Doctoral Dissertation

Metabolomics analysis of cocoa bean (*Theobroma cacao*) fermentation to reveal conditions related to GABA (gamma-aminobutyric acid) levels

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List of Abbreviations

(*In alphabetical order*)

ANOVA	Analysis of variance
B-Lac	B-Lactose
GABA	Gamma-aminobutyric acid / 4-aminobutyric acid
Gal	Galactose
GAD enzyme	Glutamic acid decarboxylase enzyme
GC-MS	Gas chromatography–mass spectrometry
GDL	Glucono-1,5-lactone / Glucono delta-lactone
Glc	Glucose
Hi	High environmental temperature in tropical climate (≥ 30 °C)
ICCRI	Indonesian Coffee and Cocoa Research Institute
J	Farm locate in Jember, East java, Indonesia
J-Hi	Jember beans fermented in high environmental temperature
J-Lo	Jember beans fermented in low environmental temperature
M	Farm locate in Malang, East java, Indonesia
Man	Mannose
M-Hi	Malang beans fermented in high environmental temperature
M-Lo	Malang beans fermented in low environmental temperature
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
Lo	Low environmental temperature in tropical climate (± 18 °C)
Lyx	Lyxose

OPLS-R	Orthogonal Projection to Latent Structure-Regression
PCA	Principal Component Analysis
Psi	Psicose
RATA	Rate-All-That-Apply
Raff	Raffinose
Ribf	Ribofuranose
RMSEE	Root-Mean-Square-Error of Estimation
RMSECv	Root-Mean-Square-Error of Cross-validation
Sor	Sorbose
Suc	Sucrose
SOC	Single-origin chocolate
Tag	Tagatose
TCA cycle	Tricarboxylic acid cycle
Treh	Trehalose
VIP	Variable Importance in Projection

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Chapter 1

General introduction

1.1 GABA food

GABA (Gamma-aminobutyric acid) is a non-protein amino acid that naturally present in food (Diana *et al.*, 2014). It is an inhibitory neurotransmitter that plays a role in regulating mood, anxiety, and sleep (Sahab *et al.*, 2020). Some people believe that consuming foods with high GABA content can help to improve mood, reduce anxiety, and promote sleep (Nakamura *et al.*, 2009, Sahab *et al.*, 2020). Studies have shown that GABA can be beneficial as anti-insomnia, anti-depression, anti-diabetes, anti-inflammatory, and anti-hypertension (Ramos-Ruiz *et al.*, 2018). Therefore, high GABA food are more preferred and sought after (Diana *et al.*, 2014, Ramos-Ruiz *et al.*, 2018, Hou *et al.*, 2023).

Most GABA food sources are derived from plants (Kobayashi *et al.*, 2021, Hou *et al.*, 2023). The GABA content in food varies depending on the type of food, ranging from 0.2 to 404.9 mg of GABA per 100 g. The lowest reported GABA content was found in Pu'er tea with 0.0 – 4.61 mg/100 g (Hou *et al.*, 2023). The highest GABA content was found in vegetables, with tomatoes and spinach having GABA contents of 219.9 – 404.9 mg/100 g and 232.10 – 381.00 mg/100 g, respectively (Hou *et al.*, 2023). The total GABA content available can vary depending on the cooking method used (Ito *et al.*, 2019, Ma *et al.*, 2022). For example, cabbage cooked in two different methods, microwave and boiled, showed GABA losses of 82% and 59%, respectively. Proper cooking methods can also

increase the available GABA content. For example, roasting sweet potato is reported to increase the GABA content by 3 times (Ito *et al.*, 2019).

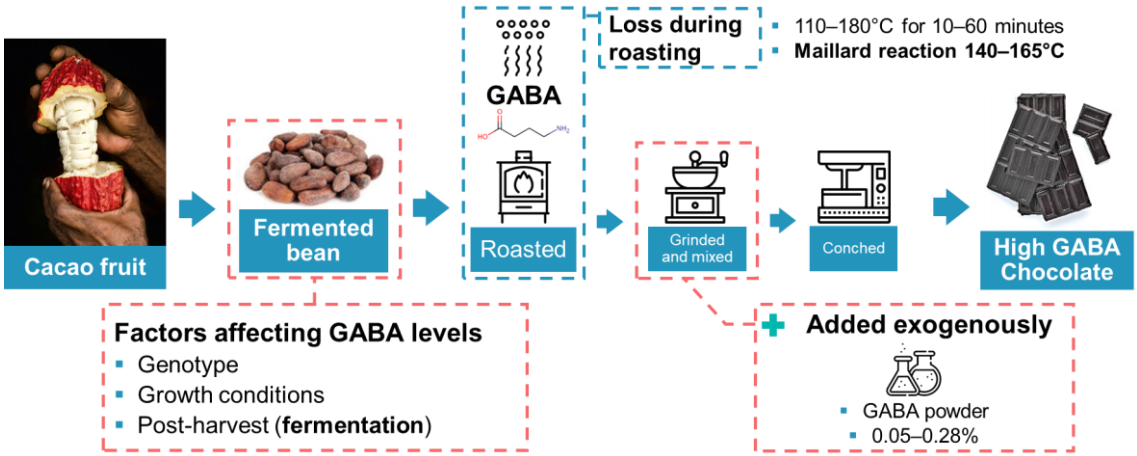

Figure 1. The chocolate-making process starts with cacao fruit until high GABA chocolate is produced.

1.2 Cocoa beans and chocolate as GABA source

Fermented cocoa beans and chocolates are both sources of GABA. Fermented cocoa beans contain 31.7 – 101.2 mg of GABA per 100 g, while chocolates contain 11.1 – 32.5 mg of GABA per 100 g (Hou *et al.*, 2023). Different GABA levels in beans are affected by genotype, growth conditions, and the post-harvest method, which is fermentation itself (Kongor *et al.*, 2016, Ramos-Ruiz *et al.*, 2018). During the chocolate-making process, some of the GABA is lost. This loss is due to the Maillard reaction, a reaction that occurs during heating (140 – 165 °C) between reducing sugars and amino acids and that gives chocolate its flavor, aroma, and brown color (Rojas *et al.*, 2022). Therefore, chocolates with high GABA content are typically enriched products that have had GABA added exogenously during production. Enriched GABA chocolates are made by adding 0.05 – 0.28% GABA powder during the grinding and mixing process after roasting (Fig.1) (Nakamura *et al.*, 2009, Koh *et al.*, 2023). The conching process

temperature and time are 65 – 75 °C for 10 – 96 hours (Afoakwa, 2016, Rojas *et al.*, 2022), so it doesn't produce Maillard reaction (Fig. 1).

1.2.1 The effect of fermentation on GABA levels

Fermentation is a natural process used to enhance GABA levels in food, which is favored by consumers (Dhakal *et al.*, 2012, Hou *et al.*, 2023). The underlying principle of microbial fermentation is that certain microorganisms, such as lactic acid bacteria (LAB) and yeast, possess high levels of GAD (glutamic acid decarboxylase) activity (Cui *et al.*, 2020, Sahab *et al.*, 2020). These microorganisms can catalyze the conversion of glutamate into GABA during the branched metabolism of GABA. A specific strain of LAB has been documented to increase GABA levels by up to 10 times under optimal conditions (Santos-Espinosa *et al.*, 2020). Additionally, GABA-producing yeasts not only elevate GABA levels in cheese but also contribute to enhancing its aroma (Li *et al.*, 2022).

Fermentation is a crucial step in the chocolate industry. It is a process in which the cocoa beans are allowed to ferment for 4 – 6 days which happens spontaneously when the fruit opened (Saltini *et al.*, 2013, Ackah and Dompey, 2021). During fermentation, the sugar and protein in the cocoa beans are broken down by microorganisms, such as yeasts and bacteria (Aprotosoaie *et al.*, 2016, Kongor *et al.*, 2016, De Vuyst and Leroy, 2020). This process produces a variety of compounds, including reducing sugars, amino acids, and volatile compounds (Schwan and Wheals, 2004). These compounds give chocolate its characteristic flavor and aroma (Afoakwa *et al.*, 2008, Rojas *et al.*, 2022). The exact mechanism by which fermentation affects GABA levels is not fully understood.

However, it is thought that the microorganisms that break down the sugar and protein in the cocoa beans may also produce enzymes that break down GABA.

1.2.2 The influence of environmental temperature on fermentation

The fermentation of cocoa beans is affected by three factors: environmental temperature, time, and type. Environmental temperature is reported to affect the fermentation temperature, which can in turn affect the fermentation process(Camu *et al.*, 2008, Calvo *et al.*, 2021). The fermentation process takes about 5 – 7 days(Saltini *et al.*, 2013, Kongor *et al.*, 2016, Rojas *et al.*, 2022). If the beans are under fermented, they will have an astringent taste. If the beans are over fermented, they will have an off-flavor note (Aprotosoaie *et al.*, 2016).

There are two main types of fermentation methods for cocoa beans: heap and wooden box (Camu *et al.*, 2007, Racine *et al.*, 2019). The heap method is the traditional method of fermentation. The beans are piled in a heap and covered with banana leaves or other materials (Racine *et al.*, 2019). This method can increase the possibility of fungal contamination (Guehi *et al.*, 2010, Afoakwa, 2016). The wooden box method is a more modern method of fermentation. The beans are placed in a wooden box and covered with a lid (Racine *et al.*, 2019). This method is more controlled and less contaminated with better fermentation result (Guehi *et al.*, 2010, Afoakwa, 2016).

Fermentation in low environmental temperature is a natural method to enrich GABA levels (Yu *et al.*, 2023). Low environmental temperature treatment during *takuan-zuke* production can increase GABA levels by up to 18.6-fold (Kobayashi *et al.*, 2021).

However, the effect of low environmental temperature on GABA levels in cocoa beans during fermentation is still unknown.

1.2.3 The effect of chocolate making process on GABA levels

A report has shown that the interaction of GABA with sugar affects its stability and availability (Lamberts *et al.*, 2008). When GABA is dissolved in water and heated to 110 degrees Celsius, it is stable and no GABA is lost. However, when sugar is added to the GABA solution, GABA is lost. GABA reacts with sugars to form Maillard products, which results in a 10% loss of GABA after heating for 4 hours at 110 °C. A similar trend has also been observed in soy milk that has been sweetened with sugar (Le *et al.*, 2020).

It is known that the origin of the beans and the fermentation process can affect the metabolite profile (Kongor *et al.*, 2016). Reports that have focused on the GABA content of fermented beans from different origins have shown that the GABA content varies (Marseglia *et al.*, 2014). The chocolate-making process further reduces the GABA content due to GABA loss during the Maillard reaction (Koh *et al.*, 2023). Therefore, fermented beans from different origins could also have different rates of GABA loss. However, it is still unknown whether the rate of GABA loss will be different if different fermented beans are used to make chocolate.

1.3 Metabolomics approach

Metabolomics, a comprehensive study of metabolites, which are small molecules produced by living organisms, is considered the omics study that is most closely related to the phenotype (Putri and Fukusaki, 2014). Metabolomics has been widely applied for

evaluating the metabolite profile of food, such as quality control, food fraud authentication, and food origin discrimination (Cambrai *et al.*, 2017, Fukusaki *et al.*, 2018). Metabolomics studies can also be used to investigate the effect of different environmental factors on naturally produced metabolites (Karimi *et al.*, 2020). These wide ranges of applications made metabolomics a valuable tool for researching the metabolite profile of cocoa beans (Herrera-Rocha *et al.*, 2023). It has been employed to identify flavor-related metabolite changes during fermentation (Fang *et al.*, 2020, Febrianto and Zhu, 2020), analyze the chemical composition of fermented cocoa beans from various origins (Febrianto and Zhu, 2022), and the changes of the volatile compound during the roasting process (Wiedemer *et al.*, 2023).

Gas chromatography-mass spectrometry (GC-MS)-based analyses are commonly utilized in food metabolomics due to their robustness (Putri *et al.*, 2019). GC-MS-based metabolomics is particularly well-suited for identifying small molecular metabolites such as acids, alcohols, catecholamines, sugars, and amino acids (Fiehn, 2016). This capability is especially valuable for detecting sugars and amino acids, which are crucial metabolites involved in generating the chocolate flavor in cocoa beans (Aprotosoaie *et al.*, 2016, Kongor *et al.*, 2016, Fang *et al.*, 2020). Previous reports have demonstrated the successful application of GC-MS-based analysis in studying the effect of production conditions on the metabolite profile of *tempeh* (Kadar *et al.*, 2020). Therefore, employing GC-MS-based metabolomics would be advantageous in elucidating the conditions during cocoa bean fermentation that are related to GABA levels.

1.4 Objective and strategy

Therefore, the objective of this research is to uncover the condition of cocoa bean fermentation related to GABA levels based on the metabolomics approach. To achieve this objective, three strategies were performed:

1. Study the effect of low environmental temperature on GABA levels in cocoa beans
2. Determine the optimal fermentation conditions that increase GABA levels
3. Determine the influence of sugar on fermented cocoa beans to GABA levels during the chocolate-making process

1.5 Thesis outline

This thesis is structured into five chapters. Chapter 1 provides an overview of cocoa beans and chocolate as sources of GABA, highlighting the factors that influence their GABA levels. It also outlines the objectives of this study.

Chapter 2 explores the application of the metabolomics approach to investigate the relationship between environmental temperature and GABA levels in cocoa beans during fermentation.

Chapter 3 focuses on investigating the optimal environmental conditions that promote increased GABA levels during cocoa bean fermentation.

Chapter 4 examines the GABA levels of fermented cocoa beans before and after the chocolate-making process, aiming to identify conditions that minimize GABA loss.

Finally, Chapter 5 presents the conclusions drawn from this study and proposes future perspectives based on the findings.

Chapter 2

GABA levels in cocoa beans are correlated with low environmental temperature

2.1 Introduction

Raw and fermented cocoa beans from different environmental temperatures are needed as samples to study the effect of low environmental temperatures on GABA levels in cocoa beans. Indonesia was selected as the origin country for providing the samples. Indonesia is a cocoa producer country that has a well-established cultivation and fermentation system (Anita-Sari *et al.*, 2018). The country's wide range of climates, as classified by the Köppen climate classification (Beck *et al.*, 2018), makes it an ideal location for this study. The results of this study will provide valuable information about the relationship between environmental temperature and GABA levels in cocoa beans during fermentation.

2.2 Materials and Method

2.2.1 Sample materials

Samples were collected the foster farms of the Indonesian Coffee and Cocoa Research Institute (ICCRI, Jember, Indonesia) from six cities across six provinces of Indonesia, as indicated in Table 1. All samples (*Trinitario* variety) underwent the same cultivation and fermentation process. The cocoa pods were opened, and the beans were extracted and divided into two batches. The first batch was sun-dried until it reached a moisture content of 7 – 7.5% and then stored at -30 °C for later analysis. The second batch

underwent a fermentation period of 96 hours, with the beans being turned after the first two days. After the fermentation process, the beans were sun-dried to a moisture content of 7 – 7.5% and stored at -30 °C. Detailed information regarding GPS (global positioning system) coordinates and environmental temperature was available for each origin location (Table S1). Environmental temperature data was sourced from the Meteorology, Climatology, and Geophysical Agency of Indonesia (http://dataonline.bmkg.go.id/home) and was based on average data from 2017 to 2019.

Table 1. Places of origin of Indonesian raw and fermented cocoa beans

Code	Place of origin		Altitude*	Temperature (ºC)		
	City	Province		Max	Min	Ave
4	Madiun	East Java	611	24.6	24	24
7	Gunung Kidul	Yogyakarta	171	31.8	23	26
12	Enrekang	South Sulawesi	595	29.2	18	23
14	Central Maluku	Maluku	13	30.2	24	27
15	Jembrana	Bali	27	30.5	23	26
18	North Lombok	West Nusa Tenggara	336	31.9	24	27

Max = maximum; Min = minimum; Ave = average; Environmental temperature data were collected from the Meteorology, Climatology, and Geophysical Agency of Indonesia. *Altitude in meter above sea level.

2.2.2 Extraction and derivatization of metabolites

The extraction method used in this study followed a previously described procedure but with some modifications and optimization (Putri *et al.*, 2019). Three cocoa beans from different locations were combined and homogenized to extract the metabolites.

In a 50 mL tube, cocoa beans and a metal cone were added and the tube was tightly sealed before being cooled with liquid nitrogen. The cocoa beans were ground twice using a Multi-beads shocker (Yasui Kikai, Osaka, Japan) at 2,000 rpm for 40 seconds. A 2 mL tube was then used to hold 5 milligrams of the homogenized sample powder. All samples were analyzed in triplicate (n = 3). To extract the metabolites, a mixed solvent consisting of methanol, chloroform, and ultrapure water in a ratio of 2.5:1:1 (v/v/v) was added to the 2 mL tubes containing the sample powder. An internal standard (ribitol) was also included. Blank samples containing only the mixed solvent were prepared as well. After incubation for 30 minutes at 37 °C and 1,200 rpm, followed by centrifugation at 4 °C and 10,000 × g for 3 minutes, the supernatant (600 μL) was transferred to a new 1.5 mL tube. Ultrapure water (400 μL) was added to the tube, which was then vortexed and centrifuged. From the resulting aqueous phase, 200 μL was transferred to a new 1.5 mL tube and covered with a holed cap. For pooled quality control (QC) samples, another 200 μL of the aqueous phase from each sample was mixed and transferred to separate tubes with holed caps. The samples were evaporated, lyophilized overnight, and then subjected to derivatization. To perform derivatization, 100 μL of methoxyamine hydrochloride (20 mg/mL in pyridine) was added to the lyophilized samples. The sample mixture was incubated at 30 °C and 1,200 rpm for 90 minutes in a thermomixer. Subsequently, 50 μL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was added, and the resulting solution was incubated at 37 °C and 1,200 rpm for 30 minutes. All samples from the first and second sets were extracted, derivatized, and analyzed simultaneously.

2.2.3 GC-MS-based metabolite profiling

After the derivatization process, GC-MS analysis of all samples was conducted using a GCMS-QP2010 Ultra instrument (Shimadzu, Kyoto, Japan) equipped with a 0.25 μm InertCap 5 MS/NP column (GL Sciences, Tokyo, Japan). Prior to the analysis, the mass spectrometer was tuned and calibrated. For each sample, one microliter of the derivatized sample was injected in split mode (25:1 v/v) at an injection temperature of 270 °C, in a random order. The carrier gas used was helium, flowing at a linear velocity of 39 cm/s and a flow rate of 1.12 mL/min. The column temperature was initially set at 80 °C for 2 minutes, then increased by 15 °C/min until reaching 330 °C, where it was held for 6 minutes. The transfer line temperature was maintained at 310 °C, while the ion source temperature was set at 280 °C. Electron ionization (EI) was employed to generate ions at 0.94 kV. Mass spectra were recorded at a rate of 10,000 μ/s within a mass range of *m/z* 85 – 500. Prior to the first sample injection, a standard alkane mixture (C8 – C40) was injected. Additionally, co-injection with authentic standards, including isocitric acid + citric acid, saccharic acid, malic acid, ethanolamine, (GABA), succinic acid, and glycerol (obtained from Sigma-Aldrich, St. Louis, MO, USA; Tokyo Chemical Industry, Tokyo, Japan; and Wako Chemical, Osaka, Japan), was performed to verify the presence of important metabolites.

2.2.4 Data processing

The initial raw data obtained were converted to the AIA file format using the GC-MS solution software packages provided by Shimadzu (Kyoto, Japan). To process the data, peak alignment, peak filtering, and tentative annotation were carried out using MS-

DIAL ver. 4.92 (Riken, Kanagawa, Japan). The annotation was based on the spectral database GL-Science DB (InertCap 5MS-NP, predicted Fiehn RI), which was downloaded from the official website of MS-DIAL. To confirm the tentative annotations, a comparison was made with the data in the NIST library (NIST/EPA/NIH EI-MS Library) included in the GC-MS solution software packages. In both the MS-DIAL and GC-MS solution software packages, a minimum similarity value of 80% was used for the annotations. The relative intensity of each annotated metabolite was normalized using an internal standard (ribitol). Metabolites with a relative standard deviation (RSD) of less than 30% within the quality control (QC) samples were selected for further analysis.

2.2.5 Multivariate data analysis

The processed data underwent principal component analysis (PCA) to generate the metabolite profiles of Indonesian cocoa beans. Subsequently, orthogonal projection to latent structure regression (OPLS-R) was employed to identify the metabolites that exhibited correlations with the environmental temperature. OPLS-R was selected due to its ability to incorporate orthogonal variables into correlated information, reducing multiple factors to a limited number of latent variables (Bylesjö *et al.*, 2007). The software used for conducting PCA and OPLS-R models was SIMCA-P+ version 13.0.1 (Umetrics, Umea, Sweden), with auto-scaling and no transformation applied. In the models, the normalized relative intensity of the annotated metabolites served as the explanatory variable (x-variable) for PCA and OPLS-R, while the environmental temperature was considered the response variable (y-variable) solely for the OPLS-R model. Based on the OPLS-R model, variable importance in projection (VIP) scores were computed for each metabolite, which played a crucial role in identifying highly correlated x-variables and

constructing significant models (Mabuchi *et al.*, 2019). Statistical analysis of the annotated metabolites was conducted using analysis of variance (ANOVA) with Tukey's post hoc test, utilizing JASP Version 0.17.1 (JASP Team, Amsterdam, Netherlands). Bar graphs were plotted using R software version 3.6.2 and Rstudio version 1.3.1093, along with the ggplot2 package.

2.3 Result and discussion

2.3.1 Environmental temperature affects metabolite profile of cocoa beans

Indonesia was chosen as the origin of cocoa beans due to its unique geographical characteristics. Cocoa production areas in Indonesia are primarily comprised of smallholder farms, which are widely dispersed across various geographic regions (McMahon *et al.*, 2015). The climate of Indonesia climate can be categorized into four main groups with a total of 10 subgroups, as classified by the Köppen-Geiger climate classification system (Beck *et al.*, 2018). As a result, environmental factors such as rainfall, temperature, and altitude, which are crucial for the survival and well-being of plants (Manalebish Debalike Asfaw *et al.*, 2019), also vary. This variation leads to differences in the availability of water, soil nutrients, air, and sunlight, depending on the specific location where cocoa trees are cultivated.

Metabolomics analysis was conducted to investigate the influence of origin on the metabolite profiles of fermented cocoa beans. Specifically, *Trinitario* fermented cocoa beans (Table 1) were analyzed to minimize the impact of other factors, such as variety. In Indonesia, the predominant cocoa tree variety is *Trinitario*, which is a hybrid resulting from the crossbreeding of Criollo Venezuela and Forastero Venezuela (Anita-Sari *et al.*,

2018). This hybrid variety exhibits disease resistance, possesses fundamental chocolate characteristics, and imparts a wine-like aroma. The fermentation process for all cocoa beans lasted 96 hours, which is considered an optimal duration for fermentation (Saltini *et al.*, 2013).

A total of 71 metabolites were tentatively identified in the raw and fermented bean samples. Principal component analysis (PCA) demonstrated distinct separation between raw and fermented cocoa beans, accounting for 42.9% of the variance (Fig. 2A). Raw cocoa beans exhibited a higher sugar profile, whereas fermented cocoa beans showed an elevated amino acid profile. It is suspected that environmental factors may influence the sugars level by affecting how plants respond to abiotic stress during cocoa bean development (Wang *et al.*, 2016). Consequently, differences in sugar content might exist prior to fermentation (Fig. 2B). During fermentation, sugars undergo hydrolysis into reducing sugars (Megías-Pérez *et al.*, 2018), which explains the decrease in sucrose and raffinose level (Fig. 2B). Notably, after fermentation, sucrose levels were higher in origin 18, indicating a slower sucrose hydrolysis rate compared to other places of origin.

Amino acids play a crucial role as flavor precursors in the production of desired chocolate flavors during the Maillard reaction, in conjunction with oligopeptides and reducing sugars (Schwan and Wheals, 2004). Amino acids are produced through protein degradation during fermentation. The protein content may differ depending on the growth and fermentation location of cocoa beans (Fang *et al.*, 2020), responding to variations in sugars and proteins associated with biotic and/or abiotic stresses. This phenomenon is illustrated by the bar graphs depicting threonine and leucine, which also exhibit variations

based on the place of origin (Fig. 2B). These differences might be attributed to variations in protein hydrolysis rates, influenced by the environmental conditions.

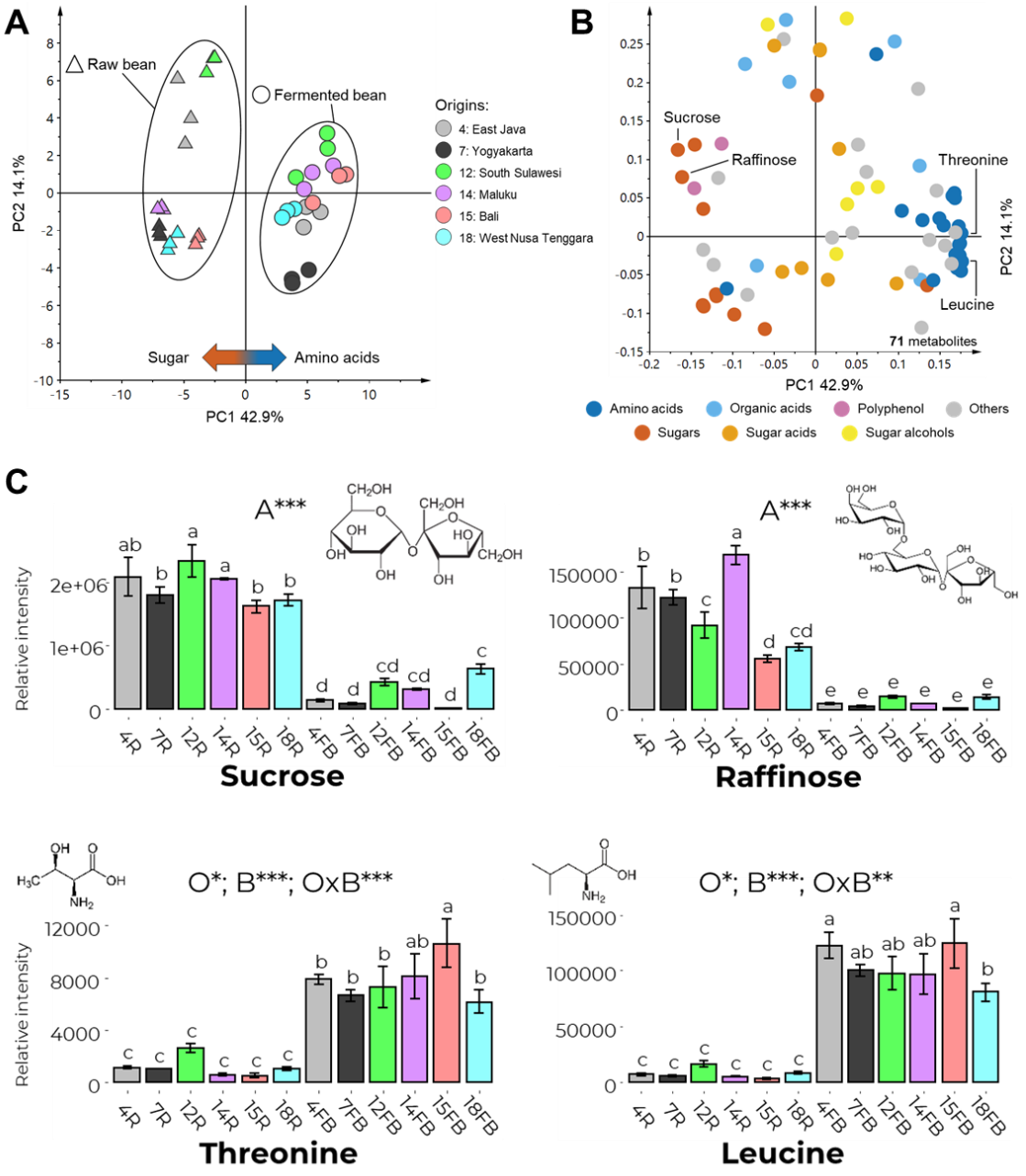

Figure 2. The PCA result of GC-MS analysis of raw and fermented cocoa beans from six different origins. A total of 71 annotated metabolites were used for PCA. Further details on the places of origin of the samples are presented in Table 1. (A) Score plot of the PCA result. (B) Loading plot of the PCA result; a complete

list of annotated metabolites is shown in Table S2. Metabolites mentioned in the loading plot are contributing metabolites to PC1 and PC2. (C) Bar graphs represent the relative intensity of metabolites mentioned in the loading plot. The horizontal axis represents the sample and the vertical axis represents the relative intensity after normalization with internal standard. Bar graphs with different letters indicate a significant difference between samples for a given metabolite. An asterisk in each metabolite bar graph indicates the level of significance (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

2.3.2 Low environmental temperature correlate with GABA levels of cocoa beans during fermentation

In this research, temperature data (maximum, minimum, and average) were collected from six different locations where raw and fermented cocoa beans originated (Table 1). Altitude data are also provided (Table 1), although altitude indirectly affects plants but directly influences the variation in environmental temperature among these six locations. This relationship is explained by the lapse rate, which refers to the rate at which temperature decreases with increasing altitude (Jacobson and Jacobson, 2005). As shown in Table 1, locations 4 and 12 have higher altitudes and lower environmental temperatures compared to the other regions. However, it is worth noting that lower altitudes may also have lower temperatures due to factors such as shade, sunlight exposure, and wind. Therefore, the correlation analysis focused solely on the relationship between the metabolite profile and environmental temperature. The environmental temperature was treated as the response variable or "y" variable in the OPLS-R analysis. The 71 annotated metabolites (listed in Table S2) were used as explanatory variables (x variables) in the OPLS-R models, which were constructed to analyze the maximum temperature, minimum temperature, and average temperature data (Fig. 3).

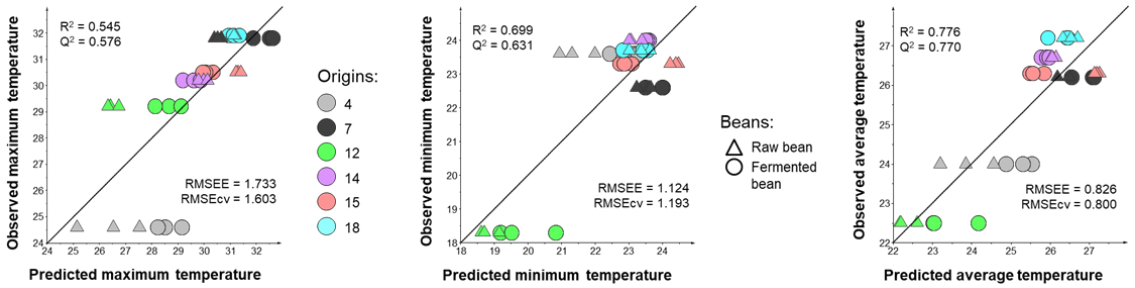

Figure 3. The OPLS-R models of maximum temperature, minimum temperature, and average temperature. The relative intensity of annotated metabolites was employed as the explanatory variable (x-variable), while the environmental temperature (Table 1) was the response variable (y-variable). Numbers are codes that represent the place of origin of the samples (4: Madiun, East Java; 7: Gunung Kidul, Yogyakarta; 12: Enrekang, South Sulawesi; 14: Central Maluku, Maluku; 15: Jembrana, Bali; 18: North Lombok, West Nusa Tenggara). Further details of the locations are presented in Table 1. Triangles indicate raw cocoa bean samples. Circles indicate fermented cocoa bean samples.

The constructed models demonstrated strong quantitative and predictive performance, meeting the criteria of $R^2 \geq 0.65$ and $Q^2 \geq 0.50$ for all models (Mabuchi *et al.*, 2019). R^2 represents the squared correlation coefficient between the explanatory and response variables in a regression, while Q^2 is a reliable measure of model predictability (Alexander *et al.*, 2015). To ensure the validity and accuracy of the models, a CV-ANOVA analysis was conducted at a significance level of 5% ($p < 0.05$). Based on this evaluation, statistically significant models were obtained for the response variables of minimum and average temperatures (Table 2). Additionally, VIP scores were calculated for each metabolite, with a VIP score ≥ 1.0 indicating importance in the model (Mabuchi *et al.*, 2019). The VIP score of each metabolite from the significant OPLS-R models was calculated and is shown in Fig. 4A. The VIP scores reveal four metabolites: GABA, glycerol, ethanolamine, and succinic acid. These four metabolites show a tendency of negative correlation with the minimum and average temperatures, suggesting that lower

minimum and average temperatures may potentially lead to higher levels of these metabolites.

Table 2. Evaluation of the 3 models obtained from OLPS-R analysis.

y Variable	A	N	R^2X(cum)	R^2Y(cum)	Q^2 (cum)	*p*-value
Maximum temperature	1 + 1 + 0	36	0.539	0.545	0.576	1.665E-05
Minimum temperature	1 + 1 + 0	36	0.541	0.699	0.631	2.097E-06
Average temperature	1 + 1 + 0	36	0.554	0.776	0.77	1.687E-09

A = number of models. N = number of samples used in producing the models. Cross-validated predictive residuals (CV-ANOVA) analysis was employed to obtain *p*-values.

Origins 12 exhibited the lowest average temperature and minimum temperatures compared to the other five locations, with values of 22.5 °C and 18.3 °C, respectively (Table 1 and Fig. 3). These conditions influence GABA synthesis in plants, known as the GABA shunt, which has been reported to increase after exposure to low temperatures (Wallace *et al.*, 1984, Carillo, 2018). Temperature has been shown to impact lipid metabolism by hydrolyzing triacylglycerols through lipase activity, resulting in glycerol production (Nakagawa *et al.*, 2020). Additionally, abiotic stress can lead to the release of ethanolamine as a result of phospholipid decomposition in plant cell membranes (Rajaeian and Ehsanpour, 2015). Succinic acid is present in both raw and fermented cocoa beans and serves as a crucial intermediate in the tricarboxylic acid (TCA) cycle through sugar catabolism (De Vuyst and Leroy, 2020). During the development of raw cocoa beans, succinic acid is necessary for the TCA cycle to generate energy and prepare against stressful conditions (Wang *et al.*, 2016). The results indicate that the intensity of succinic acid increased in certain fermented cocoa beans while decreasing in others. This

difference may be attributed to varying rates of the TCA cycle influenced by temperature variations. The bar graph depicting GABA, succinic acid, glycerol, and ethanolamine highlights that origin 12 exhibited high relative intensities in both raw and fermented beans (Fig. 4B). Detailed observation on the bar graph of GABA reveals that the intensity of GABA in cocoa beans of origin 12 increases after the fermentation. Thus, this study suggests that low environmental temperatures affect the GABA shunt, leading to increased levels of GABA, which, in turn, affects the intensities of succinic acid, glycerol, and ethanolamine in cocoa beans (both raw and fermented).

GABA shunt is mainly involved in the plant growth and development stage (Sita and Kumar, 2020). GABA accumulation occurs in response to various stress conditions, including heat, cold, drought, flood, salinity, and biotic stress (pests and diseases) (Ramesh *et al.*, 2017). GABA has been demonstrated to mitigate the harmful effects of reactive oxygen species (ROS), which are generated in large quantities during stressful situations (Ansari *et al.*, 2021). ROS can cause damage to plant cells and tissues, so the scavenging of these species helps protect plants from harm (Li *et al.*, 2021). Among the different stress conditions, cold stress or low temperature conditions are particularly significant in elevating GABA levels (Ansari *et al.*, 2021, Li *et al.*, 2021, Zhang *et al.*, 2022, Hou *et al.*, 2023). Therefore, it is crucial to investigate the impact of low environmental temperature on cocoa bean fermentation.

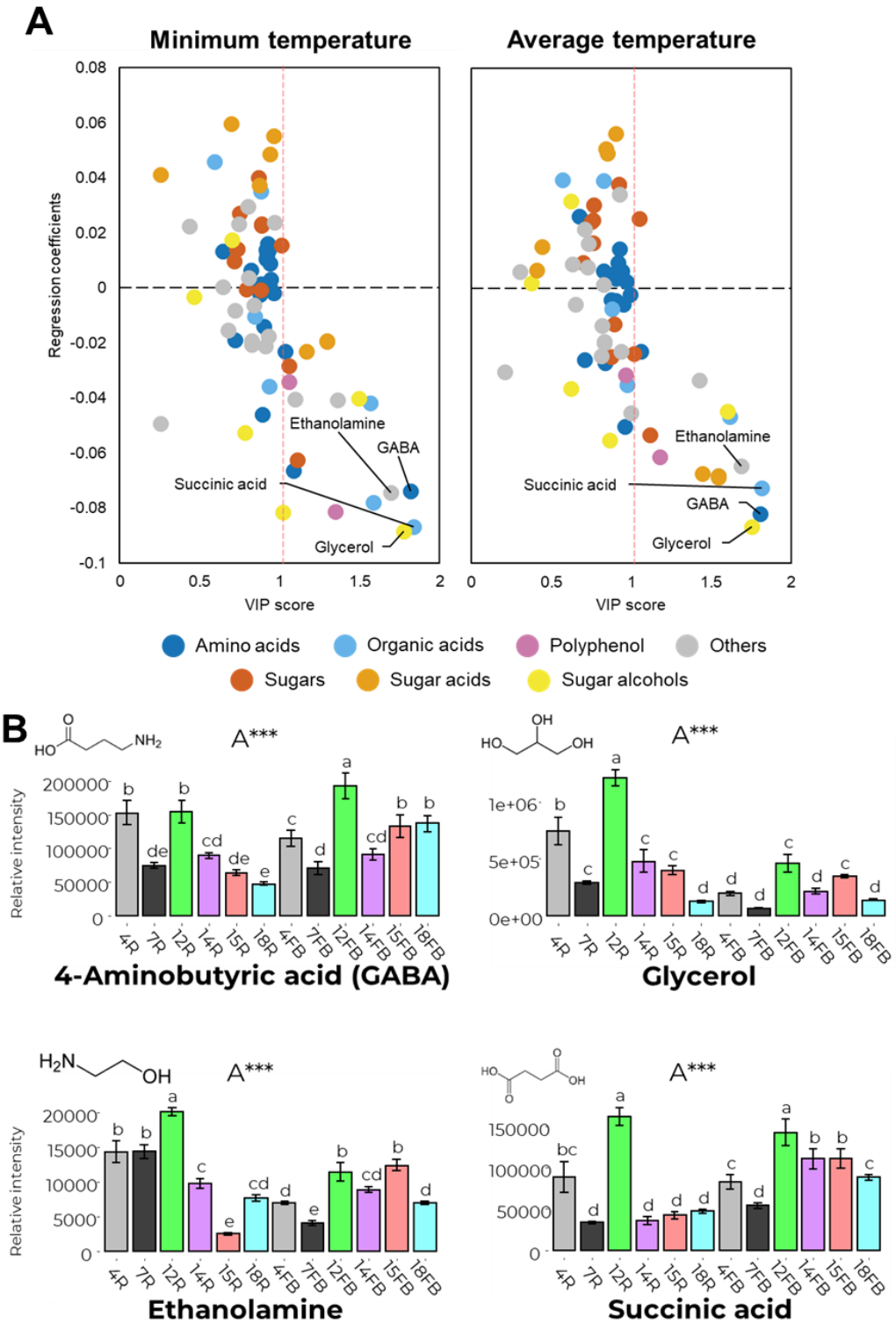

Figure 3. The four highest VIP score metabolites that correlated with the OPLS-R model of minimum and average temperatures. (A) VIP score plot of all 71 annotated metabolites. Metabolites mentioned in the plot

are metabolites with highest VIP score. (B) Bar graphs represent the relative intensity of the four VIP metabolites mentioned Fig. 3A. The horizontal axis represents the sample and the vertical axis the relative intensity after normalization with internal standard. Letters after sample code numbers indicate sample differences (R: raw cocoa beans; FB: fermented cocoa beans). Bar graphs with different letters indicate significant differences between samples for a given metabolite. Source of variation and asterisk in each metabolite bar graph indicate significant differences for a given variation (O: Place of origin; B: Bean (R/FB); OxB: Interaction of O and B; A: All variations) and level of significance (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

2.4 Conclusion

This study observed an increase in GABA intensity after fermentation in origin 12, which experiences the lowest minimum and average temperatures. This suggests a potential relationship between this phenomenon and the GABA shunt in plants, as GABA levels were found to be high both before and after fermentation. Further investigation into the optimal fermentation conditions that promote elevated GABA levels is necessary to ensure the replicability of this finding.

Chapter 3

Optimum fermentation condition to increase GABA levels

3.1 Introduction

The previous chapter suggested the possible correlation between environmental temperature and GABA levels in cocoa beans, demonstrating that fermentation in low environmental temperatures further enhances GABA levels. To ensure the smooth implementation of this finding in the chocolate industry, it is crucial to validate its repeatability. Therefore, this chapter aims to investigate the optimum environmental temperature and time of cocoa beans fermentation that increases GABA levels.

In this chapter, daily observation of fermentation was conducted to monitor changes in GABA levels. The results are expected to uncover the optimal conditions for cocoa bean fermentation that promote increased GABA levels. The samples used in this experiment still come from a *Trinitario* variety, but now only one specific clone is selected. This clone is cultivated in two different farms owned by ICCRI, with distinct environmental temperatures categorized as high and low. An environmental temperature of ≥ 30 °C is considered a high temperature that can cause heat stress in the plant (Ullah *et al.*, 2022). A day with ≥ 30 °C is also considered a hot day (Mikovits *et al.*, 2019). Whereas an environmental temperature of ≥ 18 °C is considered a low temperature in tropical climates based on the Köppen climate classification (Beck *et al.*, 2018). Swapped fermentation was implemented in the two farms to mitigate the influence of uncontrolled variables like soil, light, and rainfall, which can impact GABA levels (Ansari *et al.*, 2021, Li *et al.*, 2021). The fore mentioned factors did indeed affect the GABA levels during

fruit growth (Ramesh *et al.*, 2017, Sita and Kumar, 2020, Ansari *et al.*, 2021). Swapping was done to reduce that effect. For example, beans that grew in a higher environmental temperature were fermented in both high and low environmental temperatures after swapping. The same conditions applied to beans that grew at a lower environmental temperature. Regardless of the initial GABA levels of the raw beans, the analysis focused on the GABA level changes after fermentation, as each bean experienced fermentation in both high and low environmental temperatures. Therefore, it is possible to examine whether the increase in GABA levels remains consistent when the beans are transferred to a different temperature condition before fermentation.

The dynamic nature of cocoa bean fermentation, as revealed in the reports, emphasizes the significance of controlling and monitoring ambient temperatures to ensure the production of high-quality cocoa beans (Calvo *et al.*, 2021). Hence, environmental parameters will be recorded daily throughout the experiment.

3.2 Materials and method

3.2.1 Samples materials

The samples utilized in this chapter consisted of the *Trinitario* variety, specifically the Sulawesi 1 clone. Sulawesi 1 is one of the clones developed by ICCRI, known for its high yield and resistance to pests and diseases. This versatile clone is cultivated across various ICCRI farms throughout Indonesia (ICCRI, 2019).

All samples underwent identical cultivation and fermentation processes. The fermentation took place at two different locations (Table S1). The first location was a

farm situated in Kaliwining, Jember, Indonesia. Jember farm has an altitude of approximately 54 meters above sea level, with maximum, minimum, and average temperatures of 35.2 ºC, 21.8 ºC, and 28.0 ºC, respectively. The second location was in Sumber Asin, Malang, Indonesia, at an altitude of around 600 meters above sea level. The respective maximum, minimum, and average temperatures recorded at Malang were 29.1 ºC, 20.0 ºC, and 24.1 ºC.

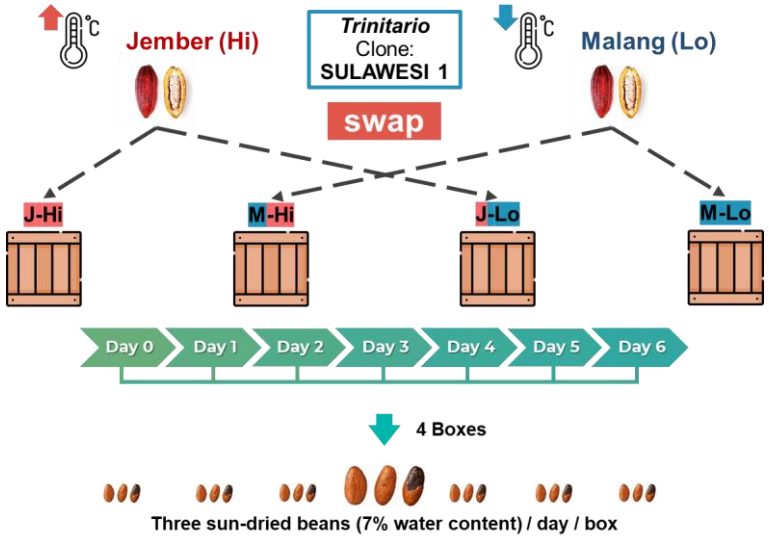

Figure 4. The workflow of swapped fermentation in Jember and Malang. The letters code above the box icon represents the resulting swapping procedure (J = Jember; M = Malang; Hi = high environmental temperature; Lo = low environmental temperature; e.g., J-Hi = Jember beans fermented in high environmental temperature).

The fermentation process took place during the 2022 harvest season. Cocoa fruits were collected, and the beans were fermented on their respective farms. However, some beans were also taken and fermented on another farm, resulting in a swap. As a result, there were four distinct fermentations, as depicted in Fig. 4. A small-scale fermentation method developed by ICCRI was employed for the fermentation process (Tunjung-Sari

et al., 2021). Each fermentation box comprised three biological replicates, with each replicate consisting of 5 kg of beans. To ensure proper identification of the biological replicates, clear separation was maintained between each replication. The fermentation period spanned seven days, starting from day 0 to day 6. Various parameters, including ambient temperature, humidity, fermentation temperature, and fermentation pH, were recorded daily. Cocoa beans were collected each day and subsequently sun-dried until their water content reached 7%. Subsequently, the dried cocoa beans were sent to Osaka University for GC-MS analysis and data analysis.

3.2.2 Extraction and derivatization of metabolites

Three cocoa beans from each day and each fermentation box were combined and homogenized to extract the metabolites in a 50 mL tube with a metal cone using a Multi-beads shocker. The homogenized sample powder (5 mg) was transferred to a 2 mL tube. Triplicate samples (n = 3) were extracted using a mixed solvent of methanol, chloroform, and ultrapure water (2.5:1:1 v/v/v) containing an internal standard. After incubation and centrifugation, the supernatant (600 μL) was collected. From the aqueous phase, 200 μL was transferred to a new tube for analysis. Pooled QC samples were also prepared by combining 200 μL of the aqueous phase from each sample. The samples underwent dry centrifugation for 2,5 hours followed by derivatization using methoxyamine hydrochloride and MSTFA. All samples were processed and analyzed simultaneously.

3.2.3 GC-MS-based metabolite profiling

GC-MS analysis was performed using a GCMS-QP2010 Ultra instrument equipped with a 0.25 μm InertCap 5 MS/NP column. The mass spectrometer was tuned and

calibrated prior to the analysis. One microliter of the derivatized sample was injected in split mode (25:1 v/v) at an injection temperature of 270 °C. Helium was used as the carrier gas, flowing at a linear velocity of 39 cm/s and a flow rate of 1.12 mL/min. The column temperature started at 80 °C for 2 minutes and increased by 15 °C/min until reaching 330 °C, with a hold time of 6 minutes. The transfer line and ion source temperatures were set at 310 °C and 280 °C, respectively. Electron ionization (EI) was used for ion generation, and mass spectra were recorded from *m/z* 85 to 500 at a rate of 10,000 μ/s. Prior to the first sample injection, a standard alkane mixture (C8 – C40) was injected.

3.2.4 Data processing

The raw data obtained were converted to the AIA file format using Shimadzu's GC-MS solution software packages. MS-DIAL ver. 4.92 was then utilized for peak alignment, filtering, and tentative annotation. The annotation process relied on the GL-Science DB spectral database (InertCap 5MS-NP, predicted Fiehn RI) downloaded from MS-DIAL's official website. To confirm the annotations, a comparison was made with the data in the NIST library (NIST/EPA/NIH EI-MS Library) included in the GC-MS solution software packages. Annotations with a minimum similarity value of 80% were considered. The relative intensity of each annotated metabolite was normalized using the internal standard (ribitol). Metabolites with an RSD of less than 30% within the QC samples were selected for further analysis.

3.2.5 Multivariate data analysis

The processed data underwent principal component analysis (PCA) to generate the metabolite profiles of Indonesian cocoa beans. The software used for conducting PCA

was SIMCA-P+ version 13.0.1 (Umetrics, Umea, Sweden), with auto-scaling and no transformation applied. Statistical analysis of the annotated metabolites and recorded environmental parameters were conducted using analysis of variance (ANOVA) with Tukey's post hoc test, utilizing JASP Version 0.17.1 (JASP Team, Amsterdam, Netherlands). Bar graphs and lined graphs were plotted using Microsoft Excel 2016.

3.3 Result and discussion

3.3.1 Correlation among recorded parameters

The recorded parameters from days 0 – 6, including environmental temperature, fermentation temperature, relative humidity, and fermentation pH, were analyzed using Pearson's correlation analysis. The results indicate correlations among most recorded parameters, except for fermentation pH. There is a significant correlation between the maximum, minimum, and average temperatures, showing moderate to very strong correlation coefficients among them (Fig. 5A). Additionally, there is a significant positive correlation between fermentation temperature and these temperature variables, although it is only a fair correlation coefficient. Furthermore, the environmental temperature demonstrates a correlation with humidity, specifically for the maximum and average temperatures, with correlation coefficients of -0.608 and -0.446, respectively.

The temperature differences between Jember and Malang during the 7 days of fermentation were calculated, revealing significant variations in maximum, minimum, and average temperatures. Jember recorded the highest temperatures, with 31.1 °C, 23.1 °C, and 26.5 °C for maximum, minimum, and average temperatures, respectively (Fig. 5B). Interestingly, Malang exhibited a similar average temperature of 22.5 °C

compared to origin 12 (Fig. 5B and Table 1). The lower environmental temperature in Malang (Fig. 5C) positively correlates with the fermentation temperature in Malang, as evidenced by the lower fermentation temperature for both beans from Jember and Malang (depicted by blue lines, blue triangle, and blue circle in Fig. 5D). This observation aligns with previous reports indicating the influence of environmental temperature on fermentation temperature (Camu *et al.*, 2008, Calvo *et al.*, 2021). The data presented indicate that Jember and Malang have distinct environmental temperatures that also impact fermentation temperature. Therefore, further observations to determine the optimum day for increasing GABA levels in low environmental temperatures can be conducted by comparing them with higher environmental temperatures.

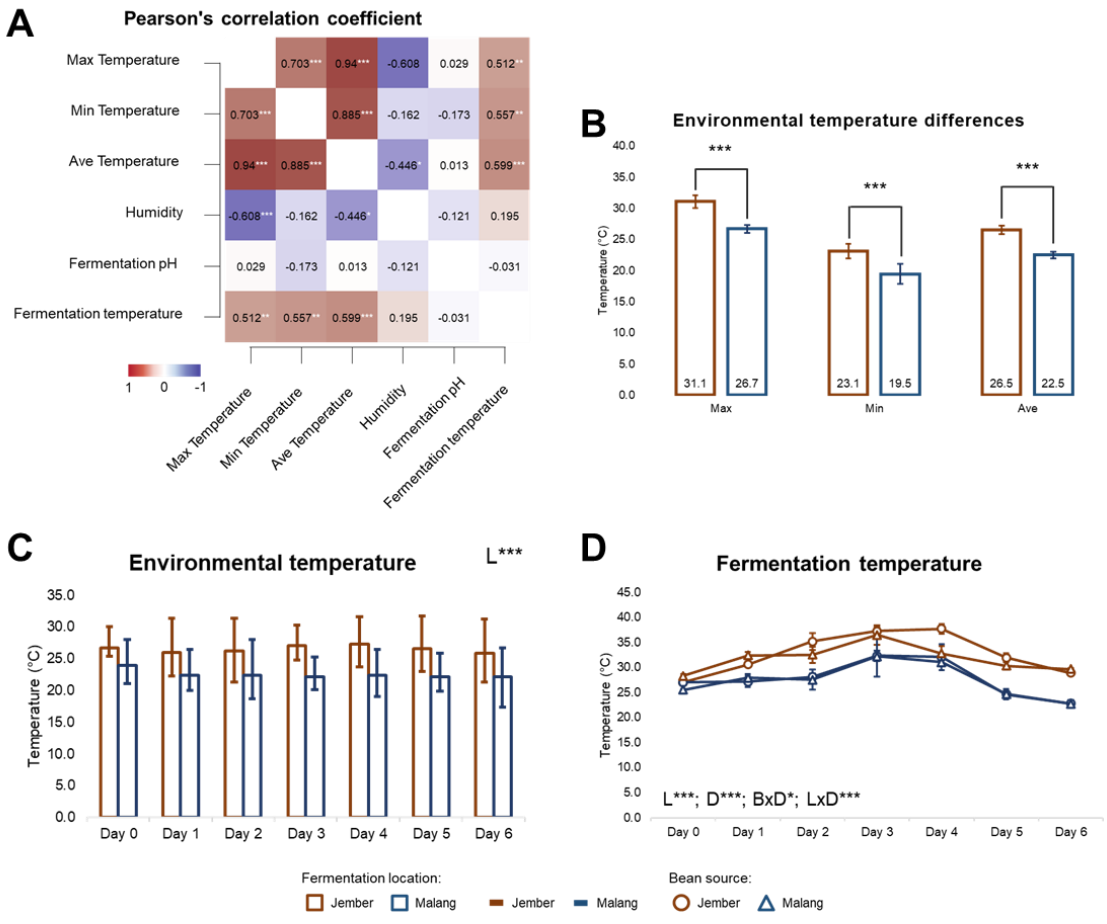

Figure 5. The statistical analysis results of recorded parameters. (A) Heatmap of Pearson's correlation coefficient of recorded parameters during swapped fermentation. Correlation coefficient: 0.1 – 0.2 = poor, 0.3 – 0.5 = fair, 0.6 – 0.7 = moderate, 0.8 – 0.9 = very strong, 1 = perfect (Akoglu, 2018). (B) The environmental temperature difference between two fermentation locations during swapped fermentation. The horizontal axis represents the temperature and the vertical axis the average of maximum temperature, minimum temperature, and average temperature. (C) Environmental temperature changes per day during swapped fermentation. The horizontal axis represents the temperature and the vertical axis the average temperature per day. (D) Fermentation temperature change per day during swapped fermentation. The horizontal axis represents the temperature and the vertical axis the fermentation temperature change per day per fermentation box. Source of variation and asterisk indicate significant differences for a given variation (L = location; D = day; LxD = interaction of L and D; A = all variations) and level of significance (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

3.3.2 Day 5 of fermentation is the optimal day for increasing GABA levels

The low environmental temperature during fermentation indeed increases GABA levels, as demonstrated by the elevated GABA levels in cocoa beans fermented in Malang (Fig. 6A). The highest increase was observed on day 5 of fermentation. Further analysis revealed that the increases on day 5 were significantly different between fermentation in Jember and Malang (Fig. 6B), indicating a significant effect of environmental temperature on GABA levels. Although GABA levels also increased in Jember fermentation, the increase was not statistically significant. Previous reports mention that both heat and cold treatments in beans can increase GABA levels, but heat treatment requires additional relative humidity treatment (Ma *et al.*, 2022, Hou *et al.*, 2023). Compared to that report, this finding further emphasizes that low temperature treatment is much easier compared to heat treatment.

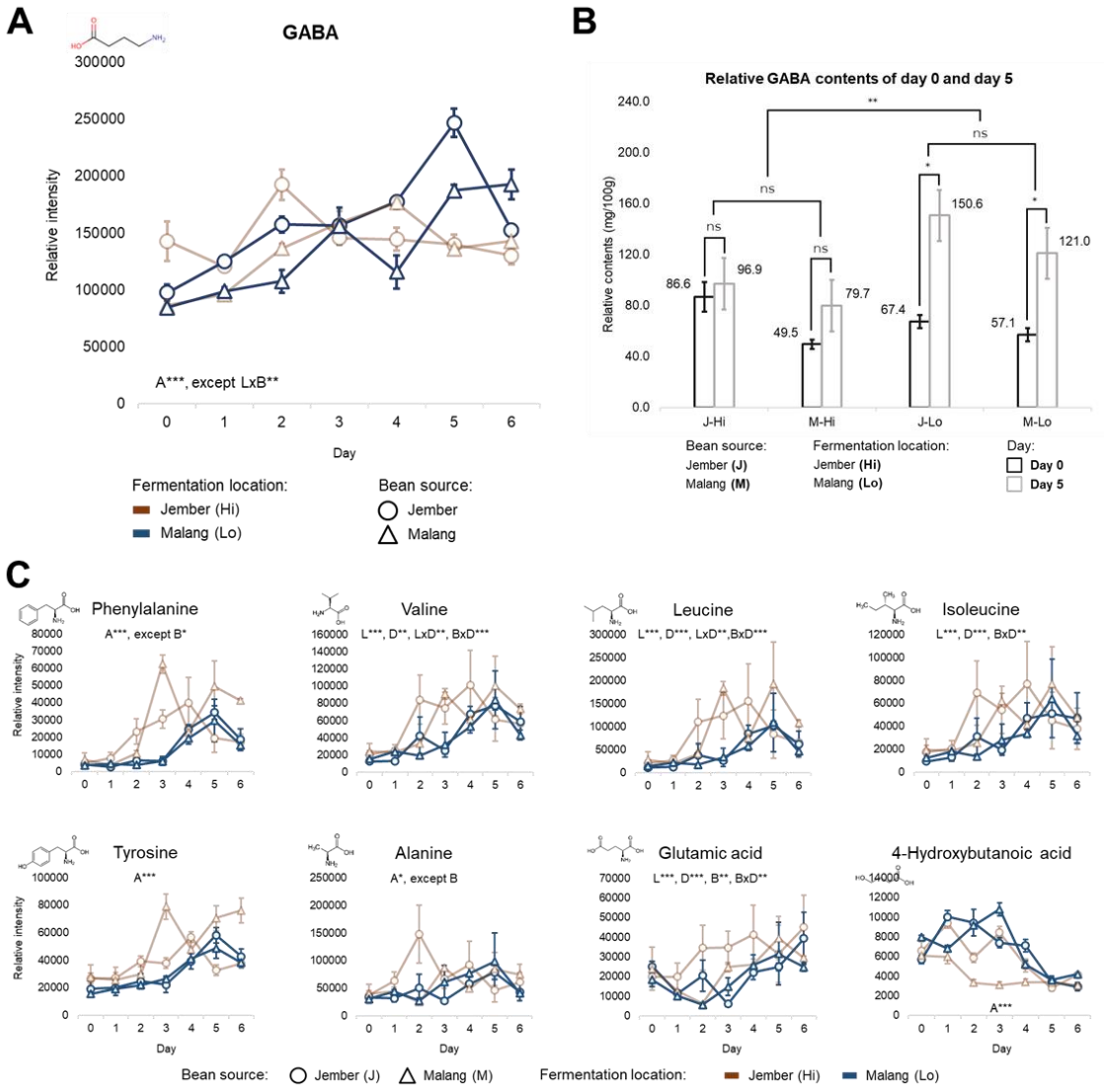

Figure 6. The GABA increase during swap fermentation and other metabolites changes. (A) The change in GABA per day per fermentation box is shown with a line chart. The horizontal axis represents the relative intensity and the vertical axis the day of fermentation. (B) Relative GABA content of GABA on day 0 and day 5. The horizontal axis represents the relative contents (mg/100g) and the vertical axis the fermentation of each box fermentation (Fig 4). (C) Other metabolites changes per day per fermentation box shown with a line chart. The horizontal axis represents the relative intensity and the vertical axis the day of fermentation. Source of variation and asterisk indicate significant differences for a given variation (L = location; B = Bean source; D = day; ?x? = interaction of ? and ?; A = all variations) and level of significance (ns = not

significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

Five days of fermentation in low environmental temperature is suggested to be the optimal day for achieving the highest GABA levels in cocoa beans. This finding is supported by the levels of other amino acids, which were also found to be highest on day 5 (Fig. 6C). However, this finding does not alter the current standard practice of cocoa bean fermentations, which typically lasts for 4 – 5 days (Saltini *et al.*, 2013). The increase in GABA levels is believed to be correlated with higher decarboxylation of glutamic acid under low environmental temperature conditions (Fig. 6C). The data also indicate that the relative intensity of glutamic acid was consistently lower in Malang compared to Jember. A similar pattern has been reported in quinoa seed fermentation (Zhang *et al.*, 2022). Additionally, GHB (4-Hydroxybutanoic acid), a precursor of GABA and associated with the GABA shunt (Fait *et al.*, 2008), was found to increase on days 1 – 3 and then decrease on days 4 – 6 with higher relative intensity in lower environmental temperature (Fig. 6C).

3.3.3 Metabolites differences between fermented cocoa beans Jember and Malang on day 5 fermentation

Further data analysis was conducted to identify the differential metabolites in cocoa beans fermented in Jember and Malang, with a focus on day 5 of fermentation. The volcano plots (Fig. 7A and 7B) of J-Hi vs J-Lo and M-Hi vs M-Lo revealed 6 differential metabolites: trehalose, galacturonic acid, galactaric acid, lactic acid, malic acid, and tartaric acid. Trehalose exhibited higher levels in beans fermented in Jember with high environmental temperatures. On the other hand, galacturonic acid, galactaric acid, lactic acid, malic acid, and tartaric acid were significantly higher in beans fermented in Malang

with low environmental temperatures. Irrespective of the bean source, these metabolites are influenced by both high and low environmental temperatures. Trehalose showed consistently higher relative intensity throughout fermentation in Jember (Fig. 7C). Initially, the relative intensities of galacturonic acid, galactaric acid, and lactic acid were lower for fermentation in Malang, but on day 5, they became higher (Fig. 7C). The relative intensities of tartaric acid and malic acid followed a similar trend during fermentation in both Jember and Malang, decreasing towards the end of fermentation. However, cocoa beans fermented in Malang exhibited higher relative intensities of tartaric acid and malic acid on day 5 (Fig. 7C).

Cocoa pulp contains a significant amount of pectin, which consists of galacturonic acid (Aprotosoaie *et al.*, 2016). Oxidation of galacturonic acid results in the production of galactaric acid (Purushothaman *et al.*, 2018). Furthermore, the conversion of galacturonic acid and galactaric acid leads to the generation of pyruvate (Chang and Feingold, 1970, Biz *et al.*, 2016), a crucial intermediate in the TCA cycle. The levels of both galacturonic acid and galactaric acid were lower in beans fermented in Jember (Fig. 7). This suggests that pectic degradation was more pronounced in Jember fermentation, likely due to the higher environmental temperatures. This observation is further supported by the higher relative intensity of trehalose, which is exclusively found in Jember fermentation. Trehalose, which is found in beans (Delgado-Ospina *et al.*, 2020), remains higher because the glycolysis pathways is from cocoa pulp degradation.

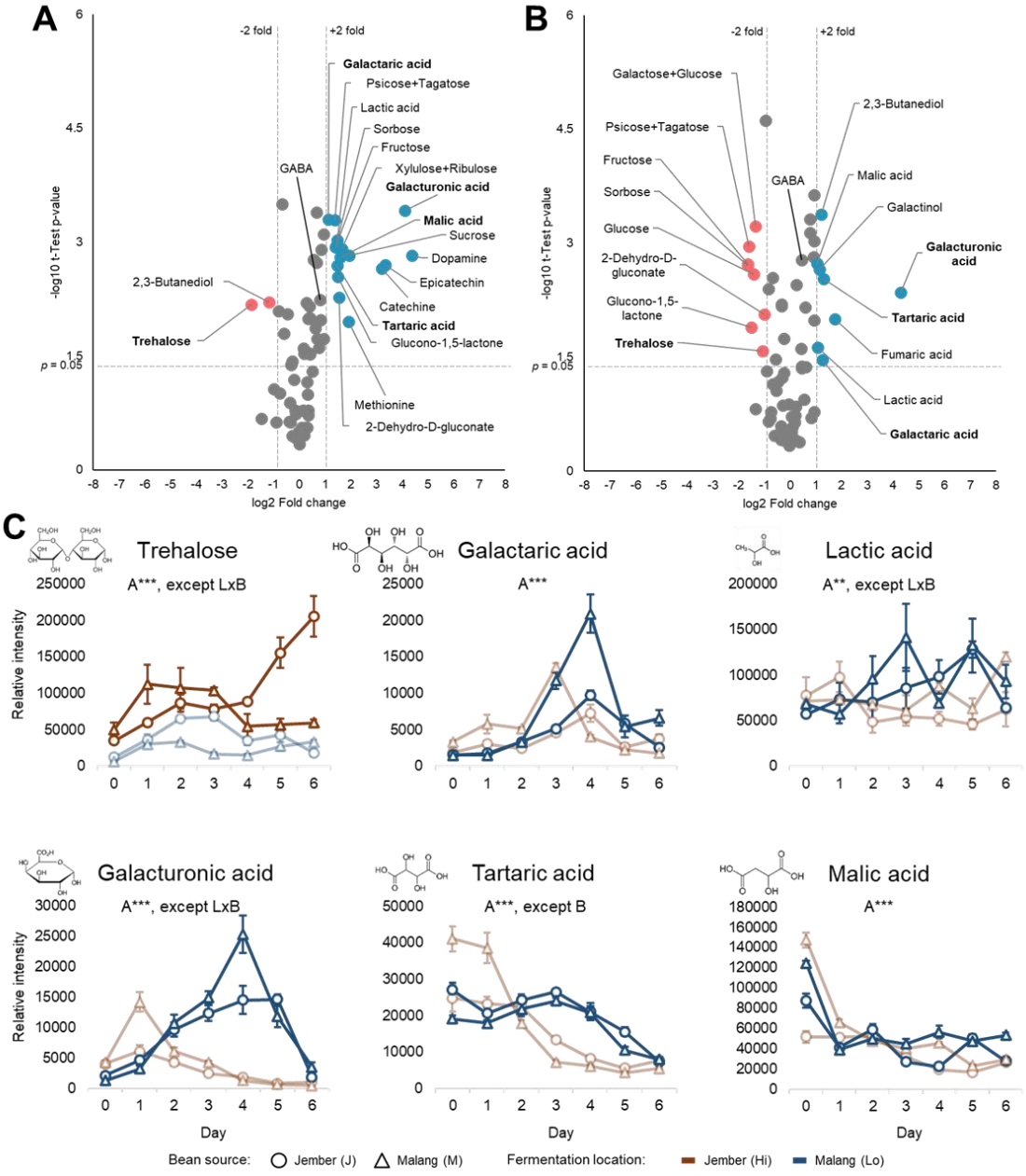

Figure 7. The differential metabolite of day 5 fermentation. (A) The volcano plot of differential metabolites of J-Hi vs J-Lo (day 5) fermentation. (B) The volcano plot of differential metabolites of M-Hi vs M-Lo (day 5) fermentation. The letters code represents the resulting swapping procedure (J = Jember; M = Malang; Hi = high environmental temperature; Lo = low environmental temperature; e.g., J-Hi = Jember beans fermented in high environmental temperature). The horizontal axis in volcano plot represents the -log10 t-Test p-value and the vertical axis the log2 Fold change. Red circle represents metabolites that 2-fold higher in cocoa bans fermented in Jember. Blue circle represents metabolites that 2-fold higher in

cocoa bans fermented in Malang. (C) The differential metabolite metabolites changes per day per fermentation box shown with a line chart. The horizontal axis represents the relative intensity and the vertical axis the day of fermentation. Source of variation and asterisk indicate significant differences for a given variation (L = location; B = Bean source; D = day; ?x? = interaction of ? and ?; A = all variations) and level of significance (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

In contrast, higher trehalose hydrolysis is assumed to occur in low environmental temperatures, as observed in Malang. The relative intensity of trehalose is consistently lower compared to Jember (Fig. 7C), potentially indicating variations in glycolysis pathways that support the TCA cycle (Aprotosoaie *et al.*, 2016, De Vuyst and Leroy, 2020). Lower environmental conditions are assumed to prolong the trehalose hydrolysis and produce more glucose, which breaks down into pyruvate, a key component of the TCA cycle. The organic acids present in fermented cocoa beans are a sign of TCA cycle activity and a result of cocoa pulp fermentation that slowly diffuses into the beans and usually appears during 24 – 36 hours of fermentation (Schwan and Wheals, 2004, De Vuyst and Leroy, 2020, Panda *et al.*, 2022). Results show that organic acid levels were higher in cocoa beans under low environmental temperature fermentation on day 5, indicating higher TCA cycle activity. GABA synthesis in plants is closely related to the TCA cycle (Ansari *et al.*, 2021, Li *et al.*, 2021). The intermediate of the TCA cycle, α-ketoglutarate, is a precursor of glutamic acid, which is decarboxylated to form GABA (Olsen and DeLorey, 1999, Sita and Kumar, 2020, Li *et al.*, 2021). Hence, higher decarboxylation of glutamic acid on day 5 of fermentation under low environmental temperature conditions was observed in Fig. 6C. Higher TCA cycle activity was reflected in lower levels of succinic acid during fermentation in low environmental temperatures,

especially after 36 hours, days 3 – 5 (Fig. 8). Succinic acid has been reported to be constantly lower during 24 – 36 hours of fermentation because it is needed for the TCA cycle (Rodriguez-Campos *et al.*, 2011, Zhang *et al.*, 2020). A similar phenomenon of lower succinic acid levels during the increase of GABA levels was reported in *takuan-zuke* dehydration process (Kobayashi *et al.*, 2021). Therefore, cocoa bean fermentation in low environmental temperatures may promote TCA cycle activity in the beans towards day 5 fermentation, thereby enhancing GABA levels in fermented cocoa beans.

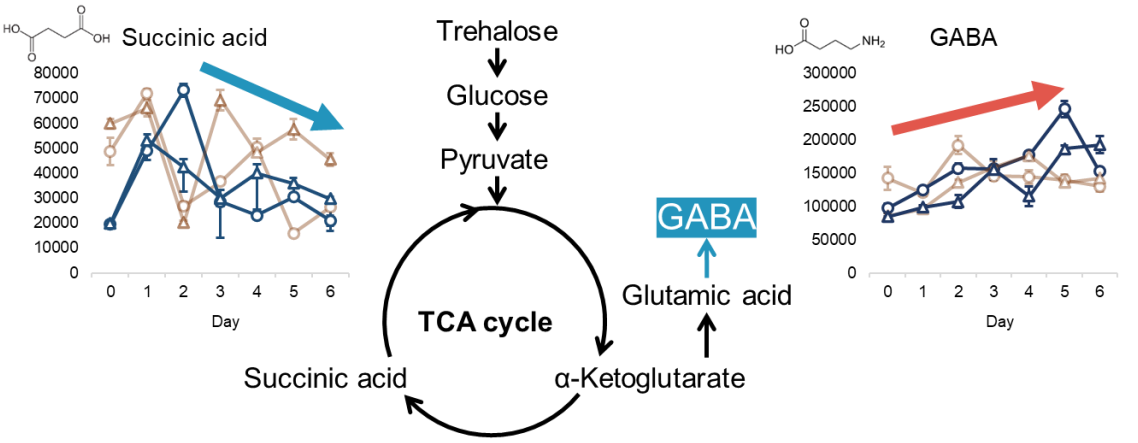

Figure 8. Succinic acid and GABA levels during cocoa bean fermentation in low environmental temperature condition.

3.4 Conclusion

Day 5 of fermentation in low environmental temperatures is the optimal time to achieve the highest GABA levels. This finding applies not only to beans grown in such conditions but also to those transferred to low environmental temperatures. Further investigation is required to examine the GABA levels in cocoa beans after they have been processed into chocolate, as it is crucial to fulfil the objective.

Chapter 4

The sugar composition of fermented cocoa beans affects the GABA levels of SOC (single-origin chocolate)

4.1 Introduction

The preceding chapters have examined the impact of low environmental temperatures on GABA levels during cocoa bean fermentation. Although fermented cocoa bean is a key ingredient in the chocolate industry, fermented cocoa bean is not the final product. Chocolate manufacturers further process the fermented beans they acquire. During the chocolate-making process, there is a loss of GABA due to the Maillard reaction during roasting. The previous discussion has also highlighted the influence of sugar addition on GABA loss during heating (Lamberts *et al.*, 2008). In this chapter, fermented cocoa beans sourced from five different regions in Indonesia were collected and used to produce chocolate. The chocolate-making process was carefully controlled to ensure uniformity in the treatment of each cocoa bean. The roasting time and temperature were kept constant, with only a 30% sugar addition as an additional ingredient. The resulting chocolates are referred to as SOC (single-origin chocolate), indicating that the beans used to make the chocolate come from a specific origin. A total of five SOCs were produced. Furthermore, metabolomics analysis was done to investigate the GABA levels of fermented cocoa beans before and after they are made into single-origin chocolates (SOC).

4.2 Material and methods

4.2.1 Samples materials

The samples required for this chapter were provided in collaboration with Pipiltin Cocoa of Rosso Bianco Co., located in Jakarta, Indonesia. Fermented cocoa beans (*Trinitario* variety) were sourced from foster farms associated with Pipiltin Cocoa, situated in five provinces across Indonesia: Aceh, Bali, East Java, East Nusa Tenggara, and West Papua (Table S1). These provinces are collectively referred to as the sample origins. The chocolate-making process adhered to Pipiltin Cocoa's standard operating procedures. The roasting time and temperature, quantity of added sugar cane, and conching time and temperature were kept consistent for all samples. Sugar cane was added at a ratio of 30% of the total weight to produce 70% dark chocolate, the final chocolate product. The roasting time duration ranging from 10 to 60 minutes and the temperature ranging from 110 to 180 °C (Aprotosoaie *et al.*, 2016, Rocha *et al.*, 2017). Sugar addition occurred during the mixing stage after roasting and prior to conching. Conching was conducted for a duration of 10 to 96 hours at temperatures ranging from 60 to 70 °C (Afoakwa, 2016). Consequently, no Maillard reaction occurred during the conching process.

4.2.2 Extraction and derivatization of metabolites

Three fermented cocoa beans from each origin were combined and homogenized to extract the metabolites in a 50 mL tube with a metal cone using a Multi-beads shocker. For the chocolate samples, 10 mg of chocolate from each origin was crushed using a Multi-beads shocker prior to extraction. The homogenized sample powder (5 mg) was

transferred to a 2 mL tube. Triplicate samples (n = 3) were extracted using a mixed solvent of methanol, chloroform, and ultrapure water (2.5:1:1 v/v/v) containing an internal standard. After incubation and centrifugation, the supernatant (600 µL) was collected. From the aqueous phase, 200 µL was transferred to a new tube for analysis. Pooled QC samples were also prepared by combining 200 µL of the aqueous phase from each sample. The samples underwent dry centrifugation for 2,5 hours followed by derivatization using methoxyamine hydrochloride and MSTFA. All samples were processed and analyzed simultaneously.

4.2.3 GC-MS-based metabolite profiling

GC-MS analysis was performed using a GCMS-QP2010 Ultra instrument equipped with a 0.25 µm InertCap 5 MS/NP column. The mass spectrometer was tuned and calibrated prior to the analysis. One microliter of the derivatized sample was injected in split mode (25:1 v/v) at an injection temperature of 270 °C. Helium was used as the carrier gas, flowing at a linear velocity of 39 cm/s and a flow rate of 1.12 mL/min. The column temperature started at 80 °C for 2 minutes and increased by 15 °C/min until reaching 330 °C, with a hold time of 6 minutes. The transfer line and ion source temperatures were set at 310 °C and 280 °C, respectively. Electron ionization (EI) was used for ion generation, and mass spectra were recorded from *m/z* 85 to 500 at a rate of 10,000 µ/s. Prior to the first sample injection, a standard alkane mixture (C8 – C40) was injected.

4.2.4 Data processing

The raw data obtained were converted to the AIA file format using Shimadzu's GC-MS solution software packages. MS-DIAL ver. 4.92 was then utilized for peak alignment,

filtering, and tentative annotation. The annotation process relied on the GL-Science DB spectral database (InertCap 5MS-NP, predicted Fiehn RI) downloaded from MS-DIAL's official website. To confirm the annotations, a comparison was made with the data in the NIST library (NIST/EPA/NIH EI-MS Library) included in the GC-MS solution software packages. Annotations with a minimum similarity value of 80% were considered. The relative intensity of each annotated metabolite was normalized using the internal standard (ribitol). Metabolites with an RSD of less than 30% within the QC samples were selected for further analysis.

4.2.5 Multivariate data analysis

The processed data underwent principal component analysis (PCA) to generate the metabolite profiles of Indonesian cocoa beans. The software used for conducting PCA was SIMCA-P+ version 13.0.1 (Umetrics, Umea, Sweden), with auto-scaling and no transformation applied. Statistical analysis of the annotated metabolites and recorded environmental parameters were conducted using analysis of variance (ANOVA) with Tukey's post hoc test, utilizing JASP Version 0.17.1 (JASP Team, Amsterdam, Netherlands). Bar graphs and lined graphs were plotted using Microsoft Excel 2016.

4.3 Results and discussion

4.3.1 GABA levels differed between fermented beans and SOC

The PCA analysis of the metabolite profiles of fermented cocoa beans is presented in Fig. 9A, revealing a distinct separation based on their GABA levels. Fermented cocoa beans originating from Aceh, Bali, and West Papua exhibited higher GABA levels.

Additionally, the sugar profiles of these beans demonstrated noticeable differences, as depicted by the orange circles dispersed across the loading plot in Fig. 9B.

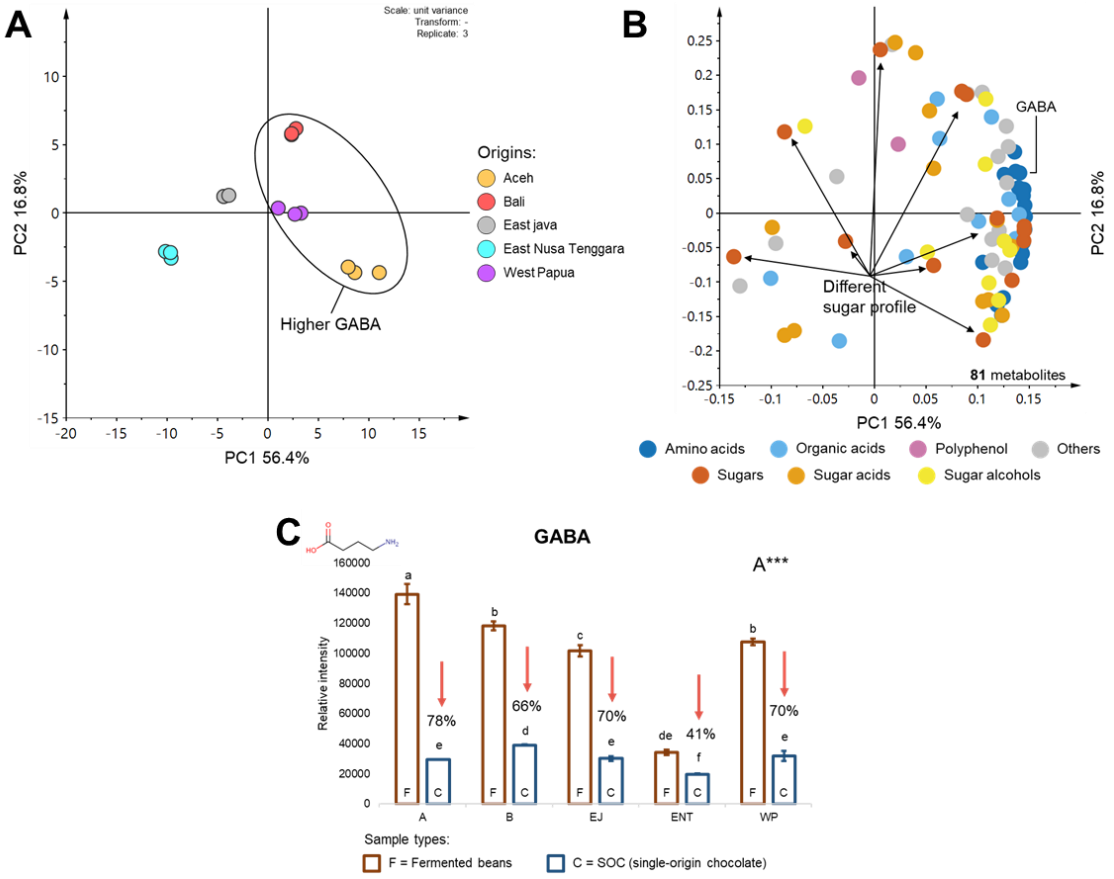

Figure 9. The PCA result of GC-MS analysis of fermented cocoa beans from five different origins and bar graph of GABA loss. A total of 81 annotated metabolites were used for PCA. (A) Score plot of the PCA result. (B) Loading plot of the PCA result; a complete list of annotated metabolites is shown in Table S4. (C) Bar graphs represent the GABA loss on fermented cocoa beans after chocolate-making process. The horizontal axis represents the sample and the vertical axis represents the relative intensity after normalization with internal standard. Letters in horizontal axis represent the place of origin of the samples (A: Aceh; B: Bali; EJ: East Java; ENT: East Nusa Tenggara; WP: West Papua; Table S1). Bar graphs with different letters indicate a significant difference between samples for a given metabolite. Source of variation and asterisk indicate significant differences for a given variation (O = origin; S = sample types; OxS =

interaction of O and S; A = all variations) and level of significance (ns = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

After the chocolate-making process, the levels of GABA decrease, and the rates of decrease vary among the different origins (Fig. 9C). Striking changes can be observed in the beans and SOC from Aceh and Bali. Interestingly, Aceh, which initially had the highest GABA levels in its fermented beans, did not maintain the same levels after being made into SOC. On the other hand, Bali, which had the second-highest GABA levels in its fermented beans, transformed into SOC with the highest GABA levels. Aceh experienced a loss of 78% in GABA levels, while Bali had a loss of 66%. The samples from East Nusa Tenggara recorded the lowest GABA loss. It is well-established in the literature that the metabolite profiles of cocoa beans and chocolate can differ, even when the cocoa variety is the same, depending on the location of growth and fermentation (Herrera-Rocha *et al.*, 2023). Moreover, variations in flavor of chocolate produce in same way can arise from differences in the fermentation process, even if the fermentation systems are similar (Kouassi *et al.*, 2022). Hence, the presence of differences among the samples in this study is anticipated.

4.3.2 Sugar in fermented cocoa beans related to GABA loss

The Maillard reaction occurs when reducing sugars and amino acids react during heating at temperatures ranging from 140 to 165 °C. Among the annotated metabolites, the reducing sugars involved in this reaction are mannose, glucose, lyxose, sorbose, galactose+glucose, and B-lactose. Although sucrose is a non-reducing sugar, it indirectly participates in the Maillard reaction. During heating, sucrose undergoes inversion, releasing fructose and glucose that are then involved in the Maillard reaction (Zhang *et*

al., 2009). To assess the relationship between GABA loss percentage and sugars involved in the Maillard reaction, Pearson's correlation coefficient was calculated (Fig. 10A). The analysis revealed that GABA loss correlated with sucrose, mannose, sorbose, and B-lactose in fermented cocoa beans. Sucrose exhibited a significantly negative correlation with GABA loss, and the correlation coefficient was remarkably strong at -0.829. Mannose, which is the C-2 epimer of glucose, showed a significant positive correlation, whereas glucose itself did not. These differences contributed to the variations in GABA loss observed after the chocolate-making process. Aceh that high in mannose has more GABA loss compared to Bali (Fig. 10B and Fig. 9C). Previous reports have shown that mannose is more reactive than glucose (Yaylayan and Forage, 1992, Hu *et al.*, 2018), suggesting that the GABA/mannose interaction results in a higher GABA loss compared to the GABA/glucose interaction.

Additionally, the intensity of sucrose, which is highest in fermented beans from East Nusa Tenggara (ENT) (Fig. 10B), was associated with the lowest rate of GABA loss at 41%. Lamberts also reported that GABA solution with sucrose exhibited the lowest GABA loss during heating. Therefore, based on these findings, it is indicated that the GABA/mannose interaction is the primary factor contributing to the high GABA loss during chocolate-making process.

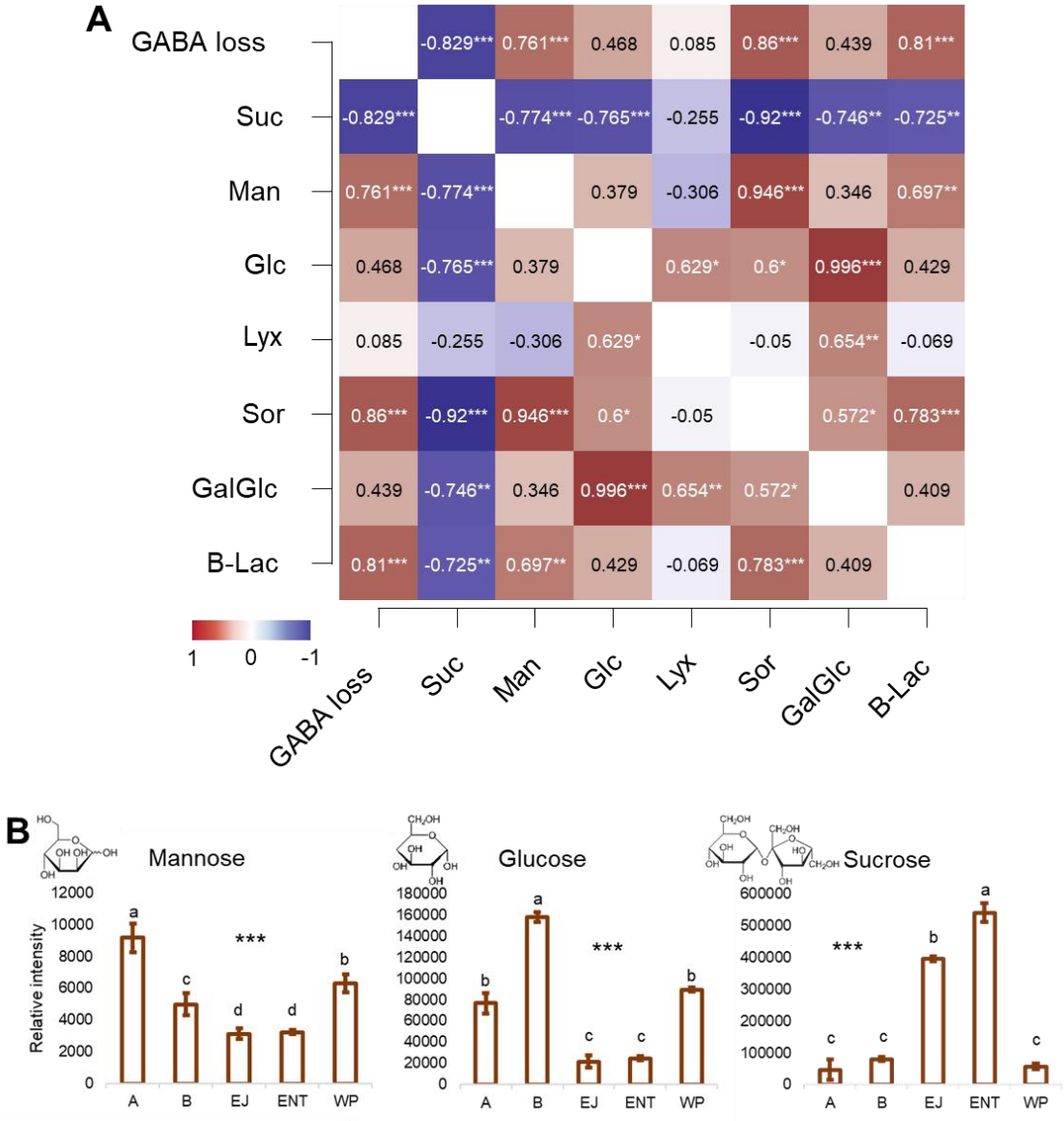

Figure 10. The correlation analysis results of GABA loss and sugars in fermented cocoa beans. (A) Heatmap of Pearson's correlation coefficient of GABA loss and sugars in fermented cocoa beans. Correlation coefficient: 0.1 – 0.2 = poor, 0.3 – 0.5 = fair, 0.6 – 0.7 = moderate, 0.8 – 0.9 = very strong, 1 = perfect (Akoglu, 2018). (B) Bar graphs represent the relative intensity of mannose, glucose, and sucrose. The horizontal axis represents the sample and the vertical axis represents the relative intensity after normalization with internal standard (A: Aceh; B: Bali; EJ: East Java; ENT: East Nusa Tenggara; WP: West Papua; Table S1). Bar graphs with different letters indicate a significant difference between samples for a

given metabolite. An asterisk in each metabolite bar graph indicates the level of significance (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). All data were statistically assessed using analysis of variance (ANOVA).

4.4 Conclusion

The varying sugar compositions in fermented cocoa beans result in different rates of GABA loss in single-origin chocolates (SOC) after the chocolate-making process. Beans with high mannose content experience the highest GABA loss, while beans with high sucrose content exhibit the lowest GABA loss. Consequently, when aiming to create SOC with elevated GABA levels, it is crucial to consider the sugar composition of the fermented cocoa beans.

Chapter 5

Conclusion

This research utilizes a metabolomics approach to uncover the relationship between cocoa bean fermentation conditions and GABA levels. Environmental temperature, particularly low temperatures during fermentation, significantly increases the GABA levels in cocoa beans, regardless of their source. This increase is likely associated with the higher TCA cycle activity in cocoa beans under lower temperature conditions. Farmers situated in tropical highlands can readily adopt this method to enhance the value of their fermented cocoa beans, as the fermentation period remains consistent with standard practices. However, the subsequent processing of fermented cocoa beans for chocolate production results in a reduction of GABA levels during the roasting stage. To minimize this loss, chocolate makers aiming for chocolates with high GABA levels should consider fermented cocoa beans with high sucrose or lower mannose. Future research should focus on identifying the optimal conditions for artificial low temperature fermentation and improvement with GABA-producing microorganisms, followed by the selection of cocoa clones with desired sugar compositions. Further investigation involving enzyme assays for GABA synthesis, and quantitative analysis of amino acids, sugars, and organic acids in relation to the TCA cycle would enhance our understanding of how GABA levels increase during cocoa bean fermentation in low environmental temperatures.

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List of publications

Original paper

Hanifah, A., Firmanto, H., Putri, S. P., and Fukusaki, E.: Unique metabolite profiles of Indonesian cocoa beans from different origins and their correlation with temperature, J. Biosci. Bioeng., 134, 125-132 (2022). https://doi.org/10.1016/j.jbiosc.2022.05.001

Presentations:

1. **Abu Hanifah,** Hendy Firmanto, Sastia Prama Putri, Fukusaki Eiichiro**.** Metabolite profile of cocoa beans and its correlation to environmental temperature. *(Online presentation) 74th Annual Society of Biotechnology Japan Meeting. 2022.*

2. **Abu Hanifah,** Tissa Aunilla, Sastia Prama Putri, Fukusaki Eiichiro**.** Correlation between metabolite profile and sensory profile of single-origin chocolates. *(Online presentation) 73rd Annual Society of Biotechnology Japan Meeting. 2021*.

3. **Abu Hanifah,** Hendy Firmanto, Sastia Prama Putri, Fukusaki Eiichiro**.** Metabolite profiling of fermented cacao beans from different origins of Indonesia. *(Poster presentation) Metabolomics 2020 Online meeting. 2020*.

4. **Abu Hanifah,** Hendy Firmanto, Sastia Prama Putri, Fukusaki Eiichiro**.** Metabolic profiling of cocoa cake from Forastero cacao beans by GC-MS

analysis. *(Oral presentation) 71st Annual Society of Biotechnology Japan Meeting. 2019.*

Supplementary

Table S1. GPS (global positioning system) coordinates of cocoa beans source farms

Code	Place of origin		GPS coordinates	
	City	Province	Latitude	Longitude
4	Madiun	East Java	-7.70825	111.71244
7	Gunung Kidul	Yogyakarta	-7.87095	110.54274
12	Enrekang	South Sulawesi	-3.33811	119.8302
14	Central Maluku	Maluku	-2.89448	129.75887
15	Jembrana	Bali	-8.28694	114.5051
18	North Lombok	West Nusa Tenggara	-8.34991	116.23672
Hi	Jember	East Java	-8.23222	113.60530
Lo	Malang	East Java	-8.23908	112.71149
A	Bireuen	Aceh	5.11275	96.72296
B	Tabanan	Bali	-8.45906	115.01802
EJ	Trenggalek	East Java	-8.08382	111.66076
ENT	Flores	East Nusa Tenggara	N/A	N/A
WP	Ransiki	West Papua	-1.50022	134.19359

Table S2. List of annotated metabolites of GC-MS-based analysis in chapter 2 (RSD<30%)

No	Metabolite	RI*	Quantitative *m/z*	Annotation
1	Lactic acid	1065.24	147.095	GL-sciences library, NIST11
2	Glycolic acid	1079.03	147.091	GL-sciences library, NIST11
3	Alanine	1108.21	116.104	GL-sciences library, NIST11
4	Valine	1224.27	144.143	GL-sciences library, NIST11
5	Ethanolamine	1276.17	174.100	GL-sciences library, NIST11, and STD
6	Leucine	1280.54	158.150	GL-sciences library, NIST11
7	Phosphoric acids	1283.06	299.043	GL-sciences library, NIST11
8	Glycerol	1283.96	147.098	GL-sciences library, NIST11, and STD
9	Isolecucine	1302.49	158.141	GL-sciences library, NIST11
10	Proline	1306.15	142.137	GL-sciences library
11	Glycine	1316.87	174.098	GL-sciences library, NIST11, and STD
12	Succinic acid	1317.51	147.096	GL-sciences library, NIST11, and STD
13	Glyceric acid	1341.98	147.088	GL-sciences library, NIST11
14	Fumaric acid	1349.78	245.025	GL-sciences library, NIST11
15	Serine	1371.75	188.094	GL-sciences library, NIST11
16	Threonine	1399.34	218.084	GL-sciences library, NIST11
17	Malic acid	1499.35	147.089	GL-sciences library, NIST11
18	Threitol	1517.87	147.077	GL-sciences library
19	Meso erythritol	1526.18	147.089	GL-sciences library, NIST11
20	Aspartic acid	1532.53	232.068	GL-sciences library
21	Pyroglutamic acid	1533.92	156.099	GL-sciences library, NIST11
22	4-Aminobutyric acid	1542.07	174.100	GL-sciences library, NIST11, and STD
23	Threonic acid	1579.20	147.090	GL-sciences library
24	2-Isopropylmalic acid	1594.31	147.087	GL-sciences library
25	3-Phenyllactic acid	1598.21	193.072	GL-sciences library, NIST11
26	3-Hydroxy-3-methylglutarate	1614.91	133.100	GL-sciences library
27	Glutamic acid	1631.05	246.093	GL-sciences library, NIST11
28	Phenylalanine	1641.98	218.050	GL-sciences library, NIST11
29	Tartaric acid	1658.97	147.090	GL-sciences library, NIST11
30	Asparagine	1684.48	116.107	GL-sciences library, NIST11
31	Arabionose	1688.17	103.072	GL-sciences library
32	Xylitol	1735.69	217.057	GL-sciences library, NIST11
33	Glutamine	1786.60	156.102	GL-sciences library
34	Xylonic acid	1795.18	147.083	GL-sciences library
35	Shikimic acid	1823.83	204.062	GL-sciences library

36	3,4-Dihydroxybenzoate	1834.15	193.013	GL-sciences library
37	Isocitric acid&Citric acid	1841.59	273.085	GL-sciences library, NIST11, and STD
38	Caffeine	1863.55	194.050	GL-sciences library, NIST11
39	Theobromine	1880.81	180.042	NIST11
40	Adenine	1883.71	264.081	GL-sciences library
41	Psicose	1890.19	103.085	GL-sciences library
42	Quinic acid	1892.27	345.147	GL-sciences library
43	Sorbose	1906.29	103.094	GL-sciences library, NIST11
44	Gluconolactone	1913.21	129.107	GL-sciences library, NIST11
45	Fructose	1916.43	103.093	GL-sciences library, NIST11
46	Tyramine	1931.62	174.105	GL-sciences library
47	Glucose	1934.14	205.065	GL-sciences library, NIST11
48	Lysine	1940.36	174.100	GL-sciences library, NIST11
49	Glucosamine	1948.10	203.075	GL-sciences library, NIST11
50	Galactose	1954.14	205.068	GL-sciences library, NIST11
51	Tyrosine	1958.53	218.048	GL-sciences library, NIST11
52	Mannitol	1968.78	205.071	GL-sciences library, NIST11
53	Sorbitol	1977.66	205.078	GL-sciences library, NIST11
54	Galacturonic acid	1983.02	333.106	GL-sciences library
55	Xanthine	2041.32	353.070	GL-sciences library
56	Gluconic acid	2043.89	147.096	GL-sciences library, NIST11
57	Saccharic acid	2058.71	333.114	GL-sciences library
58	Glucarate	2059.01	333.105	GL-sciences library
59	Dopamine	2103.57	174.095	GL-sciences library
60	Inositol	2132.36	217.052	GL-sciences library, NIST11
61	Tryptophan	2215.85	218.050	GL-sciences library
62	Stearic acid	2244.90	217.050	GL-sciences library, NIST11
63	Uridine	2478.86	217.074	GL-sciences library
64	5-Hydroxy tryptamine	2514.24	174.097	GL-sciences library
65	Sucrose	2706.35	361.134	GL-sciences library, NIST11
66	Guanosine	2812.23	324.117	GL-sciences library
67	Trehalose	2816.47	361.135	GL-sciences library, NIST11
68	Epicatechin	2896.31	368.131	GL-sciences library
69	Catechine	2917.17	368.126	NIST11
70	Galactinol	3076.92	204.055	GL-sciences library
71	Raffinose	3502.85	361.138	GL-sciences library

*Retention indices (RI) were calculated using standard alkane mixture (C10-C40). Peak annotation was performed by comparing the RI and their mass spectra with GL-science NIST library (NIST/EPA/NIH EI-

MS Library). Co-injection was performed to confirm some of the metabolites with authentic standard by spiking into extracted samples. Co-injected metabolites show with STD in the annotation column.

Table S3. Recorded parameters during swapped fermentation

Parameters	Location		Exp day						
			day 0	day 1	day 2	day 3	day 4	day 5	day 6
Environmental temperature (°C)	Jember	Max	30.110	31.411	31.333	30.278	31.566	31.719	31.214
		Min	25.392	22.328	21.282	24.844	23.721	23.002	21.263
		Ave	26.695	26.025	26.166	27.068	27.275	26.643	25.924
	Malang	Max	28.039	26.498	27.972	25.323	26.476	25.889	26.689
		Min	21.067	19.959	18.717	20.161	19.025	19.926	17.406
		Ave	24.006	22.340	22.391	22.163	22.352	22.194	22.102
Humidity (% RH)	Jember		92.501	91.112	91.043	96.479	93.872	86.574	89.466
	Malang		89.528	95.386	94.106	97.469	97.282	95.959	90.934
Fermentation temperature (°C)	J-Hi	R1	26.867	30.900	34.533	36.600	38.700	32.067	29.067
		R2	27.100	30.300	37.100	37.100	36.767	32.800	28.600
		R3	27.100	30.667	33.967	38.133	37.833	31.100	29.067
	M-Hi	R1	28.133	32.933	31.400	35.133	31.833	30.833	29.567
		R2	28.567	31.967	33.667	37.833	33.833	29.900	29.767
		R3	NA	NA	NA	NA	NA	NA	NA
	J-Lo	R1	27.500	25.900	27.700	32.800	29.100	24.633	22.833
		R2	26.367	28.033	28.867	33.100	34.167	25.667	23.433
		R3	27.167	27.567	27.833	31.200	32.967	23.667	22.133
	M-Lo	R1	25.433	27.133	25.400	28.200	31.800	24.800	22.233
		R2	25.400	28.567	29.467	36.233	30.733	25.367	23.033
		R3	25.800	28.067	27.867	32.300	30.633	23.967	22.900
Fermentation pH	J-Hi	R1	4.200	3.640	4.270	5.180	7.770	7.870	7.950
		R2	3.830	3.650	4.680	5.470	7.720	8.060	8.020
		R3	4.290	3.840	5.140	5.350	7.690	7.800	8.030
	M-Hi	R1	3.380	3.310	7.850	6.530	7.500	7.970	7.970
		R2	3.460	3.540	5.060	4.930	7.770	7.990	8.230
		R3	NA	NA	NA	NA	NA	NA	NA
	J-Lo	R1	4.180	4.820	3.860	6.510	7.820	8.200	8.070
		R2	4.230	3.110	3.870	6.540	7.480	7.910	8.060
		R3	4.350	3.510	4.780	4.340	7.500	8.420	8.030
	M-Lo	R1	4.490	3.290	3.320	5.150	6.710	8.310	7.870
		R2	4.120	3.160	3.860	4.620	7.740	7.890	8.400
		R3	3.420	3.130	3.730	4.870	7.040	8.090	7.950
Fermentation index	J-Hi	R1							0.640
		R2							0.710
		R3							0.480
	M-Hi	R1							0.400
		R2							0.450
		R3							NA

	J-Lo	R1							0.380
		R2							0.520
		R3							0.360
	M-Lo	R1							0.350
		R2							0.430
		R3							0.340

The letters code for location represents the resulting swapping procedure (J = Jember; M = Malang; Hi = high environmental temperature; Lo = low environmental temperature; e.g., J-Hi = Jember beans fermented in high environmental temperature). R = replicate. The observation of ambient temperature, fermentation temperature, humidity, and pH were conducted simultaneously with cocoa bean sampling. The environmental temperature and humidity parameters were recorded using a Tinytag TGP-4500 temperature & humidity data logger. The thermometer was placed in the same location as the fermentation process, approximately 1.5 meters above the ground, ensuring good air circulation and shade. For fermentation temperature and pH measurements, another thermometer and pH meter were inserted three times into different parts of the fermentation pile/stack at a depth of around 15 cm from the surface. The Erenear PH Meter and ThermoPro Thermometer Digital were used as instruments. The fermentation index (FI) was determined according to the previous method with minor modifications (Romero-Cortes *et al.*, 2013). Frozen cocoa beans were randomly selected, ground to a fine powder, and a 50 mg sample was mixed with 5 mL of MeOH:HCl (97:3 v/v). Extraction was performed at 4 °C for 16-18 hours on a rotating shaker, followed by centrifugation at 3500× g for 5 minutes, and collection of the supernatant. Absorbance measurements were taken at 460 and 530 nm using a CGOLDENWALL 722N Visible Spectrophotometer. These wavelengths were selected to represent structural properties and distributions during fermentation, with 530 nm representing a general λmax for anthocyanin spectra and 460 nm reflecting glycoside distribution (Racine *et al.*, 2019). The FI was calculated based on the ratio of absorbance at 460 nm to that of 520 nm.

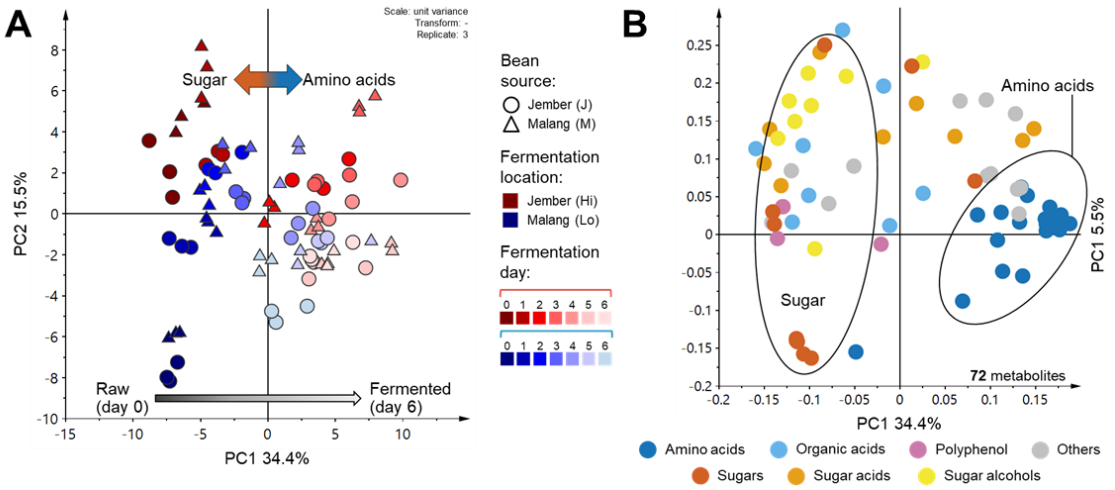

Figure S1. The PCA result of GC-MS analysis of swapped fermentation from day to day 6. A total of 72 annotated metabolites were used for PCA. Further details on the places of origin of the samples are presented in Table S. (A) Score plot of the PCA result. (B) Loading plot of the PCA result; a complete list of annotated metabolites is shown in Table S4.

Table S4. List of annotated metabolites of GC-MS-based analysis of swapped fermentation (RSD<30%)

No	Metabolite	RI*	Quantitative *m/z*	Annotation
1	2,3-Butanediol	1045.07	117.073	NIST20
2	Lactic acid	1062.75	147.052	GL-sciences library, NIST20
3	Glycolic acid	1076.41	147.053	GL-sciences library, NIST20
4	Alanine	1106.06	116.099	GL-sciences library, NIST20
5	Oxalate	1132.22	147.051	GL-sciences library, NIST20
6	Valine	1222.43	144.102	GL-sciences library, NIST20
7	4-Hydroxybutanoic acid	1237.30	147.060	NIST20
8	Ethanolamine	1275.37	174.102	GL-sciences library, NIST20
9	Leucine	1278.57	158.138	GL-sciences library, NIST20
10	Glycerol	1280.30	147.051	GL-sciences library, NIST20
11	Phosphate	1280.99	299.080	GL-sciences library, NIST20
12	1,2,3-Butanetriol	1300.10	117.072	NIST20
13	Isoleucine	1300.94	158.137	GL-sciences library, NIST20
14	Proline	1306.49	142.107	GL-sciences library
15	Succinic acid	1315.39	147.056	GL-sciences library, NIST20
16	Glycine	1316.64	174.097	GL-sciences library, NIST20
17	Glyceric acid	1338.78	147.056	GL-sciences library, NIST20
18	Fumaric acid	1346.81	245.070	GL-sciences library, NIST20
19	Serine	1368.74	204.114	GL-sciences library, NIST20
20	2,3-Dihydroxy-2-methylbutanoic acid	1390.71	117.076	NIST20
21	Threonine	1396.82	218.120	GL-sciences library, NIST20
22	Malic acid	1496.17	147.054	GL-sciences library, NIST20
23	Threitol	1513.17	147.056	GL-sciences library
24	Aspartic acid	1529.81	232.108	GL-sciences library, NIST20
25	Methionine	1530.28	176.082	GL-sciences library, NIST20
26	Pyroglutamic acid	1534.78	156.089	GL-sciences library, NIST20
27	4-Aminobutyric acid	1541.82	174.103	GL-sciences library, NIST20
28	Threonic acid	1575.47	147.058	GL-sciences library, NIST20
29	alpha-Hydroxyglutaric acid	1582.15	129.073	NIST20
30	2-Isopropylmalic acid	1594.15	275.148	GL-sciences library, NIST20
31	Glutamic acid	1628.33	246.146	GL-sciences library, NIST20
32	Phenylalanine	1643.21	218.102	GL-sciences library, NIST20
33	Tartaric acid	1655.26	147.056	GL-sciences library, NIST20
34	Asparagine	1682.89	116.086	GL-sciences library, NIST20
35	Lyxose	1684.04	103.069	GL-sciences library, NIST20
36	Xylulose+Ribulose	1699.04	147.056	GL-sciences library, NIST20
37	Xylitol	1745.74	285.070	NIST20

38	Glutamine	1785.24	156.088	GL-sciences library
39	Xylonic acid	1792.85	292.137	GL-sciences library
40	Shikimic acid	1818.77	204.086	GL-sciences library
41	3,4-Dihydroxybenzoate	1833.37	193.019	GL-sciences library
42	Isocitric acid+Citric acid	1838.83	273.100	GL-sciences library, NIST20
43	Caffeine	1871.33	194.067	GL-sciences library, NIST20
44	Adenine	1886.31	264.118	GL-sciences library
45	Psicose+Tagatose	1903.57	217.100	GL-sciences library
46	2-Dehydro-D-gluconate	1905.94	186.074	GL-sciences library, NIST20
47	Glucono-1,5-lactone	1911.30	217.100	GL-sciences library, NIST20
48	Sorbose	1912.82	217.100	GL-sciences library, NIST20
49	Fructose	1914.53	217.102	GL-sciences library, NIST20
50	Glucose	1932.11	147.050	GL-sciences library, NIST20
51	Lysine	1939.21	156.117	GL-sciences library, NIST20
52	Histidine	1941.33	154.089	GL-sciences library, NIST20
53	Galactose+Glucose	1952.50	147.050	GL-sciences library, NIST20
54	Tyrosine	1958.35	218.101	GL-sciences library, NIST20
55	Mannitol	1967.56	319.178	GL-sciences library, NIST20
56	Sorbitol	1975.33	147.057	GL-sciences library, NIST20
57	Galacturonic acid	1981.64	333.143	GL-sciences library
58	Theobromine	2015.60	237.080	NIST20
59	Gluconic acid	2043.49	147.051	GL-sciences library, NIST20
60	Glucaric acid	2059.89	333.142	GL-sciences library, NIST20
61	Scyllo-Inositol	2067.18	318.156	NIST20
62	Galactaric acid	2086.78	333.142	NIST20
63	Dopamine	2104.58	174.097	GL-sciences library
64	Inositol	2132.67	305.151	GL-sciences library, NIST20
65	Tryptophan	2220.36	218.102	GL-sciences library
66	Sucrose	2704.83	361.151	GL-sciences library, NIST20
67	Trehalose	2816.88	361.164	GL-sciences library, NIST20
68	Epicatechin	2894.14	368.157	GL-sciences library, NIST20
69	Catechine (2R-trans)-	2895.19	179.050	NIST20
70	Galactinol	3077.64	204.088	GL-sciences library
71	Juniperoside III	3503.09	362.150	NIST20
72	Raffinose	3503.29	361.158	GL-sciences library

*Retention indices (RI) were calculated using standard alkane mixture (C10-C40). Peak annotation was performed by comparing the RI and their mass spectra with GL-science NIST library (NIST/EPA/NIH EI-MS Library).

Table S5. List of annotated metabolites of GC-MS-based analysis of fermented cocoa beans and single-origin chocolate (RSD<30%)

No	Metabolite	RI*	Quantitative *m/z*	Annotation
1	2,3-Butanediol	1035.89	117.077	NIST20
2	Lactic acid	1062.73	147.061	GL-sciences library, NIST20
3	Glycolic acid	1076.38	147.069	GL-sciences library, NIST20
4	Alanine	1106.01	116.097	GL-sciences library, NIST20
5	Oxalate	1132.15	147.068	GL-sciences library, NIST20
6	Malonic acid	1206.64	147.059	GL-sciences library, NIST20
7	Valine	1222.58	144.119	GL-sciences library, NIST20
8	2-Hydroxyisocaproic acid	1240.37	103.064	NIST20
9	Ethanolamine	1275.37	174.115	GL-sciences library, NIST20
10	Leucine	1278.55	158.138	GL-sciences library, NIST20
11	Glycerol	1280.29	147.074	GL-sciences library, NIST20
12	Phosphoric acid	1280.97	300.100	NIST20
13	Phosphate	1281.00	299.082	GL-sciences library, NIST20
14	Isoleucine	1300.97	158.138	GL-sciences library, NIST20
15	1,2,3-Butanetriol	1304.11	117.065	NIST20
16	Proline	1306.48	142.103	GL-sciences library
17	Succinic acid	1315.36	147.072	GL-sciences library, NIST20
18	Glycine	1316.60	174.113	GL-sciences library, NIST20
19	1-Monoacetin	1320.23	175.071	NIST20
20	Glyceric acid	1338.83	147.071	GL-sciences library, NIST20
21	Fumaric acid	1347.04	245.081	GL-sciences library, NIST20
22	Serine	1368.82	204.128	GL-sciences library, NIST20
23	Threonine	1396.88	218.139	GL-sciences library, NIST20
24	Malic acid	1496.15	147.069	GL-sciences library, NIST20
25	Meso erythritol	1522.01	147.075	GL-sciences library, NIST20
26	Aspartic acid	1529.84	232.126	GL-sciences library
27	Pyroglutamic acid	1534.70	156.098	GL-sciences library, NIST20
28	4-Aminobutyric acid	1541.75	174.108	GL-sciences library, NIST20
29	Erythronic acid	1559.00	147.067	NIST20
30	Threonic acid	1575.39	147.068	GL-sciences library
31	alpha-Hydroxyglutaric acid	1582.20	129.086	NIST20
32	3-Phenyllactic acid	1598.97	193.111	GL-sciences library, NIST20
33	Glutamic acid	1628.49	246.142	GL-sciences library, NIST20
34	Phenylalanine	1643.24	218.112	GL-sciences library, NIST20
35	Tartaric acid	1655.32	147.065	GL-sciences library, NIST20
36	Asparagine	1683.01	116.079	GL-sciences library, NIST20
37	Lyxose	1684.58	103.060	GL-sciences library, NIST20

38	L-Norvaline	1724.10	144.117	NIST20
39	Xylitol	1733.09	217.122	GL-sciences library, NIST20
40	D-Ribofuranose	1776.24	217.094	NIST20
41	N-Acety glutamate	1789.63	156.100	GL-sciences library, NIST20
42	Xylonic acid	1792.13	292.145	GL-sciences library
43	Shikimic acid	1818.75	204.108	GL-sciences library
44	3,4-Dihydroxybenzoate	1833.57	193.037	GL-sciences library
45	Isocitric acid+Citric acid	1838.72	273.101	GL-sciences library, NIST20
46	Caffeine	1871.52	194.081	GL-sciences library, NIST20
47	Adenine	1886.33	264.117	GL-sciences library
48	Quinic acid	1890.16	345.169	GL-sciences library, NIST20
49	Psicose+Tagatose	1903.28	103.051	GL-sciences library
50	Glucono-1,5-lactone	1912.01	129.095	GL-sciences library, NIST20
51	Sorbose	1913.14	217.132	GL-sciences library, NIST20
52	L-Arabinitol	1916.52	144.000	NIST20
53	Mannose	1918.60	319.183	GL-sciences library, NIST20
54	2-Dehydro-D-gluconate	1923.40	103.050	GL-sciences library, NIST20
55	Glucose	1931.90	147.077	GL-sciences library, NIST20
56	Tyramine	1933.20	174.114	GL-sciences library
57	Lysine	1939.14	156.138	GL-sciences library, NIST20
58	Glucosamine	1945.78	203.112	GL-sciences library
59	Galactose+Glucose	1952.90	147.070	GL-sciences library
60	Tyrosine	1958.18	218.108	GL-sciences library, NIST20
61	Ethyl α-D-glucopyranoside	1961.53	204.109	NIST20
62	Mannitol	1967.57	147.073	GL-sciences library, NIST20
63	D-Galactonic acid	1970.56	275.113	NIST20
64	Sorbitol	1975.54	147.071	GL-sciences library, NIST20
65	Galacturonic acid	1981.92	333.141	GL-sciences library
66	Theobromine	2015.48	237.098	NIST20
67	Gluconic acid	2043.30	147.063	GL-sciences library, NIST20
68	Saccharic acid	2057.57	147.068	GL-sciences library
69	Glucaric acid	2059.76	333.151	GL-sciences library, NIST20
70	Scyllo-Inositol	2067.12	318.157	GL-sciences library, NIST20
71	Galactaric acid	2087.42	333.134	GL-sciences library, NIST20
72	Inositol	2132.69	305.152	GL-sciences library, NIST20
73	Tryptophan	2251.77	202.102	GL-sciences library
74	Sucrose	2705.85	361.142	GL-sciences library, NIST20
75	b-Lactose	2767.83	204.100	GL-sciences library
76	Guanosine	2809.26	324.128	GL-sciences library, NIST20
77	Trehalose	2816.76	361.163	GL-sciences library, NIST20
78	Epicatechin	2894.17	368.162	NIST20

79	Catechine (2R-trans)-	2913.14	368.165	GL-sciences library, NIST20
80	Galactinol	3077.70	204.095	GL-sciences library
81	Raffinose	3503.51	361.168	GL-sciences library

*Retention indices (RI) were calculated using standard alkane mixture (C10-C40). Peak annotation was performed by comparing the RI and their mass spectra with GL-science NIST library (NIST/EPA/NIH EI-MS Library).

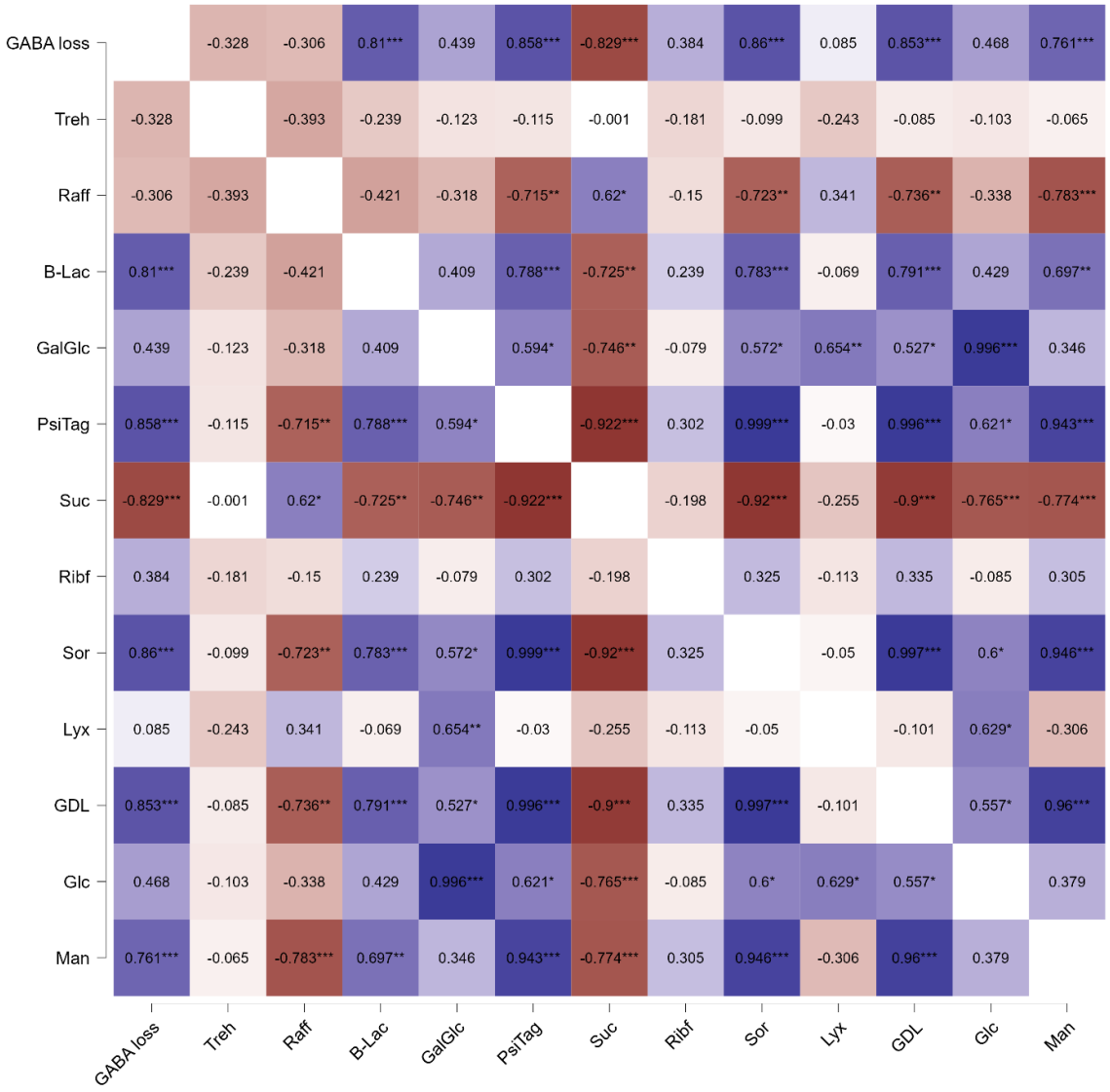

Figure S2. Heatmap of Pearson's correlation coefficient of GABA loss and all sugars annotated in fermented cocoa beans from five different origins in Chapter 4.