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Assessment of the profile of free amino acids and reducing sugars of cacao beans from local Cameroonian Trinitario (SNK varieties) and Forastero (TIKO varieties) using fermentation-like incubation

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Summary

This study investigated the profile of cacao beans from the local Cameroonian Trinitario (SNK) and Forastero (TIKO) in terms of aroma precursors (amino acids and reducing sugars) through fermentation-like incubations. Treatments consisted of incubating beans in acetic acid, 100 mmol/L for two days followed by 200 mmol/L of acetic acid for three days (Treatment T1) and in 100 mmol/L of lactic acid for two days followed by 200 mmol/L of acetic acid for three days (Treatment T2). Both treatments resulted in an increase of free amino acids content by 1.5 - 2.5 times in SNK and TIKO varieties. The ratio of the hydrophobic amino acids over the rest of amino acids showed the preponderance of T1 on the hydrophobic amino acids released in TIKO while in the SNK, some varieties displayed the highest ratio in T2. Glucose and fructose content in TIKO and SNK beans increased 2 to 3 times during incubation. Galactose and raffinose were found in unfermented beans. After incubation, raffinose was missing while at the same time a raise of galactose content could be seen. These results highlighted that acidification remains the factor inducing the releasing of free hydrophobic amino acids, the genotype being less involved.

Key words: *Theobroma cacao*; SNK and TIKO varieties; amino acids; sugars; cacao beans.

Introduction

The unique taste of chocolate attributed to fermented cacao beans after roasting is due to aroma compounds generated during Maillard reaction. These non-enzymatic reactions involve oligopeptides, free amino acids and reducing sugars which constitute the main ingredients of aroma compounds (Voigt et al., 2016; Adedeye et al., 2010). Therefore, free amino acids and reducing sugars are usually called cacao aroma precursors or flavor precursors (Biehl et al., 1985; Amin et al., 1997; Santander et al., 2019). They are released from storage proteins and sucrose respectively during the fermentation process which remains the key step to obtain high-quality cacao beans.

Cacao fermentation consists of two main steps: external and internal fermentation. External fermentation consists of microbial degradation of mucilaginous pulp rich in sugar. A succession of yeasts, lactic acid and acetic acid bacteria on mucilaginous pulp results in ethanol, lactic and acetic acids along with pulp depectinization and liquefaction. Firstly, the alcoholic fermentation takes place and slowly increases the pH (initially below 4 due to the presence of citric acid) and quickly creates ideal conditions for the growth of lactic acid bacteria (LAB). The LABs consume glucose for lactic acid biosynthesis and also enhance the temperature to a range of 35 - 40 °C. After two or three days, the manual fermentation medium stirring and the disappearance of mucilage create aeration allowing acetic acid bacteria (AAB) to grow. Through AAB activity, the ethanol previously released is oxidized (or converted) into acetic acid. This high exothermic reaction increases both the temperature up to 45 °C or more and the pH to a range of 5 - 5.5. Simultaneous to external fermentation, the second phase of fermentation is also running. The lactic and acetic acids released, penetrate the cotyledons at a first stage through hilum, later, the testa also is getting permeable (Rohsius, 2007). The migration of these two organic acids gradually lowers the internal pH of the cacao beans from 6.5 to 4.5 approximately, causes the death of the embryo, disintegrates the cell compartments, releases endogenous enzymes and finally induces degradation of phenolic compounds, sucrose into reducing sugars and storage proteins into oligopeptides and amino acids (Hansen et al., 1988; Thompson et al., 2001; Schwan and Wheals, 2004; Jespersen et al., 2005; Camu et al., 2007; Nielsen, 2007; Afoakwa et al., 2013).

It is well established that among storage proteins found in cacao beans, only from globulin namely Vicilin-class (7S) and from albumin, oligopeptides and free amino acids are generated through the enzymatic process (Voigt et al., 1994; Voigt and Biehl, 1995; Caligiani et al., 2016; Marseglia et al., 2014; Kumari et al., 2016). This biochemical reaction involves two specific endogenous enzymes, aspartic endoprotease and carboxypeptidase, and requires favourable and specific conditions for each one. Though more research remains to be done for a deep understanding of the mechanism of albumin degradation, nevertheless Biehl and Ziegleder (2003) and D'Souza et al. (2008) argued that albumin is essentially a source of oligopeptides. However, some authors proved that in Criollo genotypes albumin is the source of free amino acids (Marseglia et al., 2014). Regarding globulin, it has been demonstrated that aspartic endoprotease and carboxypeptidase induce proteolysis of vicilin-class globulin (7S) of all cacao beans varieties tested (Voigt et al., 1994; Voigt and Biehl, 1995). Finding from Voigt et al. (1994) highlighted the mechanism and revealed the necessary cooperation between both enzymes for the release of cacao-specific aroma precursors. Firstly, through aspartic endoprotease activity, few amino acids and hydrophobic peptides are generated. Thanks to carboxypeptidase, hydrophobic peptides are degraded into hydrophobic amino acids and hydrophilic

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peptides. The mixture of these two compounds displayed the typical flavor of cacao during roasting in the presence of reducing sugars (Spencer and Hoge, 1992; Voigt et al., 1993; Voigt et al., 1994a; Kratzer et al., 2008). Reducing sugars are also an essential ingredient of cacao aroma (Cerbulis et al., 1955; Barisic et al., 2019). The main reducing sugars found in fermented cacao beans are glucose and fructose. They are issued from the enzymatic hydrolysis of sucrose, which involves invertase (Caligiani et al., 2016; Mayorga-Gross et al., 2016).

Considering the specific conditions required by each endogenous enzyme involved in the production of amino acids and reducing sugars for their optimal activity, it seems obvious that obtaining a specific cacao flavour lay on the physico-chemical parameters observed during the fermentation process and on the genotype. These two aspects determine not only the variability of the amino acids and reducing sugars produced but also their amount.

Three main cacao genetic groups (Forastero, Criollo and Trinitario) have been defined to classified cacao based on morphological traits and geographical origins. Criollo was first domesticated in Central America more than 2000 years ago, while Lower Amazon Forastero variety (Amelonado type) was domesticated in Brazil. Forastero group also includes many other populations from all the Amazonian (Upper Amazon Forastero, UPA) and Orinoco regions and presents a high diversity, as revealed by many genetic studies (Loor Solorzano et al., 2012). Trinitario group is hybrid between Criollo and Forastero. In Cameroon, the 5th largest cacao producer (ICCO, 2019), cacao varieties from the three major group are cultivated. However, Trinitario variety is largely constituted by local genotypes developed since the 1950s commonly called SNK: "Selection of Nkoemvone" (Efombagn et al., 2006; Nyasse et al., 2007; Efombagn et al., 2013). This variety was widely spread in Cameroonian cacao farms and today with German cacao constitutes the main varieties (Efombagn et al., 2006). The term "German cacao" was given by farmer for traditional cacao, known for their resistance to black pod disease and because cacao was partly introduced in Cameroon during the German colonization period. The German cacao presents a high level of admixture of diverse genes (Stoll et al., 2017). Genes of Lower Amazon cacao (54%) are predominant followed by Upper Amazon cacao (33%) and Criollo (7%). Only a low content of Trinitario is found (Efombagn et al., 2008). Contrary to SNK varieties, Forastero varieties named TIKO's are less known by farmers. There are no data concerning their behaviour during fermentation, biochemical profile and agronomic traits. Yet, these varieties are present in cacao farms. To enhance Cameroonian cacao, the characterization of these two subgroups in terms of their aroma precursor release is required.

Ngouambe et al. (2019) showed that the purple and brown beans in Cameroon raw cacao contain a high level of amino acids. Interestingly, the group of hydrophobic and acidic amino acids accounted for 61.70% and 30.17% in brown cacao beans and 60.70% and 37.51% in the purple.

As natural fermentation is hard to control, lab-scale fermentation also known as fermentation-like incubation have been optimized to appreciate the changes occurring inside the cacao beans through degradation of different storage compounds induced by the penetration of lactic acid and acetic acid (Rohsius et al., 2006; Kadow et al., 2015; Eyamo et al., 2016; John et al., 2016). In view of the interesting results obtained through this technique, it could also be used to collect information on the profile of cacao beans subjected to optimal fermentation conditions. The present work aimed to assess the profile of free amino acids and reducing sugars of cacao beans from the local varieties (SNK and TIKO) of Cameroon using fermentation-like incubation. The impact of the successive or simultaneous presence of lactic acid and acetic acid at the start of the incubation process on the cacao beans aroma precursors released from the local Cameroonian varieties is highlighted.

Materials and methods

Materials

Cacao beans collection

The fruits analysed were harvested in September - October 2016 from field grown plants of each genotype conserved in cacao germplasm collection at Nkoemvone (SNK collection) Research Station of the Institute for Agricultural Research for Development (IRAD) in the South Region of Cameroon. The collection was established in the 1950s, after random selection by farmers and the breeders in the field, based on yield and assigned accession numbers prior to their transfer and their vegetative propagation in the nurseries on-station (Efombagn et al., 2009ab).

Pods are from seven local Trinitario varieties: SNK10, SNK15, SNK16, SNK48, SNK64, SNK377, SNK450; two F1 Hybrid local varieties: SNK620 and SNK624 resulting from introduced Trinitario variety crossed with Upper Amazonian variety ICS84 × UPA 337 (Nyasse et al., 2006 ; Efombagn et al., 2013); two Upper Amazonian varieties which are the introduced Forastero regularly found in Cameroonian cacao farm: IMC67 and T60/877 and two local Forastero: TIKO31 and TIKO32. The name ''TIKO'' is referred to the locality (in South West Region of Cameroon) were these varieties were selected and recognised for their tolerance to *Phytophthora megakarya* and their productivity. For each variety, about twenty full ripe (based on their characteristic color change) undamaged pods were harvested, transported to the laboratory and fermentation-like incubation was directly applied. Germinated seeds were not encountered in the pods.

Reagents and standards

Unless otherwise specified, all the chemicals and solvents used were analytical grade purchased from Merck (Darmstadt, Germany). Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Methods

Fermentation-like incubation

As presented in Tab. 1, two different treatments were applied to thirteen cacao varieties, according to Bahmann (2013) and Eyamo et al. (2016) with slight modifications. Six to seven intact fruits were harvested at the Institute for Agriculture Research for Development at Nkoemvone station (South Cameroon) and transported to the laboratory. Three independent repetitions were made (not at the same day) for each treatment and five (05) fruits were used for each fermentation-like incubation repetition. Then, at the end of the experimentation, about 20 intact fruits were managed for each variety. For fermentation-like incubation, cacao pods were washed twice for 15 minutes in sodium hypochlorite solution (3 mL/L; 0.3%) and then rinsed three times with sterilized water. After natural drying under a laminar flow hood, pods were sprayed with ethanol, flamed, and opened. Each cacao pod contained ca. 30-50 beans depending on the

Tab. 1: Experimental design of fermentation-like incubations.

Treatments	Days of incubation				
	Day 1 30 °C	Day 2 40 °C	Day 3 45 °C	Day 4 50 °C	Day 5 50 °C
T1	Acetic acid 100 mM; pH 4 3 repetitions of 5 fruits (25 seeds), n=3			Acetic acid 200 mM; pH 5 3 repetitions of 5 fruits (25 seeds), n=3	
T2	Lactic acid 100 mM; pH 4 3 repetitions of 5 fruits (25 seeds), n=3			Acetic acid 200 mM; pH 5 3 repetitions of 5 fruits (25 seeds), n=3	

variety. Twenty-five beans randomly selected from the five opened cacao pods were put into a sterilized glass bottle containing 150 mL incubation medium (100 mmol/L acetic acid or 100 mmol/L lactic acid, and adjusted to pH 4 with 1 mmol/L NaOH; (Tab. 1). After two days of incubation, the beans were transferred under the hood to a second sterilized glass bottle containing the same volume of 200 mmol/L acetic acid pH 5 and incubated for three more days. During this second phase, glass bottles were constantly shaken under laminar flow hood to increase oxygen in the media. The temperature was controlled throughout the experiments by incubation the bottles in a 30-50 °C water bath (Tab. 1). After incubation, the cacao beans were sun-dried between 8 a.m. and 5 p.m. for 7 days with stirring every three hours (final water content: 6.9%). The dried beans were stored in black plastic bags and transported to the University of Hamburg for biochemical analysis.

Biochemical analysis

Seventy five dried beans from the three incubations, resulting from each treatment and variety, were mixed and cut lengthwise with a cacao Guillotine (Type MAGRA, Swiss company TESERBA). The shells and radicles were removed. Cotyledons were coarsely crushed with a blender and the particles were stored at - 20 °C. For defatting, two grams of crushed cotyledon were milled with 10 mL n-hexane in a ball mill (Typ MM200 from Retsch, Germany) by shaking for 10 minutes at a frequency of 25/s. The homogenate was rinsed out of the ball mill with about 75 mL of petroleum ether (bp 40-60 °C), then filtered and dried in a vacuum drying oven. Defatted fine cotyledon powder was stored in -20 °C for free amino acids and sugar analysis.

Free amino acids analysis

Free amino acid contents were analysed according to Rohsius et al. (2006) with slight modifications. 0.5 g of defatted ground cacao powder was stirred at < 4 °C for 1 hour with 1.4 g of polyvinyl-polypyrrolidone (PVPP) and 50 mL of distilled (or deionized) water. The pH was adjusted to 2.5 with 50% of aqueous solution of trifluoroacetic acid (1/1; v/v). The solution was centrifuged at 2,800 g (Centrifuge Heraeus ThermoScientific Megafuge 11R, Hanau, Germany) for 10 minutes at 5000 rpm. The clear supernatant solution was filtered through a 0.45 µm filter (Multoclear, CS-Chromatography). 30 µL of each sample was lyophilized (1h; -20 °C; 0.05 mbar, Christ Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) directly in the vial and stored at -20 °C until analysis. Free amino acids extraction was repeated twice (technical repetition for extraction).

Free amino acids were separated with Reverse-Phase HPLC apparatus (HPLC pump 64 from Knauer; Degaser, Degassex DG-4400 from Phenomenex) after they had been converted to O-phthalaldehyde (OPA) derivatives. The chromatographic system consists of precolumn (LicChroCART 250-4 (Merck, Darmstadt, Germany)); separating column (Lichrospher 100 RP-18; 5 µm); an autosampler (Merck-Hitachi AS-4000) and a fluorescence photometer detector (Hitachi F-1050). Eluents were: A; containing 1.6 L sodium acetate solution/glacial acetic acid (50 mmol/L; pH 6.2), 50 mL MeOH (Lichrosolv R, gradient grade), 20 mL tetra hydrofuran (Lichrosolv R; gradient grade) and B; containing 200 mL sodium acetate solution/glacial acetic acid (50 mmol/L; pH 6.2), 800 mL MeOH (Lichrosolv R, gradient grade). The column was equilibrated with eluent A and elution gradient was (A+B=100%; v/v): (1) 2 min 100-95% A, (2) 10 min, 95-85% A, (3) 8 min, 85-60% A, (4) 5 min, 60-50% A, (5) 15 min, 50-0% A, (6) 10 min, constant 0% A, (7) 5 min, 0-100% A, (8) 20 min, constant 100% A. The conditions of elution were as follows: temperature 30 °C; flow rate. 1.3 mL/min. For the measurement by HPLC, 800 µL of borate buffer (200 mmol/L of boric acid; adjusted to pH 9.5 with concentrated KOH, boiled for 5 min) was added to each lyophilized sample. Before injection of 400 µL of OPA-reagent was added to convert the amino acids into O-phthalaldehyde derivatives. 20 µL of this mixture was finally injected into the column. After injection, the derivatization was stopped 2 min later by passing the eluent through the column. For each amino acid extraction, two injections were done (technical repetition for HPLC).

The OPA reagent was prepared 24 hours before being used. The preparation of the OPA-reagent consisted of a mixture of 100 mg of Ophtaldialdehyde (Merck, No. 11452) previously dissolved in 2.5 mL of MeOH (Lichrosolv R, gradient grade) with 22.4 mL of borate buffer (pH 9.5) and 100 µL of 2-mercaptoethanol (Merck, N° 15433). The amino acids of each sample were identified based on the retention time of commercial standards derivatized amino acids while, they were quantitated via a peak surface of chromatogram using the calibration curve from standard mixtures containing 1-10 pmol/µl of each amino acid. The standard deviation of reference substances was equal to ± 2.0% excepted for glutamic acid, glutamine, alanine, tryptophan (± 2.9 - 3.8%); arginine, asparagine, threonine and serine (± 5.7 - 7.3%); glycine (± 9.2%) and lysine (± 9.6%).

Reducing sugar analysis

Sugars were extracted and quantitated in duplicate (two technical replicates) according to Rohsius (2007) with some modifications. 100 mg of defatted powder was mixed with 1 mL of ultrapure water (type I, ELGA purelab, High Wycombe, United Kingdom) for the extraction of sugars. The solution was homogenized on a laboratory vortex for 20 s and incubated on a Thermomixer comfort (Eppendorf, Hamburg, Germany) for 1 hour at 1300 rpm and 80 °C followed by centrifugation for 10 minutes at 16060 g (Biofuge pico, Heraeus, Hanau, Germany). 300 µL of the supernatant was collected and diluted with 900 µL of ultrapure water. The solution was filtered through a 0.2 µm syringe membrane filter (PES Perfect Flow, Wicom, Heppenheim, Germany) and subjected to HPLC analysis.

The sugars were separated by ligand exchange chromatography in an isocratic mode. 10 µL of each sample was injected onto the column Rezex RCM-Monosaccharide Ca^{2+} column with 8% crosslinking (Phenomenex, Torrance, USA) established at 85 °C using a flow rate of 0.6 mL/min of ultrapure water. The chromatographic system consisted of AS-2000A autosampler; L-6200 smart pump; L-7350D column oven; L-7490 refractive index detector (all Merck Hitachi, Darmstadt, Germany) and ERC 3512 degasser (Erma, Tokyo). D-7000 HPLC-System-Management HSM version 4.1 software was used to record the data. The identification of each sugar was based on retention times of commercial standards used. The retention times of the various sugars were determined by recording the refractive index (RI). Sugars were quantitated with the peak area of each sugar in samples and calibration curve of standard solutions. Extract injection was repeated twice (technical replicate for HPLC).

Statistical analysis

All data were analysed and graphics drawn using R-studio version 0.96.122. Statistical significance was assessed by analysis of variance (ANOVA). The least significant difference (LSD) was used to separate and compare the means and significance was accepted at 5% level ($P < 0.05$).

Results and discussion

Amino acid contents

In unfermented beans (Treatment 0; T0), the total contents of free amino acids varied among varieties (Fig. 1). They ranged between 4684.98 mg/kg fat free dry material (ffdm) (SNK 10) and 7234.08

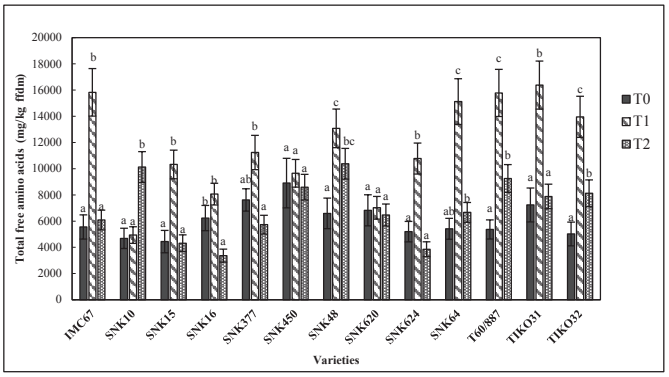
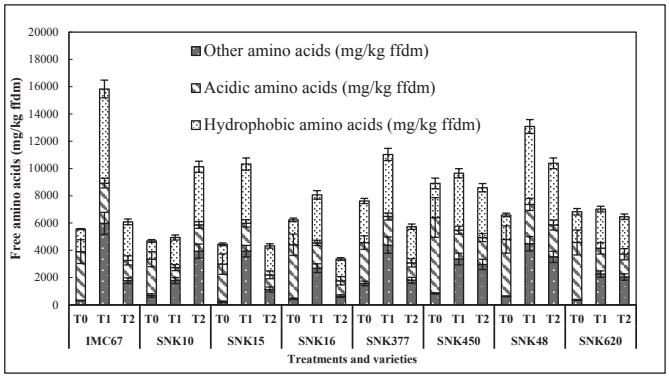


Fig. 1: Total free amino acids content in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$). γ-amino butyric acid (GABA) was detected in all cacao samples but was excluded in the analysis.



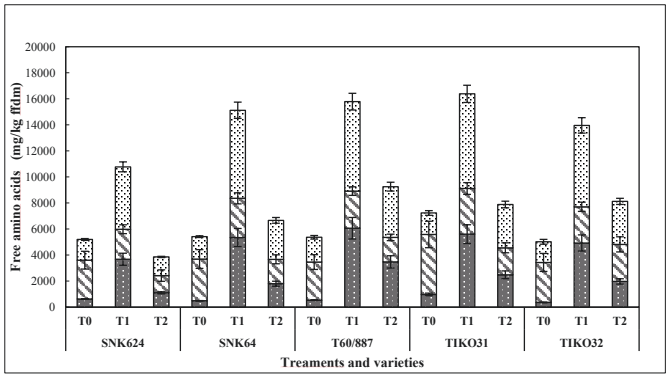


Fig. 2: Distribution of hydrophobic amino acids, acidic amino acids and other amino acids in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$). γ-amino butyric acid (GABA) was detected in all cacao samples but was excluded in the analysis.

mg/kg ffdm (TIKO31). At this stage, it was interesting to note that no significant difference was observed between SNK and TIKO as well as IMC67 and T60/877 varieties (Fig. 1). It is well known that unfermented cacao beans contain free amino acids (KIRCHHOFF et al., 1989), their content varies between 2000 to 4000 mg/kg ffdm (ROHSIUS et al., 2006). However, our results showed that the quantities found in all the varieties used were relatively high. Nevertheless, it cannot be used to determine their potential for future operations because it is well established that unfermented cacao beans do not present any aromatic compound during roasting and do not generate any cacao flavor (LOPEZ, 1986; PUZIAH et al., 1998; HURST et al., 2011). In addition, the percentage of the hydrophobic amino acid group was relativity lower than the acidic group (Fig. 2). According to Kirchoff's classification, the average ratio of hydrophobic/acidic/other amino acids is 31% / 59% / 10%. The ratio obtained showed the predominance of free acidic amino acids group unlike those reported by PUZIAH et al. (1998) where the third group (other amino acids) exhibited the highest content (30% / 18% / 52%); or those reported by ROHAN (1964) in Malaysian unfermented cacao beans where the hydrophobic are predominant (41% / 26% / 33%). Our results showed that TIKO varieties displayed highest acidic amino acids percentage 62% against 58% and 60% in SNK and IMC67 + T60/877 respectively (Fig. 2). In some particular varieties; IMC67, SNK10, SNK16, SNK40, SNK48 and TIKO31, the percentage of the acidic amino acids was 2 times higher than hydrophobic amino acids (Fig. 2). This difference could be attributed to the genetic background of each variety since analysis was done in unfermented beans. Glutamic acid was the most represented amino acid. Its content represents at least 60% of the total content of acidic amino acids in all the varieties. For hydrophobic amino acids, alanine was the most abundant amino acid. In SNK450 for example, its content represents about 50% of the total hydrophobic group (1205.41 mg/kg ffdm over 2495.19 mg/kg ffdm) (Fig. 2).

After five days of incubation in acetic acid only (Treatment 1; T1), the content of free amino acids considerably increased compared to the contents recorded in unfermented beans (T0). The total amino acid concentration doubled in all the Forastero and TIKO varieties; by 2.84; 2.94; 2.26; 2.78 for IMC67, T60/887, TIKO31 and TIKO32 respectively (Fig. 1). According to the total concentration, the SNK varieties can be divided into two groups: the varieties which doubled their total concentration namely SNK15 (10324.30 versus 4438.92 mg/kg ffdm), SNK48 (13080.60 versus 6502.49 mg/kg ffdm), SNK624 (10776.26 versus 5198.05 mg/kg ffdm) and SNK64 (15122.72 versus 5415.46 mg/kg ffdm); and those which registered a simple increase namely SNK10 (+ 5%), SNK16 (+ 30%), SNK377 (+ 48%) and SNK450 (+ 8%) (Fig. 2). The abundance of free amino acids observed is mainly due to the content of the hydrophobic amino acids which considerably increased during incubation (Fig. 2). The average ratio of hydrophobic / acidic / other was 43% / 21% / 36%. In addition, the varieties showing an exponential increase also displayed a three to four times higher content of hydrophobic amino acids. Their concentrations in Leu and Phe particularly increased in all the treatments compared to those of unfermented beans (T0). Leu was almost 10-fold higher in SNK10 (1338.15 against 137.48 mg/kg ffdm), SNK48 (1778 against 164.92 mg/kg ffdm), SNK64 (2087.51 against 217.01 mg/kg ffdm); SNK624 (1382.02 against 125.86 mg/kg ffdm) and SNK16 (1068.03 against 182.86 mg/kg ffdm). Phe was 4-8-fold higher in SNK15 (999.52 versus 194.83 mg/kg ffdm); SNK450 (953.07 versus 165.89 mg/kg ffdm); SNK48 (1335.51 versus 323.25

mg/kg ffdm); SNK624 (1116.31 versus 255.01 mg/kg ffdm) and SNK64 (1691.42 versus 208.22 mg/kg ffdm). The same trends was observed between the TIKO varieties where the hydrophobic amino acids were three and four times more abundant; 7267.80 in T1 versus 1651.21 mg/kg ffdm in T0 and 6251.20 in T1 versus 1585.24 mg/kg ffdm in T0 for TIKO31 and TIKO32 respectively. Leu and Phe were also particularly abundant; 17 and 13 times for Leu; 7 and 6 times for Phe in TIKO31 and TIKO32 respectively (Supplement 2).

As observed in the hydrophobic amino acids, incubation in acetic acid (T1) resulted in an exponential increase in the total content of the third group of amino acids (named other amino acids). In SNK15 and IMC67 particularly, this increase was 18-fold greater than (T0); 5979.29 mg/kg ffdm versus 318.73 mg/kg ffdm and 3941.47 versus 215.69 mg/kg ffdm respectively. TIKO32 and TIKO31 showed a similar evolution with an increase of 13 and 5-fold respectively (Supplement 1 and 2). Interestingly, the relative abundance of other amino acids increased compared to T0: 38% versus 4% (SNK15); 35% versus 7% (TIKO32); and 33% versus 7% (SNK16); 32% versus 7% (SNK620). Although this quantitative leap was induced by all the amino acids belonging to this group, Trp, Arg and Lys amino acids have a significant contribution. The latter represent approximately 63%, 79%, 75% and 78% of the total amount recorded in this third group in SNK15, SNK16, SNK620 and TIKO32 respectively. In addition, it should be noted that Trp, Thr and Lys which were not detected in unfermented cacao beans (T0) in IMC67, SNK10, SNK15, SNK16, SNK450, SNK620, SNK64 and TIKO32 were present after incubation (Supplement 2).

After beans incubation in lactic acid for two days followed by three days in acetic acid (treatment 2; T2), half of the varieties showed a lower amount of total free amino acids compared to unfermented cacao (Fig. 1). These latter varieties belong exclusively to SNK subgroup (SNK15, SNK16, SNK377, SNK450, SNK620, and SNK624). For the rest of the varieties, the increase recorded ranged between 1.08-fold (TIKO 31; 7883.85 versus 7234.08 mg/kg ffdm) – 2.16-fold (SNK10; 4684.98 versus 10129.96 mg/kg ffdm) higher than T0. The average ratio hydrophobic/ Acidic/ other was 42% / 28% / 30% (Fig. 2). The concentration of hydrophobic amino acids increased in all varieties excepted in some SNK varieties: SNK16 (-12.55%), SNK377 (-13%) and SNK624 (-10%) compared to T0. This reduction is mostly due to a lower content of Leu and Phe in these varieties. The amount of the other free amino acid group increased during incubation compared to T0; +20% (30% in T2 versus 10% in T0) and slightly decreased compared to T1 -6% (30% in T2 versus 36% in T1). As in T1, the content of all the amino acids belonging to this third group increased. The appearance of Lys, Trp and Thr mostly contributed to this increase. Arg also contributed significantly, its concentration was approximately 11 times higher in SNK10 in treatment 2 after incubation (1364.96 versus 124.23 mg/kg ffdm) (Supplement 3). Compared to T0, the percentage of the acidic amino acid decreased by half (28% in T2 versus 59% in T0). However, this residual rate remains relatively high compared to T1 (28% versus 21%). It is important to note that the respective contents of all the amino acids in this group dropped during this incubation. Moreover, as found in unfermented cacao beans, glutamine does not appear in IMC67, SNK15, SNK16, SNK377, SNK620 and SNK624 varieties (Supplement 3).

The changes in relative concentration of free amino acids were observed in both incubations. The free amino acids found originated from those initially present in unfermented cacao beans and mostly from proteolysis during incubation. Thus, the increase observed in incubated beans compared to unfermented beans is exclusively due to the penetration of the acids. Biehl and Passern (1982) demonstrated that only the acidification (with acetic acid) can promote proteolysis in cotyledons of cacao beans. Moreover, more studies have well demonstrated that the acidification of the cotyledons creates adequate conditions for the endoprotease activities of cacao beans. The amount of free amino acids depends on the pH within the cotyledons and therefore on the flow of acid entering (Voigt et al., 2018). Thus, the variations in total amounts of free amino acids observed between the two treatments in the same variety may be entirely due to the type and the flow of the organic acid in the cacao beans during incubation. We can assume that the two types of chronology of the acids used (treatment 1 and treatment 2) do not allow the same flow and consequently the acidification results in each case does not lead to the same pattern of proteolysis. Andersson et al. (2006) investigated the transport characteristics of the seed coat of *Theobroma cacao* and found that certain structures strongly influence the course of transport processes in the mature seed. These authors suggested that flavour precursor development in seeds is dependent to transport kinetics of water and solutes into the seeds during fermentation process. Our results showed that, incubation with acetic acid from the two first days (T1) would promote better proteolysis with a net increase of +67% compared to the average content of free amino acids released in T2. However, only the hydrophobic ratio on the rest of the amino acids is decisive.

The total amounts of amino acids recorded in incubated beans were close to those reported by Puziah et al. (1998) through natural fermentation and Kirchhoff et al. (1989) using fermentation-like incubation. These similarities highlight the fact that the experimental design well-mimicked natural fermentation and remains a useful tool of deep-understanding for the main role of lactic and/or acetic acid on biochemical reactions occurring inside the beans during fermentation process.

The ratio of hydrophobic on the rest of the amino acids (acidic and other amino acids) recorded ranged between 0.29 (TIKO31; Treatment 0) and 0.96 (SNK15; Treatment 2). Excepted SNK337, and T60/887 with 0.66 and 0.54 respectively, all the unfermented cacao beans (treatment 0) exhibited a ratio below 0.5. For T1 and T2, the ratio ranged between 0.5 - 1. However, the varieties used do not show a similar hierarchy between ratio in T1 (RT1) and T2 (RT2). Excepted for SNK624, the rest of SNK varieties showed higher ratio value in T2 than T1. On the other hand, TIKO, IMC67 and T60/877 varieties recorded highest value in T1 (Fig. 3). The difference observed between RT0 and RT1 and between RT0 and RT2 inside the varieties clearly revealed the impact of the fermentation process on hydrophobic free amino acids released and the acidic amino acids decreasing. Moreover, it highlighted the crucial role of acidification on aroma precursor's behavior during incubation or fermentation. The accumulation of hydrophobic free amino acids is explained by the cleavage characteristics of two proteases of cacao beans. The aspartic endoprotease (E.C. 3.4.23) attacks the storage proteins preferentially at sites of hydrophobic amino acids and the carboxypeptidase (E.C. 3.4.16) releases single hydrophobic amino acids (Biehl et al., 1993; Voigt et al., 1994). As a result of the different pH and temperature optima of these enzymes, proteolysis primarily depends on the fermentation conditions: duration and intensity of acidification, temperature and aeration (Ziegleder and Biehl, 1988). Thus, the total amount of free amino acids and oligopeptides liberated during hydrolysis varies considerably (De Witt, 1957; Biehl and Passern, 1982; Biehl et al., 1985; Rohsius et al., 2006). The fact that RT1 is relatively lower than RT2 in some SNK varieties is mostly due to the low rate of acidic amino acids disappearance observed in T1. The data collected revealed that T1 released more hydrophobic amino acids but few acidic amino acids are lost. The hydrophobic amino acids are the only free amino acids that contribute to chocolate flavours during roasting (Mulono et al., 2016). Thus, the ratio expressed may indicate the useful free amino acids fraction of marketed cacao samples. According to the latter results, it is obvious that for SNK and TIKO varieties, the early production of acetic acid during fermentation process is necessary for a better yielding of amino acids

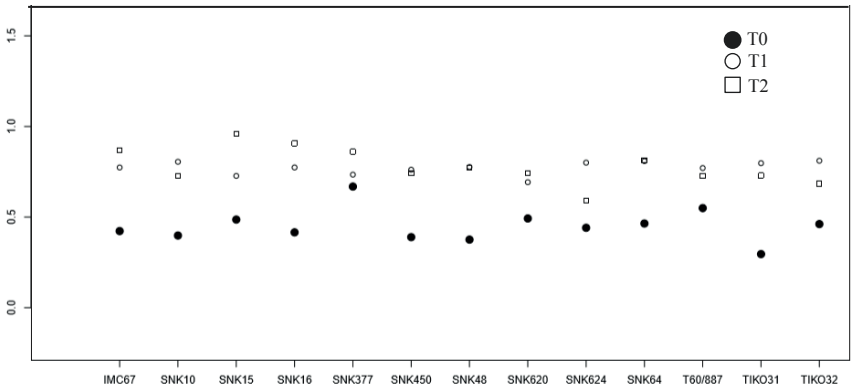


Fig. 3: Ratio of hydrophobic amino acids / acidic amino acids + other amino acids in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$). γ-amino butyric acid (GABA) was detected in all cacao samples but was excluded in the analysis.

especially free hydrophobic amino acids. Therefore, acidification of cacao beans cells by acetic acid and its driving force in lowering the cell pH impact strongly the protease activity, the free amino acid and oligopeptides release during the fermentation process.

Sugars content

In unfermented cacao beans, the concentration of sucrose ranged between 53.20 mg/g ffdm (TIKO31) – 35.56 mg/g ffdm (T60/887). These initial concentrations showed a high variability among SNK varieties. SNK620, SNK624 and SNK450 displayed higher values with 49.23 mg/g ffdm, 52.34 mg/g ffdm and 49.26 mg/g ffdm respectively. After incubation, the concentration of sucrose dropped drastically. According to the residual concentration of sucrose, the varieties studied can be divided into three groups. The first group, constituted by IMC67, TIKO31, T60/887 and SNK377 where the sucrose was completely degraded during incubation in both treatments. The second group which includes SNK64 and TIKO32, only incubation in T1 allowed 100% of degradation. It is important to note that in this group the degradation of sucrose induced by T2 was above 90%. The last group was exclusively constituted by SNK varieties (SNK10, SNK16 SNK620, SNK624, SNK450 and SNK15), where the residual content of sucrose was found in both treatments. Nevertheless, the value recorded was below 10 mg/g ffdm in both treatments (Fig. 4). It is well established that sucrose is the main disaccharide found in unfermented cacao beans. Data from SNK and TIKO varieties compared to those reported by Reccenius et al. (1972) were two to three times higher (53.20 mg/g ffdm against 18.50 mg/g ffdm). The difference displayed among varieties could be attributed to their genetic background. Since all the varieties were subjected to the same treatments, the percentage of degradation reflected the impact of treatments applied. Unlike the TIKO, IMC67 and T60/877 varieties, in the SNK varieties, the degradation of sucrose was not completed with regards to the treatment. Thus, this latter required proper fermentation practice to release more reducing sugars from sucrose. Limiting factor for the production of aroma relevant Maillard reactions being reducing sugars (Rohsius, 2007).

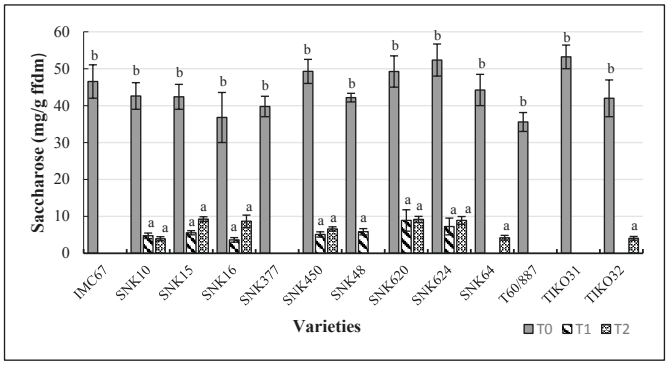
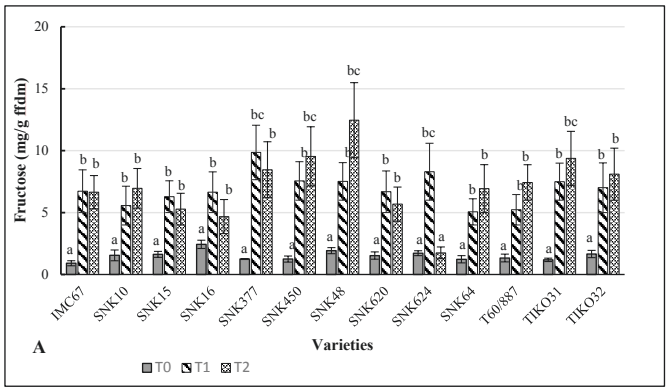


Fig. 4: Sucrose content in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$).

Before subjected to incubation, a small content of glucose was exclusively found in TIKO32 (1.93 mg/g ffdm), SNK16 (3.30 mg/g ffdm) and SNK15 (2.49 mg/g ffdm). After incubation, the concentration of glucose exponentially increased in all the varieties. In T1, it ranged between 8.16 mg/g ffdm (SNK624) – 5.40 mg/g ffdm (SNK10) and between 3.09 mg/g ffdm (SNK624) – 11.39 mg/g ffdm (SNK48) in T2. Excepted in SNK620 and SNK624, the rest of the varieties recorded their highest concentration in T2 (Fig. 5A).

In contrast to glucose, fructose was found in unfermented cacao beans in all the varieties. Its initial content ranged between 0.90 mg/g ffdm (IMC67) to 6.64 mg/g ffdm (SNK16). After incubation, as observed with the glucose released, the concentration of fructose exponentially increased in both treatments. In T1, it ranged between 6.93 mg/g ffdm (SNK377) – 9.85 mg/g ffdm (SNK64) and between 1.74 mg/g ffdm (SNK24) – 12.46 mg/g ffdm (SNK48) in T2. In TIKO varieties (TIKO31 and TIKO32), T2 displayed higher value than T1. After T2 treatment, the concentrations found were 8 times higher than those reported in unfermented cacao beans, while those recorded in T1 were only 5 times higher. As in glucose, T2 released more fructose than T1 in TIKO varieties and in some SNK varieties (SNK16, SNK620, SNK624, SNK337 and SNK15) (Fig. 5B).

Glucose and fructose are the main reducing sugars found in fermented cacao beans. In fermented traded cacao beans, Rohsius et al. (2010) reported a distribution of 0.45 mg/g ffdm : 0.67 mg/g ffdm :



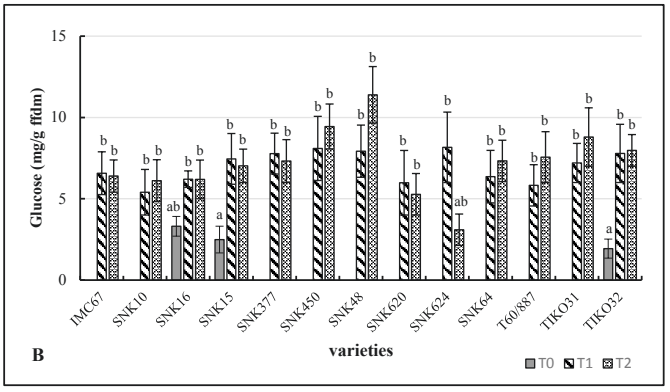
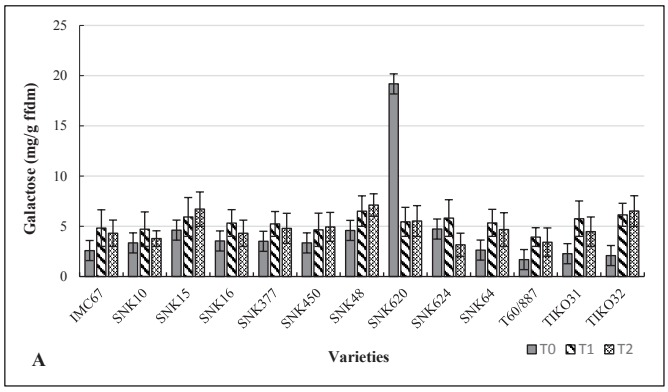


Fig. 5: Fructose (A) and glucose (B) contents in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$).



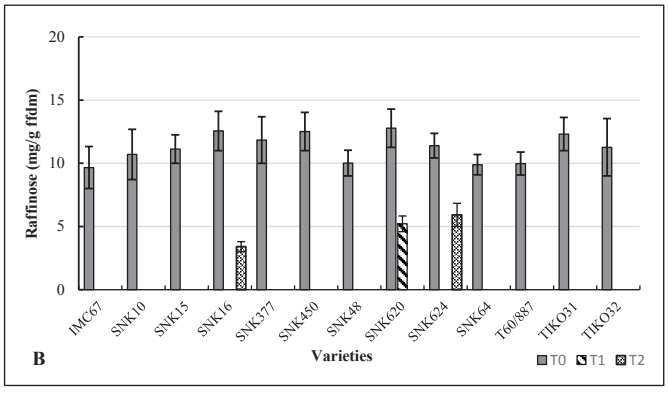


Fig. 6: Galactose (A) and raffinose (B) contents in unfermented (T0) and incubated (T1 and T2) cacao beans from different cacao varieties. T1 treatment consisted of sterile incubation of 25 cacao beans of five individual fruits in 100 mmol/L acetic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. T2 treatment consisted of sterile incubation of 25 cacao beans in 100 mmol/L lactic acid pH 4 for two days following their transfer in 200 mmol/L acetic acid pH 5 for three days and subsequent sun drying. Values are means of n = 3 (three biological replicates based on two technical replicates of extraction). Means with different letters were significantly different by Tukey ($p < 0.05$).

0.04 mg/g ffdm for sucrose : glucose : fructose. They are indispensable aroma precursors. Their carbonyl group is involved in Strecker-type reaction in Maillard reaction during the roasting process. Our results showed the preponderance of T1 over T2 in TIKO, IMC67 and T60/877 varieties. However, all the SNK varieties did not show the same trends. Surprisingly, the trend was the same as observed in the amount of amino acids released. SNK16, SNK620, SNK624, SNK377, SNK15 released more reducing sugars in T1 while SNK64, SNK10, SNK450 and SNK48 released more in T2. However, the content recorded did not correspond to those expected coming from sucrose degradation. This was due to exudation into the medium which occurs during incubation or fermentation.

Galactose and raffinose were also found in unfermented cacao beans. The concentration of raffinose was higher than galactose in all the varieties. It ranged between 12.31 mg/g ffdm (TIKO31) – 9.66 mg/g ffdm (IMC67) while galactose ranged between 4.72 mg/g ffdm (SNK624) – 1.68 mg/g ffdm (T60/887) (Fig. 6A). Contrary to the monosaccharides regularly found in cacao (glucose and fructose), the concentration of galactose did not exponentially increase after the incubation. In both treatments, the concentration recorded in each variety was barely two times higher than those obtained in unfermented cacao beans (against eight times higher in fructose). With the exception in SNK16, SNK620 and SNK624, raffinose disappeared entirely during incubation in the rest of varieties (Fig. 6B).

Raffinose is a trisaccharide containing galactose linked by α-(1-6) bond to the glucose unit of sucrose (Švejstil et al., 2013). This non-reducing sugar is found in many fruits and cereals (Jovanovic-Malinovska et al., 2013; Svejstil et al., 2015). Cerubulis (1955) is the first author who reported the presence of raffinose in the cacao beans followed by Reccenius et al. (1972). In the varieties used for this study, the content of raffinose was two to three times lower than sucrose. Their entire disappearance after incubation was due to enzymatic and/or non-enzymatic degradation into sucrose and galactose. This may justify not only the slight increase of galactose observed in some varieties after incubation but also the lower residual sucrose content found in SNK varieties.

Conclusion

The fermentation-like incubation which well-mimicked the fermentation process allows predicting the behavior of local Cameroonian cacao varieties (SNK and TIKO). The high content of free hydrophobic amino acids and reducing sugars generated in both treatments carried out demonstrated that these varieties have an irrecusable potential of aroma precursors. Thus, our data provide additional knowledge to be considered in the promotion of Cameroonian cacao. Therefore, it will be interesting to set up appropriate natural fermentations according to specific agro-ecological conditions. Otherwise, the results obtained from both treatments showed that the beginning

of fermentation (presence or absence of acetic acid, treatment 1 and treatment 2 respectively) slightly influences the result in SNK and TIKO varieties. Therefore, fermentation process allowing acidification of beans with acetic acid at the early stage is necessary for free amino acid release. Moreover, investigation on the special flavors generated from these varieties during the roasting process and also on the dynamics of the accumulation of these aroma precursors during the fermentation process will be helpful to valorize these widely spread varieties in Cameroonian cacao farms.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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Supplement 1: Hydrophobic, acidic and total free amino acids contents in unfermented cacao beans (T0) of different clones.

AA/Clones	IMC 67	SNK10	SNK15	SNK16	SNK377	SNK450	SNK48	SNK620	SNK624	SNK64	T60/887	TIKO31	TIKO32
Leu	214.92±5.00	114.41±0.51	137.48±8.06	182.86±12.42	336.92±2.54	235.62±1.25	164.92±4.04	142.79±5.68	152.86±3.04	217.01±16.61	177.24±14.54	128.68±5.71	145.77±5.08
Ala	306.29±12.18	347.22±5.38	405.47±29.47	464.47±5.08	777.09±1.64	1205.41±11.29	485.11±7.14	727.29±40.59	326.22±7.51	380.42±27.31	542.71±40.94	481.39±11.10	616.73±10.90
Phe	246.65±1.73	222.96±1.01	194.83±15.28	306.08±0.02	537.99±0.57	165.89±2.26	323.25±3.97	380.39±15.57	255.01±2.34	208.22±16.59	289.08±18.24	229.81±3.40	218.22±17.89
Tyr	278.07±3.66	340.33±5.01	315.93±26.76	458.59±8.39	685.90±1.08	281.03±4.66	418.62±3.40	589.93	351.21±3.19	276.99±19.14	419.15±28.76	521.63±1.12	320.65±5.58
Val	318.09±5.90	126.36±1.37	201.09±14.18	228.98±3.01	361.46±0.14	318.63±0.44	203.39±0.98	212.14±10.24	265.59±3.16	329.61±23.61	248.44±15.87	145.75±3.91	142.94±9.64
Ile	286.92±5.26	183.01±0.22	197.33±13.94	189.65±2.23	352.14±0.20	288.59±1.00	204.73±1.88	199.66±7.57	239.23±3.16	305.47±23.04	226.15±14.41	143.92±3.17	140.90
Total hydrophobic	***1650.97±38.47***	***1334.33±101.90***	***1452.14±99.00***	***1830.66±128.86***	***3051.52±189.73***	***2495.19±390.43***	***1800.04±130.79***	***2252.22±237.28***	***1590.15±70.02***	***1717.75±66.46***	***1902.79±137.58***	***1651.21±179.31***	***1585.24±186.28***
Asp	185.48±5.68	222.98±2.96	295.99±24.66	288.31±1.54	92.17±0.57	283.87±3.46	199.46±5.21	148.46±6.81	173.02±0.56	248.90±15.66	143.27±8.87	282.05±9.04	131.67±0.95
Glu	2090.72±119.16	1434.38±80.60	1751.63±148.47	1655.01±20.66	1090.20±3.67	2132.30±20.57	2363.19±28.51	2095.57±115.99	1693.42±8.98	1789.89±128.06	1287.61±90.57	1483.64±11.42	1636.01±1.24
Asn	991.48±16.04	672.14±7.33	723.46±55.99	1676.67±21.90	1168.89±5.56	3154.16±2.92	1272.36±14.58	1529.79±96.26	783.82±9.23	880.15±75.09	1141.76±66.45	2441.65±7.34	1004.53±4.42
His	317.81±8.69	337.76±3.72		340.41±5.74	643.03±87.96		336.30±7.95	433.65±18.25	332.94±5.00	285.27	353.55±34.58	397.46±9.10	298.99±24.47
Total acidic	***3585.51±870.94***	***2667.28±546.03***	***2771.09±748.19***	***3960.41±780.61***	***2994.30±495.10***	***5570.34±1454.84***	***4171.32±1001.08***	***4207.48±915.80***	***2983.21±682.63***	***3204.22±719.94***	***2926.20±567.58***	***4604.80±1016.41***	***3071.22±691.44***
Thr					156.17±0.357	125.64±3.45	86.81		128.08				
Trp		356.88±15.22			532.19±2.51							334.29±9.89	
Gly	102.97±12.75	85.39±2.27	103.02±4.92	121.38±3.43	141.42±5.55	169.88±5.49	125.35±6.48	103.62±7.11	107.91±3.43	117.95±3.48	130.53±17.77	108.41±14.99	120.56±6.96
Arg	121.60±4.14	124.23±0.26	112.67±7.71	210.27±3.23	408.26±8.50	240.11±1.86	175.84±4.81	160.06±8.42	109.67±0.82	137.68±6.84	142.55±12.17	259.33±3.93	149.38±2.38
Ser	94.15±15.78	116.85		112.30±6.43	129.44±1.43	135.43±7.74	102.69±0.58	102.79±7.24	107.59±47.12	112.26±4.49	102.49±29.09	110.70±22.02	94.02±7.53
Lys					*203.71±0.18*	*167.27±5.84*	*130.41±0.10*		*171.41*	*125.57*	*160.79±9.83*	*165.32±38.82*	
Total other	***318.73±14.01***	***683.37±125.16***	***215.69±6.820***	***443.97±54.13***	***1571.22±167.99***	***838.35±44.88***	***621.12±33.79***	***366.48±32.83***	***624.67±27.35***	***493.48±10.99***	***536.38±24.46***	***978.06±98.77***	***363.98±27.68***
Total free AA	***5555.23±923.42***	***4684.98±773.10***	***4438.92±854.01***	***6235.05±963.60***	***7617.05±852.82***	***8903.89±1890.15***	***6592.49±1165.66***	***6826.19±1185.91***	***5198.05±780.01***	***5415.46±797.39***	***5365.38±729.63***	***7234.08±1294.50***	***5020.45±905.41***
% AA hydrophobes	***29.71***	***28.48***	***32.71***	***29.36***	***40.66***	***28.02***	***27.30***	***33.00***	***30.59***	***31.71***	***35.46***	***22.82***	***31.57***
% AA acidic	***64.5***	***56.93***	***62.42***	***63.51***	***39.31***	***62.51***	***63.27***	***61.63***	***57.39***	***59.16***	***54.53***	***63.65***	***61.17***
% AA Others	***5.75***	***14.59***	***4.87***	***7.13***	***20.63***	***9.42***	***9.43***	***5.37***	***12.02***	***9.13***	***10.01***	***13.53***	***7.26***

Supplement 2: Hydrophobic, acidic and total free amino acids contents in cacao beans of different clones after 5 days of fermentation-like incubation in treatment 1.

T1													
AA/Clones	**IMC 67**	**SNK10**	**SNK15**	**SNK16**	**SNK377**	**SNK450**	**SNK48**	**SNK620**	**SNK624**	**SNK64**	**T60/887**	**TIKO31**	**TIKO32**
Leu	2165.31±112.66	703.92±20.14	1388.15±28.521	1068.03±18.82	1474.76±88.16	1239.40±25.16	1778.00±90.49	784.65±0.30	1382.02±1.57	2087.51±32.80	2186.07±6.73	2278.03±88.84	2016.20±39.84
Ala	646.51±0.66	283.60±13.69	481.99±9.38	318.23±4.87	482.79±4.62	515.00±0.620	669.64±13.19	314.50±2.67	477.00±4.06	757.20±10.80	775.88±0.73	916.86±24.05	782.72±4.52
Phe	1708.02±41.53	435.80±13.26	999.52±27.18	849.24±20.69	1164.81±26.70	953.07±5.21	1335.51±25.23	664.93±11.49	1116.31±4.89	1691.42±35.49	1643.15±5.99	1734.24±64.97	1478.48±5.43
Tyr	1068.88±0.11	339.99±12.58	696.83±18.50	594.78±13.55	808.39±10.43	645.41±1.01	856.06±23.81	504.22±25.92	788.71±6.18	1004.14±13.45	1018.24±5.41	1029.67±35.16	899.07±2.25
Val	773.63±22.25	269.64±9.45	469.64±8.39	400.80±9.65	495.01±9.15	484.33±4.89	636.57±11.22	357.26±3.79	602.25±2.94	719.99±16.44	736.42±2.87	785.05±29.72	640.36±3.06
Ile	542.25±22.76	169.43±3.47	310.80±7.13	286.00±2.21	331.59±12.70	334.42±4.41	439.97±12.64	246.98±0.57	424.44±2.90	503.61±6.73	510.05±4.46	523.94±20.71	434.35±2.41
Total hydrophobic	***6904.62±650.12***	***2202.39±186.78***	***4346.97±402.92***	***3517.10±315.50***	***4757.39±447.48***	***4171.65±338.30***	***5715.76±505.95***	***2872.56±211.82***	***4790.75±380.36***	***6763.89±624.06***	***6869.83±640.99***	***7267.80±661.09***	***6251.20±592.92***
Asp	259.22±0.25	97.67±5.11	177.68±2.87	156.60±7.64	191.98±7.18	217.63±1.06	257.15±8.11	171.28±2.38	181.99±9.14	253.30±7.28	261.57±0.51	271.33±1.78	231.51±7.51
Glu	1080.86±20.00	549.63±14.35	813.27±40.99	656.03±14.60	791.16±59.87	877.13±3.671	1128.73±11.13	999.00±12.51	897.27±11.00	946.03±29.57	1020.10±52.14	1108.32±10.91	959.40±27.79
Asn	900.86±6.98	304.74±11.29	625.18±9.260	461.03±4.35	658.10±8.21	644.73±8.60	1071.09±34.05	717.25±20.05	817.15±0.46	1231.37±19.388	878.37±19.07	1196.48±10.89	985.75±11.46
Gln	704.35±21.62		419.71±13.21	273.87±4.79	456.61±4.50	378.21±1.57	426.93±21.76		410.13±10.71	578.84±48.45	692.05±2.37	696.96±16.88	602.20±45.15
His				307.91								231.76	
Total acidic	***2945.30±353.28***	***952.05±226.24***	***2035.85±273.14***	***1855.46±192.82***	***2097.87±260.85***	***2117.72±291.136***	***2883.91±443.63***	***1887.54±420.83***	***2306.56±338.68***	***3009.55±426.66***	***2852.10±329.58***	***3504.87±451.726***	***2778.88±354.90***
Thr	363.25±5.03	100.64±5.32	429.43±283.68	153.78±0.30	239.61±2.16	206.91±0.37	300.59±3.76	145.20±6.82	250.09±6.61	330.12±6.01	366.65±3.05	372.77±21.59	329.84±1.16
Trp	974.49±124.86	580.53	409.67±327.01	559.77±29.35	732.40±13.89	630.94±21.43	724.44±39.50	582.41±62.34	631.98±22.89	808.71±8.62	874.37±5.10	793.28±82.81	741.69±21.03
Gly	202.03±1.31	90.42±5.43	684.94±744.09	124.14±0.29	159.45±6.15	136.42±2.85	197.51±0.73	111.75±0.82	161.74±0.07	199.24±0.50	216.35±4.64	230.62±12.56	210.43±1.73
Arg	2137.24±25.07	456.30±22.19	1372.30±28.38	850.88±15.10	1595.39±20.30	1141.24±6.86	1420.83±16.36	626.24±5.21	1252.69±15.59	1863.64±41.88	2083.87±16.96	1817.84±70.80	1618.03±19.62
Ser	400.17±3.78	107.28±15.19	236.58±27.56	166.16±13.72	236.34±6.038	230.54±18.38	277.89±9.54	135.66±5.81	247.65±5.81	354.99±16.16	449.34±9.38	437.21±32.01	372.13±2.27
Lys	1781.38±58.63	447.73±8.26	700.69±649.74	728.90±38.31	1290.78±3.56	943.97±16.49	1304.66±3.68	493.19±17.35	1046.17±5.68	1590.78±22.89	1962.08±31.31	1807.49±80.71	1518.67±9.30
Met	120.70±27.26		107.82±4.60	106.13±7.91	127.84±13.40	70.77±4.08	254.96±47.04	165.38	88.59±2.12	201.77±37.36	109.09±1.51	149.61±62.02	138.95±47.16
Total other	***5979.29±809.40***	***1782.92±221.67***	***3941.47±417.10***	***2689.78±319.64***	***4381.86±599.99***	***3360.82±427.70***	***4480.91±524.24***	***2259.85±232.52***	***3678.947±463.23***	***5349.27±693.46***	***6061.78±826.90***	***5608.85±720.23***	***4929.77±620.96***
Total free AA	***15829.22±1812.82***	***4937.37±634.70***	***10324.30±1093.18***	***8062.35±827.97***	***11237.13±1308.32***	***9650.20±1057.14***	***13080.60±1473.83***	***7019.96±865.17***	***10776.26±1182.28***	***15122.72±1744.19***	***15783.71±1797.48***	***16381.53±1833.05***	***13959.86±1568.79***
% hydrophobic AA	***43.61***	***44.60***	***42.10***	***43.61***	***42.33***	***43.22***	***43.22***	***40.91***	***43.22***	***44.72***	***43.52***	***43.18***	***44.78***
% Acidic AA	***18.60***	***19.28***	***19.71***	***23.01***	***18.46***	***21.94***	***22.04***	***26.88***	***21.40***	***19.90***	***18.06***	***21.39***	***19.90***
% others AA	***37.79***	***44.6***	***38.19***	***33.38***	***39.21***	***34.84***	***34.74***	***32.21***	***35.38***	***35.58***	***38.42***	***35.43***	***35.32***

Supplement 3: Hydrophobic, acidic and total free amino acids contents in cacao beans of different clones after 5 days of fermentation-like incubation in treatment 2.

AA/Clones	IMC 67	SNK10	SNK15	SNK16	SNK377	SNK450	SNK48	SNK620	SNK624	SNK64	T60/887	TIKO31	TIKO32
Leu	827.99±0.11	1326.41±59.01	626.05±0.44	424.90±9.91	755.21±24.23	1087.52±59.48	1386.40±73.77	731.12±1.45	273.61±13.06	875.18±55.71	1172.66±35.12	990.29±44.80	917.63±19.37
Ala	429.75±1.09	401.13±12.98	313.66±0.18	219.47±0.38	390.163±9.22	492.18±4.73	554.20±0.50	311.54±0.21	262.14±12.39	508.38±6.37	466.85±12.02	521.99±10.56	612.05±16.66
Phe	588.10±7.86	1042.85±22.85	426.60±3.91	291.97±3.75	571.94±17.37	846.29±10.82	1049.10±11.76	645.95±4.02	241.48±10.38	590.47±16.81	927.01±33.36	667.58±9.69	643.89±19.33
Tyr	402.64±5.50	738.96±19.77	341.51±2.82	308.10±3.70	422.22±9.63	575.58±3.98	713.61±6.69	478.59±3.90	289.23±11.01	418.72±5.47	632.04±13.24	501.19±3.18	512.49±12.99
Val	358.76±1.79	453.84±9.18	251.45±2.14	198.05±1.51	317.04±8.68	390.58±4.95	476.68±1.43	344.42±3.05	192.05±8.99	358.19±12.78	426.65±13.72	393.55±3.48	379.88±9.83
Ile	220.24±0.62	304.570±8.67	156.89±1.08	158.40±0.68	194.53±5.35	267.67±8.23	347.27±7.56	242.40±3.67	172.05±8.45	241.06±9.89	272.82±7.16	249.64±8.22	235.47±4.27
Total hydrophobic	***2827.50±211.19***	***4267.78±404.22***	***2116.18±161.50***	***1600.917±95.97***	***2651.12±197.44***	***3659.84±304.71***	***4527.28±392.65***	***2754.04±195.53***	***1430.59±46.78***	***2992.02±220.34***	***3898.05±339.38***	***3324.27±255.12***	***3301.44±235.40***
Asp	110.90±0.80	199.47±2.42	90.34±2.11	79.33±0.07	113.94±2.00	157.51±1.88	206.78±2.39	141.34±0.24	61.41±2.92	122.74±2.16	151.76±1.81	151.09±3.94	152.12±4.05
Glu	727.29±35.92	789.48±25.44	661.84±3.96	692.42±31.69	719.36±63.17	805.04±54.27	978.62±25.88	960.48±37.10	923.34±21.51	848.09±27.13	698.00±15.73	1006.75±0.30	1327.92±41.03
Asn	644.19±0.79	540.75±1.96	323.33±7.83	347.73±3.87	441.21±15.80	662.60±21.02	807.10±9.93	570.90±4.19	338.45±23.68	663.53±17.95	635.09±21.27	627.42±4.26	1084.12±38.38
Gln		396.96±18.68				339.70±1.07	341.08±25.94			241.81	396.33±0.00	288.29±4.65	282.77±6.69
Total acidic	***1482.39±334.47***	***1926.67±248.36***	***1075.52±287.36***	***1119.50±307.33***	***1274.52±303.04***	***1964.87±295.62***	***2333.60±368.23***	***1672.73±409.73***	***1323.22±440.03***	***1876.19±343.05***	***1881.20±248.95***	***2073.56±382.20***	***2846.95±581.81***
Thr	112.39±0.53	210.42±7.90	92.08±1.45		119.71±0.41	177.67±0.84	229.87±6.66	141.71±3.42	177.07	133.56±3.46	197.98±10.89	165.79±8.03	142.06±5.40
Gly	106.64±6.50	144.28±0.96	89.67±5.64	79.85±2.40	112.01±2.12	148.13±2.30	171.46±2.25	125.31±9.73	83.74±3.39	121.29±1.78	162.89±6.59	150.80±10.83	134.54±3.87
Ser	137.88±6.62	233.13±8.06	89.56	81.25	104.04±4.70	169.38±0.31	202.59±0.65	131.04±1.02	169.60	129.86±0.75	222.42±13.88	196.24±31.94	169.39±4.60
Arg	534.55±0.33	1364.96±47.11	445.21±1.09	257.87±0.59	580.10±16.48	1013.77±3.32	1119.87±2.74	586.17±3.90	191.09±7.79	528.76±11.32	1216.73±54.16	737.23±5.13	568.11±14.10
Lys	539.77±42.59	1181.17±15.49	410.69±22.20	226.93±9.39	488.19±12.65	840.34±59.69	996.02±7.26	448.03±15.95	152.87±12.38	491.71±4.24	1092.49±13.20	821.63±93.63	549.17±15.60
Trp	345.06	673.10			401.23±13.38	547.39±26.63	611.99±18.31	608.23±29.63	328.52±6.607	393.54±11.80	516.64±10.54	414.30±10.43	412.37±19.31
Met		128.40±34.00				69.33±0.47	186.95±14.28				67.15		
Total other	***1776.31±206.54***	***3935.50±521.97***	***1127.23±185.26***	***645.92±94.29***	***1805.31±214.64***	***2966.03±379.43***	***3518.79±410.30***	***2040.51±233.79***	***1102.91±80.26***	***1798.75±193.09***	***3476.33±471.55***	***2486.00±299.64***	***1975.66±205.34***
Total free AA	***6086.21±752.21***	***10129.96±1174.55***	***4318.95±634.14***	***3366.34±497.60***	***5730.96±715.13***	***8590.75±979.77***	***10379.68±1171.20***	***6467.30±839.05***	***3856.72±567.08***	***6666.97±756.49***	***9255.59±1059.90***	***7883.85±936.97***	***8124.05±1022.56***
% hydrophobic	***46.45***	***42.13***	***24.91***	***47.56***	***46.25***	***42.60***	***43.61***	***42.58***	***37.10***	***44.88***	***42.12***	***42.17***	***40.63***
% Acidic	***24.35***	***19.02***	***48.99***	***32.26***	***22.24***	***22.87***	***24.48***	***25.87***	***34.31***	***28.14***	***20.32***	***26.30***	***35.04***
% Others	***29.18***	***38.85***	***26.10***	***19.18***	***31.51***	***34.80***	***33.91***	***31.55***	***28.59***	***26.98***	***37.56***	***31.53***	***24.31***