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Identification of Robusta Coffee (*Coffea canephora*) Metabolite Compounds and Caffeine Content Due to Fermentation of Lactic Acid Bacteria *Lactobacillus plantarum* Using UPLC-QTOF-MS/MS

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Abstract: Robusta coffee (*Coffea canephora*) is one type of plant that has long existed in Indonesia and has high economic value. Robusta coffee beans have the potential to be developed to reduce caffeine levels by treating them with lactic acid bacteria. This study aims to determine the metabolite profile of Robusta coffee from several samples treated with *Lactobacilus plantarum* bacteria. Samples were taken from the West Java location at the Patani Coffee Plantation. Sample extraction was carried out using the Ultrasonic Assisted Extraction (UAE) method with 96% ethanol. Metabolite content analysis was carried out using UPLC-QTOF-MS/MS with a C18 stationary phase column (Okta Decyl Silica), a mixture of formic acid/water 0.1/99.9 (v/v) and formic acid/acetonitrile 0.1/99.9 (v/v) mobile phases. The results of the analysis were interpreted using Masslynx software. The results showed that the caffeine content in coffee not fermented with *Lactobacillus plantarum* was 2.40%, coffee fermented with *L. plantarum* bacteria for 6 hours had an abundance% of 1.76%, and coffee fermented with *L. plantarum* bacteria for 24 hours had an abundance% of 2.38%. LC-MS/MS analysis identified compounds 35,26, and 25 in three Robusta coffee samples. These results were not significantly different from the fermentation of Lactobacillus plantarum in Robusta coffee beans.

Keywords: Caffeine; Fermentation; History of coffee; Robusta coffee

Introduction

History of Coffee Development in Indonesia. The development of coffee in Indonesia began in the 16th century during the Dutch colonial era. In 1696, India sent Yemen or Arabica coffee seedlings to the Dutch governor in Indonesia to be cultivated, particularly in Batavia (now Jakarta). During the colonial period, the primary coffee varieties that were grown were Arabica and Robusta, which initially had low market value. Thanks to the persistence of Indonesian coffee farmers, coffee plantations gradually expanded. However, the coffee industry experienced ups and downs, especially during the political and economic crises following Indonesia's independence. After the 2000s, the coffee industry saw a significant resurgence. Since then, coffee has become a favorite beverage for many, with increasingly diverse preparations. Today, coffee is not only limited to the bitter black variety but also includes a wide range of delicious flavors and creative presentations. The spread of Arabica coffee to Indonesia was initiated by a Dutchman in the 17th century, around 1646, who obtained Arabica Mocca seeds from Arabia. These seeds were sent by the Dutch governor-general in Malabar to Batavia in 1696 (Gandul., 2010). Unfortunately, the plants were destroyed by floods. In 1699, new seedlings were imported, which then thrived in the areas around Jakarta and West Java, eventually

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spreading to other parts of the Indonesian archipelago (Wahyudi et al., 2018).

For about a century, Arabica coffee has developed as a popular crop among the people. The first coffee plantations were established in Central Java (Semarang and Kedu) in the early 19th century, and in Besuki, it wasn't until the late 1900s. Arabica coffee was the only commercial coffee variety grown in Indonesia for nearly two centuries. However, the cultivation of Arabica coffee faced a setback due to the outbreak of leaf rust (*Hemileia vastatrix*), which entered Indonesia in 1876. Arabica coffee could only survive in highland areas (above 1,000 meters), where the impact of the disease was less severe. Robusta coffee (*Coffea canephora*) was introduced to Indonesia in 1900. This variety proved resistant to leaf rust, and it requires lighter growing conditions and maintenance, while its yields are significantly higher. As a result, Robusta coffee quickly expanded and displaced other coffee varieties. Today, over 90% of Indonesia's coffee plantation area is planted with Robusta coffee (Wahyudi et al., 2018). Coffee is a popular beverage enjoyed by a large portion of the world's population, not only because of its distinctive aroma and taste but also due to its numerous health benefits. This is because coffee beans contain various chemical compounds, such as carbohydrates, proteins, minerals, caffeine, trigonelline, aliphatic acids (carboxylic acids), chlorogenic acids, fats and their derivatives, glycosides, and volatile components (Mangiwa et al., 2015).

Robusta coffee (*Coffea canephora*) originates from Congo and thrives in lowland areas up to an altitude of about 2,000 meters above sea level, with temperatures ranging from 23 to 26°C. Outside its native region, Robusta coffee can grow well in areas with an average annual temperature of 22–26°C (Rawanda et al., 2021). The caffeine content in raw Robusta coffee beans is higher compared to raw Arabica coffee beans. Raw Arabica coffee beans contain less caffeine than raw Robusta beans. Robusta coffee contains about 2.2% caffeine, while Arabica coffee contains about 1.2% caffeine (Andry et al., 2023).

Caffeine is an alkaloid found in coffee beans, tea leaves, and cocoa beans. To ensure that the coffee powder available in the market is safe and of good quality, the National Standardization Agency (BSN) has set a maximum caffeine limit of 150 mg per day and 50 mg per serving (SNI 01-7152-2006) (Mulyani et al., 2019). According to BPOM (National Agency of Drug and Food Control), the maximum caffeine limit for coffee in 2004 was 150 mg. Coffee overdose can lead to anxiety, tremors, insomnia, high blood pressure, nausea, seizures, frequent urination, rapid heart rate, digestive disturbances, acid reflux, gout, and diabetes (Mulyani et al., 2019). The health benefits of caffeine include improved focus and alertness, a potential lower risk of heart disease and diabetes, weight loss, better athletic performance, reduced hair loss, improved memory and cognitive skills, and even protection against certain types of cancer (Andry et al., 2023).

This study aims to determine the metabolite profile of Robusta coffee (*Coffea canephora*) and to identify any differences in the metabolite composition of Robusta coffee based on the treatment conditions for each sample. The samples include untreated Robusta coffee (*Coffea canephora*) and those treated with *Lactobacillus plantarum* for 6 hours and 24 hours. The analysis was conducted using Ultra Performance Liquid Chromatography - Quadrupole Time of Flight - Mass Spectrometry (UPLC-QToF-MS/MS), which is currently one of the best instruments for analyzing various compounds. UPLC-QToF-MS/MS combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. UPLC was chosen due to its superior ability to handle a wide range of applications with very high sensitivity and selectivity (Taleuzzaman M et al., 2023).

Method

Instrumentations and Materials

The materials for fermentation were *Lactobacillus plantarum*, water, MRSB (DeMan Rogosa Sharpe Broth), ethanol, and robusta coffee beans from Patani coffee plantation in Bogor Regency, West Java Province, Indonesia. Ripe coffee beans with a reddish color were harvested in August 2023, directly separated from the fruits, and washed.

Fermentation

Fermentation is carried out using wet coffee beans, to which Lactic Acid Bacteria (LAB) at a concentration of 1.89 x 10^9 cfu/g is added, with soaking times of 0, 6, and 24 hours for every 500 grams of coffee. The fermentation process follows the underwater tank fermentation method (Haile & Kang, 2019).

Extraction

Extraction is a process of obtaining desired components from a material by separating one or more components from the source material. Factors that influence the extraction process include extraction time, temperature, and the type of solvent used (Mangurana et al., 2019). The next stage is the separation process, which consists of filtration and evaporation. Filtration is carried out to separate the simplicia (raw material) from the solvent containing the bound bioactive compounds, while evaporation is performed to remove the solvent, resulting in the extracted product. The extraction

process includes several steps: sample cutting, weighing, soaking with a solvent, filtration, and separation. Cutting the sample aims to facilitate stirring and ensure better contact between the material and the solvent during the soaking process. The ground sample is then weighed to determine the initial weight of the material, allowing the yield to be calculated. The weighed material is then soaked in a solvent. The soaking process is known as maceration. Maceration is an extraction method involving the soaking of the material in a solvent, with or without stirring. The maceration process lasts for 3 x 24 hours. After maceration, the mixture is filtered using Whatman filter paper to separate the liquid extract from the residue. The liquid extract is then evaporated using a rotary evaporator at a temperature of 45-50°C and a speed of 65-90 rpm to obtain a concentrated extract.

Analisis LC-MS/MS

Identification of Secondary Metabolites Using LC-MS/MS

Identification of Metabolites refers to the method developed by (Karomah et al., 2019). The software for compound discovery processes LC-MS/MS data in RAW format. The workflow is selected for untargeted metabolomics processing, where the statistics of undetected compounds are not identified using a local database. Files in RAW format are added to the workflow, with options that include selecting spectra, aligning retention times, detecting unknown compounds, grouping unknown compounds, predicting compositions, searching mass lists, filling gaps, normalizing areas, and marking background compounds. The unknown compounds detected are processed using an m/z tolerance of 5 ppm and a minimum peak intensity of 2,000,000. For mass list searches, a manual database is used, followed by running the analysis to identify compounds in the extract. This approach facilitates the discovery of metabolites by comparing observed mass spectra to known data, identifying molecular structures, and categorizing compounds in the extract

Results and Discussion

The results of LC-MS/MS analysis can describe the differences in the % abundance of compounds in robusta coffee.

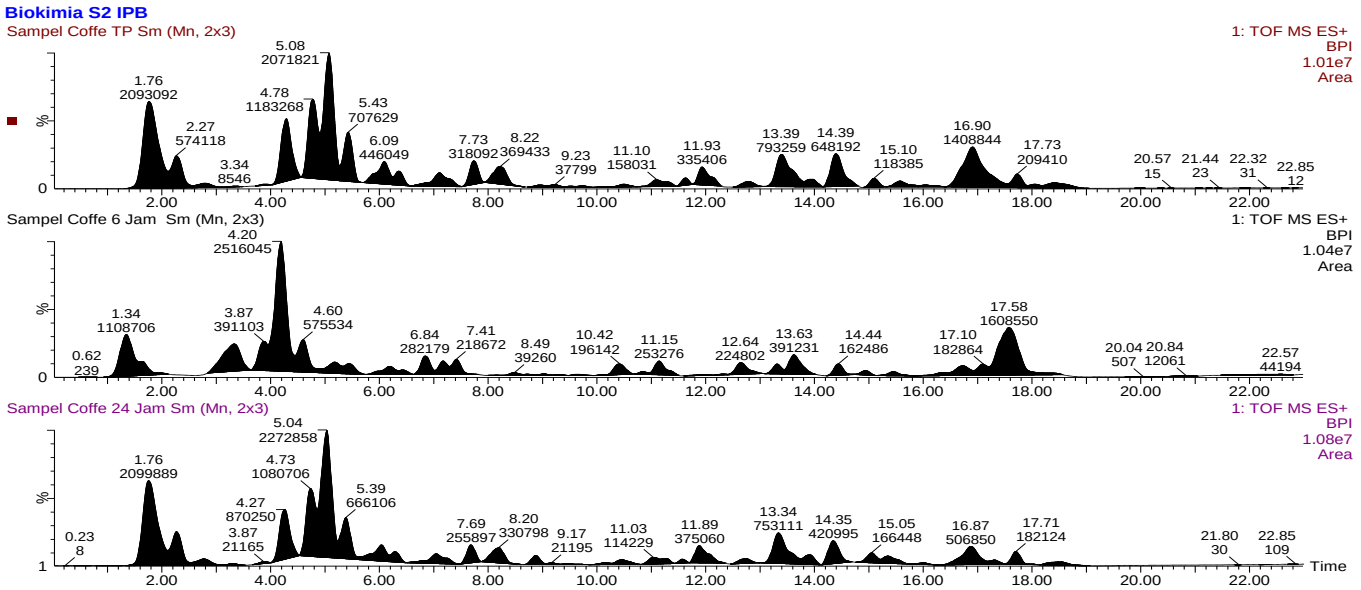

Figure 1. Results of UPLC-QTOF-MS / MS of robusta coffee *(Coffea canephora)*

Table 1. Prediction of interpretation of chromatogram data of Robusta coffee that is not fermented with *Lactobacillus plantarum*

Rt (minutes)	m/z pubchem	formula	Compound name	relative abundance (%) 0 hour
1.76	138. 0661	$C_6H_8N_3O$	1-Amino-3-carbamoylpyridinium	14.97
1.76	998. 0302	$C_{66}H_{110}O_6$	Triacylglycerol	14.77
2.27	220. 1305	$C_9H_{14}N_7$	1-[2-(4,6-Diamino-1,3,5-triazin-2-yl)ethyl]-2-methyl-1H-imidazol-3-ium	4.10
2.79	275. 1481	$C_{11}H_{22}N_3O_5$	epsilon-(gamma-Glutamyl)lysine	0.69
3.34	294. 1678	$C_{13}H_{27}O_7$	18-Crown-6-methanol	0.06

Rt (minutes)	m/z pubchem	formula	Compound name	relative abundance (%) 0 hour
3.34		$C_3H_5N_{10}$		0.08
3.90	310. 1285	$C_{15}H_{20}NO_6$	N-[(6,7-Dihydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methyl]-2-hydroxy-N-(2-hydroxyethyl)ethanaminium	0.08
4.29	163. 0395	$C_9H_7O_3$	2-oxo-3-phenylpropanoate	7.43
4.73	147. 0531	$C_5H_9NO_4$	Glutamic acid	8.22
4.78	163. 0395	$C_9H_7O_3$	2-oxo-3-phenylpropanoate	8.46
5.08	195. 0876	$C_8H_{11}N_4O_2$	1-{[5-(2-Methyl-2-propanyl)-1,2-oxazol-3-yl]carbonyl}-1,2-triazadien-2-ium	14.81
5.43	177. 0530	$C_6H_5N_6O$	5-[(2Z)-2-(2-Furylmethylene)hydrazino]tetrazol-1-ide	5.06
6.09	499. 4435	$C_{25}H_{22}O_{11}$	3,4-Dicaffeoyl-1,5-quinolactone	3.19
6.09	342. 1162	C12H22O11	D-Sucrose	3.90
6.36	498. 1135	$C_{21}H_{19}N_6O_9$	N′-{(Z)-[4-(2,4-Dinitrophenoxy)-3-methoxyphenyl]methylene}-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydro-5-pyrimidinecarbohydrazide	1.35
7.10	512. 1291	$C_{22}H_{21}N_6O_9$	7-(3,4-Dimethyl-1-piperazinyl)-1-(2,4-dinitrophenyl)-6-nitro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid	2.54
7.73	367. 1299	$C_{20}H_{19}N_2O_5$	4-(1-Benzyl-2-oxo-3-pyrrolidinyl)-5-[(2Z)-3-hydroxy-2-butenoyl]-1H-pyrrole-2-carboxylate	2.27
7.10	194. 0804	$C_8H_{10}N_4O_2$	**Caffeine**	2.49
8.22	309. 0869	$C_{17}H_{13}N_2O_4$	3-Carbamoyl-1-[2-oxo-2-(2-oxo-2H-chromen-3-yl)ethyl]pyridinium	2.64
8.94	363. 2176	$C_{21}H_{31}O_5$	(5ξ)-6-O-[2-(Cyclohexylmethyl)-4,6-dimethylphenyl]-2,4-dideoxy-D-glycero-hexonate	0.25
9.23	479. 2671	$C_{29}H_{38}NO_5$	Cytochalasin B	0.27
9.52	277. 2173	$C_{18}H_{29}O_2$	linolenate	0.10
9.72	519. 2853	$C_{31}H_{39}N_2O_5$	4-(3-{(2R)-3-{Hydroxy[(2R)-2-methyl-2,3-dihydro-1-benzofuran-5-yl]methylene}-2-[4-(2-methyl-2-propanyl)phenyl]-4,5-dioxo-1-pyrrolidinyl}propyl)morpholin-4-ium	0.14
10.11	446. 2875	$C_{30}H_{40}NS$	1-(5-Cyclohexylpentyl)-3-[(mesitylmethyl)sulfanyl]quinolinium	0.05
10.48	403. 2016	$C_{25}H_{27}N_2O_3$	1-(5-{4-[(2-Acetylphenyl)carbamoyl]phenoxy}pentyl)pyridinium	0.33
11.10		$C_{30}H_{45}N_6O_9$		1.13
11.63		$C_{23}H_{25}N_8O_2$		0.56
12.79	279. 2329	$C_{18}H_{31}O_2$	(Z,Z)-9,12-Octadecadienoate	0.73
13.39	353. 2697	$C_{21}H_{37}O_4$	3-Ethyl-4-(9-hydroxy-4,6,8,10-tetramethyl-7-oxododec-4-en-2-yl)oxetan-2-one	5.67
13.96	279. 2317	$C_{18}H_{31}O_2$	gamma-Linolenic acid	0.95
14.39	180. 0633	$C_6H_{12}O_6$	D-(+)-Glucose	4.47
14.39	279. 444	$C_{18}H_{31}O_2$	(Z,Z)-9,12-Octadecadienoate	4.63
14.39	354. 0950	$C_{16}H_{18}O_9$	Chlorogenic acid	4.73
15.10	484. 3638	$C_{26}H_{44}N_8O$	2-[4-(4-[(3,5-Diisopropylphenyl)amino]-6-{[3-(dimethylamino)propyl]amino}-1,3,5-triazin-2-yl)-1-piperazinyl]ethanol	0.85
15.58	515. 4207	$C_{32}H_{55}N_2O_3$	(2β,3α,5α,16β,17β)-17-Acetoxy-3-hydroxy-16-(1-methyl-1-piperidiniumyl)-2-(1-piperidinyl)androstane	0.80
16.05	513. 4050	$C_{32}H_{53}N_2O_3$	(2β,3α,5α,8ξ,9ξ,14ξ,16β)-3-Acetoxy-16-(1-methyl-1-piperidiniumyl)-17-oxo-2-(1-piperidinyl)androstane	0.26
16.90		$C_{40}H_{55}N_8O$		10.07
17.73	470. 3845	$C_{26}H_{47}N_8$	N~2~,N~2~-Dibutyl-N~4~-[4-(diethylamino)phenyl]-N~6~-[3-(dimethylamino)propyl]-1,3,5-triazine-2,4,6-triamine	1.50
18.04		$C_{41}H_{68}NO_6$		0.43
18.41	683. 5124	$C_{42}H_{69}NO_6$	4-{[(3β)-28-{[(2S)-1-hydroxy-4-methylpentan-2-yl]amino}-28-oxoolean-18-en-3-yl]oxy}-2,2-dimethyl-4-oxobutanoic acid	1.22

Table 2. Prediction of interpretation of chromatogram of Robusta coffee data fermented with *Lactobacillus plantarum* 6 hours

Rt	m/z pubchem	formula	Compound name	Relative Abundance (%) 6 hours
0.62				0.00
1.34		$C_{11}H_{23}N_4O$		10.50
3.32	147. 0531	$C_5H_9NO_4$	Glutamic acid	7.41
3.32	163. 0502	$C_8H_7N_2O_2$	2-Methoxycarbonylbenzenediazonium	7.73
3.87	163. 0502	$C_8H_7N_2O_2$	2-Methoxycarbonylbenzenediazonium	3,71
3.87	355. 1248	$C_{14}H_{19}N_4O_7$	1-Hydroxy-2-[N′-(3-hydroxy-2-methoxy-2,3,4,4a,11,11a-hexahydropyrano[3,2-b][1,5]benzodioxepin-4-yl)carbamimidoyl]-1-oxohydrazinium	3.71
3.87	180. 0633	$C_6H_{12}O_6$	D-(+)-Glucose	3.57
4.20	998. 0302	$C_{66}H_{110}O_6$	Triacylglycerol	17.14
4.20	195. 0988	$C_7H_{11}N_6O$	4-Amino-1-[(2E)-2-cyano-2-(hydroxyimino)ethyl]-3,5-dimethyl-4H-1,2,4-triazol-1-ium	23.84
4.60	177. 0658	$C_9H_9N_2O_2$	3-(4-Methylphenyl)-5-oxo-2,5-dihydro-1,2,3-oxadiazol-3-ium	5.45
5.17	499. 1470	$C_{23}H_{23}N_4O_9$	(2S)-2-Ammonio-4-{4-[(1Z)-2-({(3S)-1-[(R)-carboxylato(4-hydroxyphenyl)methyl]-2-oxo-3-azetidinyl}amino)-N-hydroxy-2-oxoethanimidoyl]phenoxy}butanoatato(8-)	2.29
5.45	516. 1532	$C_{28}H_{25}N_2O_8$	12-Methyl-13-(4-O-methylhexopyranosyl)-12,13-dihydrofuro[3,4-c]indolo[2,3-a]carbazole-5,7-dione	1.67
6.18		$C_{24}H_{25}N_4O_9$		1.70
6.42		$C_{23}H_{25}N_6O_8$		0. 52
6.84	367. 1513	$C_{18}H_{19}N_6O_3$	(4S)-2-Amino-4-[4-(2-amino-2-oxoethoxy)-3-methoxyphenyl]-4,10-dihydro-1H-[1,3,5]triazino[1,2-a]benzimidazol-5-ium	2.67
6.84	342. 1162	$C_{12}H_{22}O_{11}$	D-Sucrose	2.87
7.17		$C_{21}H_{13}N_2O$		1.91
7.41	351. 1564	$C_{18}H_{19}N_6O_2$	2-[3-(5H-imidazol-1-ium-1-yl)propylamino]-4-(4-methoxyphenyl)-6-oxo-3H-pyrimidine-5-carbonitrile	2.07
8.16	195. 0988	$C_7H_{11}N_6O$	4-Amino-1-[(2E)-2-cyano-2-(hydroxyimino)ethyl]-3,5-dimethyl-4H-1,2,4-triazol-1-ium	0.02
8.49	479. 2896	$C_{27}H_{38}N_5O_3$	N-(4-Methoxybenzyl)-N′-[2-(4-methyl-1-piperazinyl)-2-(1-methyl-1,2,3,4-tetrahydro-6-quinolinyl)ethyl]ethanediamide	0.37
8.73	295. 2380	$C_{17}H_{31}N_2O_2$	(1R,3aS,10aS,10bS)-1-(3-Carboxypropyl)-2,2-dimethyldecahydro-1H,4H-pyrido[3,2,1-ij][1,6]naphthyridin-2-ium	0.11
9.02	480. 3009	$C_{32}H_{38}N_3O$	2-[3-(2-Aminophenyl)-3-oxopropyl]-3-(benzylamino)-2-methyl-1-(2-phenylethyl)-5-propyl-2H-pyrrolium	0.16
9.32	446. 3165	$C_{29}H_{40}N_3O$	(3Z,5Z)-3,5-Bis[4-(diethylamino)benzylidene]-1,1-dimethyl-4-oxopiperidinium	0.11
9.65	403. 2240	$C_{23}H_{27}N_6O$	(4aS,9bR)-2,8-Dimethyl-5-{[5-(4-methylphenyl)-2H-tetrazol-2-yl]acetyl}-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indol-2-ium	0.18
10.09		$C_{17}H_{39}N_6O$		0.05
10.38	194. 0804	$C_8H_{10}N_4O_2$	**Caffeine**	1.76
10.42	633. 3644	$C_{35}H_{53}O_{10}$	4-(7″,12″-Diacetoxy-4,10″,13″-trimethylhexadecahydrodispiro[cyclohexane-1,3′-[1,2,4,5]tetroxane-6′,3″-cyclopenta[a]phenanthren]-17″-yl)pentanoat	1.86
10.84	445. 2346	$C_{25}H_{29}N_6O_2$	1-Methyl-1,4-bis{[3-(4-methylphenyl)-1,2,4-oxadiazol-5-yl]methyl}piperazin-1-ium	0.31
11.15		$C_{15}H_{35}O_4$		2.40

Rt	m/z pubchem	formula	Compound name	Relative Abundance (%) 6 hours
11.93	353. 2911	$C_{19}H_{37}N_4O_2$	1-[2-({[(3R,4S)-3-Ethyl-1-(isopropylcarbamoyl)-4-piperidinyl]acetyl}amino)ethyl]pyrrolidinium	0.13
11.93		$C_{18}H_{42}NO_2$		0.07
12.64		$C_{15}H_{35}O_4$		2.13
13.32	500. 3937	$C_{26}H_{53}N_4O_5$	2-Methyl-2-propanyl 2-[(2S)-1-({(2S)-1-[(5-hydroxy-4-undecanyl)amino]-3-methyl-1-oxo-2-butanyl}amino)-3-methyl-1-oxo-2-butanyl]hydrazinecarboxylate	1.32
13.63		$C_{15}H_{35}O_4$		3.71
13.63	354. 0950	$C_{16}H_{18}O_9$	Chlorogenic acid	3. 87
14.44		$C_{15}H_{49}N_{16}S$		1.54
14.94		$C_{29}H_{55}N_8$		0.55
15.45	512. 4229	$C_{34}H_{57}O_3$	(17β)-3-Oxoestr-4-en-17-yl palmitate	0.68
16.35		$C_{34}H_{64}N_3OS_2$		0.59
16.73		$C_{29}H_{56}O_3Cl$		2.16
16.73		$C_{33}H_{61}O_5Cl_2$		2.16
17.10	569. 4417	$C_{32}H_{56}N_7O_2$	2,5,7-Tris(2-methyl-2-propanyl)-L-tryptophyl-N-isopropyl-L-argininamide	1.73

Table 3. Prediction of interpretation of chromatogram of Robusta coffee data fermented with *Lactobacillus plantarum* for 24 hours

Rt	m/z pubchem	formula	Compound name	Relative Abundance (%) 24 hours
0.23		$C_7H_{11}N_6O$		0.00
0.53		$C_7H_{11}N_6O$		0.00
0.86		$C_7H_{11}N_6O$		0.00
1.23		$C_7H_{11}N_6O$		0.00
1.74		$C_8H_8N_5O_3$	2-Azido-N-(3-nitrophenyl)acetamide	5.41
1.76	195. 0988	$C_7H_{11}N_6O$	4-Amino-1-[(2E)-2-cyano-2-(hydroxyimino)ethyl]-3,5-dimethyl-4H-1,2,4-triazol-1-ium	16.53
2.27	180. 0633	$C_6H_{12}O_6$	D-(+)-Glucose	4.42
2.27	354. 0950	$C_{16}H_{18}O_9$	Chlorogenic acid	4.70
2.27	220. 1305	C9H14N7	1-[2-(4,6-Diamino-1,3,5-triazin-2-yl)ethyl]-2-methyl-1H-imidazol-3-ium	4.74
2.77		$C_{11}H_{22}N_3O_5$		1.13
3.30	295. 1652	$C_{15}H_{23}N_2O_4$	N-[3-Ethoxy-1-(3-nitrophenyl)-3-oxopropyl]-1-butanaminium	0.15
3.87	310. 1285	$C_{15}H_{20}NO_6$	N-[(6,7-Dihydroxy-4-methyl-2-oxo-2H-chromen-8-yl)methyl]-2-hydroxy-N-(2-hydroxyethyl)ethanaminium	0. 17
4. 27	163. 0400	$C_9H_7O_3$	Phenylpyruvate	6.85
4.73	147. 0531	$C_5H_9NO_4$	Glutamic acid	8.22
4.73	163. 0402	$C_9H_7O_3$	Phenylpyruvate	8.51
5.04	998. 0302	$C_{66}H_{110}O_6$	Triacylglycerol	14.97
5.04	163. 0400	$C_9H_7O_3$	Phenylpyruvate	17.89
5.39	177. 0557	$C_{10}H_9O_3$	4-Methoxycinnamate	5.24
6.05		$C_{25}H_{23}O_{11}$		2.87
6.29		$C_{21}H_{19}N_6O_9$		1.15
7.03		$C_{22}H_{21}N_6O_9$		2.16
7.69	367. 1299	$C_{20}H_{19}N_2O_5$	4-(1-Benzyl-2-oxo-3-pyrrolidinyl)-5-[(2Z)-3-hydroxy-2-butenoyl]-1H-pyrrole-2-carboxylate	2.01
8.20	309. 0869	$C_{17}H_{13}N_2O_4$	3-Carbamoyl-1-[2-oxo-2-(2-oxo-2H-chromen-3-yl)ethyl]pyridinium	2.60
8.20	194. 0804	$C_8H_{10}N_4O_2$	Caffeine	2.38
8.88	363. 2176	$C_{21}H_{31}O_5$	(5ξ)-6-O-[2-(Cyclohexylmethyl)-4,6-dimethylphenyl]-2,4-dideoxy-D-glycero-hexonate	1.03
9.17		$C_{19}H_{40}N_7O_3S$		0.17

Rt	m/z pubchem	formula	Compound name	Relative Abundance (%) 24 hours
9.45	277. 2173	$C_{18}H_{29}O_2$	Linolenate	0.13
10.15	377. 2333	$C_{22}H_{33}O_5$	(8E,10Z,14E,16Z,18E)-7,13,20-Trihydroxy-8,10,14,16,18-docosapentaenoate	0.23
10.46	434. 2901	$C_{25}H_{40}NO_5$	4-[(2R)-2-Hydroxy-3-{[(1S,5S)-3,3,5-trimethylcyclohexyl]oxy}propyl]-4-[4-(methoxycarbonyl)benzyl]morpholin-4-ium	0.81
11.03		$C_{30}H_{45}N_6O_9$		0.90
11.28	273. 2528	$C_{13}H_{32}N_5O$	N-[1-(Dimethylamino)-2-propanyl]-N′-(3-ethoxypropyl)-N-ethylcarbonohydrazonic diamide	0.58
11.58	445. 2121	$C_{27}H_{29}N_2O_4$	(1S)-6-(Benzyloxy)-1-(3,4,5-trimethoxyphenyl)-2,3,4,9-tetrahydro-1H-β-carbolin-2-ium	0.31
11.89		$C_{12}H_{31}N_4O_4$		2.95
12.75	353. 2697	$C_{21}H_{37}O_4$	3-[(4-Hexadecanyloxy)carbonyl]-3-butenoate	0.71
13.04	293. 2122	$C_{18}H_{29}O_3$	(9Z,11Z)-11-[(3S)-3-Pentyl-2-oxiranylidene]-9-undecenoat	0.02
13.34	279. 2329	$C_{18}H_{31}O_2$	(Z,Z)-9,12-Octadecadienoate	5.93
13.92		$C_{30}H_{49}N_3O_4$		1.36
14.35	279. 2329	$C_{18}H_{31}O_2$	(Z,Z)-9,12-Octadecadienoate	3.31
14.35	342. 1162	$C_{12}H_{22}O_{11}$	D-sucrosa	3.87
15.34		$C_{38}H_{19}NSBr$		1.31
16.00	513. 4050	$C_{32}H_{53}N_2O3$	(2β,3α,5α,8ξ,9ξ,14ξ,16β)-3-Acetoxy-16-(1-methyl-1-piperidiniumyl)-17-oxo-2-(1-piperidinyl)androstane	0.19
16.24		$C_{36}H_{82}N_7O_{11}$		0.00

The identification of active compounds using UPLC-QToF-MS/MS for analyzing the compound content in Robusta coffee (*Coffea canephora*) was carried out using the ACQUITY UPLC system (Waters instrument). The stationary phase used was C18 or ODS (Octadecyl Silica), which is capable of separating compounds with high, medium, and low polarity. The mobile phase was a mixture of formic acid and water (0.1/99.9 v/v) and formic acid with acetonitrile (0.1/99.9 v/v), with a gradient elution system, where the ratio of the two solvents changes over time. The elution results will then enter the MS detector. The sample entering the MS system will be converted into water droplets that drip through a needle and will acquire a positive charge, as the ion source used is positive ESI (Electrospray Ionization). The separation results will appear as chromatograms, which can then be processed using Masslynk version 4.1 to obtain the m/z spectra of each peak from the chromatogram. (Mutiah et al., 2019).

The chromatogram results of the extract, which is Robusta coffee (*C. canephora*), are shown in Figure 1. Each peak in the chromatogram represents the presence of a single compound. The chromatogram is processed using Masslynx version 4.1 to obtain the m/z spectra, allowing the prediction of the molecular formula from the interpretation. The predicted molecular formula is then used to search for the compound name with the help of the ChemSpider website. When entering the molecular formula into ChemSpider, the number of hydrogen atoms (H) is reduced by 1. This is because the positive ESI ion source adds a proton (H^+) to the compound, and therefore, the m/z value should be corrected by subtracting the actual mass of hydrogen (1.0078). For some target compounds whose molecular formulas are already known, the m/z spectra can be directly checked in ChemSpider to confirm the identification from the chromatogram.

The metabolite profiles obtained from the interpretation results (Table 1) show differences in the number and type of compounds. Specifically, there are 35 compounds in Robusta coffee without *Lactobacillus plantarum* bacteria treatment, 26 compounds in samples treated with *L. plantarum* bacteria for 6 hours, and 40 compounds in samples treated with *L. plantarum* bacteria for 25 hours. The composition of compounds in plants is influenced by two factors, namely internal factors and external factors (Heuberger et al., 2013). Internal factors that affect the composition of compounds include genetic and physiological variations, while external factors such as geographical conditions (altitude), climate, humidity, light intensity, temperature, nutrient intake, and radiation also play a significant role (Verma and Shukla, 2015). These factors cause differences in the number of compounds found in each sample. Tables I, II, and III show the various compounds contained in Robusta coffee from three different samples with different treatments and durations. Each Robusta coffee sample shows a dominant or primary compound.

In coffee samples not fermented with *L. plantarum*, the dominant compound was 1-{[5-(2-Methyl-2-propanyl)-1,2-oxazol-3-yl]carbonyl}-1,2-triazadien-2-ium with a retention time of 5.08. In coffee samples fermented with L. plantarum for 6 hours, the dominant compound was 4-Amino-1-[(2E)-2-cyano-2-(hydroxyimino)ethyl]-3,5-dimethyl-4H-1,2,4-triazol-1-ium with a retention time of 4.20. In coffee samples fermented with L. plantarum for 24 hours, the dominant compound was Phenylpyruvate with a retention time of 5.04.

Based on the interpretation data, it is known that Robusta coffee contains caffeine. The results of the analysis showed that caffeine in Robusta coffee samples, with an abundance of 2.40%, was present in samples that were not fermented by *Lactobacilus plantarum* bacteria. In coffee samples fermented by *L. plantarum* for 6 hours, the abundance was 1.76%, and in samples fermented by *L. plantarum* for 24 hours, the abundance was 2.38%. The MS spectrum of caffeine is presented in Figure 2.

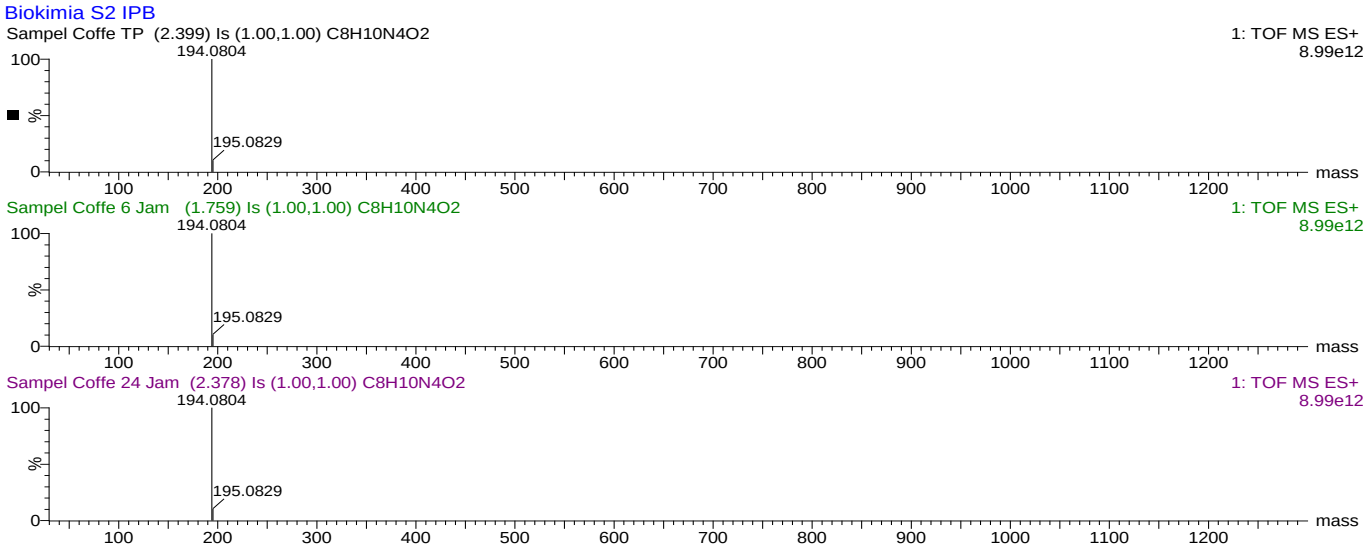

Figure 2. Spectrum and structure of the compound caffeine ($C_8H_{10}N_4O_2$)

Previous studies have shown that caffeine provides benefits such as increased concentration, improved mood, weight loss, better physical and sports performance, and free radical scavengers. Coffee contains caffeine as a former of the aroma and taste of coffee. According to SNI 01-2907-2008, coffee that is suitable for consumption has a maximum caffeine content of 2%. Coffee that has a caffeine content of more than 2% will be harmful to health, such as being able to increase blood pressure and heart rate. The results of the interpretation of the caffeine test are in Figure 1 proving that fermentation with the addition of microorganisms can reduce caffeine levels in coffee beans.

Based on research by Adrianto *et al*., (2020), it was stated that during fermentation, glucose levels which are substrates for Lactobacillus plantarum will be broken down into organic acids, so that the mucilage layer on the coffee beans will be degraded. Degradation of the mucilage layer makes it easier for water to penetrate the coffee beans through the horny skin layer. Water that penetrates the pores of the coffee causes caffeine to dissolve. This is related to the nature of caffeine which is soluble in water and binds water molecules. The amount of dissolved caffeine causes caffeine levels to decrease. The results of research by Siregar *et al*. (2020) also stated that during the fermentation process, microorganisms degrade caffeine to obtain a carbon source through caffeine demethylation. Caffeine demethylation is able to break the bonds of complex caffeine compounds into free caffeine compounds with a smaller size and are easy to move. These complex caffeine compounds are broken down into dimethyl xanthine, such as: theobromine, paraxanthine, and theophylline. The decrease in caffeine levels is also influenced by the length of fermentation time. The presence of proteolytic bacterial activity that produces quite high levels of protease enzymes causes the caffeine content in coffee beans to decrease (Farida *et al*., 2013).

Based on research by Usman *et al*. (2015), coffee beans on the outside have a mucus layer consisting of 80% pectin and 20% sugar. This layer becomes a substrate for the inoculum. Then the substrate will be reduced by the inoculum causing water to easily enter the beans through the horny skin in the form of pores. Water that seeps into the coffee beans causes caffeine to dissolve. This is due to the nature of caffeine which is dissolved in water. This is explained by Ridwansyah (2003) that the solubility of caffeine in water is due to

binding one water molecule. In addition, the decrease in caffeine content is caused by the esterification process which results in the breakdown of the complex caffeine compound into chlorogenic acid. The caffeine compound becomes free with a small size and molecular weight and becomes easy to move to diffuse through the cell wall and dissolve in water. This decrease in caffeine levels is caused by the activity of lactic acid bacteria. The decrease in caffeine levels is also influenced by the length of fermentation time. The presence of proteolytic bacterial activity that produces quite high levels of protease enzymes causes the caffeine content in coffee beans to decrease with increasing fermentation process (Adrianto *et al*., 2020).

Caffeine, a non-volatile component in green coffee beans, is a nitrogen-containing metabolite widely known to have an arousal effect. Although there is ongoing debate about whether consuming caffeine Although caffeine is beneficial or detrimental to human health, several studies have suggested that caffeine has positive effects, such as increased alertness, improved mood and focus, and the ability to stay awake (de Mejia & Ramirez-Mares, 2014). In addition, it can improve memory consolidation (Borota *et al*., 2014). In a study (Kim *et al*. 2022), caffeine levels after fermentation were highest in the first *Pediococcus pentosaceus, Lactobacillus palntarum, Saccharomyces cerevisiae*, before fermentation, *Leuconostoc mesenteroides* and lowest in *Candida parapsilosis*. These results suggest that the caffeine content of green coffee beans can be controlled differently through the fermentation process using different starter microbes. Caffeine is a purine derivative with a ketone group conjugated at carbons 2 and 6 of the purine ring. It is commonly found in tea, coffee, chocolate, sodas, and energy drinks. Excessive consumption of caffeine can lead to effects such as nervousness, anxiety, tremors, insomnia, hypertension, nausea, and seizures (Jee *et al*., 2020).

Conclusion

The research results show that there are differences in the metabolite compound content in *Coffea canephora* (Robusta coffee) with varying treatments. There were 35 compounds identified in coffee samples without *Lactobacillus plantarum* bacteria, 26 compounds in coffee samples treated with *L. plantarum* for 6 hours, and 25 compounds in samples treated with *L. plantarum* for 24 hours. The main compound found in the coffee is caffeine, with a % of 2.38% in coffee samples not fermented with *L. plantarum*. In coffee samples fermented with *L. plantarum* for 6 hours, the % was reported to be 1.76% and in coffee samples fermented with L. plantarum for 24 hours, the abundance was 2.40%. Based on the results, it shows that there are various types and numbers of identified compounds and a decrease in caffeine levels during the 6-hour fermentation time of *L. plantarum*.

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The authors declare no conflict of interest.

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