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Assessing the Impact of Yeast Fermentation in Dry Processing on Coffee Quality from *Coffea arabica* cv. Caltimor, *C. arabica* cv. Bourbon and *C. canephora* cv. Robusta

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Abstract

This study aims to investigate the influence of yeast fermentation and inoculation methods on the quality of coffee across 3 different coffee cultivars. Fermenting *Sacchoromyces cerevisiae* SafAle s-33 on *Coffea arabica* cv. Caltimor via bucket inoculation outperforms spray inoculation across all aspects including microbial cell adhesion, the chemical profile, reducing sugar content and sensory analysis conducted by a Q-grader. The PCA analysis revealed distinct profiles of organic acids and volatile compounds in roasted beans in yeast fermentation and natural fermentation of all 3 coffee varieties. Particularly, volatile compounds such 1-(4-Methoxyphenyl)octan-1-one, 8-Oxabicyclo[3.2.1]oct-6-en-3-one and 5,6-Dimethoxy-2-(2-hydroxyethyl-1-thio)-3 trimethylsilyl methyl-1,4-benzoquinone and, furfuryl ethyl ether and 5-methyl furfural were detected only in yeast fermented bean. The malic acid also contained a higher amount in roasted coffee beans from the yeast fermentation of all 3 coffee varieties than the natural fermentation. Those compounds present a notably positive influence on the sensory aspects of the coffee i.e. sweet caramel, dry fruit, vanilla and hazelnut-like flavors. In addition, roasted beans from the yeast fermentation of *C. arabica* cv. Caltimor and *C. canephora* cv. Robusta displayed higher sensory scores when compared to those from natural fermentation, conversely in *C. arabica* cv. Bourbon, beans from both yeast fermentation and natural fermentation displayed similar quality levels. Our study concluded that the commercial yeast strain within the beer and wine industry proved to be an alternative source for coffee fermentation. Nevertheless, the selection of the coffee variety plays a crucial role when utilizing *S. cerevisiae* SafAle s-33 as a starter.

Keywords: Coffee, Yeast fermentation, *Saccharomyces cerevisiae*, Dry process, HS-GCMS, LC-MS/MS

Introduction

Coffee is one of the most consumed beverages in the world. It is rich in flavor and aroma due to the presence of multiple volatile and non-volatile compounds available inside the coffee cherries and beans [1]. Aromatic types of arabica coffee (*Coffea arabica*) beans provide fine-flavored coffee of various aromas i.e., flower, fruit, honey, chocolate, caramel or even toasted bread. The major volatile compounds associated with the flavor and consequent acceptance of the beverage are 5-hydroxymethylfurfural, benzeneacetaldehyde, 6-methyl-3,5-dihydroxy-4H-pyran-4-one, γ -butyrolactone, Linalool, (3,4-Dihydro-2H-pyrrol-5-yl)-ethanone, (E)-β- Damascenone and 2-acetylpyrrole [2,3].

Different post-harvest processing including dry, wet and semi-dry result in different tastes and quality of coffee due to changes in the metabolic reaction and chemical composition of coffee beans [4-7]. The dry coffee processing is the oldest post-harvest process. In this process, coffee cherries will be washed and spread on the ground to dry by sunlight for 10 to 25 days or until the beans' moisture decreases to 11 - 12 %. Natural fermentation occurs when the pulp and mucilage of coffee are broken down enzymatically by microorganisms [8]. Coffee mucilage contains polysaccharides such as pectin, starch and cellulose, which can prolong the drying process of coffee beans. In some cases, prolonged drying time can increase the possibility of microbial growth, which can impact the final quality of coffee [9]. Yeasts, lactic acid bacteria, and mesophilic bacteria metabolize pulp carbohydrates into organic acids and volatile compounds, which are essential for coffee quality [10-14]. Thus, microbial fermentation is gaining wide popularity as a method for producing specialty coffee [9,15-20]. For instance, fermentation of *C. arabica* cv. Catuaí Amarelo by direct inoculation with *Saccharomyces cerevisiae* CCMA 0543 provided the volatile compounds corresponding to caramel flavor [20]. Similarly, the inoculation of *S. cerevisiae* CCMA 0543 on *C. arabica* cv. Catuaí Vermelho resulted in the presence of several volatile compounds such as 4-ethenyl-1,2-dimethoxybenzene, heptadecanol, 4-hydroxy-2-methylacetophenone and 1-butanol,2-methyl [19]. Fruity flavors including apple and cherry compounds were present in roasted coffee beans following inoculation with *Candida parapsilosis* CCMA 0544 [19].

Dry coffee processing is the least sophisticated process and requires the longest time compared to other processes. Nonetheless, this method provides good quality coffee, which results in higher prices than those generated from semi-dry or wet processing [21]. Our study compared 2 methods 1) direct spray and 2) inoculation in polystyrene buckets to inoculated *S. cerevisiae* SafAle s-33 that are typically used in the beer industry. The microorganism count, the profile of volatile and non-volatile compounds, and their impact on the sensory of 2 coffee varieties of *C. arabica* cv. Caltimor and *C. arabica* cv. Bourbon, and *C. canephora* cv. Robusta was evaluated to reveal the influence of microbial and inoculating methods on the quality of the coffee.

Materials and methods

Coffee cultivars used in the study

The coffee cherries were harvested manually from a coffee farm located in Doi Pangkhon, Chiang Rai, Thailand (latitude: 18° 56'38" S; longitude: 46° 59'33" W with an elevation of 1,250 - 1,500 m above sea level). The coffee cherries of *Coffea arabica* cv. Caltimor (Caltimor) was harvested in November 2021, while cherries of *C. arabica* cv. Bourbon (Bourbon) and *C. canephora* cv. Robusta (Robusta) was harvested in January 2022.

Yeast strain used in the study

Commercial yeast strains *Saccharomyces cerevisiae* SafAle s-33 (Fermentis, Lille, France) that is currently being used in the beverage industry was grown on yeast extract peptone glucose (YEPG) broth medium (20 g/L glucose (Himedia, Mumbai, India), 10 g/L peptone soy (Gibco, MI, USA) and 10 g/L yeast extract (Gibco, MI, USA)). The cultures were incubated in a shaker incubator at 30 °C, 120 rpm for 48 h. The cells were recovered by centrifugation (8,000 rpm for 10 min) and then resuspended in sterile distilled water by incremental volumes of sterile distilled water until reaching 1×10^7 cells/mL [19].

Yeast inoculation

Coffee cherries were washed with water before being used in any fermentation process. For yeast fermentation by spray inoculation, yeast suspension (2.5 L of 1×10^7 cells/mL) was sprayed directly on the coffee cherry and then spread onto the tray. As for yeast fermentation by bucket inoculation, yeast suspension (2.5 L of 1×10^7 cells/mL) was added into 200 L of polystyrene bucket with 50 kg of coffee cherries. The pH in the inoculated bucket was measured every 12 h, after the pH of coffee cherries reached pH 3.0, the inoculated cherries were transferred to a tray. Both control treatments and natural fermentation were performed by substituting yeast suspension with the same amount of sterile water (**Figure S1)**.

There were 4 treatments in Caltimor cherries which were natural fermentation by spraying coffee cherries with sterile water (control; CSC), yeast fermentation by spray inoculation method (CSYI), natural fermentation in bucket inoculated with sterile water (control; CBC), yeast fermentation by bucket inoculation method (CBYI). Only the bucket method was used for yeast inoculation of Bourbon and Robusta. Therefore, only 2 treatments were performed in each cultivar including natural fermentation in bucket method with sterile water in Bourbon (BBC) and Robusta (RBC). Similarly, 2 treatments in yeast fermentation by bucket method in Bourbon (BBYI) and Robusta (RBYI).

Coffee processing

Cherries of all treatments were arranged in layers (5 - 8 cm) to ferment and dried by sun exposure. Cherries were turned in daily. Once moisture reached 30 %, coffee cherries were separated into 2 trays then spread into thinner layers (2 - 3 cm) and dried for 8 - 11 days or until moisture was lower than 11 %. Coffee beans were then hulled to remove the parchment skins and stored at room temperature. The hulled green coffee samples were subjected to medium roasting for 8 min at 220 °C using a coffee roaster (Model: Roest-s100 plus, RØST COFFEE, Norway; **Figure S1**)

Evaluation of microbial population and reducing sugar in fermented coffee beans, Caltimor

Scanning Electron Microscope (SEM) analysis

Five g of cherries from the following treatments: CSC, CSYI, CBC and CBYI were collected at 72 h post-inoculation and stored at −20 °C. The preparation and observation of samples using an SEM were performed at the Scientific and Technological Instrument Centre (STIC), Mae Fah Luang University. The fermented coffee beans were cut longitudinally and immersed in a fixative solution (4 % glutaraldehyde), and then serially dehydrated with ethanol (15 min in each wash) as follows; 25 % ethanol, 30 % ethanol, 50 % ethanol and 70 % ethanol stored at 4 °C overnight and after that, dehydrated with 75 % ethanol, 90 % ethanol and 100 % ethanol. Samples were dried and placed on aluminum stubs covered with aluminum foil with carbon strips placed on top. The dried specimens were sputtered in a gold bath followed by microscopic observation. The stubs with gold were placed on the TESCAN MIRA's SEM. The microscope was turned on and the images were digitally generated and recorded. The images were observed with increasing variables, using a working condition of 5 keV and a 9 - 10 mm working distance.

Quantification of the yeast population

Ten g of cherries from the following treatments: CSC, CSYI, CBC and CBYI were collected at 72, 120 and 168 h post-inoculation. The specific primers for quantitative polymerase chain reaction (qPCR), SC-5fw/(5'-AGGAGTGCGGTTCTTTGTAAAG-3)/SC-3bw (5' TGAAATGCGAGATTCCCCT-3'), were used for yeast quantification. The DNA concentration in the samples was limited to 50 ng per analysis. The qPCR was carried out using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., California, USA). Each reaction comprised 4 µL 5xHOT FIREPol EvaGreen qPCR Mix Plus (ROX; Solis Biodyne, Tartu, Estonia), 10 pmol of each primer (Invitrogen, São Paulo, SP, Brazil), and 2 µL template DNA extracted from coffee beans, for a total volume of 20 µL. The mixture was heated to 95 °C for 12 min, followed by 39 cycles of denaturation at 95 °C for 15 s and annealing/extension at 62 °C for 20 s. The cycling temperature was then increased by 1 °C every 5 s from 65 to 95 °C to obtain the melting curve. For the standard curves were prepared from samples containing a known number of yeast cells. The *S. cerevisiae* SafAle s-33 was cultivated in YEPG broth at 28 °C for 24 h. DNA was extracted using the DNA yeast extraction method and serially diluted (1:10) from 10^3 to 10^8 cells/mL. The qPCR was performed with SC-5fw/SC-3bw as described above. All analyses were performed in triplicate.

Quantification bacterial population

Ten g of cherries from the following treatments: CSC, CSYI, CBC and CBYI were collected at 72, 120 and 168 h post-inoculation. Samples were prepared according to Martinez *et al.* [22] with some modifications. Ten g of fermented coffee cherry were added to flasks with 50 mL of sterile 10 % (w/v) peptone water (Gibco, MI, USA), and incubated in an orbital shaker (200 rpm; 1 h at 28 °C). For bacteria quantification, each culture was diluted 20 times and then plated in triplicate. The population count was determined by spread on selective media for lactic acid bacteria (LAB), mesophilic bacteria, acetic acid bacteria and actinobacteria. The deMan, Rogosa, Sharpe agar (MRS agar; 68.2 g/L (Himedia, Mumbai, India)) and nutrient agar (NA; 28 g/L (Himedia, Mumbai, India)) were used for the quantification of LAB and mesophilic bacteria, respectively. Aaronson's medium (2 g/L potassium nitrate (Loba, Mumbai, India); 0.8 g/L casein (Fluka, Milwaukee, USA); 2 g/L sodium chloride (RCI Labscan, Bangkok, Thailand); 0.04 g/L iron (II) sulfate heptahydrate (Loba, Mumbai, India); 2 g/L dipotassium hydrogen orthophosphate (Qrec, Newzealand); 0.05 g/L magnesium sulfate heptahydrate (Qrec, Newzealand); 0.02 g/L calcium carbonate (VWR, Pennsylvania, USA); 20 g/L agar (Himedia, Mumbai, India)) was used for actinobacteria counts. Glucose, yeast extract and calcium carbonate agar (GYC) media (50 g/L Glucose (Himedia, Mumbai, India); 10 g/L yeast extract (Gibco, MI, USA); 5 g/L calcium carbonate (VWR, Pennsylvania, USA); 20 g/L agar powder (Himedia, Mumbai, India)) was used for acetic acid bacteria counts. The MRS and NA plates were incubated at 37 °C for 2 - 3 days, while GYC and Aaronson's media were incubated at 30 °C for 3 - 7 days.

Quantification of reducing sugar

Three g of cherries from the following treatments: CSC, CSYI, CBC and CBYI were collected at 72 and 216 h post-inoculation. Sample preparation for reducing sugars analysis was performed as described by Evangelista *et al.* [17]. Briefly, the sample was ground into a fine powder in a mortar and pestle with liquid nitrogen. Each sample was homogenized in Falcon tubes with 20 mL of 16 mM perchloric acid and Milli-Q water by vertexing at room temperature for 10 min. Extracts were centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was adjusted pH to 2.11 using a 200 mM perchloric acid solution and re-

centrifuged under the same conditions. The second supernatant was filtered through a 0.22 μm cellulose acetate membrane and stored at −18 °C until analysis.

Reducing sugar concentration was determined using the dinitrosalicyclic acid (DNS) method described by Miller [23]. Generally, 1 mL sample was mixed with 1 mL DNS reagent. The tubes were incubated in a boiling water bath for 5 min and then were cooled at room temperature. Eight mL of distilled water was mixed, and the optical density of the samples was measured at an absorbance of 540 nm. The concentration of 1.0 mg/mL of glucose solution was prepared as a working standard stock solution. The standard glucose curve (0 - 0.2 mg/mL) was plotted and used to estimate equivalent glucose values.

Chemical analysis of fermented coffee beans, Caltimor, Bourbon and Robusta

Organic acids by LCMS

Organic acids in fermented coffee beans from the following treatments: CSC, CSYI, CBC, CBYI, BBC, BBYI, RBC and RBYI were determined by Liquid Chromatograph Tandem Mass Spectrometer (LC-MS/MS). Two sets of samples were used 1) green beans (10 g) that were collected at 216 h post-inoculation; 2) roasted beans from all treatments. The samples were analyzed using Shimadzu Nexera Ultra high-performance liquid chromatography (UHPLC) system equipped with 2 pumps (LC-3-AD), Column Oven (CTO-30) and an Auto-sampler (SIL-30AC). The separation for all samples was achieved by using X-Bridge BEH RP C18 column (2.1×100 mm^2, 2.5 μm waters) - the mobile phase of 10 mM ammonium format in water as solvent A and acetonitrile as solvent B. The gradient elution condition followed: 0 - 3.0 min, 2 - 40 % B; 3.0 - 4.5 min 40 - 95 % B; 4.5 - 5.5 constant 90 % B; 5.5 - 6.0 min 90 - 2 % B and 6 - 12 min constant 2 % B. The Mass Spectrometer was performed on an LC-MS/MS 8060 system (Shimadzu), equipped with an Electrospray Ionization source (ESI) operated in positive electrospray ionization and multi-reaction monitoring (MRM) transition and compound-dependent parameters, such as voltage potential Q1, Q3 and collision of individual analyst. Parameters for MRM detection in the positive mode were as follows: Nebulizer gas: 3 L/min; heating gas: 10 L/min; drying gas: 10 L/min; interface temperature: 400 °C; desolvation line temperature: 250 °C; heat block temperature: 400 °C. Mass transitions were monitored at 50 ms dwell times and unit mass resolutions and individual parameters are listed in **Table S1**. LabSolutions LCMS version 5.90 (shimadzu) was used for data collection and quantitation.

Analysis of volatile compounds by HS-SPME/GC-MS

Volatile compounds were determined by headspace solid-phase micro-extraction (HS-SPME), followed by gas chromatography-mass spectrometry (GC-MS), according to Evangelista *et al.* [17]. Two-gram roasted beans from the following treatment: CSC, CSYI, CBC, CBYI, BBC, BBYI, RBC and RBYI were macerated in liquid nitrogen before being transferred into the sample bottle. The samples were analyzed using an Agilent model 6890 GC interfaced to an Agilent 5973 mass selective detector instrument (Agilent, Santa Clara, United States) with the headspace method. The samples were heated at 80 °C for 30 min under headspace conditions. The samples were passed onto the GC at 40 °C for 6 min, which was ramped to 80 °C at the rate of 3 °C/min and then ramped to 200 °C at the rate of 10 °C/min which was held for 10 min. The runtime for GC was set for 40 min before passing to MS. The volatile compounds were identified by comparing the retention times with those of standard compounds injected under the same conditions of the samples and compared with data from the spectra library (W11N17).

Sensory test by cupping method

The sensory test by a Q-grader followed the guidelines created by the Specialty Coffee Association of America (SCAA). The sample was roasted within 24 h of cupping and allowed to rest for at least 8 h. The roast level for cupping was measured between 30 min and 4 h after roasting using the coffee ground to the SCAA standard grind for cupping and measured at room temperature.

Statistical analysis

Data of volatile compounds were statistically analyzed by principal component analysis (PCA) using an online metaboanalyst program (https://www.metaboanalyst.ca/MetaboAnalyst). A m*n matrix was built with the relative areas, where n was the value of each identified peak of the volatile compound and m was the different treatments of strains plus control.

Results and discussion

Influence of inoculate method on microbial growth and reducing sugar of fermented Caltimor coffee cherries

Fermentation is an important post-harvest process that improves the flavor, aroma and overall sensory quality of coffee. The different microorganisms and inoculation methods could lead to different flavors and aromas of coffee due to the production of volatile compounds and organic acids. Thus, inoculating microorganisms as a starter in fermentation plays an important role in this post-harvest process [8]. Medina-Pradas *et al.* [24] defined starter culture as a living microorganism added to the beginning of fermentation to induce specific changes in the chemical composition and sensory properties. A wide range of microorganisms such as yeasts, filamentous fungi, lactic acid bacteria, acetic acid bacteria and enterobacteriaceae have been used as a starter in coffee fermentation [25-28]. Yeast is one of the most popular starters for coffee fermentation because of its ability to produce enzymes that contribute to the increased concentration of carbon sources and acid. Yeast promotes the degradation of coffee mucilage and pulp by producing pectinolytic enzymes, such as poligalacturonase, pectin lyase and pectin methylesterase. Poligalacturonase catalyzes hydrolyze 1 - 4 glycosidic bonds into pectic acid (poligalacturonic acid), while pectin lyase catalyzes pectin breakage by transelimination, releasing unsaturated galacturonic acids and pectin methylesterase, which is responsible for the de-esterification of the methoxyl group of the pectin, forming pectic acid and methanol [16].

The *S. cerevisiae* SafAle s-33 was used in the dry processing of Caltimor coffee beans with 2 inoculation methods (spray and bucket methods). The SEM demonstrated that yeast and bacteria adhere to the surface of coffee beans (**Figure 1**). After 72 h post-inoculation, coffee from CSYI and CBYI had an abundance of yeast cells. Bacterial cells were also presented in CSYI and CBYI. In contrast, fewer cells of yeast and bacteria were exhibited on CSC and CBC (**Figure 1**). The yeast and bacterial populations in coffee cherries were quantified as expected, *S. cerevisiae* was dominant during the fermentation of both inoculated treatments. At 168 h post-inoculation, the yeast population in CSYI was 6.110 and 7.385 log CFU/g in CBYI treatment, while the population of yeasts in CSC and CBC were only 0.503 and 1.190 log CFU/g, respectively (**Figure 2**). At 168 h post-inoculation, the population of LAB in CSC and CBC only ranged from 0.038 - 0.810 log CFU/g; but a high number of LAB population present in both inoculated treatments (CSYI = 2.579 log CFU/g, CBYI = 2.494 log CFU/g; **Figure 3(a)**). Interestingly, at 168 h post-inoculation, a higher mesophilic bacteria population was observed in both treatments, CSC and CSYI (1.122 log CFU/g), than in 2 treatments of bucket method (CBC = 0.204, CSYI = 0.572 log CFU/g; **Figure**

3(b)). The populations of acetic acid bacteria (0.002 - 0.218 log CFU/g) and actinobacteria (0.018 - 0.315 log CFU/g) were low throughout the fermentation process (**Figures 3(c)** and **3(d)**).

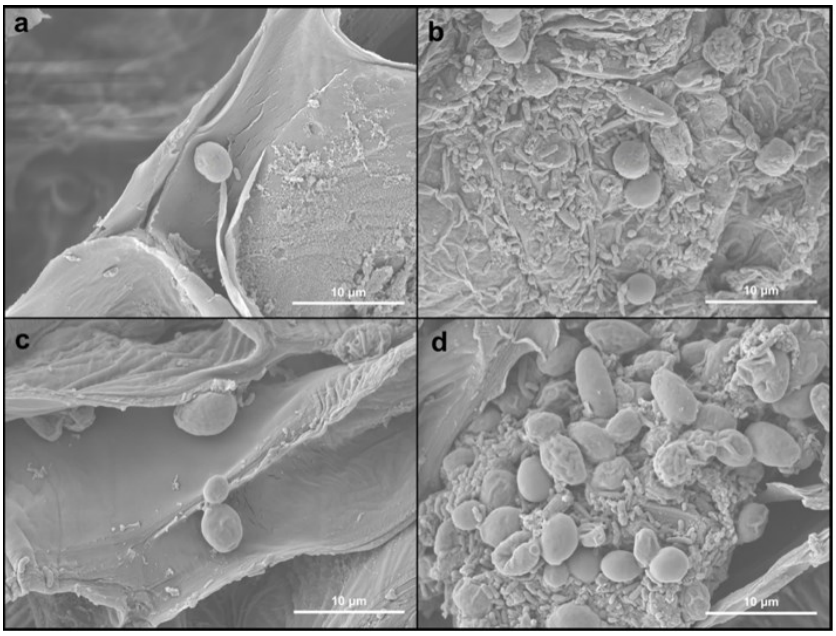


Figure 1 The SEM analysis of Caltimor cherries in (a) CSC; (b) CSYI; (c) CBC; (d) CBYI (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation).

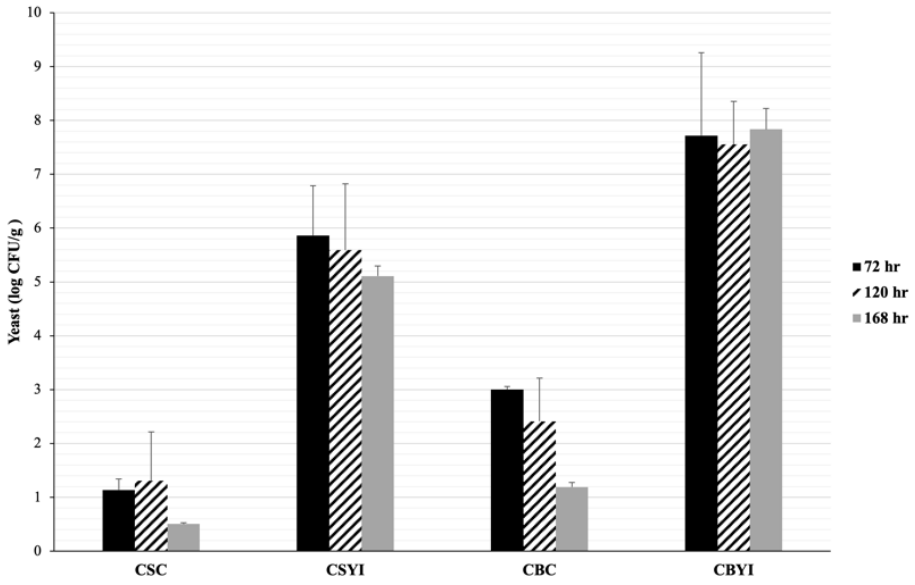


Figure 2 The population of the *S. cerevisiae* SafAle s-33 in Caltimore coffee cherries from 4 fermentation treatments (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation).

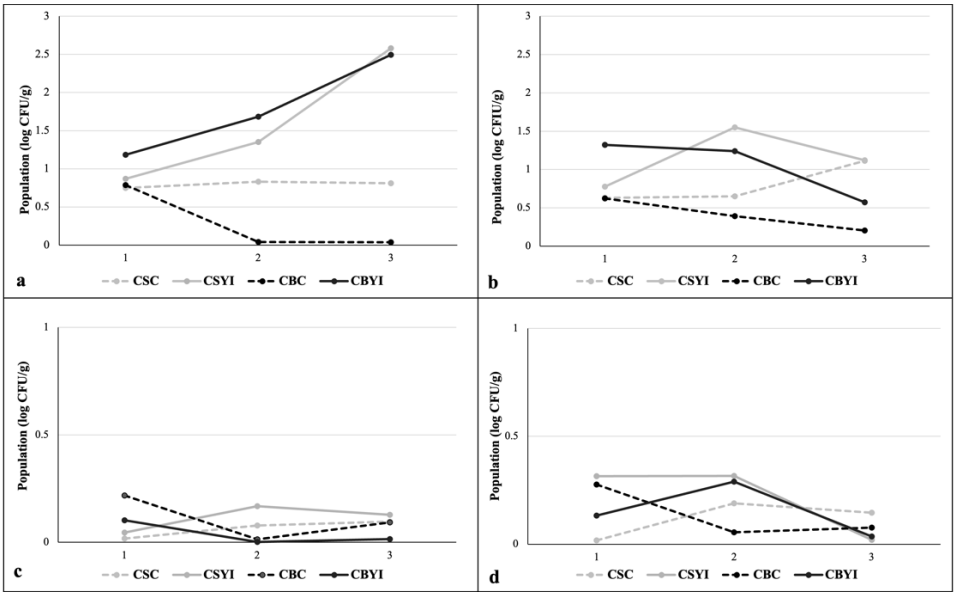


Figure 3 The microbial population of (a) lactic acid bacteria; (b) mesophilic bacteria; (c) acetic acid bacteria; and (d) actinobacteria on Caltimor coffee cherries at 72, 120 and 168 h post-inoculation (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation). The bars represent the standard deviation of the mean.

Various factors such as moisture and temperature of the coffee beans can impact the extent of colonization and the colonizing species [16]. The dominant microbe in this study were yeast and LAB. A high population of *S. cerevisiae* was observed throughout the coffee process (72, 120 and 168 h post-inoculation) and slightly higher in bucket inoculation compared to spray inoculation. This suggested that conditions in a bucket inoculation support the growth *S. cerevisiae* more effectively than spray inoculation. Although, bacteria were not intentionally inoculated into the coffee processing; however, fermentation by yeast using both inoculation methods led to an increase in the LAB population at 120 h post-inoculation and continued increasing at 168 h post-inoculation. In contrast, the LAB population in natural fermentation (CSC and CBC) declined after 120 post-inoculations. This could be attributed to yeast activity, as it breaks down mucilage and releases substances conducive to the growth of LAB. LAB produces an organic acid and a volatile compound that enhances the flavor, aroma and quality of coffee. Ribeiro *et al.* [29] highlighted that *Leuconostoc mesenteroides* CCMA 1105 and *Lactobacillus plantarum* CCMA 1065 produce volatile compounds such as Isovaleric acid; 2,3-butanediol; phenethyl alcohol; β-linalool; ethyl linoleate; and ethyl 2-hydroxypropanoate these compounds have potential to enhance the sensory quality of the brewed coffee. Similarly, yeast diffuses from the pulp to the bean and persists after roasting providing aroma and flavor to the roasted coffee beans [30-32].

At the beginning of fermentation (72 h post-inoculation), the reducing sugar concentration in Caltimor cherries from CBC and CBYI ranged from 2.23 to 2.33 mg/g, which was slightly lower than in the CSC and CSYI (3.90 to 5.27 mg/g; **Figure S2**). However, at 216 h post-inoculation, a similar amount of reducing sugar concentration in both treatments from spray methods was detected in a coffee sample (CSC = 3.90 mg/g and CSYI = 5.27 mg/g; **Figure S2**). In contrast, after 216 h post-inoculation, the increase in sugar concentration was detected in both bucket inoculation methods, especially in the yeast-fermented coffee (22.3 mg/g; **Figure S2**). The increase of reduced sugar content in yeast fermentation coffee by bucket

inoculation throughout the fermentation period may be due to the sugars released from the endosperm to the outside. Sugar is important in the Maillard reaction and caramelization in the roasting process [33]. Therefore, the amount of reducing sugars inside the yeast-fermented coffee bean especially when inoculated by bucket method, influences the coffee beans' color, aroma and flavor during roasting.

Influence of inoculated method on chemical compounds in fermented Caltimor, Bourbon and Robusta coffee beans

Seven organic acids (acetic, citric, lactic, quinic, malic, fumaric and tartaric) were detected in the green beans of all treatments. However, variations in both concentration and profiles of organic acids among the green bean treatments were evident through PCA analysis (**Figure 4**), illustrating distinctions arising from the diverse inoculation methods employed in this study. The sample in CBYI was on the positive side of the principal component (PC1) and (PC2); while CBC was on the negative side of the principal component. On the other hand, CSC and CSYI were clustered next to each other (**Figure 4**).

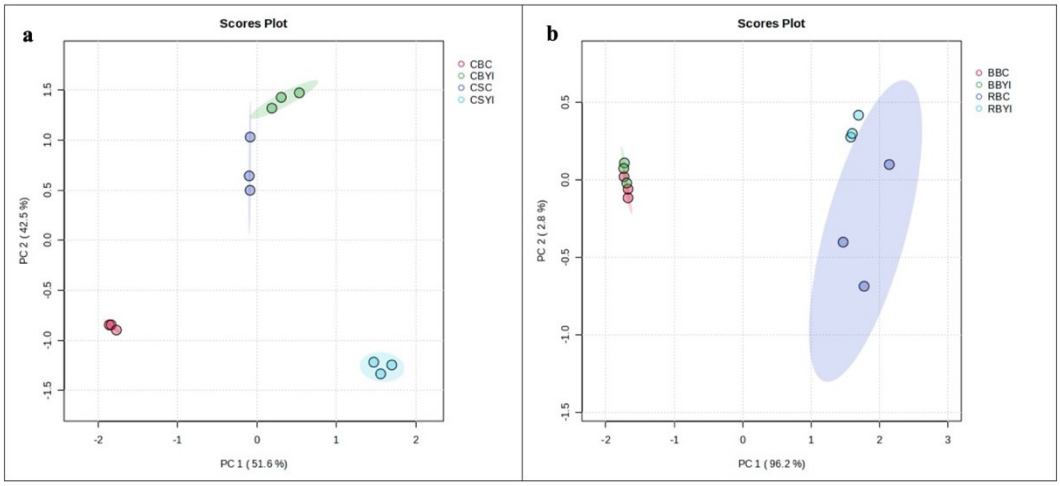


Figure 4 Principle component analysis of the organic acid in green coffee (a) Caltimor beans from CSC, CSYI, CBC and CBYI treatment, (b) Bourbon beans from BBC and BBYI and Robusta from RBC and RBYI (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation; BBC = Bourbon, control in bucket inoculation (natural fermentation); BBYI = Bourbon, yeast fermentation by bucket inoculation; RBC = Robusta, control in bucket inoculation (natural fermentation); RBYI = Robusta, yeast fermentation by bucket inoculation).

Out of 7 organic acids found in green beans, 3 compounds including fumaric, acetic and tartaric acid were absent from roasted beans (**Figures 5** and **6**). The concentration of the organic acids in roasted beans was different among treatments. Citric and lactic were higher in CSC and CSYI. On the other hand, malic acid was present in high concentration in yeast fermentation by bucket inoculation (CBYI = 59.794 mg/mL; CSYI = 47.988 mg/mL; CBC = 38.722 mg/mL; CSC = 34.91 mg/mL; **Figure 6**).

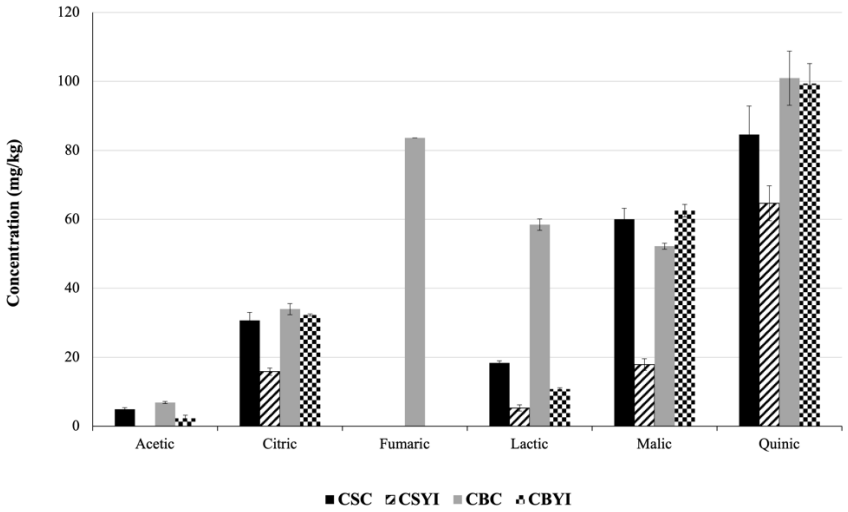


Figure 5 The concentration of acetic, citric, fumaric, lactic, malic and quinic in Caltimor coffee beans at 216 h post-inoculation in CSC, CSYI, CBC and CBYI (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation). The bars represent the standard deviation of the mean.

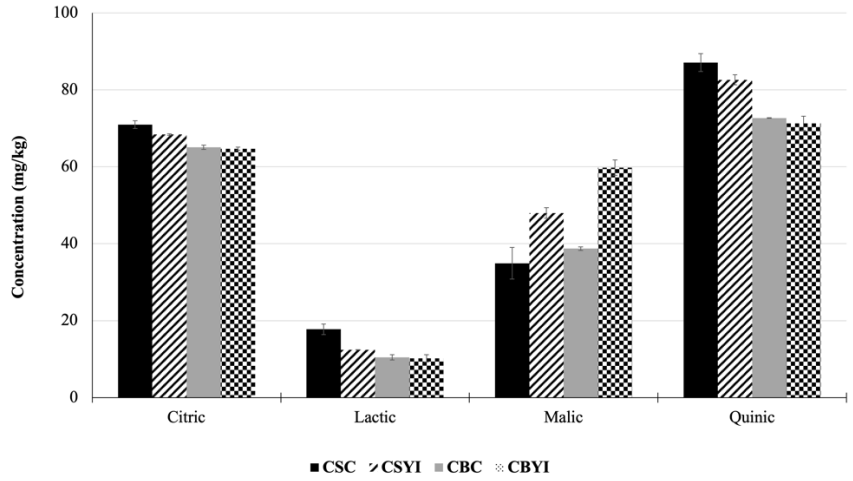


Figure 6 Concentration of citric, lactic, malic and quinin in roasted coffee beans during natural fermentation in CSC, CSYI, CBC and CBYI (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation). The bars represent the standard deviation of the mean.

A total of 68 volatile compounds were detected by SPME/GC-MS, those compounds were classified by their chemical groups including acids, alcohols, aldehydes, ester, furans, furanones, ketones, phenol, pyran, pyrazines, pyridine, pyrroles and others (**Table 1**). In general, CBC and CBYI provided a higher number of volatile compounds compared to CSC and CSYI (**Figure 7(a)**). Furanones and phenol were detected in coffee beans from CBC, but these 2 groups were absent in coffee beans in CBYI. In addition, the number of aromatic compounds, furans and pyran were increased in CBYI, while the amount of alcohol was decreased.

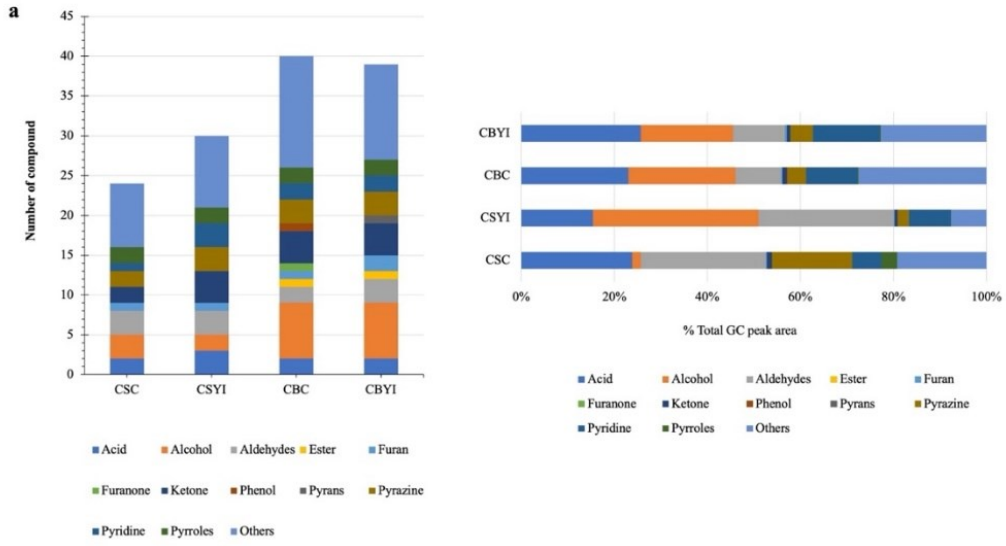
Table 1 Volatile classes identified by headspace GC-MS of roasted coffee beans at different conditions.

Compound	GC peak area %			
	CSC*	CSYI*	CBC*	CBYI*
Acid				
2-Cyclopropyl-3,3-dimethylbutanoic Acid	-	11.20 ± 2.45	25.18 ± 4.23	20.02 ± 2.08
Benzoic acid, 3-(2-furanylmethoxy)-2-nitro-, methyl ester	0.15	-	-	-
Methanesulfinic acid methyl ester	-	3.74 ± 1.80	-	-
Methylenecyclopropanecarboxylic acid	86.40	24.56 ± 13.80	83.78 ± 7.05	71.92 ± 5.52
Alcohol				
(1S,4S)-3,3-Dideuterio-4-methylcyclohexan-1-ol	2.67	-	-	-
(2SR,5RS,3Z)-5-Cyclohexyl-5dimethyl(phenyl) silylpent-3-en-2-ol	0.15	-	-	-
[(1R,2R)-2-(hydroxymethyl)cyclohexyl] methanol	-	-	0.22 ± 0.04	0.08 ± 0.02
1,3-Propanediol, 2-methyl-, dipropanoate	-	-	-	0.18 ± 0.07
1H-Imidazole-4-methanol	-	-	47.85 ± 47.68	0.20 ± 0.11
3-(isopropylamino)butan-1-ol	3.67	2.32 ± 0.00	4.48 ± 0.66	4.90 ± 0.13
6-Methylenebicylo[3.2.1]hoctan-1-ol	-	-	0.13 ± 0.04	0.11 ± 0.01
(2R)-2-(2-iodanylethyl)oxirane	-	-	0.35 ± 0.26	-
2-Hexen-4-ol, 5-methyl-	-	-	0.08 ± 0.02	0.09 ± 0.00
Hexanol-5-D2	-	95.52 ± 3.94	55.13 ± 23.96	58.34 ± 9.05
Aldehydes				
2-Furancarboxaldehyde, 5-methyl-	23.79	-	23.54 ± 2.27	17.16 ± 0.39
2-Methyl-2,3-octadienal	-	-	-	0.07 ± 0.00
3-Furaldehyde	58.36	27.46 ± 0.14	-	-
5-Methyl furfural	-	11.31 ± 0.10	-	-
Malonaldehyde	12.11	38.30 ± 2.50	22.81 ± 5.61	19.08 ± 1.30
Ester				
beta.,D-Xylopyranose Tetrabenzoate	-	-	0.08 ± 0.00	0.10 ± 0.00
Furan				
2-Phenoxy-4-(phenylsulfonyl)-5-(p-tolyl)furan	0.72	-	0.97 ± 0.20	-
Furfuryl ethyl ether	-	-	-	0.85 ± 0.02
5-Methy furfural	-	-	-	0.97 ± 0.55
2-Furylmethyl formate	-	0.34 ± 0.00	-	-
Furanone				
3-(1-methoxydodec-11-enyl)-5-methylene-2-furanone	-	-	0.14 ± 0.00	-
Ketone				
1-(4-Methoxyphenyl)octan-1-one	-	0.27 ± 0.05	-	0.13 ± 0.03
2-(Phenylthio)-2,4-dimethylcyclobutan-1-one	-	-	0.11 ± 0.04	-

Compound	GC peak area %			
	CSC*	CSYI*	CBC*	CBYI*
2,2-dimethyl-4-oxidanyl-hexan-3-one	4.06	-	1.74 ± 1.27	-
2-ethyl-1-(1-methyl-1H-pyrrol-2-yl)-1-butanone	0.09	-	-	-
3,4,5,6,7,8-Hexahydro-1H-quinolin-2-one	-	1.31 ± 0.01	2.50 ± 0.01	2.06 ± 0.05
5,6-Dimethoxy-2-(2-hydroxyethyl-1-thio)-3-trimethylsilylmethyl-1,4-benzoquinone	-	0.09 ± 0.00	-	0.10 ± 0.04
5-[2-(1,3-Dioxolan-2-ylethyl]-5-hydroxy-7-oxabicyclo[4.1.0]hept-3-en-2-one	-	-	0.10 ± 0.01	-
8-Oxabicyclo[3.2.1]oct-6-en-3-one	-	0.16 ± 0.04	-	0.47 ± 0.01
Phenol				
3-Azidophenol	-	-	0.87 ± 0.03	-
Pyrans				
Dihydropyran	-	-	-	0.18 ± 0.04
Pyrazine				
Pyrazine	8.22	2.13 ± 0.46	7.98 ± 1.55	7.62 ± 1.24
Pyrazine, 2-ethyl-6-methyl-	-	1.11 ± 0.24	2.30 ± 0.1	2.04 ± 0.31
Pyrazine, ethyl-	5.41	3.40 ± 0.76	8.65 ± 3.24	7.65 ± 1.53
Pyridine				
2,6-Dimethyl-5-aminopyridine	-	1.20 ± 0.23	-	-
3-Pyridinamine	-	15.13 ± 2.91	42.37 ± 5.47	32.93 ± 2.00
Pyridine	22.70	8.23 ± 0.91	9.43 ± 2.74	18.38 ± 0.51
Pyrroles				
1H-Pyrrole, 1-(2-furanylmethyl)-	-	0.10 ± 0.03	0.20 ± 0.01	0.17 ± 0.02
1H-Pyrrole-3-carboxylic acid, 2,5-Dihydro-2-methyl-,Monohydrochloride	0.70	0.17 ± 0.00	0.71 ± 0.00	0.54 ± 0.05
5,6-Dihydro-4H-pyrrolo[1,2-b]pyrazole	11.61	-	-	-
Others				
(3R*,5S*,6R*)-3-Isopropyl-6-methyl-6-phenyl-5-(ptoluidino)-1,2,4-trioxane	3.53	0.29 ± 0.28	0.36 ± 0.30	0.79 ± 0.00
(E)-3-Hydroxy-3-methyl-1-butenyl N,N-diethylcarbamate	12.29	6.39 ± 0.54	12.51 ± 2.63	10.47 ± 0.53
(S)-(+)-4-sec-Butylpyrazole	-	0.14 ± 0.01	0.36 ± 0.05	0.35 ± 0.02
(S)-N-Benzyl-1-(2,2-dimethyl-[1,3]dioxolan-4-yl)methanamine	-	-	0.33 ± 0.05	-
1,4-Cyclohexadiene	-	-	8.48 ± 0.37	-
1,4-Dihydrosiline	-	-	75.66 ± 9.27	49.03 ± 4.41
1-Nitroso-1-(2'-phenylethyl)-hydrazine	-	-	-	0.23 ± 0.01
1-Piperidino-2-(2-methoxy-4-nitrophenoxy)ethane	-	0.10 ± 0.05	-	-
1-Propyl-2,2-dimethoxyethylamine Hydrochloride	-	-	0.07 ± 0	-
2-(1-cyclohex-3-enyl)acetonitrile	11.58	-	-	-
2,3-Bis[(adamantylcarbonyl)ethynyl]bicycle [2.2.1]hepta2,5-diene	-	0.29 ± 0.01	-	-

Compound	GC peak area %			
	CSC*	CSYI*	CBC*	CBYI*
2-Propanamine, 2-methyl-N-[(phenylthio) methylene]	1.91	0.69 ± 0.08	2.24 ± 0.05	1.86 ± 0.05
2-Thiophenepropanal	-	-	-	0.08 ± 0.01
3-azido-2-hydroxy-N-(4-methoxyphenyl) propane-1-sulfonamide	-	-	-	0.10 ± 0.02
5-Bromo-3-Methylidene-1-methoxycyclohexane	-	0.07 ± 0.01	0.05 ± 0	0.11 ± 0.01
Bicyclo[3.3.1]nonane	-	-	0.48 ± 0.05	-
Cyclohexanol, 1-(2-propenyl)-	10.48	-	-	-
Diethyl 2-(m-methoxybenzyl)malonate	1.41	-	1.3 ± 0.14	-
Ethyl bromide	-	11.85 ± 2.78	24.85 ± 2.9	16.20 ± 0.27
Methyl 5-(4-methoxyphenyl)-2,2,4,4-tetramethyl-3,5-dioxopentanoate	0.12	-	-	-
Propanamide, 2,2-dimethyl-	-	-	-	0.69 ± 0.03
tert-Butyl (4S,1R,2'E)-4-(1'-Hydroxyhexadec-2'-enyl)-2,2-dimethyl-3-oxazolinecarboxylate	28.09	-	0.63 ± 0.16	-
tert-Butyl 2-hydroxy-1-methylethylcarbamate	-	1.09 ± 0.00	2.65 ± 0.06	1.43 ± 0.35

*CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation. Data are shown as the mean. All samples were analyzed in triplicate. ± = standard deviation. - = not detected.



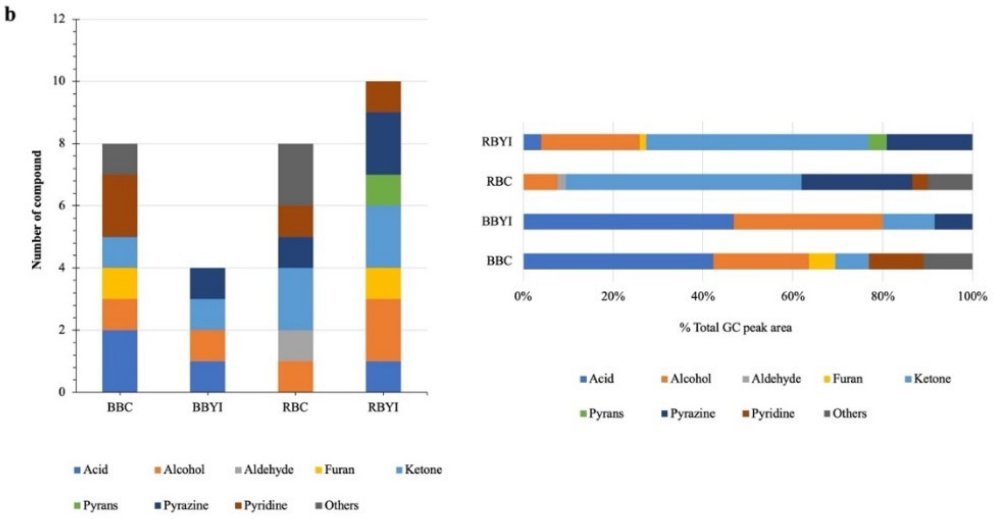


Figure 7 Volatile analysis of roasted coffee bean, the number of compounds in each group (left) and their total area (right) in (a) CSC, CSYI, CBC and CBYI coffee bean (b) RBYI, RBC, BBYI and BBC coffee bean (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation; BBC = Bourbon, control in bucket inoculation (natural fermentation); BBYI = Bourbon, yeast fermentation by bucket inoculation; RBC = Robusta, control in bucket inoculation (natural fermentation); RBYI = Robusta, yeast fermentation by bucket inoculation).

Interestingly, some compounds were detected only in CSYI and CBYI or only in CBYI (**Table 1**). For instance, volatile compounds that are classified as ketones including 1-(4-Methoxyphenyl)octan-1-one, 8-Oxabicyclo[3.2.1]oct-6-en-3-one and 5,6-Dimethoxy-2-(2-hydroxyethyl-1-thio)-3 trimethylsilyl methyl-1,4-benzoquinone were present in beans from CSYI and CBYI (**Table 1**). In addition, furfuryl ethyl ether and 5-methyl furfural were detected only in CBYI. Furfuryl ethyl ether and 5-methyl furfural occur naturally during fermentation, particularly by *S. cerevisiae* [34]. These compounds, belonging to furans and aldehydes, play a role as Maillard reaction products, often contributing to the flavor profile of various foods and beverages. For instance, 5-methyl furfural is a component of the distinctive flavors of tamarind, Dutch coffee and barrel-aged wines [35-37]. Since, the furfuryl ethyl ether is characterized by a sweet, spicy and nutty flavor profile, while 5-methyl furfural offers a sweet, caramel-like taste with hints of maple [38]. Thus, with a combination of these compounds with the other especially 1-(4-Methoxyphenyl)octan-1-one, 8-Oxabicyclo[3.2.1]oct-6-en-3-one and 5,6-Dimethoxy-2-(2-hydroxyethyl-1-thio)-3 trimethylsilyl methyl-1,4-benzoquinone are main attributes to the sensory descriptions which are sweet caramel, dry fruit, vanilla and hazelnut-like flavors to yeast-fermented coffee.

The *S. cerevisiae* SafAle s-33 is also used to inoculate Bourbon as well as Robusta. Similar to CSC and CSYI, the sample from BBC and BBYI were clustered next to each other, while RBC and RBYI were on the positive side of PC2 (**Figure 4**). Coffee beans from BBC and BBYI treatments exhibited relatively high concentrations of citric and malic acids compared to coffee beans from both RBC and RBYI. In contrast, fumaric is more predominant in Robusta than Bourbon (**Figure 8**). Although, an organic profile was similar between spontaneous fermentation and yeast fermentation in both Bourbon and Robusta; however, the volatile compounds in BBYI and RBYI were different from natural fermentation (BBC and RBC) as shown in **Figures 7** and **8**. Furans and pyridine were not present in coffee beans from BBYI, while

pyrazine was only present in those coffee beans (**Figure 7**). Interestingly, the amount of alcohol in BBYI was increased, while the same group of compounds decreased in CBYI. The yeast inoculation also changed the volatile profile in Robusta beans especially the total concentration of volatile acids and pyrans were increased after inoculation with the yeast *S. cerevisiae* SafAle s-33 (RBYI; **Figure 8**).

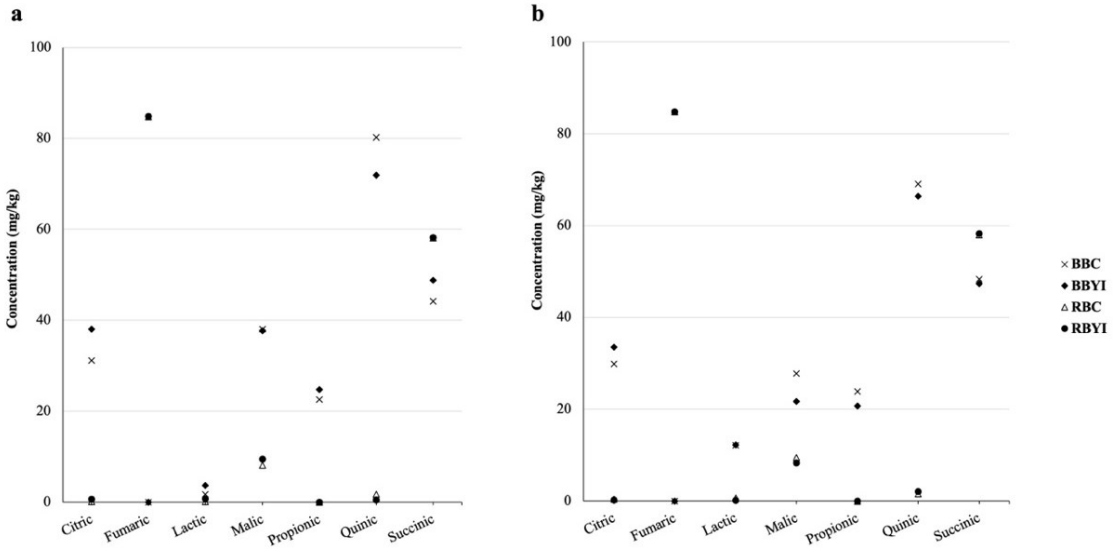


Figure 8 Concentration of citric, fumaric, lactic, malic, propionic, quinic and succinic acid in (a) green bean (b) roasted coffee beans from BBC, BBYI, RBC and RBYI (BBC = Bourbon, control in bucket inoculation (natural fermentation); BBYI = Bourbon, yeast fermentation by bucket inoculation; RBC = Robusta, control in bucket inoculation (natural fermentation); RBYI = Robusta, yeast fermentation by bucket inoculation.

The volatile and non-volatile compounds directly affect the sensorial quality of coffee. The PCA analysis exhibited a different profile of organic acids and volatile compounds in roasted beans from yeast fermentation and natural fermentation (control). Roasted coffee beans from yeast fermentation by both inoculation methods contained a higher amount of malic acid than the natural fermentation or control treatments. This could be explained by pulp and mucilage of coffee being degraded by microbial pectolytic enzyme action leading to an increase in the content of alcohol and organic acids, both of which contribute to the enhancement of overall sensory properties [39,40]. As expected, roasted coffee beans from yeast fermentation by bucket inoculation method exhibited a higher amount of malic acid compared to the spray inoculation because the condition of bucket treatment is more favorable to yeast growth as mentioned above. Malic acid is a major contributor to the fruitiness, especially the apple notes of a brewed coffee's flavor profile.

The volatile compounds include aldehydes, ketones, alcohol, esters, pyrazines, furans, acids, nitrogen compounds and phenolic volatile compounds, which are components of the coffee aroma profile. The production of volatile compounds in coffee beans differed depending on the yeast species, coffee variety, genetic variability, environmental condition, geographical origin and pre- and post-harvest processing [15,31]. Fermented green coffee beans with different yeast strains led to different metabolic processes and secretion of different extracellular enzymes, therefore exhibiting different flavor precursors and volatile profiles [20]. In this study, SPME/GC-MS detected 68 volatile compounds in the roasted coffee beans

including acids, furans, ketones, pyran, pyrazines, pyridine and others. More importantly, furfuryl ethyl ether and 5-methyl furfural were only found in *S. cerevisiae* SafAle s-33 inoculation by bucket inoculation method. The 5-Methyl furfural is a metabolite produced by *S. cerevisiae* and has a role as a Maillard reaction product, an acetolactate synthase inhibitor, and a flavoring agent. In terms of physical description, it is a colorless liquid, spicy-sweet, warm and slightly caramel odor [41].

Influence of yeast inoculation on coffee quality of Caltimor, Bourbon and Robusta

The cupping score of coffee in this study was higher than 80, which is considered specialty coffee by SCAA. The overall cupping score of roasted beans from CSC and CBC was the same (81.00). On the other hand, the overall cupping score from the CBYI treatment (83.25) was slightly higher than the CSYI treatment (82.25) due to better contribution in body, aftertaste, balance and overall performance (**Table S2**). However, there is no differentiation between the overall cupping score in both treatments in the Bourbon coffee bean. However, the difference scores between coffee from yeast fermentation and natural fermentation of Robusta and Caltimor are 8.17 and 1.75, respectively. This data suggested that yeast fermentation is efficient in improving the quality of coffee, especially in both cultivars especially Robusta (**Table S2**).

Overall, our results suggested *S. cerevisiae* SafAle s-33 and inoculation by bucket method are efficient in improving the quality of Caltimor and Robusta. Robusta from yeast fermentation has a higher overall cupping score than Robusta from natural fermentation, this could be a subsequence form difference in its organic acid profile. Prakash *et al.* [42], reported *C. canephora* (CXR variety) inoculated with *S. cerevisiae* as a starter showed higher sensory scores than in the un-inoculated method. On the other hand, there is no significant difference in organic acids or overall sensory cupping score between yeast fermentation and natural fermentation in both green beans and roasted beans of Bourbon. According to Bressani *et al.* [15], Bourbon amarelo coffee varieties that pulped natural processing with *S. cerevisiae* CCMA 0543 inoculated do not provide a different sensory analysis from natural processing treatment.

Conclusions

Based on the findings of this study, incorporating yeast strain *S. cerevisiae* SafAle s-33 using appropriate inoculation methods can enhance the aroma, flavor and overall quality of coffee. However, the choice of coffee variety becomes pivotal when employing *S. cerevisiae* SafAle s-33 as a starter. For instance, superior coffee bean quality might not necessitate enhancement, but it could significantly benefit lower-quality varieties such as Robusta. Crucially, *S. cerevisiae* SafAle s-33, is typically utilized in beer fermentation, but this study proved that they could enhance the quality of at least 2 different coffee cultivars. Consequently, exploring commercial yeast strains within the beer and wine industry holds promise for elevating coffee quality, developing unique characteristics, and notably, presenting an easier alternative source of *S. cerevisiae*.

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Appendixes

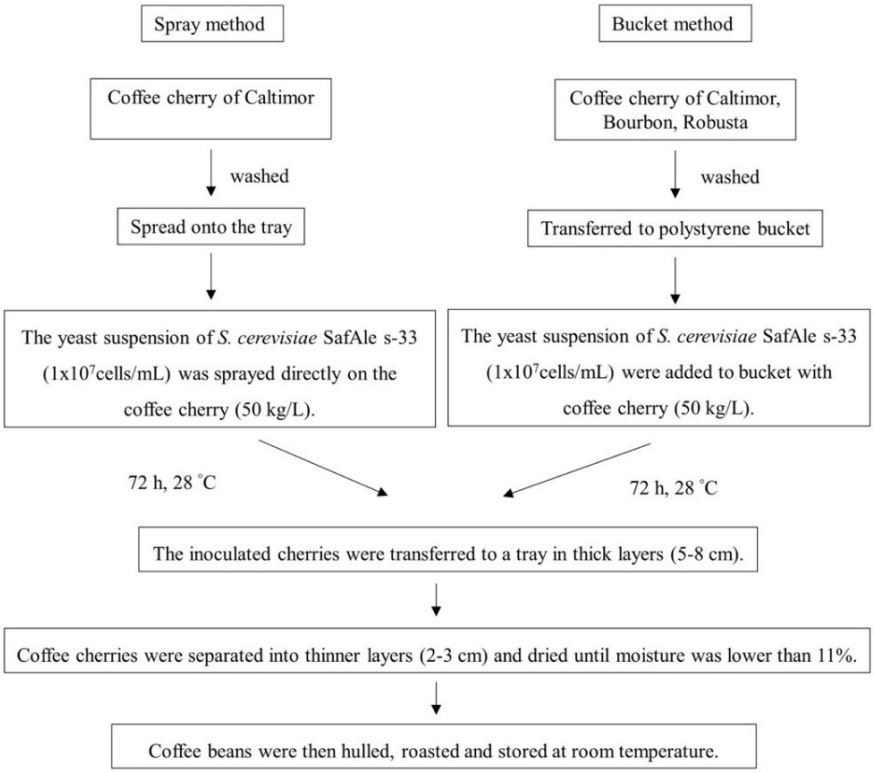

Figure S1 Processing flowchart of dry coffee process with yeast fermentation. As for natural fermentation or control treatments were performed for both inoculate methods by substituting yeast suspension with the same amount of sterile water.

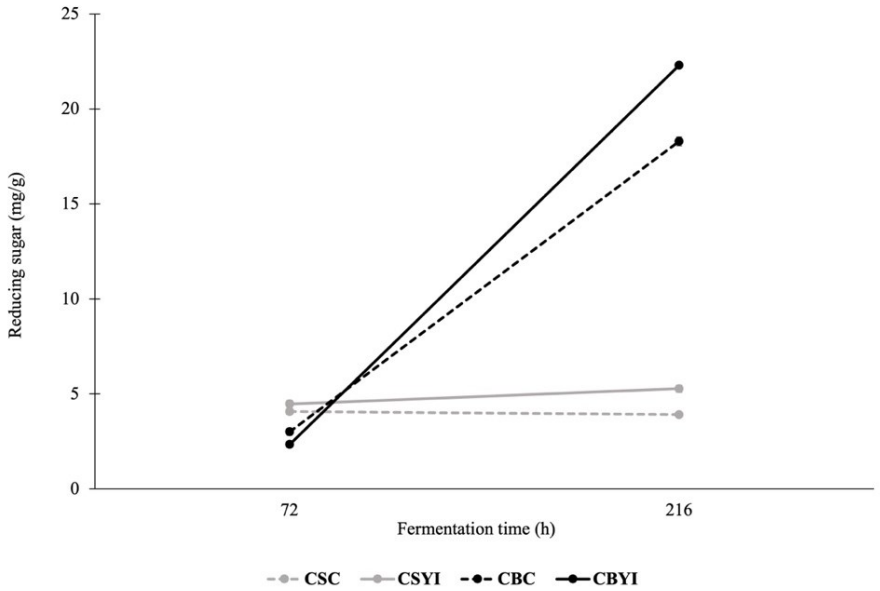

Figure S2 The concentration of reducing sugar in Caltimor cherries at 72 h and 216 post-inoculations from 4 treatments (CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation).

Table S1 Mass transitions dwell times, unit mass resolutions and individual parameter from LC-MS/MS.

Analytes	MRM transition m/z (Q1-Q3)	Polarity	Q1 (V1)	Ce (V)	Q3 (V)	Dwell Time (ms)	RT
Acetic acid	62.15 → 45.70	-	13.0	24.0	16.0	50	1.840
Citric acid	191.00 → 111.05	-	23.0	13.0	22.0	50	1.825
Fumaric acid	115 → 70.95	-	13.0	11.0	23.0	50	2.052
Lactic acid	89.0 → 42.95	-	10.0	14.0	14.0	50	1.997
Malic acid	132.95 → 115.00	-	15.0	16.0	11.0	50	1.825
Quinic acid	191.05 → 85.05	-	23.0	22.0	28.0	50	1.873
Tartaric acid	149.05 → 86.95	-	10.0	13.0	27.0	50	1.792

Table S2 The score of sensory tests following the guideline created by SCAA and cupping.

Treatment*	Aroma	Flavor	Acidity	Body	Aftertaste	Balance	Uniformity	Clean cup	Sweetness	Overall	Total
Caltimor											
CSC	7.25	7.25	7.50	7.25	7.25	7.25	10.00	10.00	10.00	7.50	81.00
CSYI	7.50	7.50	7.50	7.50	7.25	7.50	10.00	10.00	10.00	7.50	82.25
CBC	7.25	7.25	7.50	7.25	7.25	7.25	10.00	10.00	10.00	7.50	81.00
CBYI	7.50	7.75	7.50	7.50	7.50	7.75	10.00	10.00	10.00	7.75	83.25
Bourbon											
BBC	7.50	7.50	7.50	7.75	7.25	7.75	10.00	10.00	10.00	7.50	82.75
BBYI	7.75	7.75	7.75	7.50	7.50	7.75	10.00	10.00	10.00	7.50	83.50
Robusta											
RBC	7.00	6.75	6.75	6.50	6.20	6.60	10.00	10.00	6.75	6.75	73.30
RBYI	7.75	7.75	7.75	7.75	7.25	7.70	10.00	10.00	7.75	7.77	81.47

*CSC = control in spray inoculation (natural fermentation); CSYI = yeast fermentation by spray inoculation; CBC = control in bucket inoculation (natural fermentation); CBYI = yeast fermentation by bucket inoculation; BBC = Bourbon, control in bucket inoculation (natural fermentation); BBYI = Bourbon, yeast fermentation by bucket inoculation; RBC = Robusta, control in bucket inoculation (natural fermentation); RBYI = Robusta, yeast fermentation by bucket inoculation