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The effect of microbial community in the self-induced anaerobic fermentation on the aroma precursor development of geothermal coffee

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Coffee drying is an important stage for character development of green beans, and it is usually conducted in direct sunlight. Thus, this method is very dependent on the weather conditions. Geothermal drying can be a substitute method to address the limitations of conventional drying. However, geothermal drying is very fast, whereas the translocation of chemical compounds into the green bean is not ideal. Process adjustment was established by applying 5 days of Self-Induced Anaerobic Fermentation (SIAF) without adding water before the geothermal drying, to enhance the sensory quality. The whole process was referred to as 'geothermal coffee processing'. This study aimed to provide knowledge about the role effect of microorganisms involved in the SIAF on the formation of chemical compounds responsible for the geothermal coffee aroma. Samples were extracted to obtain microbial DNA, which was then analyzed using shotgun metagenomics. The species and microbial enzymes were identified by comparing the Open Reading Frames (ORF) with the databases. Furthermore, non-volatile metabolites in fermented green beans were analyzed using LC-MS/MS. The results showed that *Tatumella* sp. JGM118, *Hanseniaspora uvarum*, and *Leuconostoc pseudomesenteroides* were the dominant species. This microbial community secreted enzymes, such as glycoside hydrolases (GH), transaminases, and L-lactate dehydrogenase, as indicated by their ORFs in the samples. Moreover, these enzymes catalyzed the production of non-volatile metabolites in the coffee beans. The non-volatiles were dominated by chlorogenic acid, amino acids, and carboxylic acids, none of which were detected before SIAF. These metabolites were aroma precursors in the geothermal coffee drink after the roasting process. Therefore, it was confirmed that the microbial community in SIAF contributed to the production of geothermal coffee aroma precursors.

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Sustainability spotlight

The application of geothermal coffee processing—which involves Self-Induced Anaerobic Fermentation (SIAF) without water addition and geothermal drying—addresses the limitations of conventional coffee processing, including its dependence on weather conditions, time, water use, and labor. Water submersion is not applied during fermentation, resulting in less water being used. The geothermal heat facilitates coffee drying in the dry-house, allowing UV to pass through at higher temperatures and lower relative humidity, creating faster processing times, with less labour required than with direct sunlight drying. Moreover, the microbial community involved in the SIAF enhances the development of aroma precursors in the geothermal coffee drink.

Introduction

Traditionally, green coffee beans are produced by either a wet or dry process. The wet method involves aerobic and submerged fermentation of pulped coffee to produce high-quality coffee beverages.[1–3] Microbial activity eliminates coffee mucilage during fermentation. The fermented coffees are then dried under the sun to obtain green beans. In the dry process, fermentation occurs during drying. Whole coffee cherries are sun-dried on the drying floor, then peeled to obtain green beans.[3–5]

Drying under direct sunlight is a common practice in coffee processing. However, this method has several limitations, including those related to weather conditions. It causes variations in drying times and high workloads in the coffee industry.[6] The long rainy season that occurs in tropical areas can pose significant challenges for coffee farmers. The unpredictable

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weather in Indonesia makes this conventional coffee drying process less effective.[7]

Mechanical drying using the excess heat from a geothermal installation can be an alternative method that addresses the limitations of conventional drying.[7] This excess geothermal energy can be utilized with Heat Pipe Heat Exchangers (HPHE) to increase the temperature in the dry-house, where UV can still penetrate, to 50 °C with RH 30%.[8,9] The business evaluation showed a significant surplus for coffee farmers due to the accelerated drying process.[9] Conventional coffee drying generally takes up to 16 days, while geothermal drying requires only 2 to 4 days for pulped coffee and 7 to 9 days for whole coffee cherries.

Nevertheless, this acceleration of the drying process results in incomplete translocation of metabolites into coffee beans. Water content evaporates very quickly, with a reduction of more than 40% in the first 3 hours, resulting in a decrease in water activity from 0.88 to 0.62 by the end of the drying process.[7,10] This level of water activity is insufficient for the survival of vegetative microorganisms; therefore, fermentation does not occur during geothermal drying.

SIAF is a new coffee fermentation technology that produces CO_2 gradually as a result of microbial activities. Whole or pulped coffee is placed in a closed container to maintain anaerobiosis.[11,12] This process produces metabolites that impact the quality of the coffee drink. Process adjustments are applied by employing Self-Induced Anaerobic Fermentation (SIAF) before geothermal drying to produce coffee that is favourable to consumers and Q-graders. This combination of SIAF and geothermal drying has not been studied before. Therefore, existing information on the role of microbiota involved in SIAF on the aroma precursor development of geothermal coffee remains limited.

SIAF can improve the sensory quality of coffee beverages by producing favourable chemicals compared to the conventional coffee fermentations.[13] Chlorogenic acids, reducing sugars, organic acids, and amino acids are produced throughout coffee fermentation by yeasts and bacteria. These metabolites are precursors for pyrazines, aldehydes, ketones, furans, and esters during the roasting process. Sensory attributes resulting from the mentioned volatiles give roasted, nutty, and fruity notes.[14]

The microbial community involved in the SIAF was assumed to produce aroma precursors in the fermented green bean before geothermal drying. In this research, we analysed the relative abundance, diversity, and functional properties of the microbial community in the SIAF of Arabica coffee using shotgun metagenomics. LC-MS/MS was used to analyze the non-volatile metabolites resulting from the fermentation. Furthermore, the relations between the microorganisms, their enzymes, and their produced non-volatile metabolites, as the aroma precursors of geothermal coffee, were analyzed by connecting the acquired data from the shotgun metagenomics and LC-MS/MS to the KEGG and NCBI databases. This knowledge aims to provide a theoretical basis for the development and research of Geothermal Coffee Processing.

Materials and methods

Self-induced anaerobic fermentation (SIAF) of arabica coffee

All coffee cherry samples were harvested on the same day from an Arabica coffee plantation in Kamojang, Indonesia. After being washed, 240 kg of whole coffee cherries and 300 kg of pulped coffee, respectively, were placed in three 200-L fermentation barrels (Fig. 1). Every barrel was then tightly closed and equipped with an air lock. The SIAF process was conducted for five days at room temperature. The precise temperature and pH were monitored daily. The geothermal drying was further conducted to obtain the final green coffee beans. However, in this study, we only focus on the effects of the SIAF process on the aroma precursor development of geothermal coffee.

After five days of fermentation, coffee of each 100 grams and the released water from coffee cherries of each 50 grams, respectively, were collected from the top, middle, and bottom of each barrel and mixed thoroughly. The samples were then submerged with DNA Shield (Zymo Research, California, USA) for shotgun metagenomics analysis. Coffee samples of each 100 grams for non-

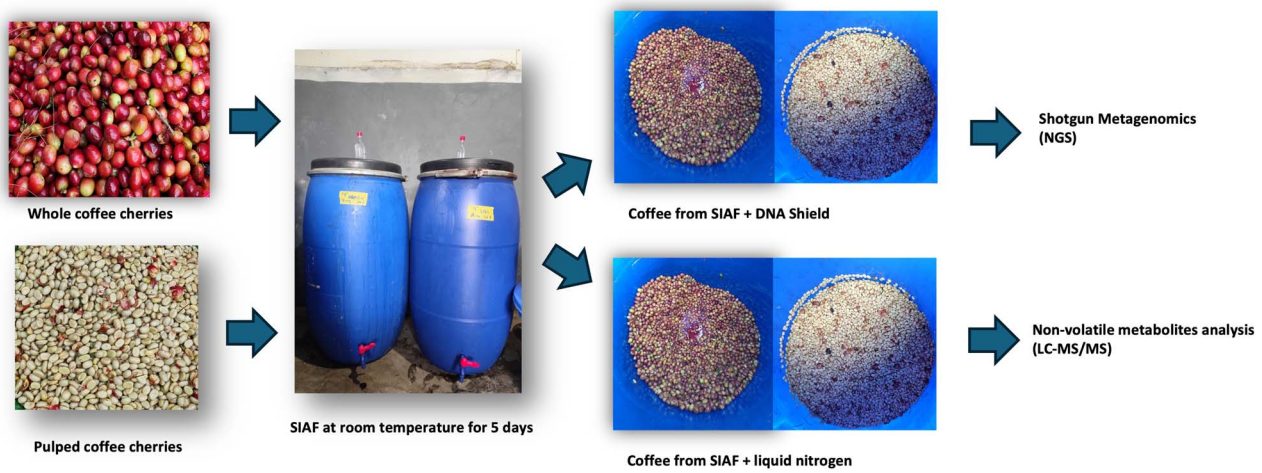

Fig. 1 Workflow of the experimental process shows that there are two different samples for shotgun metagenomics and non-volatile metabolite analysis using LC-MS/MS.

volatile metabolite analysis were also collected from the top, middle, and bottom of each barrel and mixed thoroughly. The samples were then manually peeled to obtain the green coffee beans and freeze-dried using liquid nitrogen. All composite samples were further transported in a cool box containing ice gel from the coffee processing facility to our laboratory.

Shotgun metagenomic

Shotgun metagenomics was employed to analyse the microbial community. This analysis applied the Next-Generation Sequencing (NGS) method, which provided an overview of the abundance and diversity of various microbial species and enzymes involved in the SIAF of Arabica coffee. Firstly, the samples were thawed, and then DNA extraction was conducted using the CTAB method. The DNA concentration was measured with Qubit dsDNA HS Assay Kits (Thermos Scientific, Massachusetts, USA), and the DNA integrity was measured with 4150 TapeStation (Agilent, California, USA). Further, the NGS library preparation was accomplished using an xGen DNA Library Prep EZ UNI Kit (IDT, Coralville, USA). The DNA concentration and DNA size of the samples were measured again to meet the requirements of the NGS instrument (Illumina Next-Seq 2000, San Diego, USA).[15]

The primary process that occurs in the NGS instrument was Surface-Restricted Fluorescence Sequencing (SURF-seq). This process used the principle of sequencing by synthesis. After sequencing was completed, the resulting reads were filtered with fastp (v0.23.2) and quality checked using fastQC (v0.11.9) and MultiQC (v1.13). Further steps of bioinformatics analyses include assembling reads into contigs with *de novo* assembly using MEGAHIT (v1.2.9), quality control, and visualization of contigs using QUAST (v5.0.2) and Bandage (v0.8.1).

Data transformation from FASTA file to SAM file and deletion of host DNA, coffee tree, was performed using SAMtools (v1.6). Contigs with coverage less than two and/or length shorter than 500 bp were filtered out using BBtools (BBMap v37.62). The complete genome prediction was conducted using MetaGeneMark (v3.38). The predicted ORFs with a length shorter than 100 bp were filtered out using seqkit (v2.5.1). Filtered ORFs from each sample were merged, and the redundant ORFs (percent identity = 100%) were filtered out using BBMap, generating the unique gene catalogues. Other parameters of all the tools were set to the application default.

Taxonomy assignment was performed using Diamond v2.0.15 (blastx)-MEGAN v6.24.24 (LCA, MEGAN-LR) pipeline. The curated NCBI-nr database was used. Taxonomy results were filtered to the species level. Functional binning was performed using various databases, including EC (MEGAN) and Cazy (Diamond). Mapping to gene catalogues from each sample was performed using BWA (v0.7.17-r1188). Read coverage was determined using bedtools (v2.30.0), and a count table (Reads per Kilobases – RPK) was generated using SAMtools. Taxonomy visualization was performed using Krona (v2.8.1). Downstream taxonomy and functional analysis were performed using phyloseq (v1.44.0). Completeness check was performed using BUSCO (v5.4.4).

Moreover, the Cazy database (https://cazy.org/) was used to understand the mechanism of carbohydrate-active enzymes. The detected Enzyme Commission (EC) numbers were compared to the KEGG database (https://www.kegg.jp) to recognize their cellular functions and metabolites. Alpha diversity was calculated with the Shannon-Weaver index and beta diversity with the Sørensen-Dice index. This metagenomic analysis was assisted by PT. Genetika Sains Indonesia.

Non-volatile metabolites analysis

The non-volatile metabolites were analysed using UHPLC Q Orbitrap HRMS (Thermo Fisher Scientific, Bremen, Germany).[4,16] Sample preparation was carried out by lyophilizing and then grinding the green coffee beans. The ground green bean (8.25 g) was dissolved in 150 mL of boiling water. The solution was maintained at a temperature of 93 °C while stirring using a magnetic stirrer for 1 minute. The sample solution was then cooled down and filtered using Whatman filter paper number 1. It was stored in a freezer at a temperature of −22 °C before injection.

The filtrates of ground green beans were diluted with LC-MS grade methanol (1 : 1, v/v) and filtered through a 0.22-μm PTFE membrane (Merck, KGaA, Darmstadt, Germany). A 5 μL volume of sample was injected onto an Accucore C18+ column (100 × 2 : 1 mm, 1.5 μm) (Thermo Fisher Scientific, Bremen, Germany) at 40 °C. The mobile phases were LC-MS methanol (a) and 0.05% formic acid in LC-MS water (b). The flow rate was set at 0.2 mL min^{-1}, and the gradient elution was 0 min (5% A), 0–17 min (90% A), 17–20 min (90% A), 20–23 min (5% A), and 23–30 min (5% A). Ionization mode was used in positive and negative electrospray ionization (ESI) with Q Orbitrap mass analysis. Analysis was performed at *m*/*z* 70–900, accompanied by UV detection at wavelengths of 220, 265, 272, and 320 nm.

Compound identification was performed by analysing raw data (chromatograms) using Compound Discoverer 3.2 software (Thermo Fisher Scientific) and comparing the data to online libraries (FoodDB, HMDB, NIST, Pubchem, and MzCloud). The mass spectra of the identified compounds were analysed using ThermoXcalibur 4.2 (Thermo Fisher Scientific). Identified compounds were screened using Annot (DeltaMass <5 ppm). Compound area data were normalized and displayed as heat maps.

Results and discussion

Microbial profile

The characteristics of reads and contigs in this study are shown in Table 1. Metagenomic datasets usually show 5–23% duplicate reads.[17] The whole coffee cherry SIAF sample gave a slightly higher duplication rate, which could be caused by PCR amplification during library preparation. The optimum GC content for metagenomic analysis was 50%. Lower or higher GC content than 50% was caused by lower relative sequencing coverage.[18]

The N50 for metagenomic assembly is usually 20 kbp.[19] A low N50 value might be due to low sequencing coverage, high diversity of microorganisms, and high genome complexity. Moreover, the default settings of the tools might not be optimal for the samples. However, a low N50 value was allowed for the diversity study since the contigs were aligned to the databases.

Table 1 Characteristics of the resulting reads and contigs[a]

Sample name (filtered)	Read duplication rate (%)	GC read (%)	Read length (bp)	Reads (M)	Largest contig (bp)	Total length contig (bp)	N50 (bp)	N75 (bp)	L50 (contigs)	L75 (contigs)	GC contig (%)
Whole coffee cherries SIAF	29.1	37	138	28.4	131 338	421 774 401	1303	780	83 512	190 223	36.08
Pulped coffee cherries SIAF	18.3	40	132	26.3	290 715	382 692 454	1175	742	84 334	188 740	38.39

[a] GC read is the guanine and cytosine content in the reads. The N50 and N75 were the lengths of the shortest contigs at 50% and 75% of the total assembly length. GC contig is the guanine and cytosine content in the contigs. The L50 and L75 were the counts of the shortest contigs whose length sum makes up 50% and 75% of the genome size.

The microbial community of both samples showed low alpha diversity and no overlap for beta diversity. SIAF of the whole coffee was dominated by *Tatumella* sp. JGM118 (28%), *Leuconostoc pseudomesenteroides* (18%), and yeast *Hanseniaspora uvarum* (17%), respectively. The succeeding highest species were *Lactiplantibacillus plantarum* (9%) and *Lactococcus lactis* (6%), respectively. The subordinates were species with relative abundance less than 5%, which were *Kluyvera ascorbata*, *Frateuria aurantia*, and *Serratia* sp. M24T3, *Tatumella citrea*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Pantoea* sp. Acro-835, *Tatumella* sp. UCD-D_suzukii, *Pantoea* sp. A4, *Lactiplantibacillus paraplantarum*, *Pseudomonas* sp. M47T1, *Rouxiella badensis*, and *Pantoea rodasii*.

Meanwhile, the microbial community in the SIAF of pulped coffee was dominated by *Tatumella* sp. JGM118 (42%), *Hanseniaspora uvarum* (14%), and *Leuconostoc pseudomesenteroides* (9%), respectively. *Tatumella citrea* (5%) and *Serratia* sp. M24T3 (4%) were the following two most abundant species. The subordinates were species with relative abundance less than 4%, which were *Pantoea* sp. Acro-835, *Tatumella ptyseos*, *Pantoea* sp. A4, *Frateuria aurantia*, *Tatumella* sp. JGM130, *Lactococcus lactis*, *Kluyvera ascorbate*, *Leuconostoc citreum*, *Lactiplantibacillus plantarum*, *Pseudomonas* sp. M47T1, *Tatumella* sp. UCD-D_suzukii, *Pantoea rodasii*, *Rouxiella badensis*, and *Lactiplantibacillus paraplantarum* (Fig. 2).

The final temperature showed 20.2 °C for whole cherries SIAF and 21.2 °C for pulped cherries SIAF (SI Fig. 1). The temperature of whole coffee cherries and pulped coffee cherries SIAF during 5 days increased 1.5 °C and 2.5 °C, respectively. This led to the domination of mesophilic microbiota in the fermentation process. The pH of whole cherries SIAF started at 5.5 and finished at 4.15, while the pH of pulped cherries SIAF gradually decreased from 6.0 to 4.25 on the last day (SI Fig. 2).

The temperature increase is a good sign of proper SIAF process due to the exothermic reaction of sugar conversion to organic acids and alcohols.[13] pH monitoring showed that the coffee samples were not over-fermented since pH 4 is the estimated condition of complete mucilage degradation.[20,21] SIAF occurs spontaneously due to an epiphytic microbial community. Their fermentative activity is affected by intrinsic and extrinsic factors, which interfere with the quality of final coffee beverages.[11]

The low-level oxygen available in the SIAF bioreactors was utilized by yeast, Lactic Acid Bacteria (LAB), and facultative anaerobic bacteria like *Tatumella*. The symbiotic mutualism of yeast and LAB directly affects the aroma development of coffee

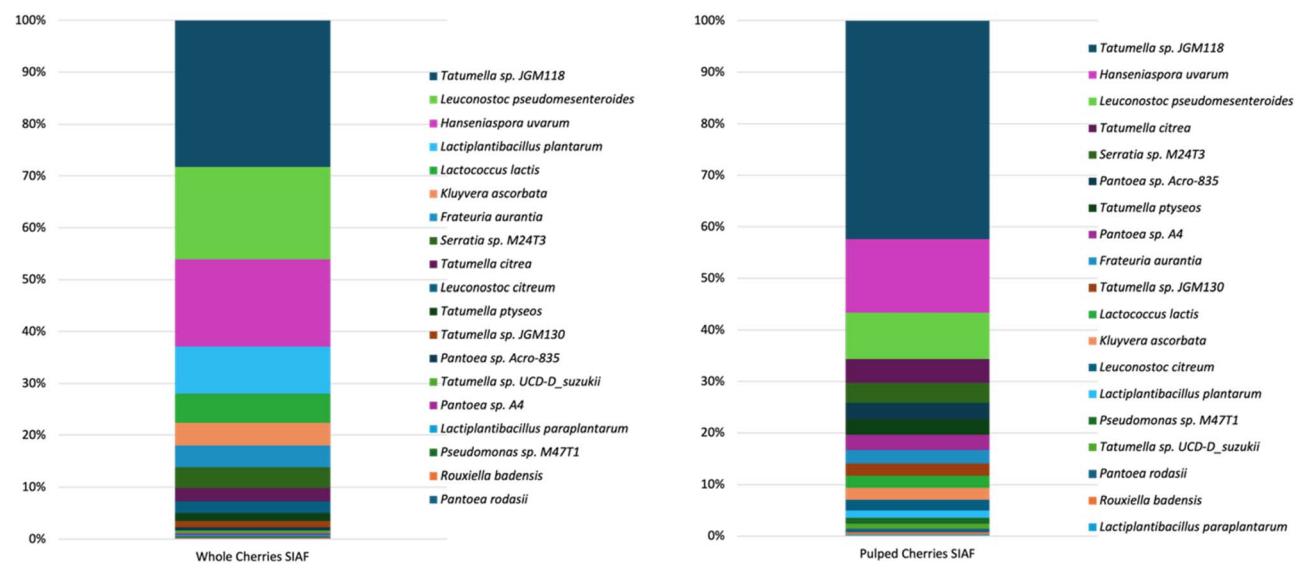

Fig. 2 Relative abundance of microbial community in the whole (left) and pulped (right) coffee cherries SIAF. *Tatumella* sp. JGM118 dominated, followed by different microbial composition and relative abundance in both samples.

drinks. Therefore, self-induced anaerobic fermented coffee is associated with improved sensory attributes. LAB delivers an acidic environment that is susceptible to yeast growth. At the same time, yeast provides soluble nitrogen compounds and vitamins that support the bacteria's growth.[13]

It was also recorded in a study from Brazil that *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, and *Leuconostoc mesenteroides* dominated Arabica coffee SIAF at mesophilic conditions.[11] The notable difference here was the domination of *Tatumella* sp. JGM118.*Tatumella* was included in the family of *Erwiniaceae* (Fig. 3). This family consists of several plant pathogens. *Tatumella ptyesos*, one of the species, was found to be the causative agent of pink disease in pineapple.[12] However, as a member of the *Enterobacterales* order, it has been mentioned that *Tatumella* have the capacity to decompose sucrose under anaerobic conditions during coffee fermentation. The intermediates of sugar degradation were further utilized through glycolysis and the TCA cycle to produce reducing sugars and organic acids, substrates of amino acid metabolism, like fructose and other byproducts.[22] These metabolites are the precursors of the Maillard reactions.[23]

Yeasts are the key players in coffee fermentation. They hold a crucial role in the development of aroma precursors in the fermented green beans. They hydrolyze macromolecules to produce reducing sugars, citric acid, amino acids, and chlorogenic acids.[24] These metabolites were translocated into the beans and affected the coffee drink quality.[20]

Besides, yeast is a volatile compound producer. The non-*Saccharomyces* yeasts deliver esters, alcohols, and fatty acids.[25] *Hanseniaspora uvarum* exhibited higher activity of pectinase, amylase, cellulase, and protease compared to other yeast species.[26] This yeast prefers fructose and glucose rather than sucrose.[21]

The LAB was represented by *Leuconostocaceae* at the beginning of coffee fermentation. Further, the abundance of *Lactobacillaceae* increases at 48 and 72 h in SIAF of pulped coffee cherries.[27] They are notable for being organic acid producers, especially lactic acid. Their activity is responsible for the degradation of coffee mucilage since pectic substances are soluble in organic acids. This acidification causes swelling of the mucilage cell wall.[26] The present simple sugars were consumed first by them before polysaccharides, resulting in organic acids *via* glycolysis and pyruvate metabolism, producing D-lactate, ethanol, and CO_2.

Frateuria aurantia[28], *Serratia* sp.,[29] and *Pseudomonas* sp. strain M47T1 (ref. 30) are beneficial for plants as a potassium-solubilizing bacterium, antifungal, and nematotoxic, respectively. While *Pantoea rodasii*[31], *Rouxiella badensis*[32], *Pantoea* sp. A4 (ref. 33) are plant pathogens, *Kluyvera ascorbata* is an emerging pathogen and a rare cause of urinary tract infection in immunocompetent patients.[34] However, it will not have any effect on consumer health since high-temperature processes are applied before consumption. Aerobic groups of fungi that produce toxins like *Aspergillus* and *Penicillium* were not found in this anaerobic environment, although their spores may still survive.[25] Temperature and humidity control during coffee drying and storage are therefore needed.

Aroma precursors

Analysis of the non-volatile metabolites using LC-MS showed that SIAF coffee is characterized by chlorogenic acid, carboxylic acid, and amino acids (Fig. 4). Before SIAF, these metabolites were not detected. Further, its concentration increased 7 to 10-fold after SIAF.

Chlorogenic acid (CGA) was the dominant metabolite in the green beans from the SIAF process. CGA is an ester of caffeic acid and quinic acid. This metabolite is produced for plant protection.[35] SIAF of whole cherries delivered a higher relative

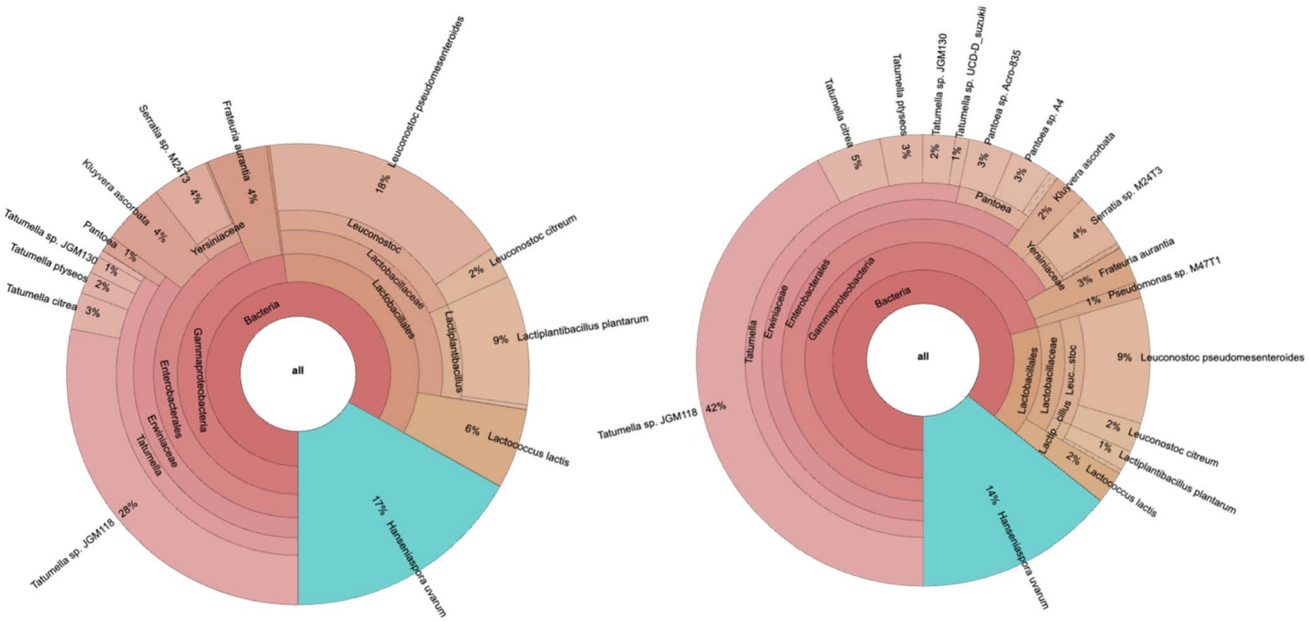

Fig. 3 Microbial diversity of whole (left) and pulped (right) coffee cherries SIAF. The taxa showed species, genus, family, order, class and domain of the microbial community.

abundance of CGA compared to SIAF of pulped cherries, since CGA was concentrated in the mucilage. During the roasting process, this phenolic compound is degraded back into quinic and caffeic acid, phenol derivatives, and lactones.[25]

In both coffee SIAF samples, groups of amino acids and peptides, groups of lactic and citric acids, and caffeine from alkaloid groups were the second, third, and fourth highest relative abundance, respectively. Other metabolite groups, such as sugars, quinic acids, and coumarin, were also identified in the fermented green beans. Lactic and citric acids are formed during SIAF. The high abundance of citric acid in the coffee SIAF was due to the microbial community's high yield of Krebs.[35]

Coffee mucilage is a suitable substrate for microbial growth. It contains sugars like sucrose, as well as organic acids like citric and malic acids.[13] During SIAF, microbial pectolytic enzymes catabolize pectin to release sugars. While microbial proteolytic enzymes also release amino acids and peptides.

Malic acid can be converted to lactic acid directly and citric acid through the TCA cycle.[25] CO_2 influences the anaerobic conditions where glycerol is produced. Glycerol is the precursor of phospholipids, which contribute to the formation of aldehydes and alcohols.[13]

Steps of the coffee processing interfered with the performance of the microbial community in coffee fermentation, although the calculated beta diversity was zero. Their performance was affected by the substrate and involved enzymes. The pulping process, for instance, changes the substrate

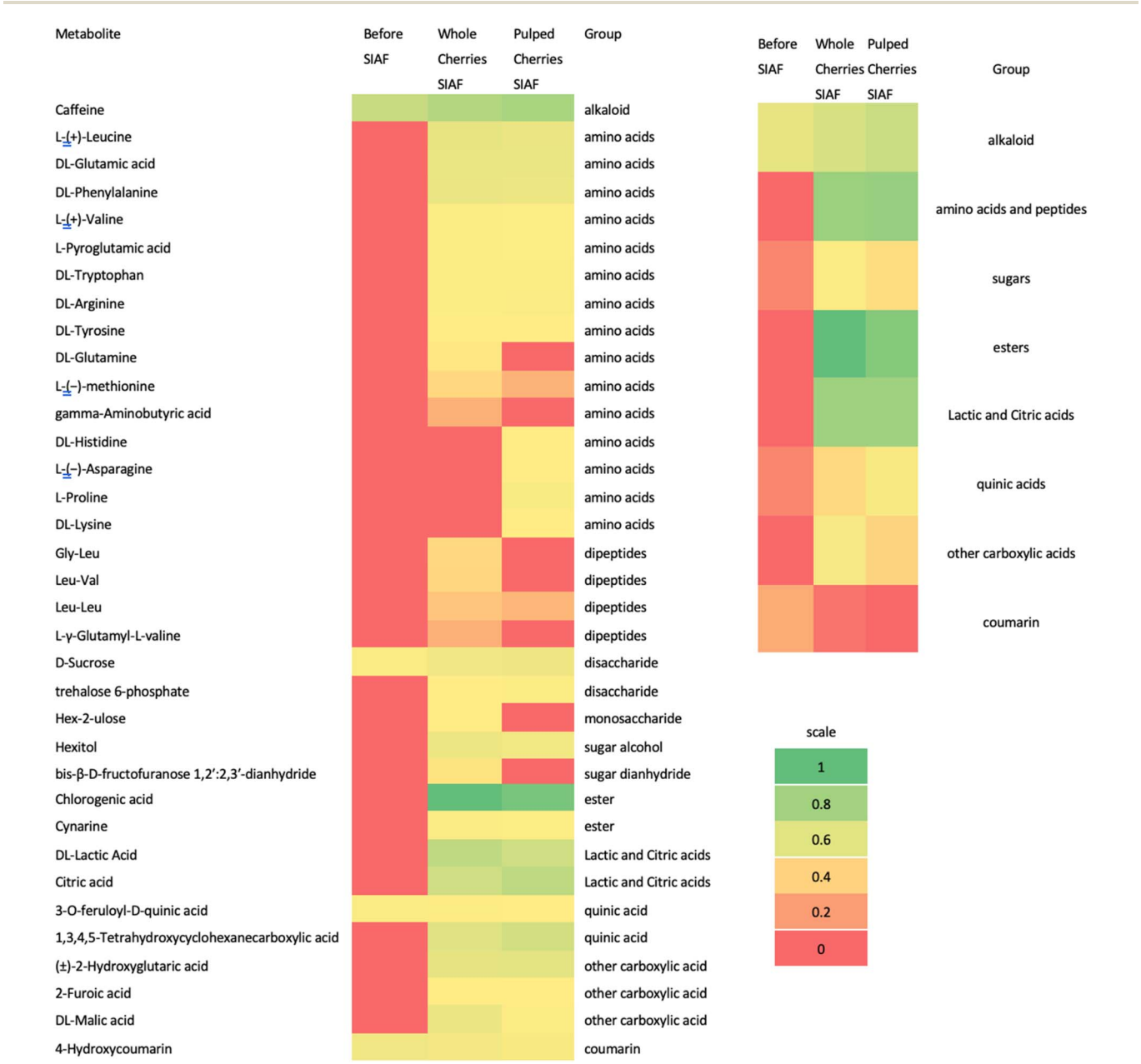

Fig. 4 Heatmap of non-volatile metabolites in the green beans from whole cherries and pulped cherries SIAF that affect the aroma development of geothermal coffee. Every compound group forms a different aroma precursor.

composition and abundance. Therefore, the produced metabolites during SIAF may differ from the whole cherry sample.[13]

Marked by the red colour in the heatmap (Fig. 4), there were several amino acids and peptides that were produced in the pulped cherries SIAF sample but not in the whole cherries, and *vice versa*. Hex-2-ulose was not found in the pulped cherries SIAF sample. This could be affected by the partial pulp removal and its transformation into Hexitol. Bis-β-D-fructofuranose 1,2′:2,3′-dianhydride might also not be detected due to the pulping process.

Lactic acid in this study was found to be higher in the sample of whole cherries SIAF, compared to the pulped cherries SIAF. Without the pulping process, a high concentration of carbohydrates, the substrate of lactic acid, was still intact in the whole coffee cherries. Lactic acid is mainly produced by LABs, which favor glucose *via* glycolysis or pyruvate metabolism. It was relevant to the higher relative abundance of *Lactobacillales* in the whole cherries SIAF sample (35%) compared to the pulped cherries SIAF sample (15%). The microaerophilic condition inside SIAF bioreactors also brought an ideal environment for LAB.

Citric acid is mainly produced by the yeast.[13] Pyruvate, as a result of sugar metabolism in the Krebs cycle, is converted into Acetyl-CoA and oxaloacetic acid, the precursors of citric acid. Citric acid was found to be higher in the pulped cherries, SIAF. Although a lower relative abundance of yeast was found in this sample, the relative abundance of *Tatumella* sp. JGM118 was higher.

Tatumella exhibited strong amylolytic activity.[23] The degraded sugars could be used as substrates for the TCA cycle. The elimination of the coffee cherries' outer skin provided direct access for the microbial community to the mucilage, which delivered a variation of citric acid concentrations between the two samples. The pulping process also resulted in microbial enzyme alterations.

Citric acid contributes to sensory attributes of the coffee drink, such as citrus note, fruity, and floral, while lactic acid gives milky, creamy, and tartness.[36] The highlighted sensory attributes of SIAF coffee beverages are citrus, honey, caramel, chocolate, and nuts. Malic acid's associated aroma is green fruit, like an apple. Coffee drink from pulped cherries SIAF has an aroma like cereal, nuts, and coconut, corroborating the lactic acid content. While coffee drinks from whole cherries, SIAF deliver notes of rum, watermelon, chocolate, caramel, and brown sugar.[36]

Proteins, polysaccharides, sugars, and chlorogenic acids are naturally occurring metabolites of coffee pulp.[20] These metabolites are coffee aroma precursors. However, fermentation alters the content of these metabolites. The mentioned metabolites are further involved in the formation of volatile compounds such as pyrazines, furans, esters, ketones, aldehydes, and acids during coffee bean drying and roasting *via* a series of complex reactions. These volatiles eventually generate the aroma of the final coffee beverages.[20]

The series of complex reactions during roasting includes caramelization, pyrolysis, dehydration, hydrolysis, enolization, cyclization, polymerization, and, mainly, Maillard. Polysaccharides and sugars are the substrates of the caramelization reaction. Phenols and guaiacol are composed *via* thermal reactions from chlorogenic acids and phenolics. Precursors of Maillard reactions are free amino acids and reducing sugars. Both metabolites interact and generate pyrazines and pyridines, which give the roasted and nutty aroma in the coffee beverages. Pyrazines and pyrroles are also produced *via* pyrolysis of hydroxyl amino acids like threonine and serine.[20]

Fermentation also influences the aroma development in the coffee bean through the translocation of microbial metabolites produced in this process into the green beans from the mucilage.[20] Chlorogenic acid, citric acid, malic acid, quinic acids, and other acids in the green beans not only contribute to the coffee acidity but also bring masking or synergistic effects to the coffee aroma. They create the cross-modal aroma-taste interaction in coffee through sensory perception.[37] Moreover, bitter-tasting subqualities of coffee (caffeine, quinic acids, tryptophan, phenylalanine) also influence the retronasal perception of its aroma.[38]

Microbial enzymes

Coffee mucilage contains 47% wet basis of pectin 39. Carbohydrate content of the mucilage was measured to be sucrose (33.15 mg g^{-1}), glucose (49.24 mg g^{-1}), and fructose (76.26 mg g^{-1}). These numbers are much higher than those for lipid (0.422 mg g^{-1}) and protein (0.996 mg g^{-1}).[40] Therefore, carbohydrate-active enzymes were significantly important in the coffee fermentation.

Since the coffee plant DNA had been removed using the bioinformatics analysis, only the ORF of microbial enzymes was further observed. Various ORFs of carbohydrate active enzymes (Cazy) were detected. Samples of whole cherries SIAF and pulped cherries SIAF distributed different variations and relative abundance of Cazymes ORF (Fig. 5). Coffee processing alters the microbial enzymes involved in coffee fermentation.

The main Cazymes that are directly involved in the degradation of carbohydrates are glycoside hydrolases (GH). While glycosyl transferases (GT) are involved in carbohydrate biosynthesis. Carbohydrate esterases (CE) are the ones that remove acyl- or alkyl-group from carbohydrates. Auxiliary Activities (AA) breakdown lignin and non-catalytic carbohydrate-binding modules (CBM) do not have enzyme activity.

In the whole cherry SIAF sample, the ORF of the GH1 family dominated with more than 45% relative abundance. The common enzyme activities of the GH1 family are β-galactosidases and β-glucosidases, which are possessed by *Lactococcus lactis*, *Lactiplantibacillus plantarum*, and *Hanseniaspora uvarum*.[41,42] β-galactosidases are one of the pectinases.[43] Pectinases break down pectin of the coffee mucilage to produce sugars, organic acids, and alcohols. Hexitol is developed *via* fructose and mannose metabolism. The activity of this enzyme was confirmed in this study as the sugar content increased in the green bean after SIAF.

The ORF of GH13 was the second most abundant GH family in the whole cherry sample. Based on the Cazy database, there are several Cazymes that are included in this group. Enzyme α-amylase is produced by *Lactiplantibacillus plantarum*, *Pseudomonas* sp, and *Serratia* sp. Amylopullulanase originates from *Lactiplantibacillus plantarum*. Oligo-α-1,6-glucosidase is

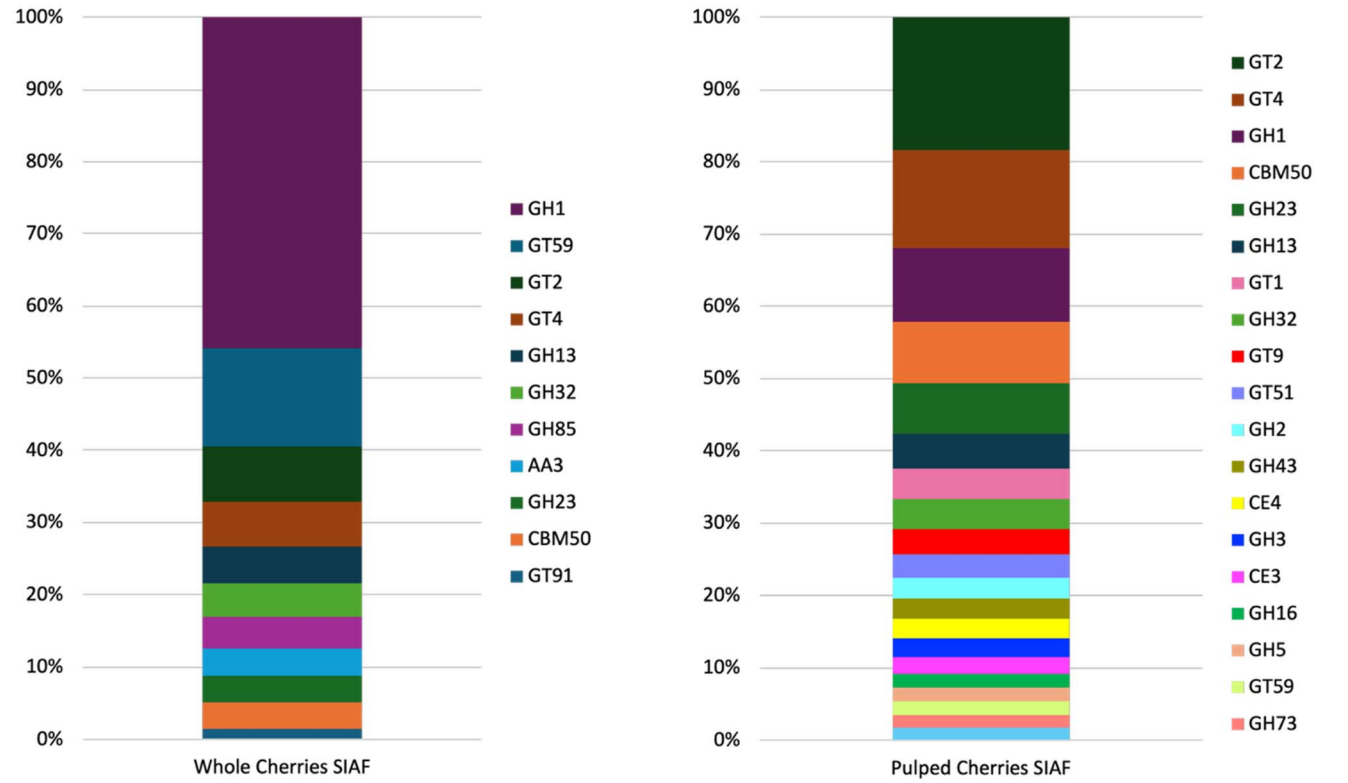

Fig. 5 Carbohydrate-active enzymes detected in the whole (left) and pulped (right) coffee cherries SIAF samples.

associated with *Lactiplantibacillus plantarum* and yeast. Sucrose isomerase originates from *Serratia* sp. and sucrose phosphorylase from *Leuconostoc mesenteroides*. Enzyme α-glucosidase is possessed by yeast.

The most abundant ORFs of the GH families in this sample were GH32, GH85, and G23. Invertase, which breaks down sucrose into glucose and fructose, is included in the GH32 family and is produced by yeast. One of the GH85 family activities is endoglycosidase. An example of the GH23 is peptidoglycan lytic trans glycosylase, which is associated with *Pseudomonas* sp.

In the pulped cherries SIAF sample, the ORF of GH 1, GH23, GH13, GH32, GH2, GH43, GH3, GH16, GH5, and GH73 families dominated the glycoside hydrolases, respectively. β-galactosidases and β-glucosidases are also included in the GH2, GH3, and GH5 families since both hydrolyse beta-glycosidic bonds. The GH43 family exhibits activity like β-xylosidase or α-L-arabinofuranosidase. Activities of GH16 are reflected by β-porphyranase and β-1,3-glucanase, which are originated from *Pseudomonas* sp., and 1,6-glucanosyltransferase from yeast. *N*-acetylglucosaminidase or autolysin from *Lactiplantibacillus plantarum* and *Lactococcus lactis* is one of the activities of the GH73 family.

The enzymes that contribute to aroma development detected from the ORF in the coffee SIAF samples are shown in Table 2. The identified EC numbers of enzymes detected from the ORF were compared to the KEGG database to understand their molecular pathways and mechanisms related to coffee aroma development.

Several of their metabolites were detected in the previous non-volatile metabolite analysis results. While the NCBI database was used to predict the producers of the microbial enzymes. The NCBI database provides information on the genomes and proteins (enzymes) of microorganisms. However, the registered genomes are incomplete genomes that were sequenced by Whole Genome Shotgun (WGS). Therefore, the microbial producers of aroma precursors can only be predicted. It is evident that all detected species in the samples participated in the production of metabolites related to aroma development.

Amino acids are produced *via* various amino acid metabolisms. Activities of proteolytic enzymes, such as transaminases and dipeptidases, were confirmed, as amino acids were produced in green beans after SIAF. Lactate is composed *via* glycolysis, and malate *via* pyruvate metabolism. The detected microbial L-lactate dehydrogenase in the samples explains that microorganisms involved in the SIAF process played an important role in the formation of lactic acid.

As of August 2025, *Serratia* sp. M24T3, *Tatumella citrea*, *Pantoea* sp. Acro-835, and *Pseudomonas* sp. M47T1 has not yet WGS-sequenced. Their ORF of enzymes is not available. The only available data are ORFs that are common in multiple species of their genus. Therefore, the predicted producers of several metabolites were mentioned as *Serratia* sp., *Tatumella* sp., *Pantoea* sp., and *Pseudomonas* sp.

The LAB belongs to *Lactococcus*, which distributes, proteolytic enzymes that enable the hydrolysis of peptides and caseins. This group also provides transaminases that degrade amino acids and transform them into alfa-ketoacids, which are

Table 2 Microbial enzymes contributed to aroma precursor development identified in the whole and pulped coffee SIAF samples[a]

EC number enzymes	Pathways	Metabolites	Reference	Predicted producers
2.6.1.42 Branched-chain-amino-acid transaminase	Valine, leucine, isoleucine metabolism	L-(+)-Leucine*		*Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*
1.4.1.13 Glutamate synthase (NADPH)	Alanine, aspartate, and glutamate metabolism; histidine metabolism	DL-Glutamic acid*		*Hanseniaspora uvarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Rouxiella badensis*, *Pantoea rodasii*, *Pseudomonas* sp.
3.5.3.8 Formimidoylglutamase				*Lactococcus lactis*
3.5.1.68 *N*-Formylglutamate deformylase				*Rouxiella badensis*, *Pantoea rodasii*, *Pantoea* sp., *Serratia* sp., *Pseudomonas* sp.
3.5.2.9 5-Oxoprolinase (ATP-hydrolyzing)				*Hanseniaspora uvarum*
3.5.1.2 Glutaminase				*Hanseniaspora uvarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Tatumella ptyseos*, *Tatumella* sp., *Rouxiella badensis*, *Pantoea rodasii*
2.6.1.9 Histidinol-phosphate transaminase	Phenylalanine metabolism	DL-Phenylalanine*		*Hanseniaspora uvarum*, *Leuconostoc pseudomesenteroides*, *Kluyvera ascorbata*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*
2.6.1 Transaminases				*Leuconostoc pseudomesenteroides*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Lactiplantibacillus paraplantarum*
2.6.1.57 Aromatic-amino-acid transaminase				*Hanseniaspora uvarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Tatumella* sp., *Pseudomonas* sp.,
2.6.1.1 Aspartate transaminase				*Tatumella* sp. JGM118, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Frateuria aurantia*, *Pseudomonas* sp.
2.6.1.66 Valine-pyruvate transaminase	Valine, leucine, isoleucine metabolism	L-(+)-Valine*		*Tatumella* sp. JGM118, *Kluyvera ascorbata*, *Tatumella* sp. *JGM130*, *Serratia* sp., *Pantoea* sp.
2.6.1 Transaminases	Tryptophan metabolism	DL-Tryptophan*		*Leuconostoc pseudomesenteroides*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Lactiplantibacillus paraplantarum*
2.6.1.9 Histidinol-phosphate transaminase	Tyrosine metabolism	DL-Tyrosine*		*Hanseniaspora uvarum*, *Leuconostoc pseudomesenteroides*, *Kluyvera ascorbata*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*
2.6.1 Transaminases				*Leuconostoc pseudomesenteroides*, *Lactiplantibacillus plantarum, Lactococcus lactis, Lactiplantibacillus paraplantarum*
2.6.1.57 Aromatic-amino-acid transaminase				*Hanseniaspora uvarum, Lactococcus lactis, Kluyvera ascorbata, Tatumella* sp., *Pseudomonas* sp.,
2.6.1.1 Aspartate transaminase				*Tatumella* sp. JGM118, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Lactiplantibacillus paraplantarum*, *Frateuria aurantia*, *Pseudomonas* sp.
3.5.1.94 Gamma-glutamyl-gamma-aminobutyrate hydrolase	Alanine, aspartate, and glutamate metabolism; arginine and proline metabolism; butanoate metabolism	Gamma-aminobutyric acid*		*Leuconostoc pseudomesenteroides*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Leuconostoc citreum*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp.

Table 2 (*Contd.*)

EC number enzymes	Pathways	Metabolites	Reference	Predicted producers
2.6.1.19 4-Aminobutyrate-2-oxoglutarate transaminase				*Lactiplantibacillus plantarum*, *Kluyvera ascorbata*, *Tatumella ptyseos*, *Rouxiella badensis*, *Pantoea rodasii*, *Pseudomonas* sp.
1.1.1.23 Histidinol dehydrogenase	Histidine metabolism	DL-Histidine*		*Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*
3.4.13.9 Xaa-Pro dipeptidase	Amino acids metabolism	Proline*		*Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp.
3.4.13 Dipeptidases	Amino acids metabolism	Gly–Leu* Leu–Val* Leu–Leu* L-γ-Glutamyl-L-valine*		*Tatumella* sp. JGM118, *Leuconostoc pseudomesenteroides*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp., *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*
1.1.1.289 Sorbose reductase	Fructose and mannose metabolism	Hexitol*		*Hanseniaspora uvarum*
1.1.1.14 L-iditol 2-dehydrogenase				*Lactiplantibacillus plantarum*, *Lactiplantibacillus paraplantarum*, *Pseudomonas* sp.
1.1.1.27 L-lactate dehydrogenase	Glycolysis	Lactate*		*Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*
1.1.1.40 Malate dehydrogenase	Pyruvate metabolism	DL-Malic acid*		*Tatumella* sp. JGM118, *Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp.
2.5.1.18 Glutathione transferase	Glutathione metabolism	Glutathione**	45	*Tatumella* sp. JGM118, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp., *Pseudomonas* sp. *Pantoea* sp., *Pseudomonas* sp.
3.4.11.2 Membrane alanyl aminopeptidase	Glutathione metabolism	Glycine**	46	*Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*
2.3.1.9 Acetyl-CoA *C*-acetyltransferase	Citrate cycle (TCA cycle)	Acetyl-CoA**	47	*Hanseniaspora uvarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Pantoea rodasii*, *Pseudomonas* sp.

Table 2 (*Contd.*)

EC number enzymes	Pathways	Metabolites	Reference	Predicted producers
1.1.1.37 Malate dehydrogenase	Citrate cycle (TCA cycle)	Oxaloacetate**	47	*Tatumella* sp. JGM118, *L. pseudomesenteroides*, *Hanseniaspora uvarum*, *L. plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp.
1.1.1 Alcohol dehydrogenases	Glycolysis	Ethanol**, acetaldehyde**, aldehyde**, ketone**	48,49	*Tatumella* sp. JGM118, *Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp.
	Pyruvate metabolism	Ethanol**, acetaldehyde**	48,49	
3.5.1.4 Amidase	Phenylalanine metabolism	Phenylacetate**	50	*Tatumella* sp. JGM118, *Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Pseudomonas* sp.
	Aminobenzoate degradation	Benzoate**	51	
	Styrene degradation	Acrylate**	51	
2.2.1.6 Acetolactate synthase	Butanoate metabolism	Acetolactate**	52	*Tatumella* sp. JGM118, *Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp.,
1.1.1.47 Glucose 1-dehydrogenase	Pentose phosphate pathway	D-glucono-1,5-lactone**	20	*Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp., *Pantoea* sp., *Pseudomonas* sp.
1.1.1.6 Glycerol dehydrogenase	Glycerolipid metabolism	Glycerol**	13	*Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Rouxiella badensis*, *Pantoea rodasii*
3.1.4.46 Glycerophosphodiester phosphodiesterase	Glycerophospholipid metabolism	Glycerol 3-phosphate**	13	*Leuconostoc pseudomesenteroides*, *Hanseniaspora uvarum*, *Lactiplantibacillus plantarum*, *Lactococcus lactis*, *Kluyvera ascorbata*, *Frateuria aurantia*, *Leuconostoc citreum*, *Tatumella ptyseos*, *Tatumella* sp. JGM130, *Lactiplantibacillus paraplantarum*, *Rouxiella badensis*, *Pantoea rodasii*, *Serratia* sp.

[a] The microbial enzymes were detected from their open reading frames. Pathways were identified from the KEGG database. *Metabolites related to aroma development were detected by LC-MS/MS and matched to the enzymes based on the KEGG database. **Metabolites related to aroma development based on references and not detected by LC-MS/MS, and matched to the enzymes based on the KEGG database. Microbial producers were predicted based on the NCBI database.

precursors, of aldehydes. *Lactiplantibacillus plantarum* has tyrosine kinases.[44] Moreover, *L. lactis* subsp. *lactis* delivers arginine dehydrolase, which releases ammonia from arginine. *Hanseniaspora uvarum* enhances the abundance of D-lysine, glutamine, isoleucyl, glycyl, prolyl, and alanyl dipeptides. However, they decrease L-tyrosine, L-phenylalanine, Leucyl, and Valyl di- and tripeptides. *H. uvarum* consumes intermediates of glycolysis and the Krebs cycle.[41]

Lactate dehydrogenase is possessed by *Lactococcus* and *Lactiplantibacillus plantarum*.[44] This enzyme converts L-lactate from pyruvate. *L. lactis* subsp.*lactis biovar. Lactobacillus diacetylactis* can produce diacetyl from citrate, which brings a buttery aroma.[53] *Hanseniaspora uvarum* possesses alcohol dehydrogenase.[41,54]

The aforementioned microorganisms were present during SIAF of whole and pulped coffee cherries in relatively high abundance (Fig. 2). The *Lactococcus* group in this study was predicted to produce transaminase, amino acid hydrolase, and dipeptidase. *Lactiplantibacillus plantarum* was found to have a transaminase that produces tyrosine. *Hanseniaspora uvarum* was predicted to have ORFs that produce glutamate synthase, glutaminase, and dipeptidases. Lactate dehydrogenase was indeed predicted to be produced by *Lactococcus lactis*, *Lactiplantibacillus plantarum,* and other microbes. Alcohol dehydrogenase was also predicted to be possessed by *Hanseniaspora uvarum* and other microorganisms. Therefore, LABs and yeasts deliver a significant contribution to the coffee aroma development (Table 2).

Enzymes responsible for producing several metabolites found in fermented green beans, such as caffeine, chlorogenic acid, and citric acid, were not detected. This explains that several metabolites in the coffee were naturally occurring or produced by plant enzymes. As chlorogenic acid was present in the SIAF green beans but not before the fermentation, it was assumed that chlorogenic acid from coffee pulp was not yet translocated to the beans, and the complex matrix was not yet degraded by microbes, so CGA could not be released. Nevertheless, enzymes which generate precursors of citric acid, Acetyl-CoA *C*-acetyltransferase for Acetyl-CoA and malate dehydrogenase for oxaloacetate, were identified.

Metabolites from the detected enzymes that were not available in the result of non-volatiles analysis could be volatiles, intermediates that had been further transformed during the fermentation process, or had not been translocated from mucilage into the green bean. Ethanol, acetaldehyde, aldehyde, and ketone from alcohol dehydrogenases are volatiles. Therefore, volatile analysis is needed for further investigation.

Glutathione could be further decomposed into glycine by the detected enzyme, aminopeptidase. Other metabolites that were not found in the fermented green beans might be transferred during coffee drying. Thus, metabolite analysis of dried green beans is suggested to gain a more comprehensive understanding.

Conclusions

Geothermal coffee processing, employing SIAF, can be an alternative method that addresses the limitations of conventional coffee processing. This study provided information on the role of microorganisms in the SIAF that affect the aroma development of final coffee beverages. The dominant species in the coffee SIAF samples was found to be *Tatumella* sp. JGM118, *Hanseniaspora uvarum*, and *Leuconostoc pseudomesenteroides*. The concentration of non-volatile metabolites such as chlorogenic acid, amino acids, and carboxylic acids, which were not detected in the green bean before SIAF, increased 7- to 10-fold after SIAF. These metabolites are aroma precursors of coffee drinks. The ORF of microbial enzymes that contribute to the coffee aroma development were identified in the samples, such as glycoside hydrolases (GH), transaminases, and L-lactate dehydrogenase. Therefore, the role of microbial community in SIAF as the producer of geothermal coffee aroma precursors was confirmed. Further, it is recommended to detect volatiles in the self-induced anaerobic fermented green bean samples. Metabolite analyses and sensory evaluation by Q-graders on the geothermal dried green beans are also suggested to get more coherent information.

Author contributions

Zikrina Istighfarah, C. Hanny Wijaya, Lilis Nuraida, Erliza Noor, and Wisnu Ananta Kusuma designed the experimentation. Zikrina Istighfarah performed the experiments and wrote the original manuscript. C. Hanny Wijaya, Lilis Nuraida, Erliza Noor, and Wisnu Ananta Kusuma validated and reviewed the final manuscript.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting the findings of this study have been included in this article and as part of the supplementary information (SI). No restrictions apply to the availability of these data. Supplementary information is available. See DOI: https://doi.org/10.1039/d5fb00673b.

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