24	3.52	2.91	3.10
2-Undecanol	0.01	-	-	-	-	-	-	-	-
1,2,3-Propanetriol	2.53	2.78	3.15	3.13	5.47	3.01	3.68	0.46	-
Furfuryl alcohol	-	-	-	-	-	-	-	-	1.60

Esters									
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	-	2.64	0.47	-	-	0.39	0.40	-	-
Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl-	-	-	47.59	-	-	-	45.15	-	-
Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl) bis [4-methyl-	-	-	-	-	2.87	-	-	-	-
5-Hydroxymethyl furfural	-	-	-	-	-	-	-	2.07	-
Ethers									
Tris(dimethylamino)-methane	-	5.00	-	-	-	-		7.00	-
Propanoic acid, 2-hydroxy-, (S)-, polymer with hydroxyacetic acid	-	-	-	-	-	-		1.22	-
Octane	-	-	-	-	-	-		10.66	-
1-Propoxyhexane	-	-	-	-	-	-	-	-	2.40
Amines									
(S)-(+)-1-Cyclohexylethylamine	-	-	-	-	33.18	-	-	-	-
Bicyclophenamine	-	-	-	-	-	43.61	-	-	-
Bicyclo[2.2.1]hept-5-en-2ylmethanamine	-	-	-	-	-	-	-	44.23	-
Ketones									
Acetoin	3.70	3.04	2.98	3.40	4.63	5.73	2.89	0.54	11.73
Amide									
Quinoline-3-carboxamide	1.60	-	-	1.75	-	-	-	-	-

Propionic acid was detected in 2.5% inoculum at 27.5 °C and 4 % inoculum at 25.0 °C fermentation conditions with retention peak areas of 0.60 and 1.08, respectively. In the food industry, propionic acid was commonly used as a preservative in baked goods**.** It was the main substrate involved in propanoate metabolism and would be inter-converted into 2-propyn-1-al by mitochondrial aldehyde dehydrogenase. It was an important cellular osmolyte and methyl donor that protects cells from oxidative stress by metabolising several peroxidation-derived lipids (Brocker *et al.*, 2010).

Lactic acid was detected in 4 % inoculum with a slight decrease in temperature (23.9 °C). Lactic acid was involved in the production of

nicotinamide adenine dinucleotide (NAD), which was then used in glycolysis to produce adenosine triphosphate (ATP) as an energy source (Joseph, 2012). L-lactic acid was found in the GC-MS profile in 5 % inoculum at 27.5 °C fermentation condition, and it was detectable even when the temperature was increased to 30.0 °C. L-Lactic acid is the biologically active isoform in humans as the levorotatory isomer of lactic acid. Notably, poly-L-lactic acid microfibers could facilitate robust regeneration of the vascularised central nervous system (CNS) tissue after complete spinal cord transection (Hurtado *et al.*, 2011).

Higher antioxidant activity was also linked to the presence of lactic acid bacteria during fermentation (Nisa *et al.*, 2019). In our study, strongest scavenging effects (conditions 1, 2 and 3) on the DPPH radical were due to the presence of L-lactic acid as shown in Table 2. Total titratable acid (TTA) and lactic acid concentration detected during fermentation was observed to increase with fermentation temperature (He *et al.*, 2020).

Fatty Acids

Four fatty acids detected in Thai black jasmine rice kefir were octadecanoic acid, docosanoic acid, hexadecanoic acid and carboxylic acid (Table 2). Hexadecanoic acid was detected in 5 % inoculum at 27.5 °C fermentation condition. This acid was also known as palmitic acid and was present in palm oil, palm kernel oil, butter, cheese, milk and meat. Octadecanoic acid, also known as stearic acid, was only detected in 3 % inoculum at 25.0 °C condition. A study had discovered that palmitic and stearic acid could markedly suppress granulosa cell survival with induced cell apoptosis (Mu *et al.*, 2001). Stearic acid was also incorporated into phospholipids, di- and triglycerides, cholesterol, cholesterol esters and other sterol esters.

Alcohols

Six alcohols were detected in Thai black jasmine rice kefir, including ethanol, cyclobutanol, 2,3-butanediol, 2-undecanol, 1,2,3-propanetriol and 2-furanmethanol. Ethanol and 2,3-butanediol were detected in all fermentation conditions, while 1,2,3-propanetriol was detected at all conditions except for the 5 % inoculum and 27.5 °C. 2-furanmethanol was only detected at 5 % inoculum and 27.5 °C. The alcohol cyclobutanol was detected at 2.5 % inoculum with 27.5 °C and 4 % inoculum at 23.9 °C, while the alcohol 2-undecanol was only detected at 2.5 % inoculum and 27.5 °C (Table 2). The increasing content of alcohol in the kefir was directly proportional to the increase in starter quantity (Sulistyaningtyas *et al.*, 2019). Thus, the number of starters used had potential to alter alcohol content in kefir.

Three out of the six alcohols produced by Thai black jasmine rice kefir grains had wide usage. They included ethanol, 1,2,3-propanetriol and 2, 3-butanediol (Bai *et al.* 2008; Hurtado *et al.*, 2011; Feher, 2012). These were also the three foremost Thai black jasmine rice kefir products. Bioproduction of ethanol and 2, 3-butanediol from excessive biomass was an effective alternative for chemical synthesis with economically important implications (Bai *et al.*, 2008; Celinska & Grajek, 2009). 1, 2, 3-Propanetriol, or glycerol, had long been used to reduce increased tissue pressure in glaucoma and relieve intracranial pressure (Peltola *et al.*, 2007). Glycerol could increase plasma osmolality and, thereby, decrease the volume of cerebrospinal fluid (Singhi *et al.*, 2008).

Esters

Four esters were detected, including 4H-pyran-4-one, 2,3-dihydro-3, 5-dihydroxy-6-methyl-; benzeneethanamine 2,5-difluoro-beta, 3,4-trihydroxy-N-methyl-benzene,1,1'-(1,1,2,2-tetramethyl-; 1,2-ethanediyl) bis 4-methyl-; and furfuryl alcohol (Table 2).

The most interesting aspect was that 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl- was detected in four fermentation conditions in the GC-MS profile, which included 3 % inoculum at 25.0 °C, 3 % inoculum at 30.0 °C, 4 % inoculum at 27.5 °C and 4 % inoculum at 30.0 °C. Previous studies reported that DDMP played a role as a strong antioxidant in the

Maillard reaction of natural products (Yu *et al.*, 2013; Zhou *et al.*, 2014). Recent research also confirmed on the antioxidant activity of Thai rice milk kefir (Zahid *et al.*, 2018). Moreover, DDMP modulated the expression of tumour necrosis factor-α (TNF-α) and had been shown to suppress anti-apoptotic genes related to NF-κB activation in human colon cancer cells (Ban *et al.*, 2007). DDMP from *Lactobacillus* sp. also stimulated autonomic nerve activities in rats (Beppu *et al.*, 2012). These reports concurred with the findings of studies confirming anti-inflammatory activity of Thai rice milk kefir (Deeseenthum *et al.*, 2017).

Another ester in Thai black jasmine rice kefir, benzeneethanamine, 2,5-difluoro-beta., 3,4-trihydroxy-N-methyl-, was detected under the 3 % and 4 % inoculum at 30 °C fermentation condition, while the ester benzene,1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis[4-methyl- was detected in 4 % inoculum at 25.0 °C. The ester 5-hydroxymethylfurfural appeared in the GC-MS profile in 5 % inoculum at 25.0 °C. Inoculation rates had been observed to significantly affect the production of benzene derivatives in cow milk kefir (Guler *et al.*, 2016).

Ethers, Amines, Ketones and Amides

Thai black jasmine kefir contained four types of ether, including tris-(dimethylamino)-methane; propanoic acid, 2-hydroxy-, (S)-, polymer with hydroxyacetic acid; octane; and, 1-propoxyhexane (Table 2). The ketone acetoin was detected at all fermentation conditions. Three ethers, namely tris- (dimethylamine)-methane; propanoic acid, 2-hydroxy-, (S)-, polymer with hydroxyacetic acid; and, octane were detected in 5 % inoculum at 25.0 °C, while 1-propoxyhexane was only detected in 5 % inoculum at 27.5 °C. Tris (dimethylamino) methane was also detected in 3 % at 25.0 °C.

Three amines were detected in Thai black jasmine kefir, including (S)-(+)-1-cyclohexylethylamine, bicyclophenamine and bicyclo [2.2.1] hept-5-en-2-ylmethanamine. These were detected in conditions of 4 % inoculum at 25.0 °C, 4 % inoculum at 27.5 °C and 5 % inoculum at 25.0 °C on the GC-MS profile, respectively (Table 2). The amide quinoline-3-carboxamide was detected in the GC-MS profile of Thai black jasmine kefir in 2.5 % inoculum at 27.5 °C and 4% inoculum at 23.9 °C (Table 2).

Walsh *et al.* (2016) studied changes in the microbial community structure and associated pathways related to changes in the level of volatile compounds. They found that *Acetobacter* spp. correlated with acetic acid, *Lactobacillus* spp. correlated with carboxylic acids, esters and ketones, *Leuconostoc* spp. correlated with acetic acid and 2,3-butanedione, and *Saccharomyces* spp. correlated with esters. Their results increased the understanding of microbial populations and how levels of volatile compounds could be manipulated in flavouring the kefir and improving its nutritional aspects. Table 3 summarizes the activity of some compounds.

Antioxidant Activity

Antioxidant activity of Thai black jasmine rice milk kefir, as determined by DPPH free radical scavenging, had showed significant differences between fermentation conditions (Figure 2A). However, there were no significant differences in ferric reducing/antioxidant power (FRAP) assays between the conditions. Kefir produced by Thai black jasmine rice milk under conditions 1 to 3 had antioxidant activity at more than 84 % in the scavenging of DPPH. FRAP assays showed antioxidant activity of between 1.4 and 1.7 µg $FeSO_4$/ml (Figure 2B), which concurred with the observations of Deeseenthum *et al.* (2018). Increase in total phenolic content after fermentation with kefir grains could affect DPPH radical scavenging activity (Sabokbar *et al*., 2015).

Moreover, certain natural bioactive components in kefir showed relatively slow scavenging potential because large peptides and proteins would hydrolyse slowly and display lower antioxidant activity. Differences in characteristics of the starter culture used might have strong radical scavenging activities

Table 3: Chemical composition and activity of some volatile compounds in Thai black jasmine rice milk kefir

Chemical name	M.W (g/ mol)	Formula	PubChem CID	Activity	Reference
Ethanol	47.06	C_2H_6O	12201684	Disinfectant	(Kampf *et al.*, 2010)
Acetic acid	243	$C_2H_4O_2$	176	Anti-bacterial activity	(Halstead *et al.*, 2015)
Lactic acid	90.08	$C_3H_6O_3$	612	Anti-fungal activity	(Matevosyan *et al.*, 2020)
Tris (dimethylamino) methane	145.25	$C_7H_{19}N_3$	79831	Formylation agent	(Kantlehner, 2001)
Furfuryl alcohol	98.1	$C_5H_6O_2$	7361	Anti-oxidative activity	(Yanagimoto *et al.*, 2002)
Propanoic acid	74.08	$C_3H_6O_2$	1032	Anti-fungal agent in food	(Yun & Lee, 2016)
Octadecanoic acid	284.5	$C_{18}H_{36}O_2$	5281	Flavouring agent	(Lacroix C., 2010)
Acetoin	179	$C_4H_8O_2$	179	Antimicrobial activity	(Raju *et al.*, 2013)
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	144.12	$C_6H_8O_4$	119838	Anti-oxidative activity	(Padmashree *et al.*, 2018)
Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl-	219.18	$C_9H_{11}F_2NO_3$	541614	Anti-fouling activity	(Gadhi *et al.*, 2019)
Hexadecanoic acid	256.42	$C_{16}H_{32}O_2$	985	Anti-inflammatory compound	(Aparna *et al.*, 2012)
Quinoline-3-carboxamide	172.18	$C_{10}H_8N_2O$	15561101	Anti-tumour activity	(Deronic *et al.*, 2016)

Footnote: M.W. (Molecular weight) Compound Information (National Center for Biotechnology Information, 2019).

on low-molecular-weight casein hydrolysates (Kim *et al.*, 2007; Sah *et al.*, 2015). Fermented hazelnut milk at 25 °C displayed a more significant capacity to contribute protons than fermented samples at other temperatures. This temperature provided better conditions for the microorganisms to engage in antioxidant metabolite formation (Maleki *et al.*, 2015).

The same letter (a, b...) indicates non-significant difference between mean values.

DPPH % scavenging and FRAP assay were performed as follows: (1) = 2.5% inoculum with 27.5 °C, (2) = 3.0% inoculum with 25.0 °C, (3) = 3.0% inoculum with 30.0 °C, (4) = 4.0% inoculum with 23.9 °C, (5) = 4.0% inoculum with 25.0 °C, (6) = 4.0% inoculum with 27.5 °C, (7) = 4.0% inoculum with 30.0 °C, (8) = 5.0% inoculum with 25.0 °C, and (9) = 5.0% inoculum with 27.5 °C .

Metabolic Pathway Analysis

Analyses were performed to identify the most significant metabolic pathways affected by Thai black jasmine kefir (Figure 3). Table 4 shows

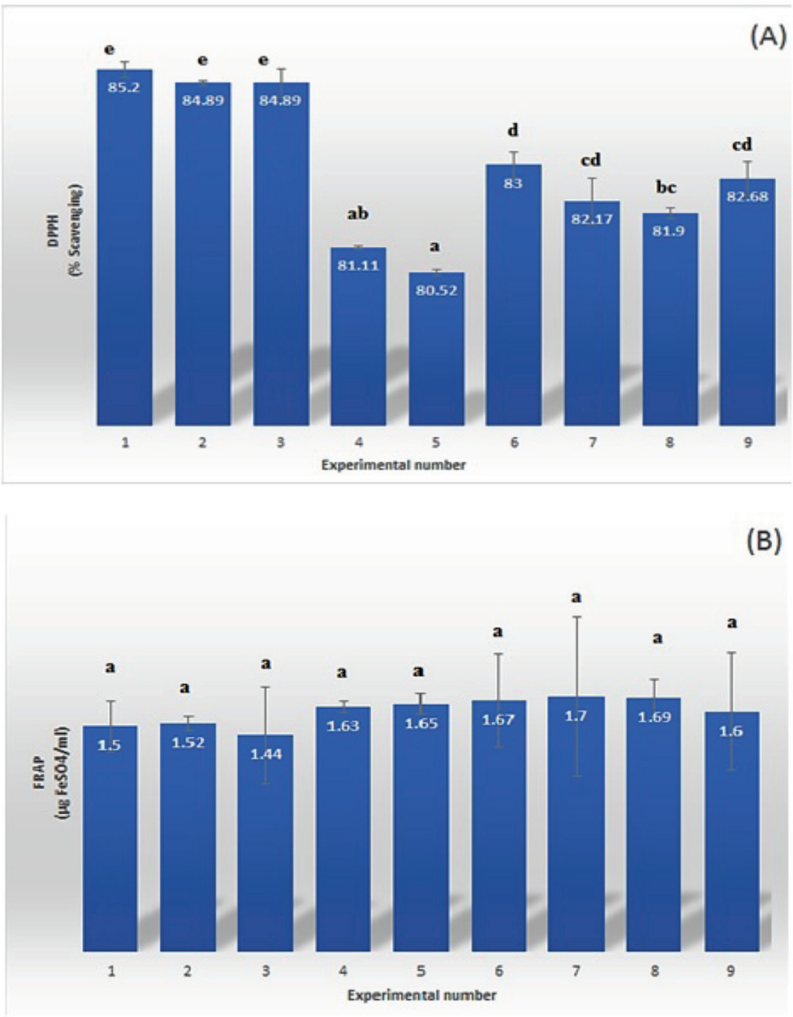

Figure 2: Antioxidant activity of Thai black jasmine kefir produced under nine fermenting conditions as measured by DPPH (A) and FRAP (B) assays

the conversion results and metabolites identified in rice kefir**.** Metabolites without a match were excluded from the pathway analysis**.** Comparing metabolites, the impact values of pyruvate metabolism, fatty acid biosynthesis, sulphur metabolism and glycerolipid metabolism were 0**.**15, 0**.**01, 0**.**06 and 0**.**12, respectively**.** Based on the p and impact values, metabolism of pyruvate, sulfur and glycerolipid were identified as pathways affected by rice milk kefir fermentation**.**

Results concurred with Walsh *et al.* (2016), which stated that pathways involving fatty acid biosynthesis had been identified in kefir and were specifically involved in the production of volatile compounds**.** Notably, most of the volatile compounds detected were formed from lipid metabolism and carbohydrate metabolism **(**Walsh *et al.*, 2016**)**, while some volatile compounds might originate from more than one metabolic pathway. Precursors might be proteolysis**-**derived amino acids, as well as fatty acids from lipolysis or carbohydrates **(**lactose,

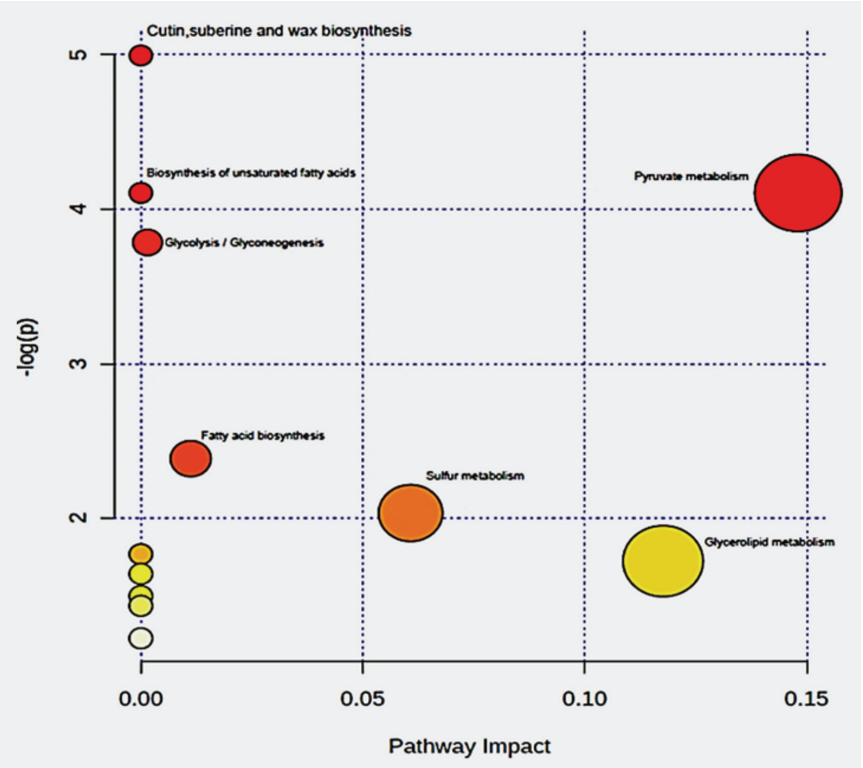

Figure 3: Metabolome view map generated using MetaboAnalyst 4.0

citrate and lactate**)** **(**McSweeney & Sousa, 2000). Based on our findings, acetic acid was present in all fermentation conditions, formed from substrates such as lactic acid and ethanol.

Most significant pathways were determined employing pathway library, over-representation analysis test and pathway topology analysis. The x-axis represents the pathway impact and y-axis represents pathway enrichment. Colour and size of the circles indicate significant changes in metabolites and impact score in the metabolic pathways, respectively.

The availability of substrates, such as ethanol and lactic acid, was considered favourable for growth of acetic acid bacteria, with primary activity of ethanol oxidation into acetic acid **(**Wang *et al.*, 2012; Illeghems *et al.*, 2013**).** The compound 4H-pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl **(**DDMP**)** was a Maillard reaction product of glucose and amino acid**.** The amino acid proline was found to play a crucial role in DDMP formation through reaction with glucose, and it was known for its catalytic action **(**Zhou *et al.*, 2014**).**

Compounds, such as cyclobutanol, 1-ethyl, benzeneethanamine,2,5-difluoro-beta, 3,4-trihydroxy-N-methyl, benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis[4-methyl,propanoicacid,2-hydroxy-,(S)-,polymer with hydroxyacetic acid, 1-propoxyhexane,(S)-(+)-1-cyclohexylethylamine, bicyclophenamine, and 1-bicyclo[2.2.1]hept-2-ylethanamine were detected in Thai black jasmine kefir for the first time. However, these compounds did not match with known pathways (Table 5). Identifying pathways of these compounds might be crucial to gain knowledge on their bioprocesses.

Recently, metabolomic studies had successfully revealed metabolic profiles of LAB-predominant fermented food **(**Mozzi *et al.*, 2013**).** Ultimately, identifying the essential organisms and metabolites for each process would increase

Table 4: Anti-oxidative compound name mapping

Compound	Match	HMDB ID	PubChem ID	KEGG ID	Comment
Acetic acid	Acetic acid	HMDB0000042	176	C00033	1
Propionic acid	Propionic acid	HMDB0000237	1032	C00163	1
Lactic acid	L-Lactic acid	HMDB0000190	61503	C00256	1
L-Lactic acid	L-Lactic acid	HMDB0000190	61503	C00256	1
Octadecanoic acid	Stearic acid	HMDB0000827	5281	C01530	1
Docosanoic acid	Behenic acid	HMDB0000944	8215	C08281	1
Hexadecanoic acid	Palmitic acid	HMDB0000220	985	C00249	1
Carboxylic acid	Carboxylate	METPA0005	NA	C00060	1
Ethanol	Ethanol	HMDB0000108	702	C00469	1
Cyclobutanol, 1-ethyl	NA	NA	NA	NA	0
2,3-Butanediol	2,3-Butanediol	HMDB0003156	262	C00265	1
2-Undecanol	2-Undecanol	HMDB0030942	15448	NA	1
1,2,3-Propanetriol	Glycerol	HMDB0000131	753	C00116	1
Furfuryl alcohol	2-Furanmethanol	HMDB0013742	7361	C20441	1
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	NA	NA	NA	NA	0
Benzeneethanamine, 2,5-difluoro-beta,3,4-trihydroxy-N-methyl	NA	NA	NA	NA	0
Benzene, 1,1'-(1,1,2,2-tetramethyl-1,2-ethanediyl)bis[4-methyl	NA	NA	NA	NA	0
5-Hydroxymethylfurfural	5-Hydroxymethyl-2-furancarboxaldehyde	HMDB0034355	237332	C11101	1
Acetoin	Acetoin	HMDB0003243	4068	C00466	1
Tris(dimethylamino) methane	Trimethylamine	HMDB0000906	1146	C00565	1
Propanoic acid, 2-hydroxy-, (S)-, polymer with hydroxyacetic acid	NA	NA	NA	NA	0
Octane	Octane	HMDB0001485	356	C01387	1
1-Propoxyhexane	NA	NA	NA	NA	0
(S)-(+)-1-Cyclohexylethylamine	NA	NA	NA	NA	0
Bicyclophenamine	NA	NA	NA	NA	0
1-Bicyclo[2.2.1]hept-2-ylethanamine	NA	NA	NA	NA	0
Quinoline-3-carboxamide	Ivacaftor	HMDB0015705	16220172	NA	1

Footnote**:** In comment column, 1 indicates an exact match and 0 indicates no match

commercial manufacturing efficiency (Bourrie *et al.*, 2016). In particular, metabolic pathway analysis might play a key role in determining the species of microorganisms involved in various pathways for a deeper understanding of cell metabolism and the likelihood of target manipulation.

Total is the total number of compounds in the pathway; Hits is the actual matched number from the data; Raw p is the original p value calculated from the enrichment analysis; Holm is the p value adjusted by Holm-Bonferroni method; FDR is the p value adjusted using False Discovery Rate; Impact is the pathway impact value.

Conclusion

The results of this study suggested that incubation temperature and inoculation rates were crucial factors that affected the production of volatile compounds by microorganisms in rice milk kefir. Metabolism of pyruvate, sulfur and glycerolipid were identified as pathways in the fermentation of Thai black jasmine rice milk kefir. Rice milk kefir showed potential as supplementary food to ameliorate multiple human diseases due to its antioxidant property. People who could not tolerate kefir produced from animal milk might benefit from rice milk kefir, which was wholly fermented from a plant source. These findings could be applied to improve fermentation processes. However, further research would be required to identify the metabolic pathways of acetic acid, ethanol, and 2, 3-butanediol produced in Thai black jasmine kefir.

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Table 5: Major changed pathways based on p value and false discovery rate

Pathway	Total	Hits	Raw P	Log(p)	Holm Adjust	FDR	Impact
Pyruvate metabolism	22	2	1.65E-02	4.10E+00	1.00E+00	5.22E-01	0.15
Fatty acid biosynthesis	56	2	9.21E-02	2.38E+00	1.00E+00	1.00E+00	0.01
Sulfur metabolism	15	1	1.31E-01	2.03E+00	1.00E+00	1.00E+00	0.06
Glycerolipid metabolism	21	1	1.79E-01	1.72E+00	1.00E+00	1.00E+00	0.12

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