Isolation, Selection, and Evaluation of Yeast and Lactic Acid Bacteria Starters for Coffee Flavor Modification

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ABSTRACT

Background: As a worldwide popular agricultural product, coffee price can be determined by flavor and quality of coffee beans, which can be modified by processing method, fermentation, varieties, and others. After harvesting, cherries go through including wet-, dry and semi-dry processing, among which the common phenomenon is the happening of fermentation. Fermentation is the key step for aroma formation in green beans, and usually be done by indigenous microbial flora and recently developed into using selected starters for achieving better aroma. The utilizing of yeast and lactic acid bacteria in coffee fermentation is most studied than other species.

Objective: In this study, we targeted at isolating strains from coffee green beans and coffee dry pulp and using them for further re-fermentation of green beans. Furthermore, with the inoculation of isolates, the modification of compounds composition in green beans was evaluated and followed by aroma profiling and sensory analysis.

Chapter 2 explained the isolation and identification of isolates. Using culture-dependent methods from the spontaneous fermentation, yeast and LAB were isolated from Colombia (Castillo var.) and Kenya (SL 28 var.) green beans and dry pulp (Geisha var.). Results: Over 70 colonies were obtained from the spontaneous fermentation, and after the application of morphology and biochemical characteristics, 30 strains were located as LAB candidates, and 40 colonies as potential yeast strains. After sequence analysis, isolates were identified, including 11 LAB which all belonged to *Enterococcus mundtii* species but different strains, and 9 yeast strains including *Rhodotorula mucilaginosa* (4 isolates) and *Wickerhamomyces anomalus* (5 isolates).

In chapter 3, isolates were screened for the lab scale fermentation process: The isolates obtained above were screened for their production of organic acids. The isolates were inoculated to a mixture of green coffee beans (Arabica, Castillo var.) and dry coffee pulp (Geisha var.) with water, and fermentation was carried out for 24, 48, and 72 h with 2 repeats. Seven organic acids (OAs), including acetic, citric, formic, lactic, malic, quininic, and succinic acids were measured with high performance

liquid chromatography (HPLC). Results: All seven OAs were detected in samples, among which quinic acid was the most predominant acid while succinic acid gave least concentration. The highest OA producing strains were selected from each species, labeled as Y12bR from *Rhodotorula mucilaginosa*, Y18W from *Wickerhamomyces anomalus*, and L4 from *Enterococcus mundtii*, and to be used for final fermentation.

In chapter 4, multiple growth factors were evaluated: To further maximize metabolites and fermentation potential, 3 different growth factors were applied to optimize fermentation conditions, including additional sucrose (1% w/w), different status of pulp, and different inoculum. Results: Both highest and lowest OA producing isolates were evaluated for the impact of the first two factors on OA concentration. The additional sucrose, which was expected to simulate fresh pulp, led to a general decrease in OA production; this may be caused by carbon source preference. Among 7 OAs, quinic acid showed significant decrease with the addition of sugar. Briefly outlined, the use of over-ripe pulp showed significantly lower total OA concentration along with the whole fermentation time, mainly due to the decrease of quinic acid and citric acid. Finally, a higher inoculation (8 log cfu ml-1) did not efficiently increase OA production during fermentation. Overall, these results showed that fermentation using ripe pulp without additional sucrose at an inoculation of 7 log cfu ml-1 was sufficient for effective fermentation.

In chapter 5, final fermentation with selected isolates from the screening process for mono-inoculation of either yeast and co-inoculation of yeast and LAB was carried out for further fermentation ability evaluation by monitoring changes of ①pH and ②microbial population, ③chemical analysis in fermented green beans and ④roasted beans, and ⑤sensory analysis with Colombia (Castillo var.) and Kenya beans (SL. 28 var). Results: ①A significant decrease from 0 to 24 h was observed in the pH of both beans. At same fermentation time, no significant differences were observed in pH between different inoculations. At the end of fermentation, the final pH of 4.9 was obtained, which is similar to the pH generally observed at the end of the commercial yeast fermentation. ②the yeast population first increased from 0 to 48 h and then showed a decreasing trend

from 48 to 72 h. LAB population in the inoculated samples showed close to maximum values at 24 h of fermentation. ③the concentrations of chlorogenic acid (CGA), caffeine, OAs, and volatile compounds in coffee beans were monitored throughout fermentation. CGA was significantly ($p<0.05$) affected by fermentation time but changed differently between the Colombia and Kenya beans. Briefly, the inoculation of *W. anomalus* in Colombia and the inoculation of *R. mucilaginosa* in Kenya beans helped with increase CGA by fermentation time. For Colombia beans, concentration of CGA differed greatly with different inoculations. For Kenya beans, inoculated groups presented higher CGA than non-inoculation at 24 h. Co-inoculation of LAB showed significant difference ($p<0.05$) compared to mono-inoculation. Co-inoculation of *R. mucilaginosa* and LAB affected CGA content in both beans similarly, with a constant increase of CGA throughout fermentation. Caffeine concentration was significantly ($p<0.05$) different in Colombia samples after 24 h of fermentation when compared to 48 h and 72 h, although there was no significant difference among different inoculations. All seven OAs were detected in each treatment. Acetic, citric, formic, lactic, and malic acids were found in higher concentrations in inoculated beans than non-inoculated. Acetic, citric and quinic acids production in Colombia beans was significantly influenced by time. Formic and succinic acids were detected in all treatments, but their concentrations were relatively stable during fermentation. The concentration of lactic acid was significantly ($p<0.01$) affected by the co-inoculation of LAB and time. The highest lactic acid was observed in the co-inoculation of *R. mucilaginosa* and LAB at 72 h in both beans. In green fermented Colombia beans, 104 volatiles were detected, including mainly 55 hydrocarbons, 20 esters and 14 alcohols. In Kenya beans, 98 volatile compounds were detected, including 42 hydrocarbons, 18 alcohols and 20 esters. In both beans, hydrocarbons were the most abundant in numbers as well as total concentrations. Fermented beans presented high abundance of alcohols and esters, in particular multiple esters were detected at 72 h, with mono- and co-inoculation of *W. anomalus*. In conclusion, inoculation contributed to a variety of chemical compositions both in numbers and in concentration, and consequently could result in unique aroma precursors in green beans. ④in total, 107 and 108 compounds were identified in roasted Colombia and Kenya beans

respectively, including alcohols and esters as predominant, and aldehydes, furans, and others. In Colombia beans, more than 2 folds of alcohols were found in 24 h co-inoculation with LAB than other time, while at 72 h there were more varieties of alcohols. Aromatic alcohols such as phenylethyl alcohol (rosy and honey), 2,3-butanediol (cocoa, fruity and buttery) and 1-nonanol (fresh, clean and floral) were only detected in inoculated beans. Presentation of furfural (sweet) and 5-methyl-2-furancarboxaldehyde (caramellike), was found richest in *W. anomalus* inoculated beans. Co-inoculation of LAB showed richer and more types of easters during fermentation. In Kenya beans, longer fermentation time led to more distinguished aromatic esters (e.g., hexadecanoic acid ethyl ester) which increased with longer fermentation time. 2,5-dimethyl-4-hydroxy-3(2H)-furanone (caramel and honey flavor), was found richer in inoculated Kenya beans than non-incubated beans during whole fermentation process. Ketones were detected over 2 times high in 24 h fermented mono- and co-*W. anomalus* fermented Colombia beans than other time and inoculation. Compounds like 2,5-dimethyl-pyrazine (nutty, roasty, and cocoa-like) were only found in inoculated groups. Specifically, in Kenya beans, 2-furfurylthiol (coffee-like), 2,3-dimethyl-pyrazine (nutty and roasted) and butanoic acid-2-methyl-pentyl ester were only found in *W. anomalus* inoculated beans, and 9,12-Octadecadienoic acid ethyl ester was only found in 48 h *R. mucilaginosa* inoculated beans. ⑤in sensory evaluation, both best cups were given to co-inoculated beans. Fermentation time significantly ($p<0.05$) affected perceptions of acidity, body, sweetness, aftertaste, flavor, balance, and uniformity in Colombia beans. For Kenya beans, comparing to non-inoculate beans, higher score in aroma and flavor was given to 4 fermented beans at 24 and 72 h. Inoculation of yeasts gave various fruity and floral notes by time.

In chapter 6, commercial yeast species (Saccharomyces cerevisiae) were applied to coffee fermentation. By using one of the most investigated yeasts (*Saccharomyces cerevisiae*) as a comparable stater, we monitored CGA and caffeine concentration change at 24, 48 and 72 h fermentation, CGA and caffeine were monitored as being important indicators for coffee quality as well as healthy impacts and flavor contribution. Caffeine stayed stable during fermentation in respective inoculation. CGA decreased significantly by fermentation time, and reached lowest at 72 h.

While at each fermentation time, no significant difference in CGA concentration was observed.

As a conclusion, coffee flavor and chemical composition were successfully modified by using indigenous isolates in re-fermentation with green coffee beans. Using starters can have very potential and may facilitate controlled methods for achieving different flavor and quality of coffee, and further bring unexplored economic value and pleasant flavor specially in non-coffee belt countries.

Key words:

Coffee fermentation; coffee pulp; lactic acid bacteria; yeast; flavor modification

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Abbreviations

LAB: lactic acid bacteria;

OA(s): organic acid(s);

MRS: deMan, Rogosa, and Sharpe agar;

YEPD: yeast extract peptone glucose;

PCR: polymerase chain reaction;

ITS: internal transcribed spacer;

HPLC: high-performance liquid chromatography;

YR: mono-inoculation of *Rhodotorula mucilaginosa*

YW: mono-inoculation of *Wickerhamomyces anomalu*

YRcoL: co-inoculation of *Rhodotorula mucilaginosa* and *Enterococcus mundtii*

YWcoL: co-inoculation of *Wickerhamomyces anomalu* and *Enterococcus mundtii*

Chapter 1 Introduction

Overview

Coffee is a worldwide famous beverage as well as one of the most traded agricultural products. By checking the increasing total consumption, it is not hard to find the request of good quality coffee all around the world and especially for regions have coffee drinking culture but not suitable for coffee planting. Coffee, itself is a complicated matrix of hundreds of chemicals. Coffee quality can differ by different processing methods, origins, varieties, roasting and packaging, etc. In this chapter, the basic knowledge of coffee was introduced, from coffee processing and microbiology that related to coffee environment to coffee chemistry. Getting to know how microbial flora involved in fermentation and their respective metabolites affect coffee quality, furthermore the general understanding of coffee compounds composition in various status.

1.1 Coffee process introduction

Coffee (family *Rubiaceae*, genus *Coffea*) is a worldwide famous beverage, and very important as agricultural product. The total consumption has increased significantly over recent years, with the annual world coffee consumption estimated to be over 166 million 60-kg bags in 2020/2021, especially demands from non-coffee planting counties increased (by International Coffee Organization, ICO). Coffee is grown and harvested in coffee belt countries (Fig. 1), with Brazil (32 %), Vietnam (18 %), Indonesia (6 %), Colombia (6 %), Ethiopia (5 %) as the top 5 producers [1]. On the other hand, the consumption of coffee is concentrated in non-coffee belt countries such as the United States, Germany, and Japan. Japan relies solely on import to obtain coffee beans, ranking in 3rd place among all importing countries with over 7 million 60-kg bags in 2020/2021 according to International Coffee Organization [2].

Coffee beans are processed in local environments mostly by wet- (also named washed), dry-(also called natural method), and semi-dry (also called pulped natural method) process methods, during which fermentation occurs (Fig. 3). During processing, environmental microbial flora, including coffee cheery, leaves, soil, water and others, lead to the fermentation happen and more flavor related chemicals are produced by using mucilage from the coffee cherry. The coffee cherry or fruit includes different layers (from outer side to inner side) like outer skin, pulp, mucilage, parchment, silver skin, and finally, the coffee beans (Fig. 2) [3]. (i) In the dry process method, freshly picked coffee cherries are transferred and spread on an open and drying ground or platforms where the whole fruits are naturally fermented and dried. Usually happen under sunshine or in artificial solar drying house, processing time depends on climates of local farm, which is generally 20 to 30 days, during this period the moisture content from 60-65 % (wb) will reach about 10~12 % [4]. Therefore, this method is generally applied for Robusta coffee in countries where has long time of sunshine and less rainfall such as Ethiopia, Brazil, and Paraguay [5]. (ii) In the wet process method, the outer skin (exocarp) and part of the pulp (mesocarp) are mechanically removed by pulping machines called de-pulp step.

Parchment coffee, remaining with part of mucilage, is transferred to mostly polyethylene water tanks or concrete tanks, where the key substrate of mucilage is degraded and generate secondary metabolites which can benefit coffee flavor while the fermentation happens. Fermentative process takes 6~72 hours differently, depends on local climates [6]. After fermentation, the coffee beans are then removed from the tanks and sun-dried. This method is mostly applied in regions such as Central America, Colombia and Hawaii with Arabica coffees. (iii) In the semi-dry (pulped natural), the coffee pulp and part of or all the mucilage are removed mechanically, which similar pulped like the wet process. Then the pulped beans are transferred to a drying platform where the beans are fermented and dried up to 11~13% of moisture content. This drying step may take 10~15 days depends on surrounding environments. Semi-dry is mostly occurred in Brazil [4, 7, 8].

1.2 Coffee microbiology

The main objective of primary coffee processing, which can be done in any of the processing methods mentioned above, is to remove the mucilage of the coffee cherries [6, 10]. Whichever processing method the cherries go through, fermentation is the common reaction and plays key role during processing. By microbial activities, the nutrition mainly sugars in mucilage and pulp can be digested and broken down, more flavor- related metabolites (e.g., alcohols, esters and organic acids) are gained via fermentation. The indigenous microorganisms are responsible for the fermentation during coffee processing, including yeasts (e.g., n. Candida, Pichia, Debaryomyces, Kluyveromyces and Saccharomyces species) [11, 12], and filamentous fungi (e.g., *Aspergillus*, *Penicillium* and *Fusarium* species) [13-15], mesophilic bacteria (*Lactobacillus plantarum*, *Leuconostoc mesenteroides*), lactic acid bacteria (e.g. *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Enterococcus spp.* and *Lactobacillus brevis* species) [16] and other bacteria (e.g., members of the family Enterobacteriaceae, and Bacillus spp.) [8, 9, 14, 17, 18]. Except the investigation on microorganisms which can help with coffee fermentation, the ecology was also studied to have better knowledge of the whole community. Yeast species, including *Saccharomyces marxianus* (*Kluyveromyces marxianus*), *S. bayanus*, *S.*

cerevisiae var. *ellipsoideus* and *Schizosaccharomyces* spp. *Candida guilliermondii* var. *membranifaciens*, C. *parapsilosis*, C. *pelliculosa*, *Saccharomyces cerevisiae*, *S. marxianus*, *Rhodotorula mucilaginosa* and Torulopsis fumata; lactic acid bacteria, including *Leuconostoc* (*L.pseudomesenteroides* and *L. citreum*) and *Weissella* (*W. confusa* and *W. thailandensis*), *Lactobacillus plantarum*, *Lactococcus lactis subsp. lactis*, *Enterococcus sp.* and *Enterococcus faecalis*(species); other bacterial species, including *Bacillus*, *Paenibacillus*, *Acinetobacter*, *Streptococcus*, *Pseudomonas*, *Flavobacterium*, *Proteus*, *Aerobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Tatumella*, *Paracolobactrum* and *Serratia* (*Escherichia coli*, *Bacillus cereus*, B. *megaterium*, B. *macerans* and *Klebsiella pneumoniae*); filamentous fungi, including *Aspergillus tubingensis*, *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Aspergillus sp. and Penicillium decumbens*, were most found from coffee relative surroundings.

The microbial ecology associated with coffee fermentation has been investigated for over 100 years, and virous studies have been conducted in different countries and regions where belong to ‘coffee belt’ [12, 14, 15], and released the microbial species that were found in different process. Microbial starter cultures were found to be able to improve beverage quality and reduce processing time [19]. Through screening and selection of microorganisms for coffee fermentation, isolated strains with abilities like higher production of pectinase, acids, esters, alcohols and other metabolic compounds and applied these starters in coffee fermentation for their positively influence on the quality of the final beverage [19] , afterwards making special flavor coffee drinks. Such as, in semi-dry coffee fermentation, pulped Mundo Novo and Ouro Amarelo green coffee beans were found higher score in cupping test and richer of volatile compounds produced when inoculated with Saccharomyces cerevisiae (CCMA0543) [20]. Also, stater fermentation has been used in more than arabica coffee, such as Coffea canephora (Emcaper 8151) beans were sprayed with approximately 10^7 cfu ml^{-1} of *Meyerozyma caribbica* CCMA 1738 increased more than one point in sensory evaluation as well as reduced the filamentous fungal population by 90 % in the 600-m coffees. Concluded for Conilon coffee, *Meyerozyma caribbica* CCMA 1738 showed the potential to increase the beverage quality [21].

Coffee microbial ecology has geographical characteristics in numerous studies, and related to the coffee varieties as well as the process methods in farm [22, 23]. Development of indigenous microorganism in coffee fermentation which originate from the farm or coffee itself, can not only save the process cost, and also protect the balance of natural dominant flora. Different composition and metabolism can be achieved from different coffee fermentation microflora, and consequently these metabolic products directly showed impact on the quality and flavor of coffee beans [9]. Especially, some specific species are essential for the coffee fermentation and give specific flavor to beans and develop into a good beverage [24]. Thus, it is important to conduct research in different coffee origin countries and apply the distinctively special species in virous coffee fermentation process to investigate better quality and flavor in beans.

Generally, it is very common to use fresh cherry and beans to carry on the fermentation in farm. Most of the cases of coffee fermentation papers also did in farm after harvesting, concluding microbial activities from yeast and bacterial (mainly lactic acid bacteria) utilized and degraded coffee mucilage, helped build richer volatile compounds [6, 10, 22, 25-27]. There is rare and scant research conducted in non-coffee belt countries like Japan, and at the same time Japan annul coffee consumption ranks top 5 all around the world according to ICO. The quality of coffee beans is not only determined by the bean itself, but also by the processing it undergoes post-harvest. By controlling better post-harvest processing, better quality of coffee beans can be achieved, realizing quality-controlled treatment in non-coffee belt countries.

1.2.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a general term for a class of gram-positive and nonspore-forming bacteria that can convert fermentable sugar compounds into lactic acid. It is the most representative genus of probiotics. This type of bacterial cells are rod-shaped or spherical, gram-positive, do not produce catalase, do not form endospores, have no motility or only a few have motility [28] .

Lactobacilli belong to the Lactobacillaceae family in the Eubacteriales order. Such bacteria that

have been discovered so far are divided into at least 23 genera in bacterial taxonomy, mainly including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Enterococcus*, *Bifidobacterium*, etc. [29] are mainly classified into the following four categories.

The first category is the already classified genera belonging to lactic acid bacteria, including: *Lactobacillus*, *Pedicoccus*, *Aerococcus*, *Leuconostoc*, *Streptococcus*, *Listeria*, *Brochothrix*, *Bifidobacterium*, *Sporolactobacillus* and *Bacillus*. This group is well known for food fermentation and has a long history in human food science.

The second category is some new genera created by reclassifying bacteria from the first category or/and recombining some of their genera and species, including: *Carnobacterium*, *Oenococcus*, *Weissella*, *Tetragenococcus*, *Vagococcus*, *Lactococcus*, *Gemella*, *Abiotrophia*, *Atopobium*, *Olsenella*, *Fructobacillus*, *Scardovia* and *Parascardovia*. Usage of these Lactic acid bacteria in food fermentation has been updating, but not ample.

The third category is some new genera that have been reported recently, which are closely related to the previously recognized genera of lactic acid bacteria in phylogeny and have similar phenotypic characteristics and sugar metabolism pathways. These genera include: *Aeriscardnvia*, *Alloscardovia*, *Alkalibacterium*, *Desemzia*, *Isobaculum*, *Lactoovum*, *Melisococcus*, Metascardovia and *Saccharococcus*.

The fourth category is the new genera that have been reported but not belong to any of the genera mentioned above. They belong to the lactic acid bacteria group because that description of their characteristics showed similarity to lactic acid bacteria but not belong to any existing genera and established as the new genus, including: *Halolactibacillus*, *Lachnobacterium*, *Lacticigenium*, *Marinilactibacillus*, *Pilibacter*, *Paralactobacillus*, and *Sharpea*.

Various physiological function has been proved and reported from lactic acid bacteria, including i) inherent nutritional function, providing a variety of substances beneficial to growth, such as vitamins (folic acid, niacin, vitamin B1, vitamin B2, vitamin B6, vitamin B12.etc), amino acids, digestive enzymes such as amylase and protease (and nitrosaminase, lipase and others), and other growth-

promoting substances [30]. During the metabolism of sugars and proteins, lactic acid bacteria produce compounds that give lactobacilli fermented products a special aroma and produce sticky/smothy substances (mainly mucopolysaccharides) that modify the products flavor and texture. ii) antagonism against pathogenic bacteria. Lactic acid bacteria can produce acetic acid, lactic acid, hydrogen peroxide, extracellular glycosidase and lactic acid bacteriocin and other antibacterial substances. Acetic acid and lactic acid can reduce the acidity of the growth environment, killing or inhibiting many microorganisms, especially alkaliphilic pathogenic microorganisms; peroxidation Hydrogen can inhibit the growth and reproduction of pathogenic bacteria such as Staphylococcus aureus; lactic acid bacterium has antagonistic effects on a variety of pathogenic microorganisms. (iii) More beneficial function like regulating intestinal flora, immune modulation, antioxidant effect and cholesterol degradation resulted from lactic acid bacteria were also discovered and applied in medical treatment. In this study, author only discussed with the application of lactic acid bacteria in food science such as lactic acid bacteria involvement in fermented soybean, fish, vegetable, bread, cocoa products, and alcoholic beverages.

1.2.2 Yeast

Yeast is the main indigenous group which benefit coffee flavor during fermentation. Yeast is also the groups most isolated and investigated microorganisms for its metabolic function from coffee process and coffee filed such as coffee crops, farm soil and coffee pulp and etc. Yeast has geographical and seasonal characters in different coffee planting countries and regions [11, 31-33]. Various species were found different metabolic products which affects the flavor of beans after process. Common fermentation phenomenon of the chemical changes was reported among different species, which is pectin degradation, as well as the production of organic acids, ethanol, esters and other metabolites from the carbohydrates in mucilage. Therefore, yeast is the most widely studied microbial group in different coffee fermentation.

Diverse yeast species from various genera, such as *Pichia*, *Candida*, *Hanseniaspora*,

Saccharomyces and *Torulaspora*, have been detected from coffee process and developed for their value as stater in controlled fermentation [6, 19, 34, 35]. Reports have reported that *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* and *Pichia kluyveri* as suitable species of starters in coffee flavor modification. *Torulaspora delbrueckii* reduced caffeine concentration by fermentation process. Co-inoculation of different yeast species gave higher cupping score (>86). Co-inoculation with *Saccharomyces cerevisiae* and *Torulaspora delbrueckii*, can decrease content of trigonelline significantly [34]. In Wang etc. research, they found with inoculation of *Saccharomyces cerevisiae*, and *Pichia kluyveri*, fruity esters were generated more in the fermented green coffee beans [35]. This change in *Saccharomyces cerevisiae* fermented green beans directly transferred to the roasted beans by enhancing volatile profiles formation therefore enhanced the fruity attribute in the final beverage. Mono-inoculation of *Pichia fermentans* significantly improved metabolites formation during fermentation. Fermented with *Pichia fermentans* and *Pediococcus acidilactici*, increased pulp sugar consumption during process and displayed higher production of metabolites (lactic acid, ethanol, and ethyl acetate) [36].

Besides the modification and enhancement of sensory profile, inoculation of microorganisms also benefited food safety for their antagonist activity against an ochratoxigenic strain of *Aspergillus westerdijkiae* [37].

It is widely known that the utilizer of starter cultures can increase the production/contents of volatile compounds which have positive influence on complete sensory profile and consequently improves the beverage flavor and aroma. The screening of specific microorganisms, especially yeast and lactic acid bacteria, which have been investigated most during the fermentation process is essential to understand their effects during fermentation and how to use them to improve coffee quality.

1.3 Coffee chemistry

Sensory quality of coffee depends on various factors, therefore many research has been done on chemical composition of both green coffee beans and roasted coffee beans [24]. Even though,

scientists have found it is not clear about the mechanisms of how components and flavors are interfered with each other due to its complexity within the bean and different processing. In green coffee beans, chemicals like organic acids, carbohydrates, proteins, trigonelline, and caffeine which belonging to nitrogen compounds, lipids and sulfides. Secondary metabolites including organic acids, alcohols, esters, ketones, and aldehydes that might impact the final product quality [22] were produced via fermentation. Some of those secondary metabolites like free sugars and free amino acids which were produced by microbial activities, can be transferred or taken into green beans, consequently, join formation path in later roasting process [38]. During roasting, reactions like Millard, oxidation, degradation reaction happen and form more coffee relative compounds [25]. Maillard reactions, a very important reaction for aroma and flavor formation. When temperature reaches 150~200 °C aromatic chemicals such as pyrroles, thiols, furanones, pyridines, and thiophenes can be formed during roasting via Millard reaction [39].

Organic acids, popular for contribution to the acidity to coffee beverage. Acidity, as an important sensory parameter of coffee beverage evaluation, contributes to different flavor as well as intensities. Acetic acid, can be formed during fermentation and roasting process [40], was reported to contribute acidity, fruity, wine and fermented flavor at moderate concentration [41]. While, some researchers mentioned acetic acid helped with vinegar aroma, beyond perceptible sourness, flavor like rancidity, astringency, and bitterness were also related to acetic acid, which were considered negatively to final beverage flavor [42, 43]. The formation of acetic acid can be from carbohydrates (the breakdown of sucrose) in green coffee during roasting [44]. Inoculation of lactic acid bacteria also contributed to concentration of acetic acid, together with increase of citric acid, fumaric, and malic acid after fermentation, leading to higher cupping score as well as better flavor of fruity and tropical fruits [45]. As a controversial acid, both positive and negative influence on aroma were reported, contribution to overall flavor intensity and enhanced flowery and fruity [46, 47], as well as higher concentration in Robusta than Arabica roasted beans [48]. Citric acid, another most prominent in green coffee beans, offers sour taste to coffee. Citric acid, also the precursors to other acid (e.g. citraconic, glutaric,

fumaric acids) during roasting [49], breakdown products . Higher concentration of citric acid in green coffee contributed to more citric and herbaceous sensation during roasting [16], besides, higher cupping score was given to coffee beans which contained more citric acid [50]. Fermentation with additional lactic acid bacteria can increase the content of citric acid as we mentioned before [45], also inoculation with yeast could improve the concentration of citric acid, resulting in higher cupping score [51]. Citric acid can be generated by yeast (*Meyerozyma caribbica*, *Saccharomyces cerevisiae*, *Candida parapsilosis*, and *Torulaspora delbrueckii*) fermentation of coffee beans even it naturally presents in green coffee beans and fruits [32]. Citric acid is also a precursor which can join the formation of citraconic acid during roasting [49]. In green coffee beans, Arabica has less citric acid than Robusta. During roasting, the change of citric acid differed depending on the varieties of beans, in Arabica beans, different from acetic acid which increases proportionally with roast level, citric acid decreased after roasting reached medium while reverse trend was observed in Robusta beans [44]. Formic acid, contributing to sour and bitter taste, as well as chemical, pungent and fermented aroma, naturally exists in green coffee beans and can be formed by using carbohydrates in green beans during roasting from Millard reaction [52]. Lactic acid contributes to sour taste and sensation of mouthfeel which is usually described as ‘body’ in coffee beverage. Same with acetic and formic acids, lactic acid also be formed during roasting. But the concentration of lactic acid was reported to be relatively constant during roasting [44]. By using lactic acid bacteria and/or yeasts as starter in coffee fermentation, higher lactic acid can be gained [10, 32]. Malic acid naturally presents in green coffee beans and characters sour taste in coffee beverage. As an aroma precursor, malic acid can help form of succinic, fumaric and maleic acids by degradation during roasting. Malic acid is also a metabolite from yeast and lactic acid bacteria fermentation with coffee beans [53]. Behavior of malic acid also differ between Arabica and Robusta beans before and after roasting. Green Robusta had lower malic acid than Arabica while roasted Robusta had more malic acid than Arabica [48]. Quinic acid, naturally presenting in green coffee beans, is one of the most prominent organic acids. Quinic acid contribute to sour and bitter taste in coffee beverage. Quinic acid was reported higher concentration after roasting

due to the degradation from CGAs, but study reporting quinic acid stayed stable before and after roasting was also carried [48, 54]. CGAs, a group of bioactive phenolic compounds (caffeoylquinic acids (CQA, isomers include 5-CQA, 4-CQA), dicaffeyloquinic acids (diCQA, isomers include 3,4-diCQA and 3,5-diCQA), and feruloylquinic acids (FQA)) presents in green coffee beans [55]. CGAs degrade and form lactones which impart bitterness during roasting [55]. Higher CGA was reported to be bitterness and astringency which negatively contributes cupping score [56] while higher CGA was given higher score for its perceptible bitterness and flavor modification [57]. Succinic acid, another sour taste acid also giving bitter taste, presents naturally in green coffee beans. Succinic acid can be formed form citric and malic acids degradation reaction. The transition of succinic acid can occur under the influence from microbial activity and affect the final beverage [19].

Caffeine, a major alkaloid in green coffee beans, contribute to bitterness in beverage. Green Arabica coffee beans generally have 1% (dry weight) and more caffeine in Robusta beans [58, 59]. In Arabica beans, the concentration of caffeine doesn't differ much in green and roasted coffee beans. Comparing with similar level maintaining Arabica, Robusta beans tend to increase after roasting [60]. In Casal etc. research, with roasting temperature increasing to 160 °C, the concentration of caffeine increased; while from roasting temperature of 160 to 240 °C, caffeine tended to decrease and reach lower content at 240 °C than in green beans [61].

Alcohols, can be formed during fermentation by bacteria and yeast enzymes and roasting. The alcohol metabolites can help with the growth of Lactic acid bacteria, and acids can be produced or converted from alcohols by fermentation [62]. In roasted beans, alcohols are one of the most presenting volatiles and contribute fruity, creamy, buttery (2,3-butanediol) [63], and sweetness like caramellic and burnt sugar (2-furanmethanol [64] and furfuryl alcohol [65]).

Esters, aromatic esters can be converted from amino acids in green coffee beans, easters are well known for its contribution to coffee beverage as sweet and fruity (e.g. ethyl-3-methylbutyrate) taste [66]. Fermentation during cherry process can produce easters which can help modify better coffee quality [22], roasting produces easters as well.

Aldehydes, produced mainly from roasting, fermentation can also produce aldehydes [22]. Aldehydes were reported to be related to sweet and almond aroma such like benzaldehyde, and characterized by fruity taste such as acetaldehyde [67].

Ketones, naturally presenting in coffee green beans, can be produced by fermentation during coffee processing. Ketones were most reported for their aromatic characteristics like caramel and sweet as well as buttery taste. Such as 2,3-butanedione was found to behave as fruity, sweet and buttery taste [63], 3-hydroxy-2-butanone was found to be related to taste like sweet, buttery and creamy [64].

Furans, one of the most investigated and the most presenting aromatic groups, shows a sweet, caramel and coffee-like aroma [65, 68]. Furfural, behaves sweet and almond, formed from Maillard reaction during roasting. Together with 2-acetylfuran (sweet and balsam aroma), the concentration of aromatic furan differ among Arabica and Robusta beans, the former was reported to have higher content [69]. Fermentation can help with the production of chemicals which can contribute to caramel-like taste furan [35, 70]. Caramelization reaction of sugars and amino acids, can be responsible for the formation furans and furanones during roasting [71].

Pyrazines, except caramelization reaction mentioned above, during roasting Maillard reactions and Strecker degradation are also responsible for the formation of pyrazines through utilizing sugars and amino acids [71]. As the key reaction during roasting, Maillard reactions helps with degradation of trigonelline and proteins, and together with sugars (all are present in green beans) can be broken down and form volatile chemicals of pyridines, pyrroles, and pyrazines. The pyrazines, pyrroles, and pyridines groups of compounds are well known for their attributes for aroma, such as nutty, roasted, and toasted notes in the coffee aroma [43, 65, 72, 73].

Compounds composition differ depending on coffee regions and varieties, as well as process methods. Furthermore, it’s exactly the difference in composition in green coffee beans, which mostly are aroma precursors in green coffee, contribute towards coffee aroma formation during roasting and produce featured and unique flavor [39, 43, 66, 68]. For example, studies showed the resultant green

beans have high levels of desirable microbial metabolites such as ethyl acetate, acetaldehyde, glycerol and ethanol that were correlated to fruity notes and high sensory scores of flavors, aroma, body, acidity, and uniformity after roasting [14, 26, 74, 75] .

1.4 Objectives

The isolation and accomplishment of yeast and LAB in non-coffee belt countries, as well as the usage of dry pulp as substrate in fermentation, and biochemistry of the fermentation have rarely been studied. Thus, in this study, isolation indigenous microorganisms from coffee material was carried out and developed the application in green beans fermentation, aiming to modify chemical composition in green beans by re-fermentation and furthermore produce different aroma profiles in roasted coffee beans and achieve various flavors of coffee beverage.

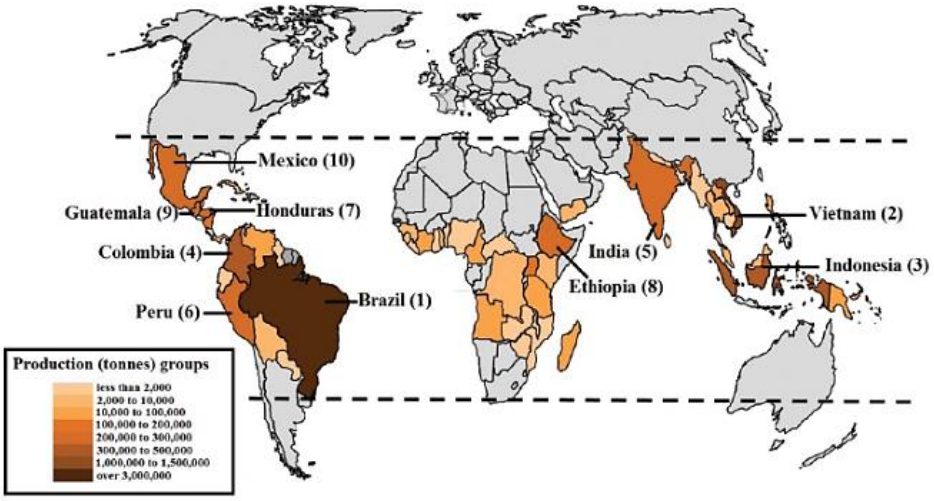

Fig. 1.1. Geographical distribution of coffee production (Source: FAO, 2014). The dashed lines indicate 'the coffee belt' zone [9].

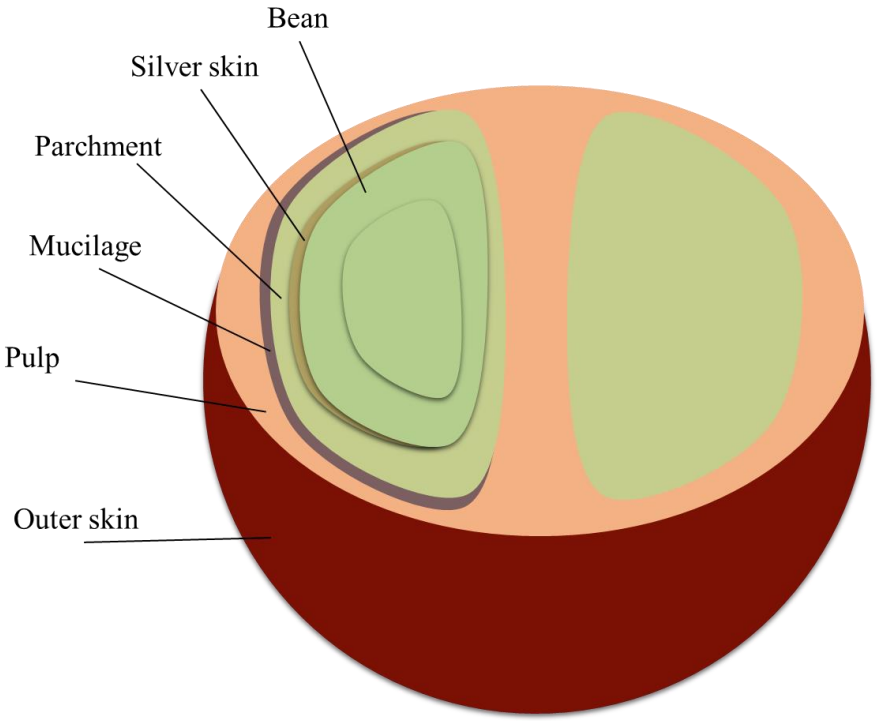

Fig. 1.2. The structure of coffee cherries.

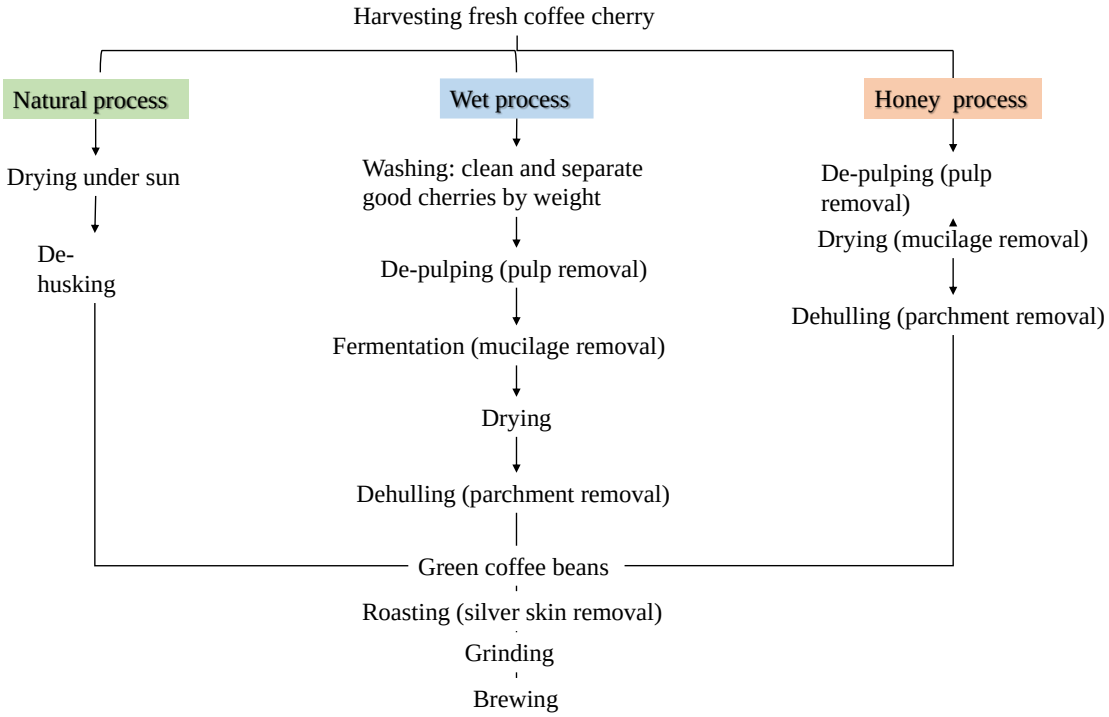

Fig. 1.3. Different methods employed to process coffee cherries, Created in Biorender.com.

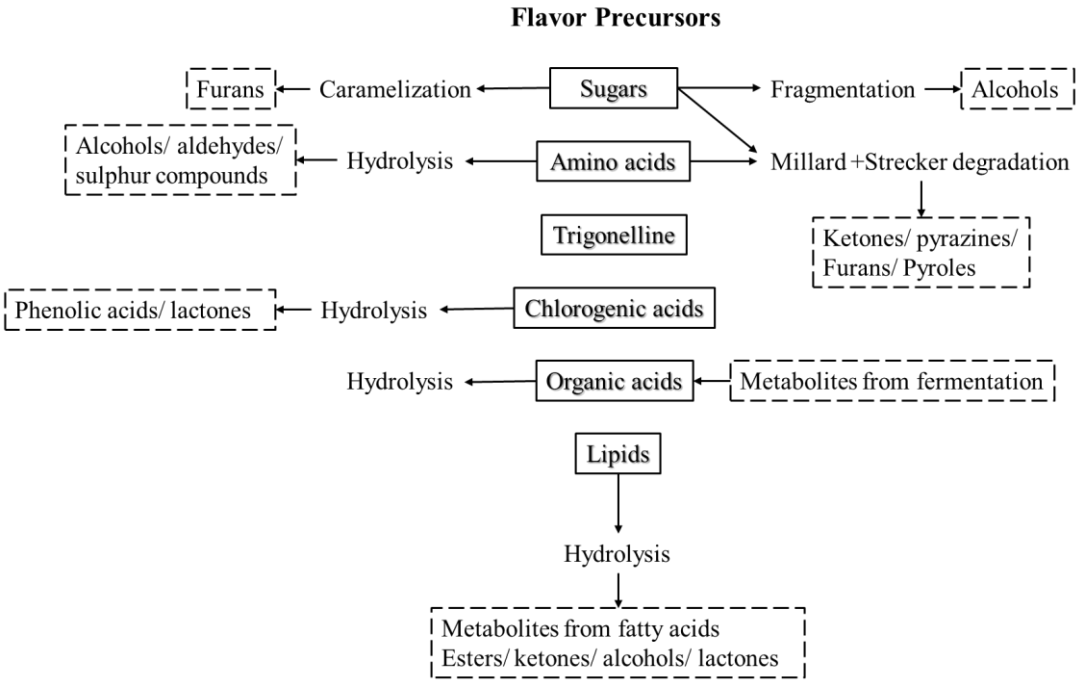

Fig. 1.4. An overview of the reactions of aroma precursors in coffee flavor formation during roasting and generation of metabolites from microbial fermentation [27].

Fig. 1.5. Specialty Coffee Association (SCA) flavor wheel ※.

※*https://notbadcoffee.com/flavor-wheel-en/*

Chapter 2 Isolation and identification of lactic acid bacteria and yeast by spontaneous fermentation

Overview

In this chapter, continuous discussion about microbiology in coffee from last chapter was spread out. Knowing yeast and lactic acid bacteria are the two most involved microorganisms during coffee processing. As the predominant microbial groups, in this chapter discussion about isolating yeast and lactic acid bacteria by using culture-dependent method from coffee was recorded. From spontaneous fermentation of coffee material, including coffee beans and coffee pulp, isolate and identify by morphology, biochemistry characteristics and sequencing. Identify isolates until species, and prepare for evaluation of their fermentation capacity in following chapters.

2.1 Material and methods

2.1.1 Coffee pulp and coffee green beans

Coffee beans (Coffea arabica, var. Castillo) from Finca Los Tres Edgaritos-Cauca, Colombia and coffee beans (Coffea arabica, var. SL N.28) from Nyeri/Karindundu, Kenya were prepared as samples. All material used in this study were harvested manually by experienced coffee picker in the farm and only mature cherries were selected, and primary separation was done by weight in flotation tanks. Floating cherries which were considered bad quality were then discarded and non-defective cherries which sank to the bottom of tanks were selected for next procedure. Drying process was carried after 48 h storage in tank and directly dried under sunshine by spreading on the drying shelf with mesh. The moisture content in wet processed beans is around 10 % before shipped to Japan. Dried ripe pulp of Geisha variety was collected from same farm in Colombia. In this study we used the same and only one type of coffee pulp in all isolation and fermentation sections consistently from start to finish.

2.1.2 Spontaneous fermentations with green coffee beans and dry coffee pulp for microbiological analysis

Sterile blue-cap bottle (250 ml) was used as the fermentation tank in this study, added 5 g green coffee beans and dry coffee pulp into the bottle in advance. Fifty ml sterilized distilled water was pre-filled into 250 ml sterile blue-cap bottle. Sterilization of distilled water and blue-cap bottles was done at 121 ◦C for 30 min in autoclave (Tomy, Eiko co., Ltd, Japan). Three fermentation bottles were prepared, including green beans from Kenya, green beans from Colombia and dry pulp. Each fermentation, 2 repeats were prepared. Incubation conditions were set to 28 ◦C for yeast isolation and 30 ◦C for lactic acid bacteria isolation, respectively for 3 days in incubator (AsOne, Japan).

2.1.3 Culture-dependent isolation, purification and maintenance of microorganisms

Medium and cultivation methods for lactic acid bacterial (LAB) and yeast were referenced former

studies [31]. In general, five grams of green coffee beans and dry coffee pulp were sampled and added into a sterile bag with 100 ml saline-peptone water (0.1% (vol/vol) peptone (Himedia, India) and 0.8% (vol/vol) NaCl (Wako, Japan), and then was homogenized in a stomacher (Asone, Japan) at normal speed for 7 min, followed vortexing (lab companion, JEIO TECH, Korea) for 2 min. Separation of big particles and liquid part was done by centrifuging at 1,000 rpm for 5 min. Samples for plate spreading were finally prepared by serial dilutions with sterile distilled water. LAB were cultured by pour plate inoculation of 100 µL on MRS agar (de Mann, Rogosa, and Sharpe agar, FujiFilm, Japan) containing 0.1% (vol/vol) cycloheximide (Merck, USA) to inhibit yeast growth and 1% $CaCO_3$. Yeasts were applied surface inoculation of 100 µl on YEPD agar (1% yeast extract (FujiFilm, Japan), 2% peptone (Wako, Japan), 2% glucose (Wako, Japan) (pH~5.6)) containing 50 mg/L chlortetracycline (Sigma, USA) to inhibit bacterial growth. All MRS plates were cultured in anaerobic jar with anaerobic bag (AnaeroPack, MGC, Mitsubishi Gas Chemical Company, Inc. Japan) then kept in the incubator (AsOne, Japan) at 30 ◦C for 48 h and all YEPG plates were directly kept in incubator at 28 ◦C for 48 h.

The colonies that appeared on the plates were purified by streaking 4 times or more on the same agar plates. The cultivation was carried out by inoculating a platinum loop of colonies from the agar plates. All purified isolates were picked and stored at −20 ◦C in corresponding broth with in advance supplement with 60% glycerol (Wako, Japan). Before experimental usage, LAB and yeast isolates were grown twice in MRS broth, and YEPG broth, and their purity was examined through streaking in MRS agar and YEPG agar, respectively.

2.1.4 Identification of lactic acid bacteria and yeast isolates

I. Identification of LAB: LAB strains were first characterized morphologically, only colonies with characteristics of cocci, coccobacilli, or rods and, chain formation were selected for next identification [76]. Colonies were picked with showing of clear zone around colonies while growing on MRS-1% $CaCO_3$ plate, for its acid producing activity. Later gram staining in conjunction with microscopic examination, and the determination of catalase activities were carried out. Only strains showed

positive gram staining results were kept. 24 h-growing broth cultures were preciously prepared and were centrifuged at 10 000 **g** at 4 ◦C for 5 min and re-suspended in filtered sterile distilled water. The catalase test was conducted by dripping one drop of hydrogen peroxide (3%) on a glass slide which was lay well mixed re-suspended culture. The catalase test is to confirm catalase enzyme producing ability from bacteria, when produced catalase enzyme contacts hydrogen peroxide, many oxygen bubbles will be formed and this bubbling phenomenon was characterized as catalase positive reaction. Otherwise, no bubbles indicate no production of catalase enzyme by the test bacterium. Therefore, the isolates, which did not give gas bubbles, were selected for subsequent activities. Gram staining positive as well as catalase negative colonies were picked and transferred into sterile ep-tubes (AsOne, Japan) at 4 ◦C to keep temporarily. Then genetic analysis was done by amplification of conserved sequence, 16S rRNA gene was targeted to amplify with reported primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1495R (5′-CTACGGCTACCTTGTTACGA-3′) [37, 38, 75].

II. Identification of yeasts: initially all yeast isolates were micro- and macro-morphologically characterized as described by [77, 78]. Subsequently, the isolates were grouped by PCR and Internal Transcribed Spacer (ITS) gene was selected for amplification. The 5.8S ITS rRNA [79] was amplified by using ITS1 (5′-TCCGTAGGTGAACCTGCG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) universal primers in this study. Instead of extracting initially total cDNA, in this study we used colony PCR (Fig. 2.1.) followed the manufacturer protocol of AmpliTaq Gold™ 360 Master Mix (Thermofisher, Japan). The PCR reaction was carried out in a 25 µl volume containing 1 U Taq DNA polymerase (Amersham Biosciences, Piscataway, NJ, USA), 2.5 µl 10 × PCR reaction buffer (Amersham Biosciences), 200 µM of each deoxynucleotide triphosphate (Amersham Biosciences), 3.0 mM MgCl2 (Amersham Biosciences), 0.8 µM of primer.

The amplified PCR products were separated by gel electrophoresis on 0.8% (w⁄ v) agarose gel and stained with ethidium bromide. The 100 bp and 1K markers (AsOne, Japan) were added as reference. Agarose gel electrophoresis was performed, and the results were visualized by Printgraph (Atto, Japan). Targeted bands were cut and purified with ExoSAP-IT™ PCR Product Cleanup

(Applied Biosystems™, Thermofisher, Japan) and sequenced by Premix analysis (FASMAC, Japan). The sequencing results obtained were aligned with sequences data in the GenBank by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2 Results and discussion

A total of 70 strains, including 53 yeasts strains and 17 of LAB strains, were isolated from the coffee beans and pulp. Those 70 were firstly identified by morphology and biochemical characteristics mentioned in session 2.1.4. The picked colonies were then sent for further sequencing analysis, and a total of 9 of yeasts and 11 of LAB were obtained and identified until species information. Strain information, nucleotide similarity, and reference ac-cession numbers of the isolates are shown in Table 2.1. All isolates revealed more than 98 % similarity in the 5.8 S rRNA and 16 S genes with published yeast and LAB strain sequences, respectively. Among the isolated yeasts, 4 isolates were identified as *Rhodotorula mucilaginosa* and 5 isolates were identified as *Wickerhamomyces anomalus*. All strains from *Rhodotorula mucilaginosa* showed colonies characteristics such as similar color of orange or red, smooth surface, and highly moist, while all strains from *Wickerhamomyces anomalus* showed colonies characteristics which had milk white color, smaller size, and dry surface (Fig. 2.4.). These yeast species were reported in other study to be fermentative and have been found in soil, fruits, vegetables and coffee [80]. In addition to their contribution to coffee fermentation, rare study has been reported but they have been shown to play a significant role in wine fermentation. For example, the co-fermentation of *Rhodotorula mucilaginosa* with *Saccharomyces cerevisiae* significantly increased the concentration of the multiple aroma compounds as well as acids [81]. *Wickerhamomyces anomalus* have also been shown to produce higher levels of esters (including acetates and ethyl esters) which supply a fruity note in wine fermentation [82]. All LAB isolates were identified as *Enterococcus mundtii*. The LAB isolates were observed different acid producing ability by giving virous transparent circles of different size and degree of transparency when growing on MRS-$CaCO_3$ plates (shown in Fig. 2.2. &2.3.). Larger size of transparent circle or more transparent zone may indicate stronger acid

producing ability.

2.3 Conclusion and summary

In this chapter, we successfully isolated and identified 11 strains of LAB (*Enterococcus mundtii*) and 9 strains of yeast (4 isolates were identifided as *Rhodotorula mucilaginosa* and 5 isolates were identified as *Wickerhamomyces anomalus*) from coffee green beans and dry coffee pulp. For all 3 species were reported in coffee fermentation and other food fermentation. Well, the application of additional inoculation of the 3 species in coffee fermentation is still rare. In this study, we did next experiments for further evaluation as potential starters in coffee fermentation.

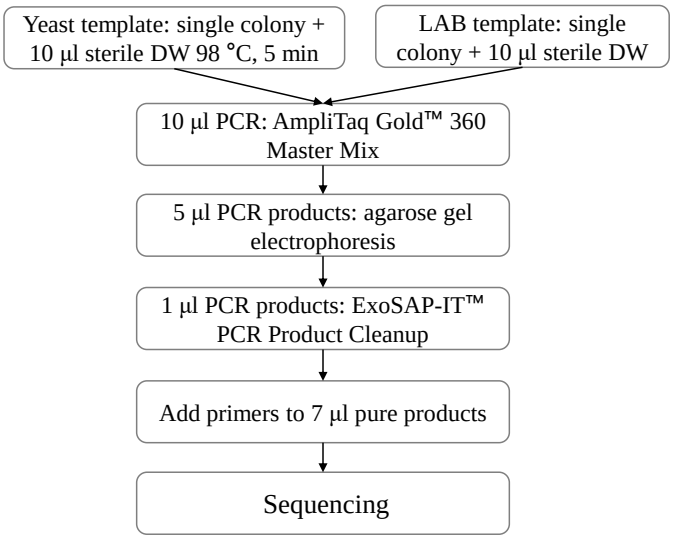

Fig. 2.1. Procedure of identification of colony.

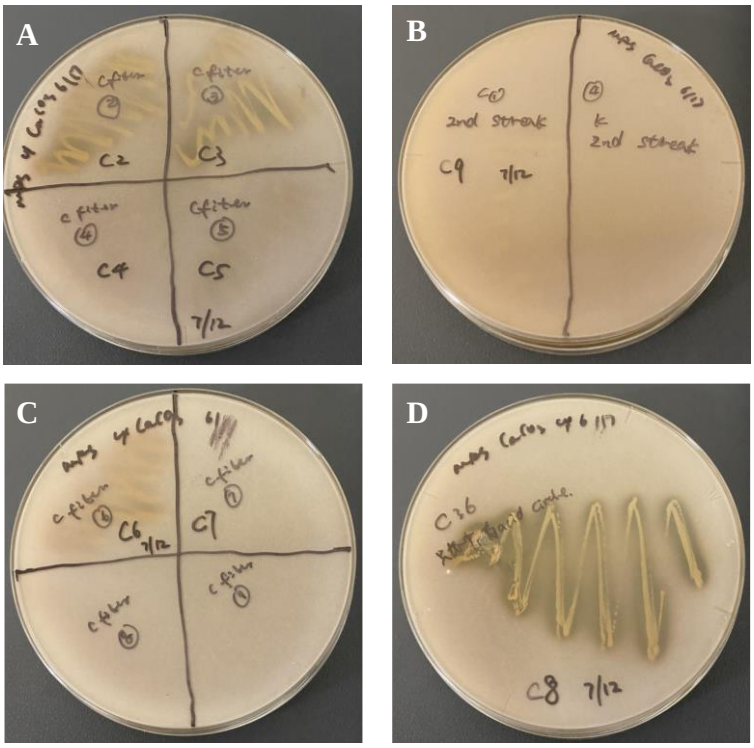

Fig. 2.2. Acid-producing circle from colonies isolated from Colombia green beans. No acid producing: A_C4, B, C_C7-9. Clear zone around colonies: A_C2, A_C3, and D_C36. Weak acid producing: A_C5 and C_C6.

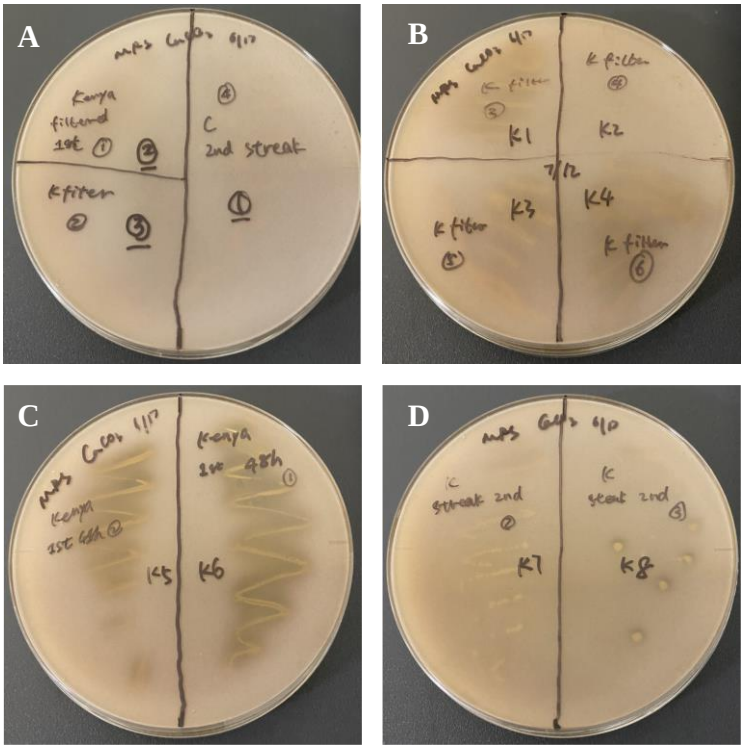

Fig. 2.3. Acid-producing circle from colonies isolated from Kenya green beans. No acid producing: A. Strong acid producing: B_K1, C_K5, C_K6 and D_K8. Weak acid producing: B_K2, B_K3, B_K4 and D_K7.

Table 2.1. Isolation results from spontaneous fermentation.

Strain label	Nucleotide Similarity (%)	Reference Accession Number	Species information
Y1	97.80	OM523876	*Rhodotorula mucilaginosa*
Y5	99.83	MK646042	
Y12a	99.82	KF953903	
Y12b	98.94	ON242334	
Y10	100.00	MZ576855	*Wickerhamomyces anomalus*
Y18	100.00	MK343437	
Y19	100.00	MZ089535	
Y38	100.00	FJ713067	
Y43	100.00	MK757882	
L4	99.39	AP019810	*Enterococcus mundtii*
L5	100.00	AB831185	
L6	99.51	MZ869125	
L6b	99.75	MZ869177	
L8	98.53	MZ869152	
L10	99.50	KC985226	
L12	99.25	MZ869176	
L14	100.00	MN636722	
L16	99.75	CP029066	
L17	99.88	MW135231	
L17b	99.75	MW135237	

Fig. 2.4. Morphology characteristics of yeast isolates *Rhodotorula* (left) and *Wickerhamomyces* (right).

Chapter 3 Screening isolates by production of organic acids

Overview

In this chapter, by using the isolates from chapter 2, evaluation of organic acids producing ability at different fermentation time was carried out. Different from fermentation process of last chapter, in this chapter the ratio of green beans and coffee pulp for the fermentation process was optimized. Monitor the change of seven organic acids, including acetic, citric, formic, lactic, malic, quinic and succinic acids, and select the highest organic acids producing strains for both yeast and lactic acid bacteria. To detect the concentration of each organic acid, method by using post-column HPLC was developed. Development and optimization of HPLC program and conditions are the main technical experiment.

3.1 Material and methods

3.1.1 Coffee beans and coffee pulp

Same green coffee beans from Kenya as session 1.2.1 were used in this chapter. Dry coffee pulp (Geisha var.) was same as session 1.2.1.

3.1.2 Fermentation process with yeast and LAB isolates

Five grams of green coffee beans and dry coffee pulp were added into 50 ml sterilized distilled water pre-filled 250 ml sterile blue-cap bottle, respectively. Sterilization of distilled water and blue-cap bottles was done at 121 ◦C for 30 min in autoclave as session 2.1.2.

LAB and yeast were precultured in 250 ml flasks of YEPD and MRS broth respectively to harvest high population approximately 10^7cfu ml^{-1}, followed by being centrifuged at 10 000 **g** for 5 min at 4 ◦C, and resuspended in sterile distilled water. For each type of fermentation, same scale of bottle was used to prepare two repeats, totally 6 fermentation bottles were used for fermentation time of 24, 48 and 72 h. Incubate fermentation bottles containing green beans and dry pulp, as well as inoculation of 10^7cfu/ml of each isolate, kept in the incubator (AsOne, Japan) at 28 ◦C for yeast isolate and 30 ◦C for LAB isolate, respectively.

3.1.3 Organic acids extraction and measurement by HLPC

OAs in yeast isolates fermented green coffee beans were sampled, extracted, and quantified at 24, 48, and 72 h of and OAs in LAB isolates fermented green coffee beans were collected at 12, 24, 48, and 72 h of fermentation. OAs produced by LAB are known to be beneficial for their coexistence with yeasts [9], OAs produced in earlier the fermentation period (12 h) were measured for the LAB isolates. The isolates were screened to select strains which present high levels of individual OA in green beans after fermentation, as well as highest total OAs concentration. For every inoculation, two repeats were prepared.

Extraction and analysis of the impacts of different inoculation were sampled from fermentation

containers at 24 h, 48 h and 72 h. The following 7 OAs: acetic, citric, formic, lactic, malic, quinic, and succinic acids (Fig. 3.1.) were analyzed by HPLC (LC-6AD Series, Shimadzu Corp., Osaka, Japan) using the ion exclusion method (Fig. 3.2.), and reverse phase HPLC method.

OAs were extracted and quantified with HPLC by following previous research [74] with some adjustments. Extraction from the fermented beans was performed in a 50 ml Erlenmeyer flask with 1 g of green beans and 3 ml of 3 mM perchloric acid solution, and the mixture was vortexed for 10 min at room temperature. The mixture was then centrifuged at 14 000 g, 4 ℃ for 10 min. The supernatant was filtered using a 0.45 μm membrane filter. The filtered extract was stored at -20 ℃ until analysis.

The measurement of OAs was developed as 2 methods, including the ion exchange post-column and reverse phase HPLC. For post-column HPLC (shown in Table 3.1), the acids were separated with a Shodex KC-811 column (8.0 mm I.D. × 300 mm) with Shodex KC-G 8B guard column (8.0 mm I.D. × 35 mm) (Resonac Corp., Tokyo, Japan). A mobile phase of 3 mM perchloric acid was used at a flow rate of 0.6 ml min^{-1} and 0.7 ml min^{-1} of coloring reagent (ST3-R, Resonac Corp., Tokyo, Japan) was added post-column. The oven temperature was set at 50 °C and UV detector at 430 nm. Quadruplicate tests were run on all samples. For reverse phage HPLC (shown in Table 3.2), OAs were eluted and separated with Shim pack VP-ODS C18 (150mm*4.6mm id 5μL) column. Two types of mobile phase were used, including A of 0.01M KH_2PO_4 solution (pH 2.50 with H_3PO_4) and B of 15% Acetonitrile. Elution program was set as 0.00–14.00 min, 0% B;14.00–14.01 min, 0–15% B; 14.01–15.00 min, 15% B; 15.00–15.01 min, 15–0% B; 15.01–20.00 min, 0% B. Total mobile phase volume was 0.6 ml min^{-1}. The oven temperature was set at 30℃, and the UV detector was set at 210 nm. Comparison of two measurement methods was done by better separation and eluted peaks, and the better method was chosen for next experiments.

3.2 Results and discussion

3.2.1 Quantification of OAs during fermentation with inoculated yeast isolates

The calibration curve was built for two methods with Shim-pack VP-ODS (150 mm L×4.6 mm

I.D.) column and Shodex KC-811 column (8.0 mm I.D.×300 mm) as shown in Table 3.3 and 3.4. The post column method with KC811 column was chosen for best OAs measurement for its better separation and elution stability of peaks for each target.

The nine yeast isolates identified in the previous section were screened to compare their ability to produce OAs. In the following results, the strains that were identified as *Rhodotorula mucilaginosa* have been labeled with the suffix 'R' (Y1R, Y5R, Y12aR, and Y12bR), while those that were identified as *Wickerhamomyces anomalus* have been labelled with the suffix 'W' (Y10W, Y18W, Y19W, Y38W, and Y43W).

OAs are known to play important roles in the sensory experience of coffee. It is widely recognized that acidity and the resulting perceived sourness are key to coffee quality [48]. There are naturally presenting OAs in coffee fruits, as well as those maintained after fermentation, such as citric, malic, quinic, and succinic acids. Being regarded as the main acids in green coffee beans, these OAs favor the sensorial characteristics of coffee [22, 74]. The seven targeted OAs measured: acetic, citric, formic, lactic, malic, quinic, and succinic acids, are considered to display pleasant and favorable flavors such as flowery, fruity, and acid flavors.

All seven OAs were detected in all beans inoculated with yeast isolates throughout the fermentation period (Fig. 3.4.(a) and Supplementary Table 1). Quinic acid was the most predominant acid in the fermented green beans while succinic acid was detected as the lowest concentration OA among the acids measured.

Although there was no clear trend in the increase or decrease of each OA, highest values were found in the later fermentation stages. Acetic acid, which gives an acidic and clean flavor to the coffee, was found to be most abundant in the Y12bR incubated beans after 72 h fermentation (1.33 ± 0.28 mg g^{-1}). Citric acid is generally considered as showing citrus and acidic flavor and can assistant with flavor modification as well as increasing cupping scores by spontaneous fermentation [32]. Highest citric acid (0.82 ± 0.22 mg g^{-1}) was detected in beans inoculated with Y12aR at 48 h. Formic acid, which gives a sour and bitter taste as well as adds a fermented aroma, was highest (0.49 ± 0.05 mg g^{-1})

in the control group (no additional inoculation) at 24 h. Lactic acid has a sour, acidic taste and is known to contribute to a ‘full body’ mouthfeel and ‘smooth’ sensations [45]. Highest lactic acid was detected in Y1R fermented beans at 72 h (2.41 ± 0.03 mg g^{-1}) and the second highest concentration was detected in Y12bR inoculated beans at 72 h (2.21 ± 0.78 mg g^{-1}). Malic acid gives out a sour taste, and together with lactic, citric, acetic, and fumaric acids, may benefit in increasing cupping score as well as increasing fruity flavor [45]. Among all fermented beans with yeast isolates, only the inoculation of Y1R, Y12aR, and Y12bR showed significant difference with control, with Y12aR inoculated beans showing the highest content of malic acid (0.57 ± 0.07 mg g^{-1}) at 48 h. Quinic acid was the most abundant among all OAs and was detected to be highest (2.36 ± 0.65 mg g^{-1}) with Y12bR inoculation at 48 h. Quinic acid not only add an acidic taste to the beverage, but along with succinic acid, also contributes to a perceptible bitter taste [48, 83]. Succinic acid showed lowest concentration among all measured OAs, with the highest detected amount in the control fermentation at 24 h.

Two-way ANOVA showed that the different *Wickerhamomyces anomalus* and *Rhodotorula mucilaginosa* strains significantly ($p<0.01$) affected the amount of quinic acid during fermentation. The content of individual OA did not show a clear increasing or decreasing trend during fermentation.

The total concentrations of the seven OAs measured are shown in the bottom row of Supplementary Table 1. After 24 h of fermentation, all inoculated groups showed higher total OA content compared to the control group (3.51 ± 0.16 mg g^{-1}), among which inoculation with Y12bR showed the highest amount (4.70 ± 0.44 mg g^{-1}). At 48 h and 72 h, the highest total OA content was detected in the beans inoculated with Y12bR, with concentrations of 4.90 ± 0.64 mg g^{-1} and 5.25 ± 0.85 mg g^{-1}, respectively.

3.2.2 Quantification of OAs during fermentation with inoculated LAB isolates

The eleven LAB isolates gained in the previous section were evaluated to compare their OAs producing ability. For LAB isolates, since they were identified as one species of *Enterococcus mundtii*,

in the following results, the strains have been labeled with the prefix ‘L’ (L4, L5, L6a, L6b, L8, L10, L12, L14, L16, L17a and L17b).

OAs produced with the inoculation of eleven LAB isolates are shown in Fig. 3.4.(b) and Supplementary Table 2. Green beans inoculated with L4 showed high concentration of total OAs throughout fermentation, with the highest content among other inoculations at 12 h and 72 h of fermentation, and the second highest content at 24 h and 48 h of fermentation. The production of OAs by LAB would be beneficial when considering their coexistence with yeasts. Researchers have found that acidification by LAB not only aids the growth of starters via offering a low pH environment, but also competes for space and nutrients with non-fermentation microbial species, thereby decreasing the generation of off-flavors [84].

3.3 Conclusion and summary

This chapter investigated the potential of new isolates as starter candidates by primarily evaluating organic acid (OA) producing ability in fermentation. The isolates were gained from coffee material itself, and by using dry coffee pulp as substrate, which was achieved as coffee by-products from coffee processing, to act as a role to provide nutrition for growth of microorganisms. Metabolites from microbial activities were collected, extracted and measured which may help modify compounds composition in green beans and consequently affect the beverage flavors of coffee beans.

Following the results of the previous chapter, a total of nine yeasts (including 5 strains belong to *Wickerhamomyces anomalus* and 4 strains belong to *Rhodotorula mucilaginosa*) and eleven lactic acid bacteria (LAB, *Enterococcus mundtii*) were obtained and identified from coffee material where the spontaneous fermentation happened with a mixture of green coffee beans and dry coffee pulp. These isolates were inoculated in sealed fermentation container where were added with a mixture of green coffee beans, dry coffee pulp, and sterile water, and fermentation was conducted while preserving the original microbial flora present in the green beans and dried pulp. Fermentation took place for 24, 48 and 72 h, with the different incubation of isolates showed influences on OAs presentation, which may

also result in different volatile profiles in green and furthermore lead to different compounds composition in roasted beans, which will be measured in the future. Besides, in this chapter only mono-inoculation was discussed, co-inoculation of yeast and LAB isolates in green beans fermentation should also be considered in future study.

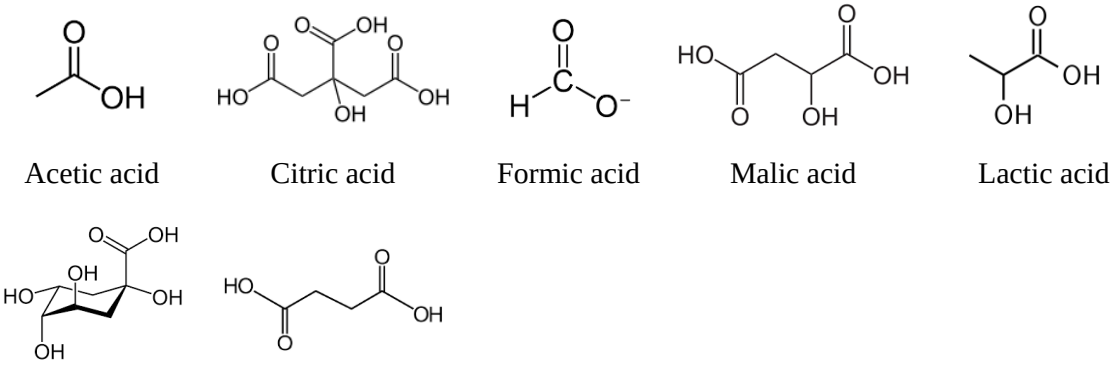

Fig. 3.1. Structure of acetic, citric, formic, lactic, malic, quinic, succinic acids and caffeine & CGA.

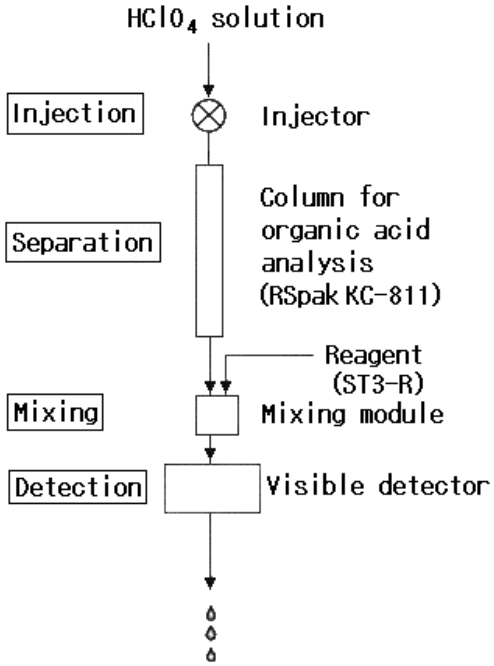

Fig. 3.2. Structure of post-column detection of HPLC with color reagent (ST3-R) [※].

※: *https://www.shodex.com/cn/dc/03/08/22.html*

Table 3.1. Condition of HPLC (organic acids)

Column	Shodex KC-811 column (8.0 mm I.D.×300 mm) with KC-G 8B guard column (8.0 mm I.D.×35 mm)
Mobile phase	3 mM $HClO_4$
Column temp.	40 ℃
Detector	430 nm
Flow rate	1 ml min^{-1} of mobile phase +0.7 ml min^{-1} of ST3-R

Table 3.2 Condition of HPLC (organic acids)

Column	Shim pack VP-ODS C18 (150mm*4.6mm id 5µL column)
Mobile phase	A- 0.01M KH_2PO_4 solution (pH 2.50 with H_3PO_4) B- 15% Acetonitrile
Column temp.	30 ℃
Detector	210 nm
Elution programmed	0.00–14.00 min, 0% B;14.00–14.01 min, 0–15% B; 14.01–15.00 min, 15% B; 15.00–15.01 min, 15–0% B; 15.01–20.00 min, 0% B.
Flow rate	0.6 ml min^{-1}

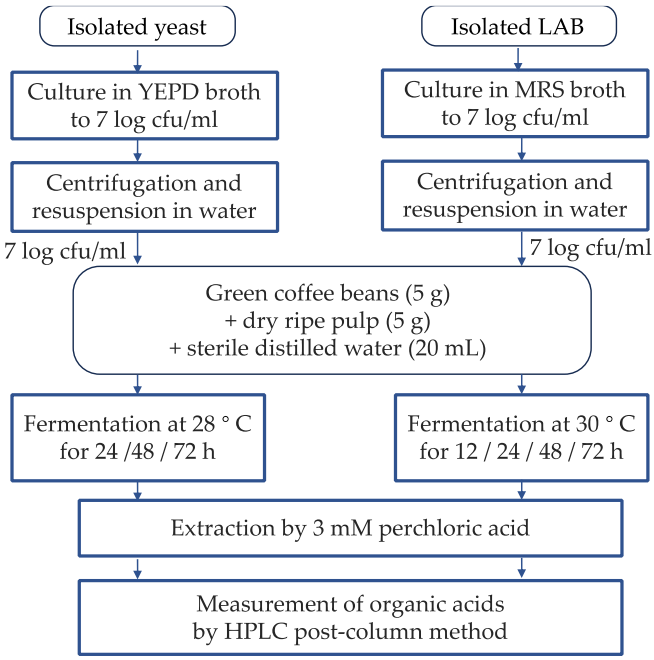

Fig. 3.3. Flow chart of fermentation process in section 3.2

Table 3.3. Regression analysis of calibration curves, Shim-pack VP-ODS (150 mm L×4.6 mm I.D.)

No.	Targets	λ, nm	Retention time(min)	Linear equation	Concentration range(mg 10ml^{-1})	Correlation coefficient (R^2)
1	Acetic acid	200	11.15	f(x)=136782*x+38907.7	2-10	0.9993728
2	Citric acid	200	14.259	f(x)=212480*x-106465	2-10	0.9981019
3	Formic acid	200	8.205	f(x)=252500*x-432864	2-10	0.9904545
4	Lactic acid	200	10.120	f(x)=157606*x-241215	2-10	0.9902416
5	Malic acid	200	8.573 15.455	f(x)=196999*x+336532	2-10	0.9973901
6	Quinic acid	200	7.546	f(x)=132623*x+53242.7	2-10	0.9991856
7	Succinic acid	200	15.588	f(x)=142825*x+299808	2-10	0.9989575
8	Caffeine	270	7.951	f(x)=104.374*x+779.606	(1-8)*10^3	0.9986037
9	CGA	320	7.119	f(x)=29746.7*x-32589.9	(1-8)*10^3	0.9998150

Table 3.4. Regression analysis of calibration curves, KC-811 column (organic acids)

No.	Targets	λ, nm	Retention time(min)	Linear equation	Concentration range(mg 10ml^{-1})	Correlation coefficient (R^2)
1	Acetic acid	200	15.212	f(x)=26851.5*x-3267.20	2-10	0.9997380
2	Citric acid	200	9.497	f(x)=49798.9*x-7447.29	2-10	0.999493
3	Formic acid	200	14.021	f(x)=43223.8*x-6478.58	2-10	0.9996732
4	Lactic acid	200	11.675	f(x)=22465.2*x-4992.38	2-10	0.9979544
5	Malic acid	200	10.661 13.785	f(x)=42045.3*x-7420.87	2-10	0.9999252
6	Quinic acid	200	10.970	f(x)=23019.4*x-3125.30	2-10	0.9998752
7	Succinic acid	200	12.503	f(x)=27947.0*x-4667.73	2-10	0.9990973

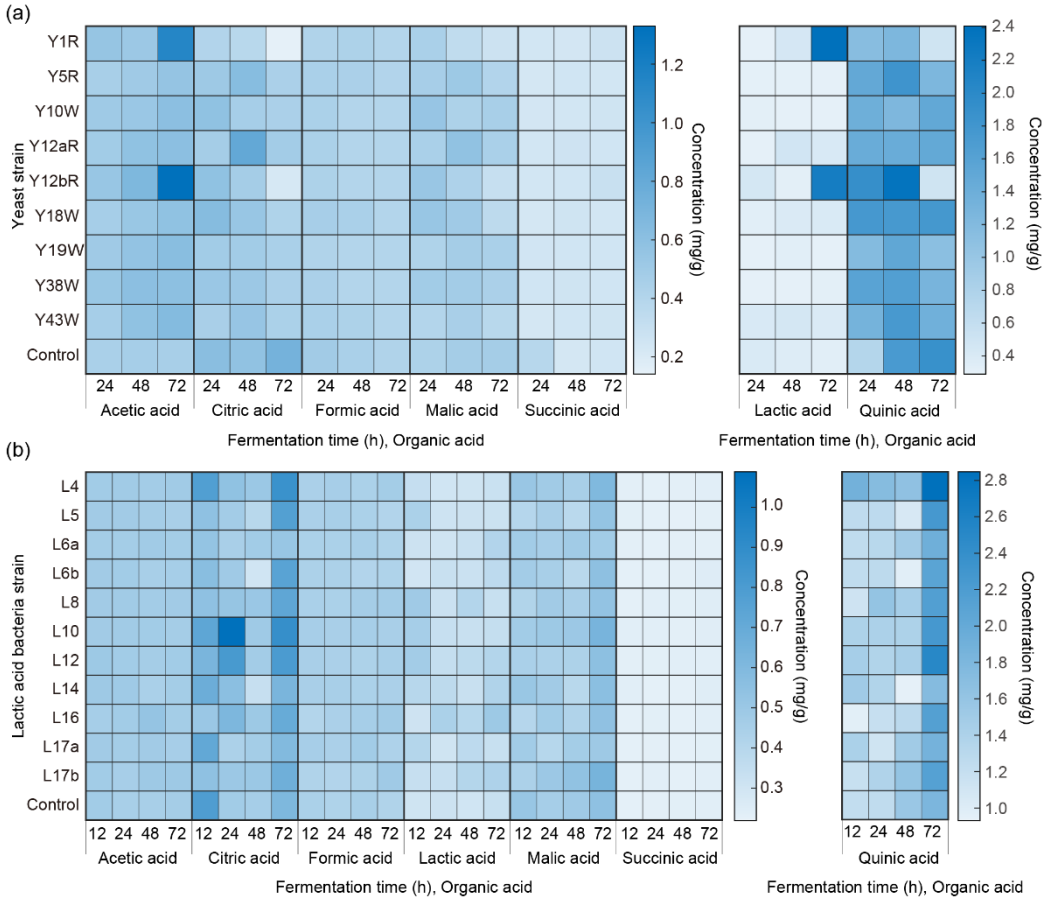

Fig. 3.4. OAs quantified in fermented coffee with (a) yeast isolates at 24, 48, and 72 h and (b) LAB isolates at 12, 24, 48, and 72 h (*n=4*).

Table 3.5. Organic acids quantified in the fermented coffee beans with different yeast isolates. Results are presented as mean ± SD (*n=4*) of organic acids (mg/g). For each row, concentration denoted with star labels are significantly different from control (* for $p < .05$, ** for $p < .01$, *** for $p < .001$)

Acids/ time (h)	Treatments									
	Y1R	Y5R	Y10W	Y12aR	Y12bR	Y18W	Y19W	Y38W	Y43W	Control
Acetic										
24	0.55±0.01*	0.46±0.01	0.51±0.04	0.49±0.01	0.54±0.10*	0.47±0.01	0.50±0.01	0.53±0.01	0.47±0.00	0.45±0.01
48	0.52±0.01	0.50±0.03	0.53±0.01	0.58±0.02*	0.67±0.05***	0.53±0.02	0.57±0.02*	0.60±0.05**	0.58±0.06*	0.47±0.02
72	1.13±0.01***	0.56±0.04*	0.61±0.02**	0.60±0.03**	1.33±0.28***	0.58±0.03**	0.62±0.03***	0.60±0.02**	0.64±0.05***	0.46±0.02
Citric										
24	0.41±0.03**	0.51±0.03	0.58±0.13	0.48±0.08*	0.58±0.15	0.64±0.05	0.49±0.05	0.53±0.10	0.45±0.03*	0.61±0.21
48	0.38±0.01**	0.63±0.02	0.47±0.04	0.82±0.22***	0.47±0.04	0.54±0.07	0.49±0.05	0.52±0.05	0.55±0.05	0.57±0.18
72	0.14±0.00***	0.45±0.12***	0.45±0.03***	0.48±0.11***	0.22±0.02***	0.42±0.04***	0.45±0.13***	0.45±0.07***	0.44±0.02***	0.72±0.19
Formic										
24	0.42±0.02***	0.45±0.01***	0.44±0.02***	0.44±0.01***	0.43±0.03***	0.46±0.01**	0.42±0.01***	0.45±0.03***	0.45±0.02***	0.49±0.05
48	0.43±0.01	0.45±0.01	0.40±0.00***	0.41±0.00***	0.40±0.00***	0.45±0.00	0.40±0.00***	0.40±0.00***	0.43±0.02	0.45±0.01
72	0.40±0.01	0.41±0.01	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00	0.42±0.02
Lactic										
24	0.29±0.02	0.30±0.01	0.31±0.01	0.29±0.00	0.45±0.11	0.33±0.03	0.30±0.01	0.29±0.01	0.41±0.04	0.40±0.11
48	0.44±0.02	0.30±0.00	0.29±0.01	0.48±0.12	0.31±0.03	0.39±0.12	0.33±0.06	0.29±0.01	0.45±0.11	0.37±0.12
72	2.41±0.03***	0.30±0.01	0.30±0.01	0.41±0.09	2.21±0.78***	0.40±0.13	0.30±0.00	0.31±0.07	0.39±0.06	0.34±0.04
Malic										
24	0.45±0.03	0.47±0.02	0.55±0.10**	0.45±0.03	0.54±0.06**	0.54±0.01**	0.42±0.00	0.49±0.06	0.41±0.05	0.43±0.04
48	0.33±0.01***	0.52±0.01	0.43±0.04	0.57±0.07**	0.43±0.04	0.50±0.05	0.48±0.02	0.48±0.02	0.45±0.05	0.48±0.05
72	0.27±0.01***	0.41±0.03	0.46±0.03	0.45±0.06	0.30±0.10***	0.35±0.05*	0.45±0.05	0.44±0.01	0.38±0.07*	0.47±0.11
Quinic										
24	1.15±0.07*	1.49±0.18***	1.38±0.09**	1.44±0.16***	1.91±0.23***	1.76±0.35***	1.18±0.14*	1.57±0.43***	1.32±0.11**	0.74±0.27
48	1.25±0.09*	1.83±0.09	1.25±0.16*	1.43±0.14	2.36±0.65**	1.75±0.40	1.52±0.40	1.64±0.32	1.74±0.03	1.73±0.41
72	0.50±0.02***	1.26±0.32**	1.50±0.12	1.49±0.38	0.49±0.19***	1.76±0.10	1.12±0.14***	1.29±0.15*	1.37±0.20*	1.88±0.70
Succinic										
24	0.24±0.01***	0.23±0.00***	0.24±0.01***	0.24±0.01***	0.24±0.01***	0.23±0.00***	0.25±0.00***	0.25±0.00***	0.23±0.00***	0.38±0.13
48	0.24±0.01	0.25±0.00	0.24±0.01	0.27±0.00	0.25±0.00	0.25±0.01	0.25±0.00	0.26±0.01	0.25±0.01	0.23±0.00
72	0.28±0.03	0.24±0.01	0.26±0.01	0.25±0.01	0.29±0.02*	0.24±0.01	0.25±0.01	0.25±0.01	0.25±0.01	0.25±0.03
Total										
24	3.51±0.12	3.91±0.23	4.00±0.36	3.82±0.21	4.70±0.44***	4.43±0.29**	3.56±0.19	4.11±0.57*	3.74±0.17	3.51±0.16
48	3.59±0.13*	4.47±0.13	3.62±0.22*	4.55±0.27	4.90±0.64*	4.43±0.59	4.03±0.53	4.21±0.43	4.46±0.15	4.29±0.77
72	5.14±0.07	3.62±0.50**	3.97±0.18	4.08±0.67	5.25±0.85*	3.60±0.14	3.89±0.69**	4.00±0.22*	3.88±0.16*	4.54±0.95
Average	4.08±0.03	4.00±0.19	3.86±0.10	4.15±0.25	4.95±0.20*	4.15±0.23	3.83±0.26	4.11±0.17	4.03±0.01	4.11±0.42

Table 3.6. Organic acids quantified in the fermented coffee beans with different lactic acid bacteria isolates

Acids	Time (h)	Treatments											
		L4	L5	L6	L6b	L8	L10	L12	L14	L16	L17	L17b	Control
Acetic	12	0.47±0.01	0.48±0.01	0.46±0.01	0.47±0.01	0.47±0.01	0.48±0.03	0.49±0.01	0.49±0.02	0.46±0.01	0.47±0.01	0.47±0.01	0.47±0.01
	24	0.48±0.01	0.48±0.02	0.46±0.00	0.47±0.01	0.48±0.02	0.48±0.03*	0.47±0.00	0.49±0.01*	0.47±0.01	0.47±0.01	0.45±0.01	0.45±0.00
	48	0.47±0.00	0.46±0.01	0.48±0.02	0.45±0.00	0.47±0.01	0.47±0.01	0.46±0.01	0.44±0.01	0.52±0.11**	0.47±0.01	0.49±0.01*	0.45±0.01
	72	0.48±0.01	0.45±0.01	0.47±0.00	0.46±0.01	0.47±0.01	0.46±0.02	0.47±0.01	0.46±0.02	0.46±0.00	0.46±0.02	0.49±0.02	0.46±0.00
Citric	12	0.78±0.23	0.55±0.07***	0.52±0.07***	0.57±0.01***	0.55±0.04***	0.73±0.06	0.63±0.09*	0.68±0.04	0.50±0.01***	0.71±0.10	0.55±0.02***	0.79±0.01
	24	0.54±0.05	0.47±0.03	0.45±0.03	0.48±0.04	0.52±0.04	1.09±0.10***	0.81±0.08***	0.56±0.03	0.62±0.01*	0.43±0.00	0.49±0.01	0.47±0.03
	48	0.50±0.01	0.39±0.03	0.47±0.03	0.30±0.07*	0.50±0.05	0.48±0.04	0.47±0.02	0.34±0.02*	0.49±0.01	0.46±0.02	0.5±0.01	0.46±0.02
	72	0.86±0.20***	0.77±0.14**	0.51±0.15	0.75±0.29*	0.73±0.14	0.88±0.06***	0.80±0.22**	0.63±0.01	0.69±0.01	0.59±0.01	0.66±0.00	0.61±0.03
Formic	12	0.44±0.01	0.44±0.00	0.44±0.01	0.44±0.02	0.44±0.02	0.46±0.01*	0.45±0.01	0.45±0.02	0.41±0.01***	0.45±0.01	0.42±0.02*	0.44±0.01
	24	0.46±0.01***	0.45±0.02***	0.44±0.01	0.43±0.01	0.44±0.01	0.45±0.01**	0.44±0.02*	0.45±0.02***	0.44±0.01*	0.44±0.01*	0.42±0.01	0.42±0.01
	48	0.44±0.02	0.44±0.01	0.45±0.01	0.41±0.01***	0.46±0.01	0.46±0.00	0.43±0.01*	0.44±0.00	0.46±0.01	0.47±0.00*	0.43±0.01*	0.45±0.00
	72	0.46±0.01***	0.41±0.01	0.42±0.02	0.42±0.01	0.47±0.01***	0.45±0.01**	0.45±0.01***	0.44±0.01*	0.48±0.01***	0.43±0.01	0.48±0.01***	0.42±0.01
Lactic	12	0.34±0.01	0.44±0.02***	0.32±0.02	0.30±0.01	0.48±0.03***	0.46±0.03***	0.47±0.06***	0.39±0.03**	0.31±0.01	0.40±0.02***	0.34±0.02	0.31±0.01
	24	0.30±0.02	0.31±0.01	0.30±0.01	0.33±0.02	0.32±0.03	0.34±0.02	0.35±0.02	0.37±0.01*	0.44±0.01***	0.30±0.01	0.34±0.02	0.32±0.01
	48	0.31±0.02	0.32±0.02	0.33±0.01	0.32±0.02	0.41±0.10***	0.33±0.01	0.38±0.05**	0.33±0.03	0.40±0.04***	0.37±0.02**	0.40±0.03***	0.30±0.00
	72	0.33±0.01	0.32±0.02	0.42±0.12**	0.37±0.06	0.33±0.02	0.35±0.02	0.41±0.05**	0.42±0.02**	0.49±0.01***	0.33±0.01	0.43±0.04***	0.33±0.06
Malic	12	0.51±0.06	0.40±0.02***	0.47±0.03	0.47±0.02	0.42±0.02***	0.47±0.03	0.44±0.02**	0.51±0.00	0.40±0.01***	0.47±0.00	0.43±0.02**	0.51±0.01
	24	0.48±0.03	0.45±0.04	0.45±0.03	0.45±0.02	0.48±0.03	0.49±0.04	0.43±0.04	0.48±0.01	0.47±0.01	0.40±0.01*	0.50±0.00	0.46±0.03
	48	0.45±0.02	0.38±0.02***	0.48±0.03	0.39±0.01***	0.45±0.01	0.50±0.01	0.43±0.02*	0.39±0.02***	0.42±0.01*	0.46±0.01	0.54±0.01*	0.48±0.01
	72	0.60±0.05*	0.53±0.06	0.47±0.10**	0.55±0.10	0.53±0.05	0.63±0.03***	0.55±0.08	0.56±0.01	0.54±0.01	0.49±0.02*	0.63±0.01***	0.54±0.01
Quinic	12	1.89±0.71***	1.25±0.17	1.24±0.17	1.24±0.11	1.13±0.12	1.40±0.20	1.46±0.18	1.52±0.07	0.95±0.04	1.42±0.06	1.18±0.1	1.23±0.05
	24	1.73±0.18*	1.26±0.09	1.31±0.16	1.27±0.20	1.61±0.16	1.41±0.22	1.37±0.15	1.37±0.03	1.19±0.50	1.11±0.02	1.38±0.05	1.24±0.08
	48	1.65±0.12	1.05±0.15*	1.50±0.20	0.96±0.05**	1.46±0.07	1.40±0.05	1.44±0.05	0.93±0.03**	1.29±0.06	1.51±0.02	1.61±0.09	1.55±0.01
	72	2.85±0.43***	2.26±0.60*	1.91±0.50	2.09±0.72	2.16±0.44	2.26±0.32*	2.51±0.64***	1.74±0.50	2.14±0.04	1.87±0.11	2.13±0.09	1.81±0.15
Succinic	12	0.23±0.00	0.23±0.00	0.23±0.00	0.22±0.00	0.22±0.00	0.23±0.00	0.23±0.00	0.24±0.00	0.23±0.00	0.23±0.01	0.22±0.00	0.22±0.00
	24	0.22±0.02	0.23±0.00	0.22±0.00	0.22±0.00	0.24±0.01	0.23±0.01	0.23±0.00	0.23±0.01	0.23±0.01	0.23±0.00	0.24±0.01	0.23±0.01
	48	0.23±0.01	0.22±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.24±0.00	0.22±0.00	0.23±0.01	0.22±0.00	0.23±0.00	0.23±0.00
	72	0.24±0.01	0.23±0.01	0.23±0.00	0.25±0.03	0.25±0.02	0.24±0.01	0.24±0.01	0.25±0.01	0.24±0.00	0.23±0.01	0.24±0.00	0.24±0.01
Total	12	4.65±0.98**	3.78±0.28	3.69±0.28	3.70±0.15	3.72±0.23	4.22±0.30	4.16±0.35	4.26±0.05	3.14±0.16*	4.10±0.11	3.56±0.08	3.83±0.29
	24	4.20±0.27**	3.65±0.15	3.64±0.19	3.66±0.28	4.08±0.25*	4.49±0.36***	4.10±0.24*	3.94±0.04	3.80±0.47	3.31±0.12	3.82±0.07	3.51±0.27
	48	4.04±0.15	3.26±0.18*	3.93±0.25	3.07±0.11**	3.98±0.18	3.87±0.06	3.85±0.04	3.10±0.02**	3.80±0.18	3.92±0.07	4.14±0.09	3.93±0.00
	72	5.82±0.66***	4.96±0.84*	4.43±0.81	4.89±1.20*	4.93±0.64*	5.27±0.45***	5.43±0.99***	4.49±0.49	5.05±0.06**	4.40±0.14	5.06±0.08**	4.22±0.29
Average		4.68±0.90	3.91±0.77	3.92±0.52	3.83±0.88	4.18±0.58	4.46±0.61	4.39±0.80	3.95±0.59	3.95±0.75	3.93±0.43	4.14±0.59	3.87±0.34

Results are presented as mean ± SD (n = 4) of organic acids (mg/g). For each row, concentration denoted with star labels are significantly different from control (* for $p < .05$, ** for $p < .01$, *** for $p < .001$)

Chapter 4 Evaluation of OAs production with different fermentation factors

Overview

OAs production with the inoculation of eleven LAB isolates and nine yeast isolates were carried out from last chapter. After statistics analysis, not every organic acid behaved significantly different in various inoculation. To maximum the effects from the microbial metabolites, in this chapter different growth factors of yeast were studied and monitoring of how these factors influence on organic acid production individually was also operated. It is essential in thoroughly eliciting important factors for well-controlled coffee fermentation and allowing optimization thereof as a prelude to further studies. Insight into the impacts of inoculation dose, better stimulation of fresh coffee pulp and two types of coffee pulp on fermentation.

4.1 Material and methods

4.1.1 Coffee beans and coffee pulp

Coffee beans from Finca Los Tres Edgaritos-Cauca, Colombia (Coffea arabica, Castillo var.). Dry pulp of Geisha variety was used for fermentation. Coffee cherries from different ripen degree, including ripe (bright red color) and over ripe (dark red color), were picked by experienced pickers in Finca Los Tres Edgaritos-Cauca and later collected after de-pulped by pulper machine. Drying in solar house for 2 weeks after depulped until totally dry. Packed in polyethylene bags and shipped to Japan.

4.1.2 Fermentation process with selected yeast under different growth factors

After screening (chapter 3), the following studies were carried out to optimize fermentation conditions. (1) Sucrose, as an important simple sugar in coffee cherry, as well as a selective carbon source for yeasts (hydrolyzed into simple sugars such as fructose or glucose by extracellular enzymes and utilized by yeasts), was added as an extra energy source to the fermentation bottle at a concentration of 1 % (w/w). (2) To evaluate the effect of different inoculation volumes, higher population of 8 log ml^{-1} were added at the start of fermentation. (3) Over ripe pulp was used instead of ripe pulp as an alternative fermentation substrate. Each condition was evaluated with several selected isolates and all other conditions were kept constant. The OA content in the beans was measured as explained earlier. In this chapter, fermentation took place in 250 ml sterile blue-cap bottles with 5 g of green beans, 5 g of dry ripe pulp, and 50 ml of distilled water, at 28 °C from 24 to 48 h. Two bottles were prepared as repeats for each growth factor at each fermentation time.

4.1.3 Organic acids extraction and measurement by HLPC

Same OAs, including acetic, citric, formic, lactic, malic, quinic and succinic acids were monitored. OAs in the coffee beans were sampled, extracted, and quantified at 24, 48, and 72 h of fermentation under different factors as explained in section 3.1.3.

4.1.4 Statistical analysis

Analysis of variance (ANOVA) and post-hoc Tukey HSD analysis were performed with SPSS version 28.0.1.1 (IBM corp. New York, USA).

4.2 Results and Discussion

The effect of the following fermentation factors: additional carbon source (sucrose), higher inoculation population, and different fermentation substrates (over ripe coffee pulp) were evaluated based on the concentration of OAs produced during fermentation (Fig. 4.1.). Since metabolites from microbial activity have been demonstrated to affect the final OA composition during fermentation of green coffee [22, 74], fermentation conditions that would influence microbial activity were evaluated.

An approximated 60 % reduction of total simple sugars (glucose, fructose, and sucrose) is reported to occur during fermentation due to microflora activity [31]. Therefore, an additional carbon source (1 % sucrose) was added to the initial substrate to stimulate the fresh cherry sugar composition with the expectation that it may enhance fermentation. For this evaluation, the strains with the highest and lowest OA production were used, which were Y5R and Y12bR, and Y19W and Y18W as representative strains for *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus*, respectively. Fig. 4.1.(a) compares fermentation with and without sugar and shows the total OAs in the fermented beans along with the individual OA composition. Generally, the additional sucrose resulted in lower total OA production for beans inoculated with *Rhodotorula mucilaginosa* strains. In particular, quinic acid showed significant decrease with the addition of sugar. Similar effects were observed for *Wickerhamomyces anomalus* inoculated beans, with the exception of Y19W inoculated beans. The Y19W strain produced the lowest content of OAs in the initial screening step, and it is interesting to observe that strains from same species may have different preferences for carbon sources. The addition of sucrose did not increase OA content in the control group either, which was fermented without the addition of inoculates. Various other fermentable sugars (e.g., fructose and glucose, originally exist in coffee pulp) could be considered as alternative carbon sources for coffee fermentation [85].

To enhance metabolic production from the yeast isolates, a higher inoculation concentration was added to the initial substrate (Fig. 4.1.(b)). Isolates that showed highest OA production, namely, Y12bR and Y18W, were used. Conventionally, an inoculum of 6 to 8 log cfu ml^{-1} [33, 35] is used for fermentation starters. Higher inoculum (8 log cfu ml^{-1}) was shown to increase certain acids while decreasing others. Generally, higher inoculation did not efficiently increase OAs production during whole fermentation, which could be explained by energy and space competition from large population. A similar phenomenon has been reported previously; yeast plateaued at around 8.1 log cfu ml^{-1} during fermentation [35]. Besides, since achieving a higher population requires more culture time and cost, an inoculum of 7 log cfu ml^{-1} was sufficient.

Finally, the use of over-ripe pulp was compared to ripe pulp as a substrate for OA production (Fig. 4.1.(c)). The same isolates used for evaluating the effect of sugar addition were used. For all yeast isolates, the use of over-ripe pulp significantly decreased total OA concentration, mainly due to the decrease of quinic and citric acids. The difference was generally larger for the *Rhodotorula mucilaginosa* isolates than *Wickerhamomyces anomalus*.

Overall, these results showed that fermentation using ripe pulp without sucrose and the inoculation of 7 log cfu ml^{-1} was sufficient for effected fermentation.

4.3 Conclusion and summary

In this chapter, alternative fermentation conditions were explored to maximize OA production during fermentation. Alternative fermentation conditions such as the addition of carbon source, different inoculum dose, and different status of coffee pulp were evaluated in coffee fermentation. On the basis of OAs produced, the following conditions were sufficient for effective fermentation: no additional sucrose, inoculation of 7 log cfu ml^{-1}, and the use of ripe pulp as the substrate.

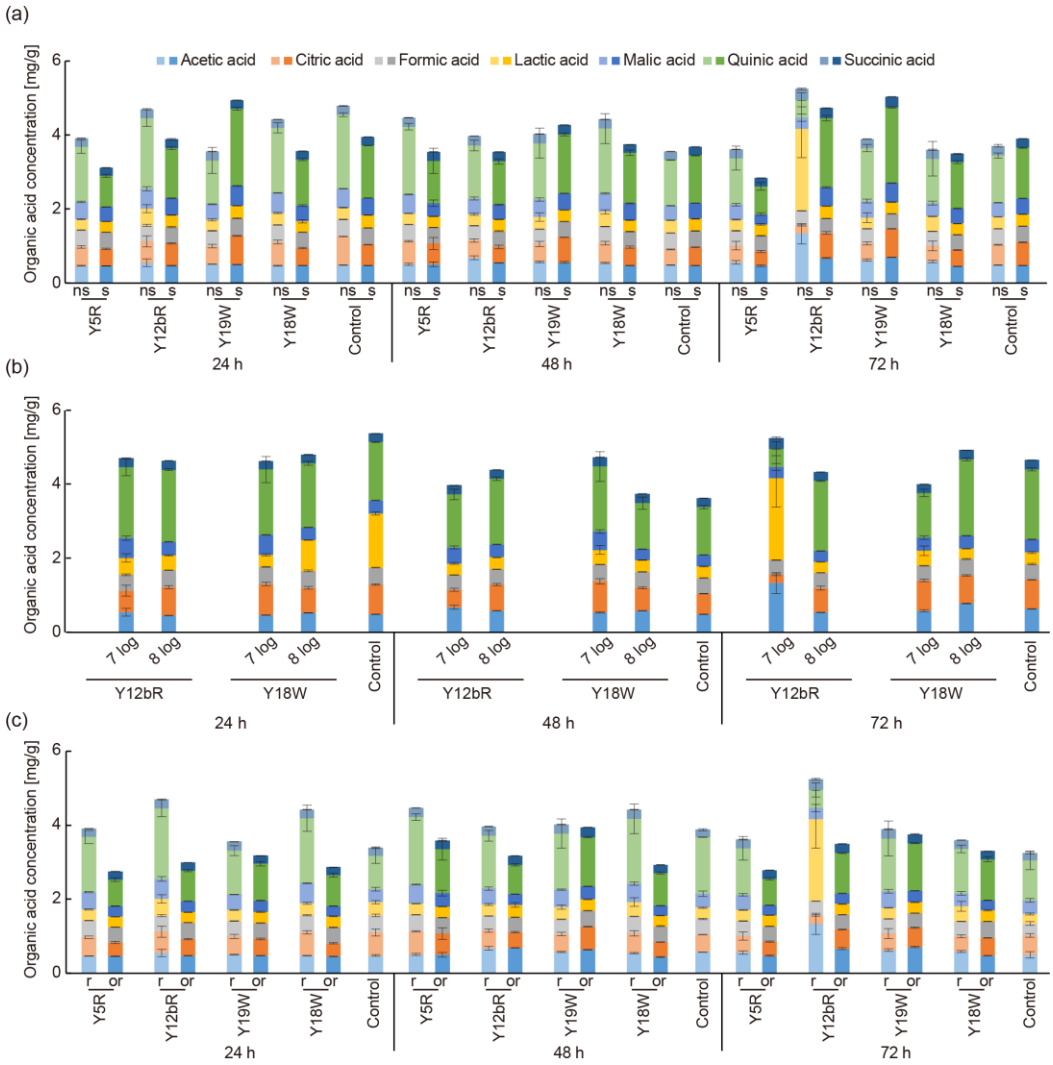

Fig. 4.1. OAs quantified in fermented coffee at 24, 48 and 72 h with (a) additional carbon source, where samples with and without added sucrose are labelled ‘s’ and ‘ns’, respectively; (b) different inoculum dose of yeast isolates; and (c) fermentation with different types of pulp, where ‘r’ and ‘or’ stand for ripe and over ripe pulp, respectively.

Chapter 5 Evaluation of OAs & volatiles and sensory analysis in 2 varieties of beans fermentation with selected isolates

Overview

The optimum combination was found at 7 log cfu ml^{-1} of inoculation and ripe pulp for higher organic acids production. In this chapter, the optical fermentation conditions together with screened high organic acid producing yeast strains of YR (*Rhodotorula mucilaginosa*) and YW (*Wickerhamomyces anomalus*) and lactic acid bacteria strain with the label of LAB (*Enterococcus mundtii*) for fermentation in two different varieties of green coffee beans was continued. More comprehensive study needs to be implemented for application of isolated starters in different coffee beans. In addition to the evaluation of organic acids and volatile compounds in fermented green beans, chemicals composition in roasted coffee beans need to execute. Apart from chemical analysis, practical application of self-isolated starters should be done based on sensory assessment.

5.1 Material and methods

5.1.1 Coffee beans and coffee pulp

Coffee beans (arabica, var. Castillo) from Finca Los Tres Edgaritos, Cauca, Colombia and coffee beans (arabica, var. SL N.28) from Nyeri/Karindundu, Kenya were prepared as samples. The wet-processed beans contained approximately 10% moisture content. Dried ripe pulp of Geisha variety was collected from same farm in Colombia. YR (*Rhodotorula mucilaginosa*) and YW (*Wickerhamomyces anomalus*) were selected from chapter 2, and LAB (*Enterococcus mundtii*) was selected for their higher OA producing ability. They were purified by spreading plate culture, and then picked and re-cultured in YEPG and MRS broth for yeast and LAB respectively. Cells were harvest when the population reach around 7 log cfu ml $^{-1}$.

5.1.2 Fermentation process with selected yeast and LAB isolates

The fermentation process in this section proceeded in two different scales depending on the analyte. For the evaluation of OAs, chlorogenic acid (CGA), and caffeine in the fermented green beans, and for measurement of pH and microbial count, fermentation was performed with 5 g of green beans, 5 g of dry pulp, and 20 ml of distilled water. For the evaluation of volatile compounds in the roasted beans and sensory evaluation, an enlarged scale of 60 g of beans, 60 g of dry pulp, and 240 ml of water in 500 ml Erlenmeyer flasks was used. The inoculation concentration was kept constant, as explained below.

The following five inoculation conditions were tested using isolates that were obtained in a previous study (chapter 2): (a) mono-culture with yeast species *Rhodotorula mucilaginosa* (YR); (b) co-culture with *Rhodotorula mucilaginosa* and LAB (YRcoL); (c) mono-culture with yeast species *Wickerhamomyces anomalus* (YW); (d) co-culture with *Wickerhamomyces anomalus* and LAB (YWcoL); no additional inoculation (Control). The LAB were of the species *Enterococcus mundtii*. All treatments were carried out with both Kenya and Colombia beans, and all experiments were set-up in

duplicate. The isolates were inoculated at a concentration of 7 log and 6 log cfu ml^{-1} for the yeast and LAB, respectively.

All groups were cultured at 28 °C for 24, 48, or 72 h, resulting in 30 fermentation conditions (5 inoculation conditions, 2 types of beans, 3 fermentation durations). The fermented green coffee beans were collected from the fermentation bottle at the end of the fermentation process and stored at −20 °C until analyses.

5.1.3 pH monitor and microbial counting during fermentation

The pH of the fermented solution was monitored every 24 h with a pH meter (Horiba, Japan). Microbial counts of yeast and LAB were also performed every 24 h during fermentation. For the microbial count, 2 g of fermented beans were taken out of the fermentation bottle and added to a bottle containing 8 ml of sterilized saline-peptone water. After mixing for 20 min at 150 rpm in a sterile shaker, ten-fold dilutions were prepared. The yeasts were counted on Yeast and Mold Count Plate (Petrifilm, 3M) after 2 days of incubation at 28 °C. The LAB were counted by spread plating in triplicate on LAB count agar plates (Biokardiagnostics, France) and incubating for 2 days at 30 °C.

5.1.4 Chemical analysis of fermented beans by HPLC

The effects of different inoculations and fermentation time on OAs, caffeine, and CGA in the fermented green beans were analyzed. The following seven OAs: acetic, citric, formic, lactic, malic, quinic, and succinic acids were analyzed by HPLC (Shimadzu, Kyoto, Japan) using the ion exclusion method. CGA and caffeine were analyzed by HPLC reverse-phase method. The details of the analysis are explained below.

5.1.4.1 Analysis of OAs

OAs were extracted and quantified with HPLC using the same method reported in a previous chapter. One gram of fermented green beans was mixed with 3 ml of 3 mM perchloric acid solution in a 50-ml Erlenmeyer flask, followed by 10 min of vortexing at room temperature. The supernatant was acquired from the mixture by centrifugation at 14,000 g, 4 °C for 10 min, and was filtered using a 0.45

μm membrane filter. The filtered extract was stored at −20 °C until analysis.

The OAs were measured using HPLC with the post-column method. The acids were separated with a KC-811 column (8.0 mm I.D.×300 mm) and a KC-G 8B guard column (8.0 mm I.D.×35 mm) (Shodex, Japan). A mobile phase of 3 mM perchloric acid was used at a flow rate of 0.6 ml min^{-1} and 0.7 ml min^{-1} of coloring reagent (ST3-R, Shodex, Japan) was added post-column. The oven temperature was set at 50 °C and UV detector at 430 nm. Duplicate tests were run on all samples.

5.1.4.1 Analysis of CGA and caffeine

For the analysis of GCA and caffeine, fermented beans were measured to 2.0 g and extracted with 10 ml of methanol at room temperature for 30 min while stirring at 1000 rpm. Centrifugation at 14000 g for 10 min at 4 °C was followed by filtration using a 0.45 μm membrane filter. Shim-pack VP-ODS column (4.6 mm I.D.×150 mm, 5 μm) with GVP-ODS guard column (4.6 mm I.D.×50 mm) (Shimadzu, Japan) was used for separation in HPLC. The HPLC program information show in Table 5.1. A mobile phase of A: 10 mM phosphate buffer (adjusted pH to 2.6) and B: Acetonitrile at 1 ml min^{-1} were used for elution using the following flow program: 0–5 min 10%B, 5–15 min 10%–70%B, 15–18 min 70%B, 18–18.01 min 70–10% B, 18.01–24 min 10% B. The oven was set at 40 °C and the UV detector was set at 270 nm for caffeine and 320 nm for CGA.

5.1.5 Volatiles analysis of fermented green beans by GC·MS

Fermented green beans were collected and extracted using dichloromethane (Wako, Japan) for volatile compounds [86]. Ground coffee beans were measured to 2.0 g and extracted at room temperature with 10 ml of dichloromethane while vortexing for 2 min. The extraction was filtered through a 0.45 μm membrane filter and transferred to an autosampler vial (Osaka Chemical Co., Ltd., Japan). 0.2 mg ml^{-1} of 1-decanol was added into the vial as an internal standard. The measurement was carried out by GC·MS-QP2010 Plus system (Shimadzu, Japan) with RESTEK Rtx-5MS (30 m×0.25 mm I.D.×0.25 μm) column. The oven temperature was maintained at 50 °C for 2 min, raised to 220 °C at 10 °C min^{-1}, raised to 300 °C at 5 °C min^{-1}, and finally kept at 300 °C for 5 min. The injector and

detector were maintained at 230 and 240 °C, respectively. The helium carrier gas was maintained at a flow rate of 1.3 ml min^{-1}. The identification of the compounds was carried out by comparison mass spectra with the National Institute of Standards and Technology (NIST) mass spectral library (NIST 05) [17, 87]. For each treatment, two repeats were prepared, and all analysis were done in duplicate.

5.1.6 Drying and roasting of fermented beans

Fermented green beans were dried to 10–12% moisture content at 45 °C in a wind oven WFO-600SD (EYELA, New York, USA). All beans were roasted one week before the cupping test and aroma compound analysis. The beans were roasted in a roaster (IKAWA model PROV3, London, Great Britain, UK) for 8 min with a maximum roasting temperature of 220 °C to obtain medium roast. After roasting, all beans were stored at room temperature for one week in a sealed package made by PET resin, aluminum and polyethylene.

5.1.7 Volatiles analysis of roasted beans by GC·MS

Volatile compounds were collected from roasted beans using dichloromethane (Wako, Japan) as the solvent [86], as described in 5.1.5. The extracts were analyzed using GC·MS using the same conditions as described in 5.1.5.

5.1.8 Sensory analysis

Sensory analysis was carried out by a panel of 5 experts with Q-grader certificates according to SCAA standards [88]. The panelists evaluated 10 sensorial attributes: fragrance, flavor, aftertaste, acidity, body, uniformity, balance, sweetness, cleanliness and overall impression. 50 g of roasted beans were ground well by Kalita KCG-17(AG) coffee grinder (Kalita, Japan). Coffee powder (8.25 g) was infused with hot water (150 ml) for 4 min using coffee cupping bowl (200 ml). The panelists scored each sample based on the sensorial attributes shown above using a scale of 6 (low intensity) to 10 (high intensity) points with 0.25-point increments. Characteristic flavors of each coffee were also recorded.

5.1.9 Statistical Analysis

Analysis of variance (ANOVA, $p<0.05$) and post-hoc Tukey HSD analysis was performed with SPSS version 28.0.1.1 (IBM corp.).

5.2 Results and discussion

5.2.1 pH monitor and microbial counting

The microbial population of yeast and LAB at 0, 24, 48, and 72 h of fermentation are shown in Fig. 5.1.(A) and (C) for inoculation on Colombia coffee beans, and in (B) and (D) for inoculation on Kenya coffee beans. Since the objective of this study was to observe the collective effect of the whole microorganism community rather than to investigate contributions from single strain, enumeration was done on total LAB and yeasts.

The yeast population generally increased from 0 to 48 h but decreased from 48 to 72 h. This increase followed by a decrease is similar to results reported on semi-dry processed coffee beans [8]. The yeast populations at 24 h of fermentation were much higher for the inoculated groups compared to the control group, but this difference became smaller with prolonged fermentation time.

LAB population in the inoculated samples showed close to maximum values at 24 h of fermentation. Interestingly, this was also the case for when only yeast was inoculated (sample YW), which may result from synergistic growth of endogenous LAB by inoculated yeasts. The initial quick increase in LAB population may result from the hydration of dry pulp providing nutrition for bacterial growth. Although the population in the control sample was much lower than the inoculated samples at 24 h, the final population at 72 h reached similar values for all samples (6.97 ± 0.22 log cfu g^{-1} in Colombia beans and 7.22 ± 0.09 log cfu g^{-1} in Kenya).

Changes in pH during fermentation are shown in Figure 1(E) and (F) for the Colombia and Kenya beans, respectively. No significant differences were observed between different inoculations. The dry pulp and coffee beans were naturally acidic, and the pH values before fermentation were 4.55 and 4.75

for the Colombia and Kenya coffee, respectively. The pH was similar with pulp of coffee red Catuaí 99 (Coffea arabica L.) which ranged from 4.5 to 4.7 [89]. For both beans, a significant decrease from 0 to 24 h was observed. For Colombia beans, the co-inoculated beans of YRcoL showed a continuous drop from 24 to 48 h, while pH increased for the other samples. The pH decrease from 48 to 72 h may be caused by acids from microbial activities, although the results are only partially explained from the OA measurement. The final pH value of approximately 4.9 is similar to the pH generally observed at the end of the commercial fermentation [90]. The moderate pH decrease in the inoculated samples may be because of the relatively weak ability of the *Enterococcus mundtii* strains to lower pH compared to other species of LAB [91]. For the Kenya beans, pH dropped significantly from 0 to 24 h, but was relatively constant after 24 h.

5.2.2 Chemical analysis in green beans

The green beans were measured for CGA, caffeine, OAs, and volatile compounds after fermentation for 24, 48, and 72 h as explained in 2.4. The results are shown in Fig. 5.2.

CGA behaves as indicator of coffee quality in several ways; for its important role as an aroma precursor, as well as its biological importance as an antiviral and antioxidant chemical [92-94]. The concentrations of CGA in coffee beans were significantly ($p<0.05$) affected by fermentation time but changed differently between Colombia and Kenya coffee beans (Fig. 5.2.(A), (B)). Broadly outlined, CGA increased by fermentation time with inoculation of YW in Colombia beans while the same increase was observed with inoculation of YR in Kenya beans. This increase could be caused by the metabolisms of different yeasts which can lead to the hydrolysis of macromolecules and generation of CGAs [27].

For Colombia beans, concentration of CGA differed greatly with different inoculations. The highest CGA concentration was found in YWcoL at 72 h, while the lowest was found in YRcoL at 24 h. Fermentation patterns between YR and YW were significantly different ($p<0.05$), and all inoculations except for YWcoL significantly ($p<0.05$) affected CGA content when compared to the control.

Compared to mono-inoculation with YW, CGA in the co-inoculated YWcoL was lower in concentration at 24 h of fermentation, a phenomenon also observed in previous research [95].

For Kenya beans, inoculated groups gave higher CGA than control at 24 h, but this was reversed at 48 and 72 h, where the control sample showed highest amounts of CGA. Co-inoculation of LAB showed significant difference ($p<0.05$) compared to the coffee beans that were inoculated with yeast only. Co-inoculation of YR and LAB (YRcoL) to the Kenya beans affected CGA content in a similar way to Colombia beans, with a constant increase in CGA during fermentation. CGA is generated during the esterification of quinic acid, resulting in some cinnamic acid derivatives (such as caffeic acid, ferulic and p-coumaric acid), where 5-O-caffeoylquinic acid is the most abundant derivative [90]. Conversely, microorganisms can decarboxylate CGAs and produce various alkyl and vinyl phenols [95]. Considering the well-known importance of CGA for flavor formation, it seems that a higher CGA level may give better cupping. However, reverse conclusions have also been shown, where higher CGA content was observed in lower quality samples [96].

Caffeine content in inoculated and control samples are shown in Figure 2(C) and (D). In the Colombia beans, caffeine concentration was significantly ($p<0.05$) different in samples after 24 h of fermentation when compared to 48 h and 72 h, although there was no significant difference among different inoculations. The caffeine concentration generally increased with fermentation time, with the exception of YR and YWcoL which showed a decrease from 48 to 72 h. YRcoL showed the lowest concentration of caffeine among all inoculations on Colombia beans at 24 h and the highest concentration at 72 h. No significant difference was found for inoculation nor fermentation time in Kenya beans.

Fig. 5.2.(E) and (F) show the concentrations of OAs detected in fermented green beans at 24, 48, and 72 h for Colombia and Kenya beans, respectively. OAs are considered to contribute to the final acidity of coffee beverage, furthermore, acidity helps enhance the sweetness of coffee drink [97]. For both beans, acetic, citric, malic, lactic and quinic acids were shown to be significantly ($p<0.05$) affected by fermentation and inoculation when analyzed by two-way ANOVA. Among these acids,

quinic acid, which has been shown to positively correlate with coffee body, sweetness, and astringency [98], was the most abundant in most inoculations and fermentation times. Quinic acid has also been reported to strongly correlate with bitter taste [98]. Highest concentrations were observed in YR inoculated Colombia beans at 48 h and YW inoculated Kenya beans at 24 h of fermentation.

Acetic acid is considered to contribute to the acidic flavor of coffee, as well as enhancing some desirable characteristics such as floweriness and fruitiness [47]. Richest acetic acid was found in YWcoL at 72 h in both beans. Acetic acid increased with fermentation time in all treatments with the exception of Colombia beans inoculated with YR in which concentration decreased at 72 h.

Citric acid contributes to citric and herbaceous perceptions and can serve as precursors to other acid degradation products. Higher levels of citric acid resulting from fermentation have been reported to be related to a higher cupping score [51]. Highest citric acid was detected in YW fermented Colombia beans at 48 h and YW fermented Kenya beans at 24 h. Citric acid production in Colombia beans was significantly influenced by time and YW inoculation. At 72 h, citric acid dropped concentration in all inoculated treatments when compared with control. Similar decrease was also observed with yeast starters (*Saccharomyces cerevisiae*, *Candida parapsilosis* and *Torulaspora delbrueckii*) in on-farm fermentation [34]. In Kenya beans, only YWcoL inoculation showed significant difference in citric acid concentration when compared to the control group.

Formic and succinic acids were detected in all treatments, but their concentrations were relatively stable during fermentation.

Lactic acid has been reported to contribute to noticeable fruity flavor (such as banana-like and orange-like flavor) and smooth sensations of full bodied coffee [45]. The concentration of lactic acid was significantly ($p<0.01$) affected by the co-inoculation of LAB. The highest lactic acid was observed in YWcoL at 72 h in both beans. Lactic acid was also affected by fermentation time and high concentrations were not detected until 72 h, which may indicate that longer fermentation times could be considered in future research with *Enterococcus mundtii* to achieve more lactic acid.

Malic acid is known as an aromatic precursor and was shown to exist in highest concentrations in

beans fermented for 48 h, in YW inoculated Colombia beans and YRcoL inoculated Kenya beans. The concentration of malic acid decreased at 72 h, which may be explained by the conversion of malic acid to lactic acid by bacterial activity [16], an explanation supported by the high concentrations of lactic acid at 72 h.

Not only do OAs contribute to acidity or sourness but can also provide characteristics such as bitterness and smooth mouthfeel to the coffee beverage. Formic, quinic and succinic acid contribute to a perceptibly bitter taste, while quinic and lactic acid contribute to the mouthfeel [48]. In general, different inoculations lead to changes in OA composition, and this would lead to different flavors in the final beverage. Although further chemical analysis is needed to investigate the correlation between constituents generated from coffee fermentation and coffee flavor, fermentation has been shown to be one of the potential methods to control specific flavors in the end product.

Fig. 5.2.(G) through (J) show the volatile compounds detected in the green beans. In Colombia beans, 104 volatiles were detected throughout the fermentation, including 55 hydrocarbons, 14 alcohols, 20 esters, 1 acid, ketone, and aldehyde. A total of 98 volatile compounds were detected Kenya beans, including 18 alcohols, 20 esters, 3 ketones, 1 acid and an aldehyde. The complete composition of volatile compounds in fermented beans is shown in Supplementary file (S-table 1). The concentrations of each group are shown in Fig. 5.2.(H) and (J).

In fermented Colombia beans, hydrocarbons were the most abundant in numbers as well as total concentrations. The fermented beans also contained high concentrations of alcohols and esters, with higher concentrations of esters at 72 h of fermentation. On the other hand, in Kenya bean, similar with Colombian beans, most various group is hydrocarbon during fermentation, followed by ester and alcohol. Multiple esters were detected at 72 h, with YW, YWcoL, and the control sample showing distinctively high concentrations of esters. Furthermore, alcohol, which is a key metabolite in yeasts [16], showed an increasing trend from 24 to 48 h but then dropped at 72 h. This trend is in agreement with the microbial dynamic population shown in Figure 1(B). Different inoculations contributed to a variety of chemical compositions, leading to the modification of flavor and aroma precursors in green

beans, which can be transformed into volatile compounds during roasting which consequently compose distinctive sensory profiles in the roasted bean [35, 67, 99].

5.2.3 Chemical analysis in roasted beans

The complete list of compounds detected are shown in the Table 5.1. In total, 107 chemicals were identified in roasted Colombia beans (Fig. 5.3.A and 5.3.C), including 7 ketones and lactones, 5 aldehydes, 13 furans, furanone and furfural, 3 acids, 11 pyrazines, pyridines and pyrans, 9 pyrazines 14 alcohols, and 19 esters. 108 chemicals were detected in Kenya beans (Fig. 5.3.B & 5.3.D), including predominant compounds 9 ketones and lactones, 11 furans, furanone and furfural, 5 acids, 2 aldehydes, 17 pyrazines and pyrans, 11 pyrazines, 6 alcohols, and 28 esters. Chemical components that could not be classified into these molecular groups have been grouped as ‘Others’. Generally, the increase in numbers and concentrations of alcohols, furans, and esters may exhibit a positive impact on the aroma and taste. Previous research has revealed that the caramel-like, sweet roasted flavor is related to a higher proportion of acids, pyridines, furans and aldehydes in Arabica coffee [100].

Alcohols are known to provide sweet, clean, and floral flavors to the coffee beverage, and yeast starters have been used in previous studies for the increase of volatile alcohol products [53]. In Colombia beans, more than 2 folds of alcohols were found in 24 h co-inoculation with LAB than other time, while at 72 h there is more varieties of alcohols. In Kenya beans, less abundance of alcohols was detected at 72 h. Phenylethyl alcohol (2-PE) which can provide rosy honey flavor to coffee was only found after 48 h fermentation in both YR and YW fermented Colombia beans, furthermore, richer 2-PE was observed in YWcoL. This may be explained in previous research that *Wickerhamomyces anomalus* could produce higher 2-PE than *Rhodotorula mucilaginosa* [101]. 2,3-butanediol, which was described as cocoa, fruity and buttery in coffee beverage was only detected in 72 h fermented Colombia beans with YRcoL and YW; 1-Nonanol, contributing to fresh, clean and floral flavor in coffee was found highest content in 72 h YR inoculated Colombia beans.

Aldehydes (Fig. 5.3., classification ‘Aldehydes’, (E, E)-2,4-decadienal, 5-hydroxymethyl-2-

furancarboxaldehyde, 5-methyl-2-furancarboxaldehyde, methylene-cyclopropanecarboxaldehyde) give fatty/orange-like, floral/chocolate notes may enhance the fruity and tropical flavor in sensory analysis due to their relatively high content, especially inoculation of YR in Kenya beans. Furfural, as well as 5-methyl-2-furancarboxaldehyde, were described as sweet and caramellike in coffee, could be related by YW since richest was found in 48 hours' YW fermented and 24 hours' YWcoL fermented Colombia beans, respectively. Kenya beans were also observed highest furfural and 5-methyl-2-furancarboxaldehyde in YWcoL inoculation (48 h). 5-methyl-2-furancarboxaldehyde, as a popular metabolite product produced by *Saccharomyces cerevisiae*, could be novelly correlated to *Wickerhamomyces anomalus*.

Esters (Fig. 5.3., classification 'Esters') are known as fruity and sweet odor in coffee which can be gained by yeast fermentation [102]. In this study, esters in Colombia beans showed higher in 24 h and 48 h fermented beans than 72 h. Inoculation of YW contributed more esters than YR from 24 to 48 h. Co-inoculated with LAB showed richer and more types of easters during fermentation. In Kenya beans, longer fermentation time gave distinguish more aromatic easters. Hexadecanoic acid, ethyl ester (fruity and creamy) gained more with longer fermentation time in Kenya beans, besides, higher content was detected in Kenya beans inoculated with YW. In Colombia beans, acetic acid, 2-phenylethyl ester (rosy and flora), a general metabolic product in yeast fermentation was only found in inoculated beans.

Furans (Fig. 5.3., classification 'Furans') are related to caramel, burnt and herbal or fruity notes in coffee sensory. 2-Furanmethanol is considered as good character of coffee flavor which usually be described as cooked sugar and burnt taste, was detected in all treatment while 24 h YWcoL fermented Colombia beans and YW fermented Kenya beans showed highest content. 2-Furanmethanol, acetate contributes to sweet and fruity banana flavor was detected higher in YW inoculated Colombia beans. 2-furanmethanol, acetate was reported to be related to metabolism of *Saccharomycetaceae*, but first time observed that it could be caused by additional inoculation of *Wickerhamomyces anomalus*. 3 furanone (buttery and caramel-like) were detected in either bean, 2,5-Dimethyl-4-hydroxy-3(2H)-

furanone, also known as furaneol, can benefit beans with caramel and honey flavor, was found richer in inoculated Kenya beans than control all along fermentation.

Ketones (Fig. 5.3., classification 'Ketones') are described as giving buttery, caramel-like, musty, mushroom-like, or fruity notes [103]. Ketones were detected over 2 times high in 24 hours' YW and YWL fermented Colombia beans than other time and inoculation. In Colombia beans, 1,2-Cyclopentanedione, 3-methyl- (sweet, caramel and coffee-like) was only detected in inoculated treatments.

Pyrazines are known as roasted, nutty and coffee-like flavor [63]. 2,5-dimethyl-pyrazine (nutty, roasty and cocoa-like) were found only in inoculated groups, 48 h fermentation showed higher than 24 or 72 h. 2,6-dimethyl-pyrazine (chocolate, cocoa and roasted nuts), 2-ethyl-6-methyl-pyrazine (flowery, fruity and hazelnut-like), and 3-ethyl-2,5-dimethyl-pyrazine (roasted flavor) were found in both beans, while getting richer with 72 hours' fermentation in inoculated Kenya beans. Pyrrole, which gives the odor of caramel, showed relatively higher with shorter fermentation time. 1H-pyrrole, 1-(2-furanylmethyl)- showed richest in 72 hours' inoculated Kenya beans with YW, which is known as hazelnut and coffee-like aroma in coffee beverage. In Colombia beans, YW fermented beans showed higher levels of pyrazines, mainly resulted by pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, isolated from microbial metabolites which has been reported for its antioxidant activity [104].

Unique compounds were produced only in YW group, in Kenya beans, 2-furfurylthiol (coffee-like), 2,3-dimethyl-pyrazine (nutty and roasted) and 2(1H)-pyridinone, which could be related to activity of additional *Wickerhamomyces anomalus*. Besides, 1-Butanol, 3-methyl-, acetate (banana-like aroma) was reported that its production was related to Pichia genus (*P. kluyveri* YC7.16. and *P. fermentans* YC5.2) species [75], was first found to be related to *Wickerhamomyces anomalus*. Also in Kenya beans, easters like acetic acid, 2-phenylethyl ester, butanoic acid-2-methyl-pentyl ester and eicosanoic acid, 2-hydroxyethyl ester were also only found in *Wickerhamomyces anomalus* inoculated groups. Compounds were only detected under inoculation of YR, such as, in Colombia beans, (E)-9-

Octadecenoic acid ethyl ester (floral odor), which could consist more proof for non-Saccharomyces yeast fermentation. In Kenya beans, 9,12-Octadecadienoic acid, ethyl ester was only found in 48 h YR inoculated beans.

In this study, not only aromatic compounds which can help coffee flavor modification but also some chemicals with special function were found (Fig. 5.3., labeled 'Others'). In Colombia beans, vitamin E which is famous for its antioxidant activity, was detected at all time with highest content at 24 h with YWcoL fermentation. Stigmasterol and campesterol are common phytosterols found in coffee which can play cholesterol-lowering effect in human intestinal. Stigmasterol showed the highest content at 48 h fermented beans with YRcoL. Campesterol was detected most in YRcoL at 48 h fermentation. Squalene contributes to immune regulation, antioxidation, anti-inflammatory response and anti-cancerous responses [105]. Squalene was found to have the highest content in 24 h fermented beans YWcoL. Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, giving antioxidant and antimicrobial activities [106], was only found in YW fermented beans. 9,12-octadecadienoic acid (Z, Z)-, methyl was detected anti-cancer function [54], was found higher content in YW inoculated groups. Compounds with beneficial characteristics were also found in fermented roasted Kenya beans, such as phytol was only detected in YW inoculated beans, which was proved with antioxidant and antimicrobial activities [107]. These nutritional compounds showed potential of coffee beans as bioactive ingredients resources and benefit human health. Besides volatiles (S-table 1&2) which are strongly relative to coffee, some non-coffee components such as benzenes were also detected. Considering those chemicals may be from extraction step e.g., tubes and vials, so those compounds that are not detected in coffee publications were deleted. This study only discussed the chemicals which were proved before from coffee researches.

5.2.4 Sensory analysis results

Inoculation of yeast and LAB to green coffee beans resulted in distinctive cupping profiles after roasting (Fig. 5.5.). For either bean, the highest cupping score were given to co-inoculated samples and

special flavor was also evaluated and recorded in Kenya and Colombia beans.

Within all fermented Colombia beans, YRcoL fermented for 48 h scored highest for total cupping score, mainly due to its high score in aroma and flavor (Fig. 5.5.). In general, 24 h fermented beans showed lower score (<80) especially control gave lowest balance, flavor and aroma. At 48 h, inoculation contributed to richer flavor, aroma and aftertaste, including fermentation of YW showed highest score in aftertaste and both YR and YRcoL gave increasing aroma behavior. With fermentation going from 48 h to 72 h, overall rating decreased, while YR and YRcoL still behaved higher score in aroma. Fermentation time significantly ($p<0.05$) affected perceptions of acidity, body, sweetness, aftertaste, flavor, balance, and uniformity, and the beans fermented for 48 h were given a relatively high score in these parameters than other fermentation times. Specific description of each cup was also recorded. 48h YR showed flat and chestnut taste, as well as good balance, YRcoL showed almond and plum flavor, YW gave plum and sweet, YWcoL gave flat and almond flavor, while control group tasted miso acidity which was described as unpleasant taste. With fermentation going on (72 h), YR showed darker and almond flavor but slightly dirty. YRcoL, YW and YWcoL showed good acidity and sweetness. Beans inoculated with YR all showed chocolate flavor. In general, 24 h fermented beans showed lower score (<80) especially control gave immature taste, which may provide clue for choosing proper future fermentation time with Wickerhamomyces anomalus. At 48 h and 72 h, all inoculated groups behaved good wine/fermented flavor. There is no significant difference by inoculation on total score or each cupping characteristic, except aftertaste of YW.

For Kenya beans (Fig. 5B/D/F), compare to non-inoculate beans, better aroma, flavor and acidity were achieved in inoculated beans respectively. YWcoL was evaluated with the highest score of 85.15±2.58 point. Even though there is no summarized trend for different fermentation time, all inoculated groups scored higher than corresponding control groups. At 24 h, higher score in aroma, flavor and acidity was given to 4 fermented beans. At 48 h, YWcoL had highest score in flavor while YW gave highest score in aftertaste. YWcoL had highest score in sweetness, which could be related to the highest concentration of furfural as well as 5-methyl-2-furancarboxaldehyde for their expression of

sweet and caramel-like taste. At 72 h, all fermented beans were given higher score in aroma and flavor than control. YWcoL and YR showed best acidity. Higher concentration of aldehyde (benzeneacetaldehyde) in YR and ester (hexadecanoic acid, ethyl ester) in YWcoL may reflect to their better aroma and flavor assessment respectively. Detailed sensory description was recorded as follow. Generally, 24 hours' fermented beans were characterized by tropical fruits; beans fermented for 48 hours were characterized by almond as well as nutty flavor; beans fermented for 72 hours were characterized by apple like flavor. According to 2-way ANOVA, 24 h was significant different from 72 h on aroma. At 24 h, YR gave fruity and sweet taste, particularly tropical fruits flavor. YRcoL was described as acidic and juicy. YW showed raspberry and sweet flavor while YWcoL gave plum and nutty flavor, as well as creamy body. Control was smooth but dropped very fast and behaved rough aftertaste. After 48 h, YR showed intense flavor including almond, plum and floral flavor. Groups of co-inoculation with LAB, flat and creamy were described by panel, although for one expert this was labeled as dirty. Conversely, non-inoculation was dark at 48 h. At 72 h, all YR beans were evaluated as green apple flavor.

5.3 Conclusion and summary

This study is the first time to investigate new flavor by fermentation with dry coffee pulp, observed chemical changes during fermentation and evaluated flavor modification. In this research, original microbial flora was reserved for maximized fermentation. *Wickerhamomyces anomalus* and *Rhodotorula mucilaginosa* were selected for their OAs produce contribution. *Wickerhamomyces anomalus* was found as one of yeasts community that are necessary for coffee beans' fermenation, including degradation activity of pectin from the surrounding plant tissue [78]. *Rhodotorula mucilaginosa* was generally found in coffee fermentation but there was no research about its coffee fermentation behavior in particular. Fermenting with screened isolates affected OAs content as well as volatile profiles from green and roasted beans, respectively. YRcoL gained lowest CGA at 24 h while YWcoL produced highest at 72 h in Colombia beans. After 48 hours' fermentation inoculated with YR

produced highest quinic acid. Co-inoculation with L4 showed different behavior in YR and YW, lower quinic acid was detected in co-inoculation of YR but revers trend happened in co-inoculation of YW at 24 and 72 h. Similar phenomenon was also found in OAs like citric and malic acid. Co-inoculation of LAB mainly influenced production of lactic acid at 72 h. Besides, *Enterococcus mundtii* was observed antimicrobial activity to Listeria, Bacillus and Aspergillus in dough LAB isolation research [91] which can be potentially applied for microbial safety during coffee fermentation. Fermentation methods also have influence on OAs production and cupping score, *Saccharomyces cerevisiae* CCMA 0543 inoculated in buckets obtained the higher score in the coffee cupping than directly spray on the surface of coffee [34]. More significant production of positive chemicals is expected in our study, further research aiming to develop efficient fermentation conditions and investigate the correlation of metabolic activities and key aromatic components is needed.

Fig. 5.1.1 Fermentation container of green coffee beans and dry coffee pulp

https://www.ikawacoffee.com/

Fig. 5.1.2 IKAWA roaster.

Table 5.1. Roasting program by IKAWA

Roaster information		
Roaster	IKAWA roaster	
Amount	~50 g	
Program*	0.00- 1:35 min	Heat up without input beans;
	- 2 min	138- 172 °C, reach stable match setting temperature;
	3:14- 4:48 min	200- 220 °C, Maillard and other reaction, color and smell changes obviously;
	-6 min	Keep at 220 °C until end
Heating	Air blowing	
Storage	1 week at room temperature before chemical analysis	

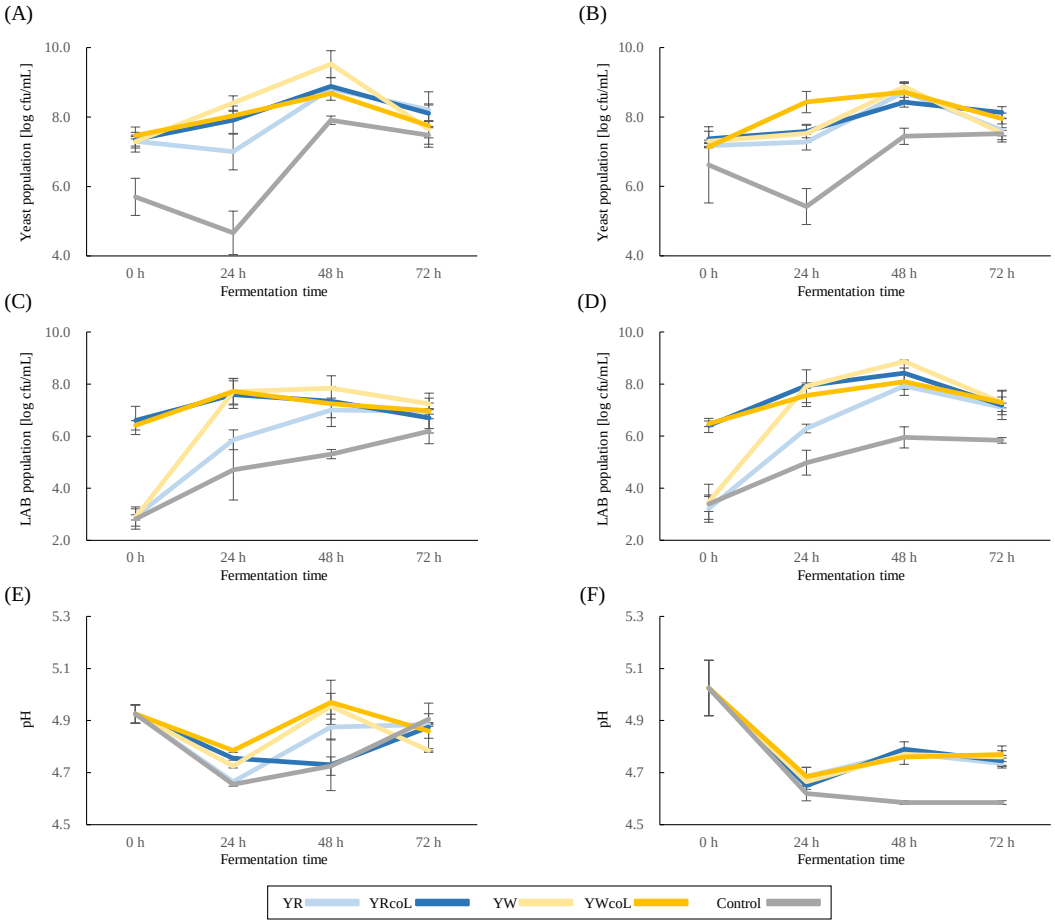

Fig. 5.1. Dynamic behavior of yeast and LAB populations and pH during fermentation of Colombia (Castillo) beans (A), (C), (E), and Kenya (SL N.28) beans (B), (D), (F). Error bars show the standard deviation of $n = 3$ samples.

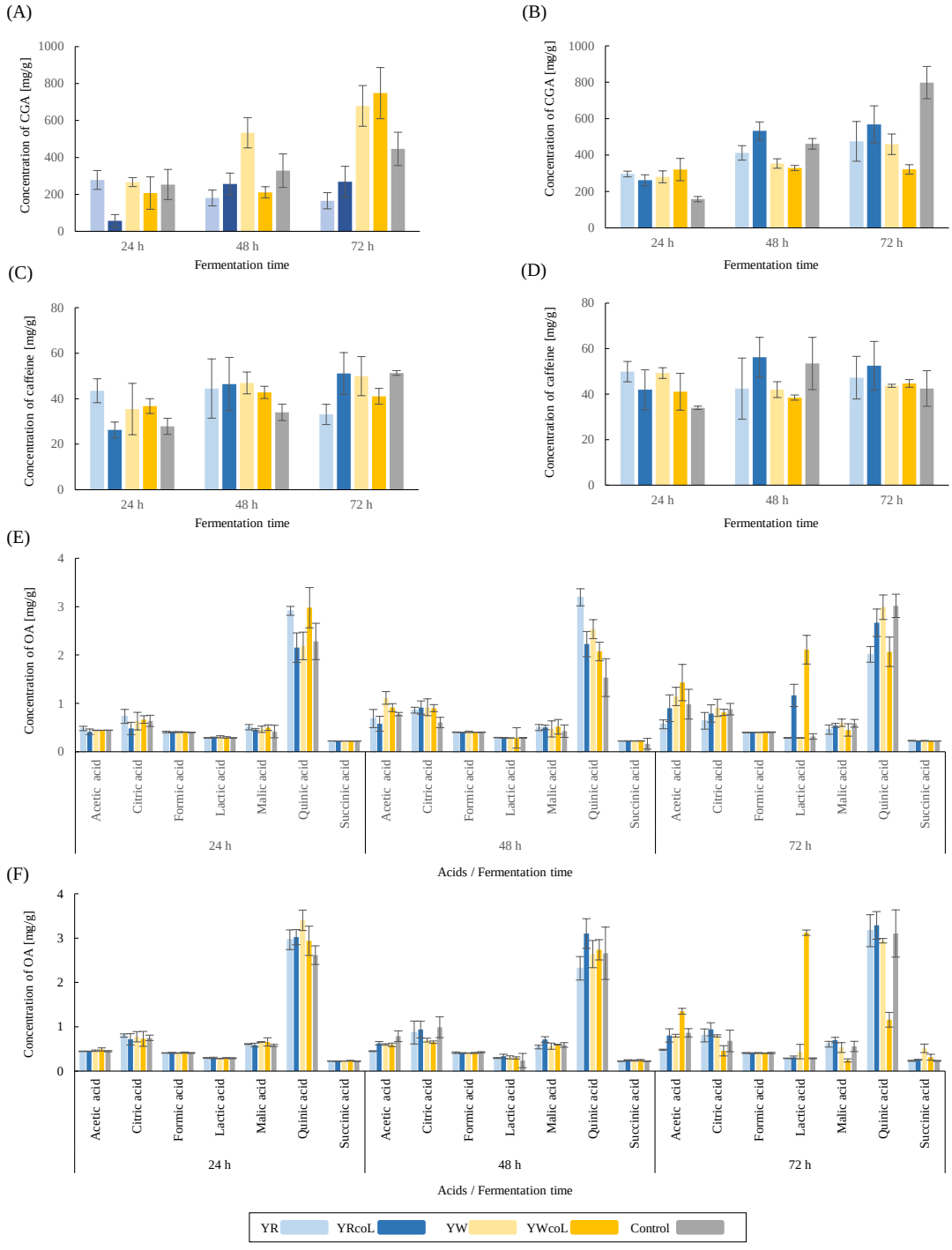

Fig. 5.2. Concentrations of chlorogenic acid (CGA), caffeine and organic acids (OAs) present in the inoculated and control fermentation of Colombia beans and Kenya beans. Error bars represent standard deviations of *n=3* samples.

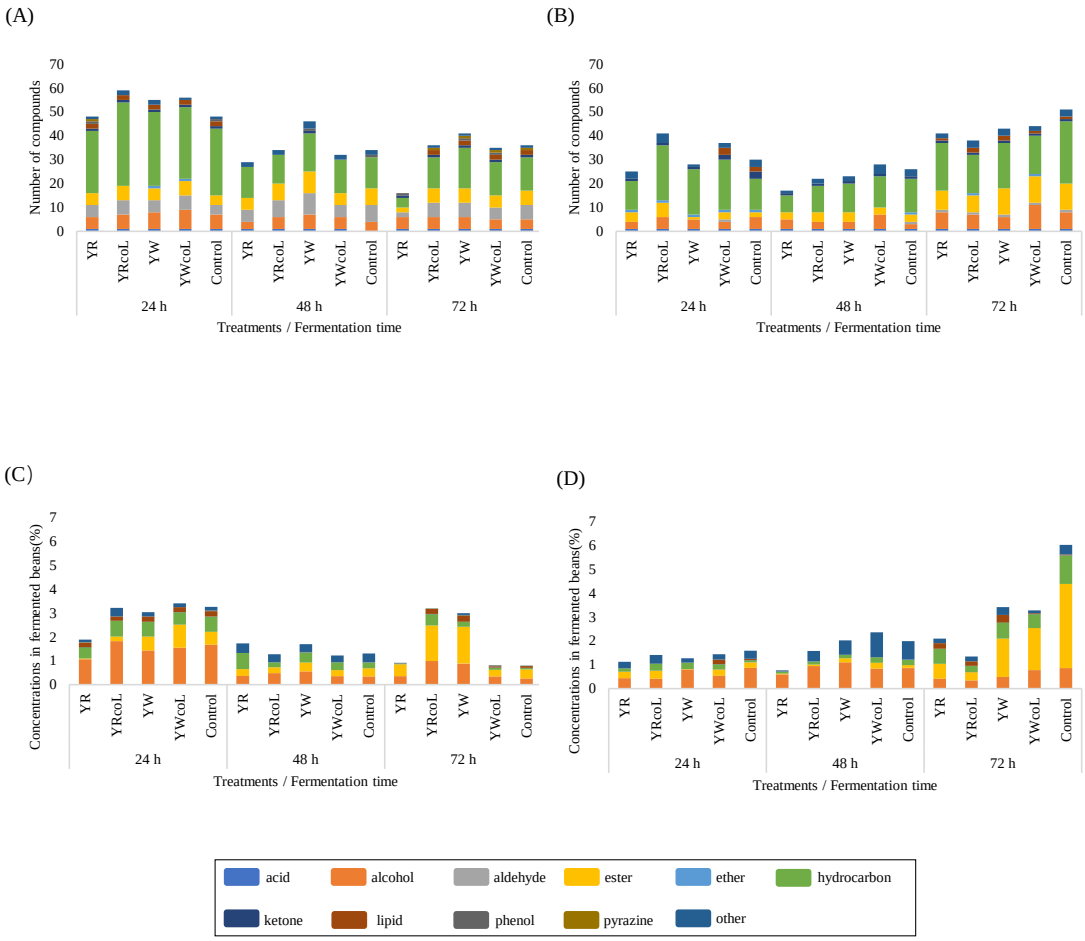

Fig. 5.3. Compounds compositions by numbers in Colombia (A) and Kenya (B) beans and by concentration in Colombia (C) and Kenya (D) beans (n=2).

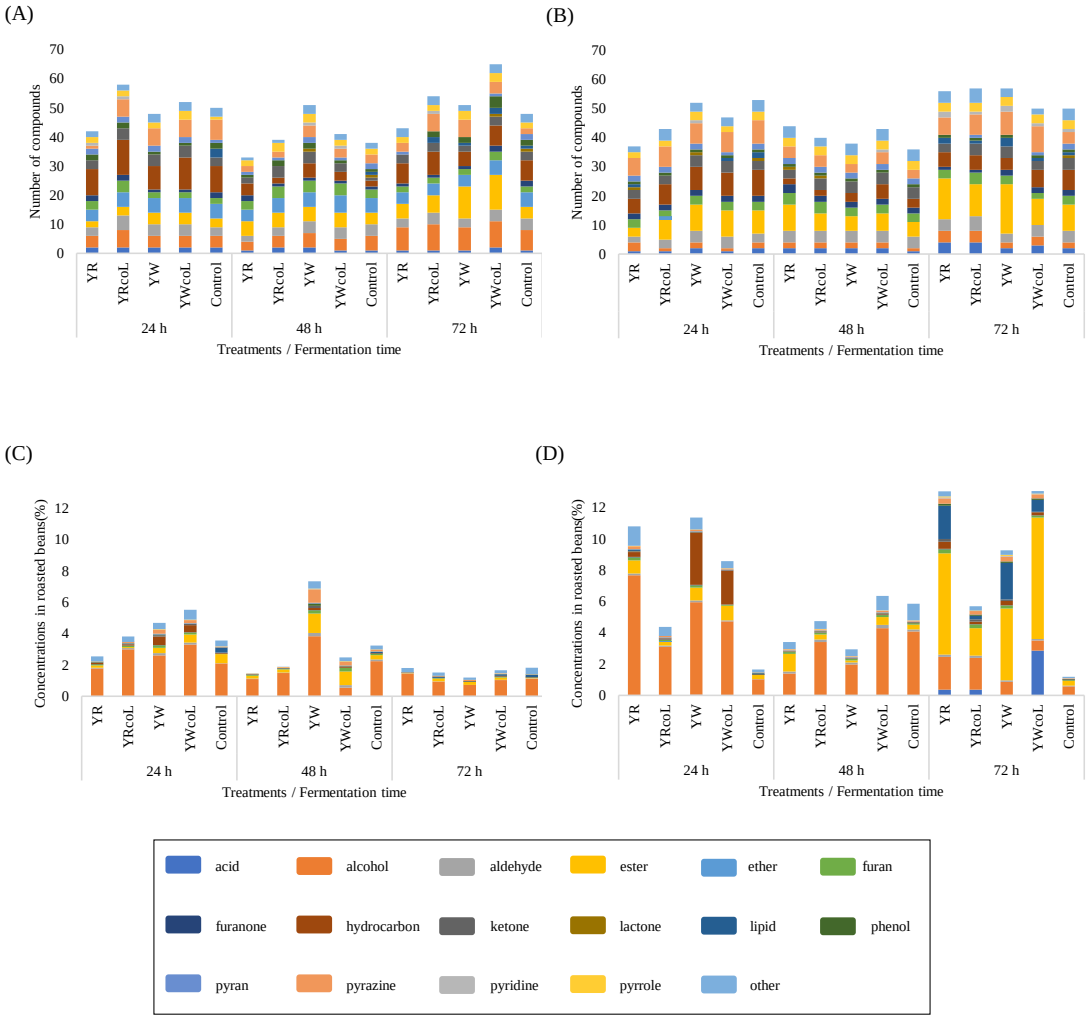

Fig. 5.4. Compounds compositions by numbers in roasted Colombia (a) and Kenya (c) beans and by concentration in Colombia (b) and Kenya (d) beans.

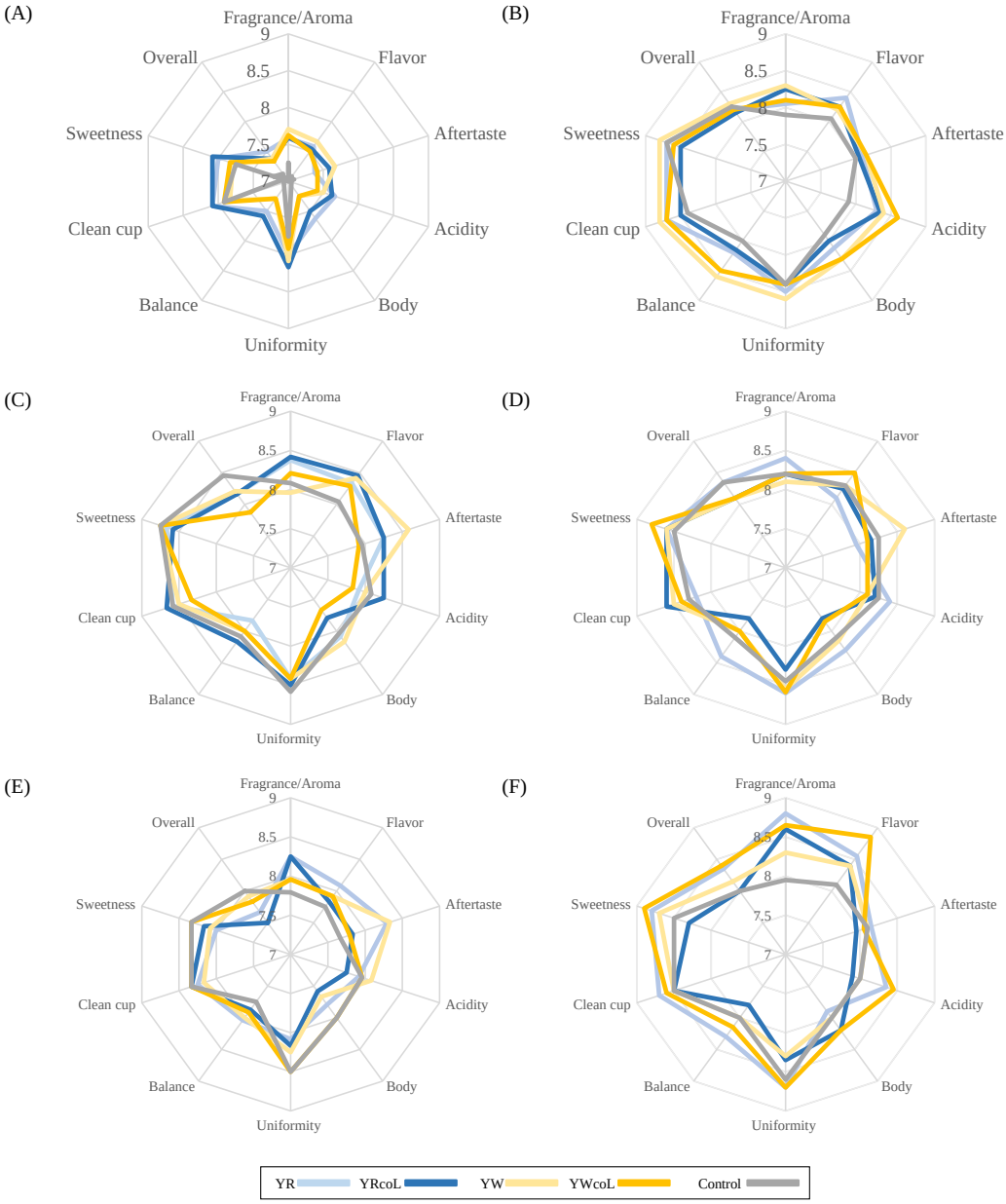

Fig. 5.5. Cupping result of different inoculation. Inoculated with YR, YRcoL, YW, YWcoL and Control at 24 h, 48 h and 72 h in Colombia beans (5A/B/C) and Kenya beans (5E/F/G).

Table 5.2 Sensory description recording from cupping Colombia beans.

Colombia	**YR**	**YRcoL**	**YW**	**YWcoL**	**Control**
24 h	Fruity	Sweet	Fruity	Special, fruity	-Immature, raw, sharp acidity, sour
48 h	Viny, chocolate, almond; -*Dark, dirty	Chocolate, acid, sweet	Plum/almond, acidic, clean, smooth; -Miso	Fermented, acidic, plum	Acidic, clean, almond; -Flat
72 h	Viny, mont blanc, nice balance	Viny, almond, plum, mandarin	Plum/almond, viny, sweet	Viny, almond	Fermented, acid; -Miso

*- stands for off-flavor.

Table 5.3 Sensory description recording from cupping Kenya beans.

Kenya	**YR**	**YRcoL**	**YW**	**YWcoL**	**Control**
24 h	Fruity, tropical, sweet	Acid, juicy	Raspberry, sweet	Plum, milky mouthfeel, nutty	Juicy; -Rough aftertaste
48 h	Intense flavor, plum, red wine	Sweet; -Flat	Juicy, mandarin; -Flat	Sugarcane, sweet, honey, orange, milky mouthfeel	-Flat
72 h	Almond, green apple	Almond; -Dirty	Champagne; -Miso	Wine; -Dirty	-Dark, smoky

Chapter 6 Comparison and evaluation of isolates with commercial starters in coffee composition modification

Overview

In this chapter, discussion about utilizing yeast isolates and commercial yeast in coffee beans re-fermentation was done by comparing and evaluating the change of CGA and caffeine under different inoculation. Yeast starter, *Saccharomyces cerevisiae*, one of the most famous species used in coffee processes. The contrast with commercial yeast can help understand fermentation capacity of isolates and potential in future application. This chapter limited comparative study at 2 detection targets for straightforward observation of concentration change instead of comprehensive analysis. Caffeine, key functional compound that benefits human health as well as the characteristic composition in coffee. CGA, conveying bitterness to coffee beverage and acting as aroma precursor. This chapter monitored these 2 targets changing during fermentation.

6.1 Material and methods

6.1.1 Coffee material and strains

Green coffee beans from Colombia were used in this chapter, Coffea arabica, var. Castillo) from Finca Los Tres Edgaritos-Cauca, Colombia. Same origin produced coffee pulp of geisha variety was added as fermentation substitute. Yeast isolates of chapter 5, R and W, were used for comparison and evaluation experiment here. The commercial strain, *Saccharomyces cerevisiae*, was purchased from Nisshin Food (Japan).

6.1.2 Fermentation process of green coffee beans

For isolates, spreading on YEPG agar plate followed by re-culture in YEPG broth until the population reach 7 log cfu ml^{-1}, and harvest cells and resuspend in sterile distilled water. For commercial yeast, directly culture yeast powder in YEPG broth and count by YEPG agar plate, collect cells until the growth reaches 7 log cfu ml^{-1}. Same procedure was done as isolates preparation. In sterile 150 ml blue-cap bottle, 20 ml of sterile distilled water containing 7 log cfu ml^{-1} of each strain was added in advance, add 5 g of green coffee beans and 5 g of coffee pulp, then cultivate in incubator at 28 ℃ for 24/48/72 h. For each inoculation, 2 repeats are prepared.

6.1.3 Evaluation of CGA and caffeine under different inoculation

Fermented green coffee beans were collected at 24/48/72 h in clean bench, and directly followed CGA and caffeine extraction by methanol reagent as mentioned in chapter 5.1.3. Extracted samples were put in 4 until analysis. Same detection method as 5.1.3 was used here by ion-exchange HPLC.

6.2 Results and discussion

Caffeine and CGA were evaluated in different inoculated beans at 24, 48 and 72 h and compared the change resulted from isolates and commercial yeast, aim to compare their ability to modify the compounds composition and furthermore to change the volatiles in roasted beans and the final

beverage flavor.

6.2.1 Evaluation of CGA and caffeine in different inoculation

Caffeine and CGA were monitored during fermentation under different starters, the results show in Fig. 6.1. Caffeine stayed stable during fermentation in respective inoculation. Similar phenomenon was observed in chapter 5. At 48 h, *Saccharomyces cerevisiae* fermented beans detected relatively higher caffeine than other inoculation but no significant difference. Caffeine is mostly investigated for its functional feature which benefits human health, for it barely contribute no flavor to coffee beverage [59, 92]. Caffeine showed lower concentration at 72 h in all beans, maybe considered by soaking effects from fermentation. CGA decreased by fermentation time, and reached lowest at 72 h. At 24 h, all treatment including control were detected relatively higher content of CGA, but no significant difference among inoculation. At 24 h, inoculated beans showed significantly high CGA than beans in 48 and 72 h. While at each fermentation time, no significant difference in CGA concentration was observed. CGA, behaves bitter aroma in coffee, also play important role as aroma precursor during roasting [54, 94].

6.3 Conclusion and summary

By comparing self isolates with *Saccharomyces cerevisiae* by fermentation with coffee pulp, continuous decrease in GCA was observed throughout fermentation. Time significantly affected CGA concentration, but no significant difference by inoculation. Well, no significant difference was found between different inoculation. CGA is well known as important aroma precursor during roasting, although for either high and low concentration of GCA have been reported to play positive role to coffee final beverage [94, 108, 109]. Further, research of chemical analysis such as organic acids, amino acids and sugars in fermented green coffee beans is necessary to have better knowledge of how microbial activity involved with fermentation and metabolites. As well as the volatile analysis in roasted coffee beans, to find out how compounds change in green beans influence on the aromatic profiles.

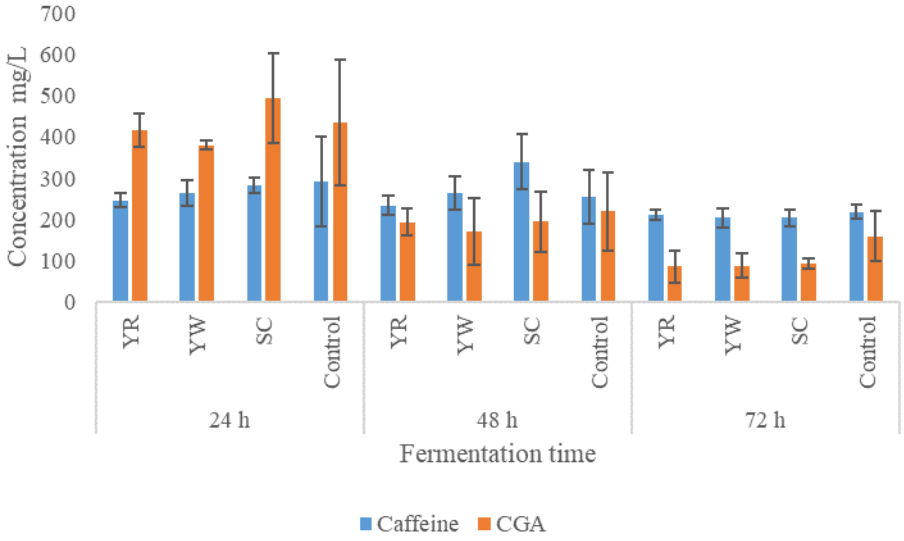

Fig. 6.1. CGA and caffeine contents in *Rhodotorula mucilaginosa*, *Wickerhamomyces anomalus* and *Saccharomyces cerevisiae* inoculated green beans at 24, 48 and 72 h.

Chapter 7 Conclusion and future outlook

Overview

In this chapter, summary of the whole work from the previous chapters was done and further plan was also mentioned based on present results of this study. Both pro and cons of author's research are dissertated here. An overall summary of research background, objective, research design, methodology, period achievements, and final results of this study.

7.1 Summary of research

In chapter 1, introduction started from general introduction of coffee. As one of the worldwide popular agricultural goods, coffee price can be affected by multiple reasons. According to ICO [2], coffee price in Jan 2023 differed from 95.98 US cents/lb for Robusta and 218.91 US cents/lb for Colombian Milds. Price is determined according to flavor and quality of coffee beans, which can be modified by processing method, fermentation, varieties, roasting, origins and other reasons. Usually there are 3 ways to process coffee after harvesting cherries, including wet-, dry and semi-dry processing. Among different methods, the common phenomenon is the happening of fermentation.

Fermentation is the key step for aroma formation in green beans. Fermentation is done by indigenous microbial flora and recently developed into using selected starters for achieving better aroma [110]. The most frequently detected and most contributing microorganisms are yeast (e.g., *Pichia*, *Sacharomyces*, and *Candida* species), lactic acid bacteria (e.g. *Lactobacillus*, *Leuconostoc* and *Streptococcus* species), filamentous fungi [14, 26, 111]. Using yeast in coffee fermentation can be tracked back more than 100 years [9]. By utilizing yeast, the pectin from mucilage layer, the important part for flavor development, can be lysed into sugars, amino acids and acids [6, 31]. These can be the rich nutrition for better growth of yeast and furthermore higher production of metabolites such as alcohols, aldehydes, pyrazines, and acids, which determine the quality/flavor and commercial value of coffee beans [112]. Lactic acid bacteria have been proved to produce organic acids, including lactic acid, acetic acid, propionic acid, citric acid and succinic acid [31, 37, 87]. Besides, lactic acid bacteria can assistance the growth of yeast and further help with generation of aromatic metabolites from yeast [53, 70].

This study targeted at isolating strains from coffee green beans and coffee dry pulp, and using them for further re-fermentation with green beans. Furthermore, with the inoculation of isolates, the modification of compounds composition can be realized and compose different aroma profiles for controlled and unique sensory perception.

In chapter 2, the isolation of yeast and lactic acid bacteria from Colombia and Kenya green beans and dry pulp were elaborated. By using culture-dependent method, first got over 70 colonies from the spontaneous fermentation. Applying morphology and biochemical characteristics, and located 30 strains as lactic acid bacteria candidates which showed cocci, coccobacilli, or rods and, chain formation, as well as catalase negative and gram staining positive. And 40 colonies as potential yeast strains for their moist, flat and round morphology features and sent for sequencing. After sequence analysis, identification isolates until species was done, including 11 lactic acid bacteria and 9 yeast strains. 11 strains of LAB all belong to *Enterococcus mundtii* species but different strais. Identified yeasts include *Rhodotorula mucilaginosa* (4 isolates) and *Wickerhamomyces anomalus* (5 isolates). All 3 species had been reported in coffee fermentation ecology research, but rare application of the 3 species in coffee fermentation [113]. Antifungal activity of *Wickerhamomyces anomalus* was reported in dough fermentation [113], this may help with inhibition of bacteria growth and cut down the generation of off-flavor compounds in coffee. Beyond coffee fermentation, *Wickerhamomyces anomalus* show capacity in enhancement of aroma in wine making, its inoculation presented higher levels of volatiles such as acetates and ethyl esters which supply a fruity note [82]. Higher production of ethyl acetate in *Wickerhamomyces anomalus* inoculated wine was also reported, in Judith's study this inoculation presented a unique aroma profile to the wines [114]. Using *Rhodotorula mucilaginosa*, co-culture with Saccharomyces cerevisiae, impact on the aroma wine was also carried out, resulting higher aromatic compounds and better sensory evaluation of floral and fruity notes [81]. The identification of *Enterococcus mundtii* was reported in coffee but no report about its application in coffee fermentation [16].

In chapter 3, screening of isolates from last chapter by monitoring the organic acids production in coffee green beans fermentation was operated. Dry pulp (harvested from Colombia, Geisha var.) and Colombia green beans (Arabica, Castillo var.) were used as fermentation substrate. Fermentation took place at 28 ℃ for yeast inoculation and 30 ℃ for lactic acid bacteria, for 24 h, 48 h and 72 h. Acetic, citric, formic, lactic, malic, quininic and succinic acids were measured. The 7 OAs are the main acids

presenting in green coffee beans, and these OAs help the determination of the sensorial characteristics of coffee [22, 74]. Acetic acid, presenting vinegar flavor, was reported that it can help with fresh and clean taste in coffee beverage at specific concentration [32]. Its production was reported to relate with metabolic activities of lactic acid bacteria and yeast [53, 115]. Citric acid, acidic and citrus odor, acting as the precursors to other acid during roasting. Citric acid can be related to lactic acid bacteria and yeast fermentation of coffee [45, 51], better cupping score were given to beans which presented higher concentration of citric acid [50]. Formic acid, naturally presenting in green beans, shows sour and bitter contribution to coffee beverage, can also help with fermented flavor as well. Lactic acid, except acidity contribution, it can also help with mouthfeel sensation. Higher malic acid, together with acetic and formic acids were reported to have higher cuppings evaluation. Malic acid can be gained through yeast and lactic acid bacteria fermentation with coffee beans [53]. Quinic acid, one of the most prominent organic acids, behaves bitter and sour, also acts as important aroma precursor. Succinic acid, acidic and bitter taste, naturally exists in green coffee beans. With higher presentation in green coffee beans, these OAs help with overall fruity and tropical fruits notes in final beverage [32, 48, 53].

In this study, all seven OAs were detected in samples, among which quinic acid was the most predominant acid while succinic acid gave least concentration. Although we monitored 24, 48 and 72 h fermented beans, for each strain from same species, there was no trend of OA producing. Finally, isolates which have high total OA for selection of isolates were picked out. By screening all 20 isolates, high OA producing strains of each species were obtained, labeled as Y12bR from *Rhodotorula mucilaginosa*, Y18W from *Wickerhamomyces anomalus,* and L4 from *Enterococcus mundtii*.

In chapter 4, to further increase metabolites and fermentation potential, 3 different growth factors were applied to optimize fermentation conditions. 1) added 1% sucrose to simulate the fermentation substrate more like fresh coffee cherry. For this evaluation, the strains with the highest and lowest OA production from last chapter were used, which were Y5R and Y12bR, and Y19W and Y18W as representative strains for *Rhodotorula mucilaginosa* and *Wickerhamomyces anomalus*, respectively. 2) comparison of different inoculum dose was carried out. Injection of 7 log and 8 log were selected for

dose evaluation. 3) same strains as mentioned in 1), both highest and lowest OA producing isolates were compared in fermentation with ripe and over-ripe coffee dry pulp (Geisha var.) to see its impact on OA concentration.

For additional sugar, general OA production decreased, this may be caused by carbon source preference. Stimulation of fresh pulp sugar composition by adding sucrose didn't help with metabolites in our fermentation. Overall OA concentration dropped when fermented with *Rhodotorula mucilaginosa* strains. Among 7 OAs, quinic acid showed significant decrease with the addition of sugar. *Wickerhamomyces anomalus* inoculated beans were also observed decrease in OA, not included Y19W inoculated beans. For higher inoculum, there was no trend behind higher inoculation or lower. At 24 h, no comparable difference in OA concentration was observed under 2 inoculation doses. Briefly outlined, higher inoculation did not efficiently increase OAs production during fermentation. This phenomenon could be caused by energy and space competition from large population. A similar phenomenon has been reported previously; yeast plateaued at around 8.1 log cfu ml^{-1} during fermentation [35]. Besides, since achieving a higher population requires more culture time and cost, an inoculum of 7 log cfu ml^{-1} was sufficient for this study. Finally, the use of over-ripe pulp was compared to ripe pulp as a substrate for OAs production. For all selected yeast isolates, the use of over-ripe pulp showed significantly lower total OAs concentration, mainly due to the decrease of quinic and citric acids. The difference was generally larger for the *Rhodotorula mucilaginosa* isolates than *Wickerhamomyces anomalus*. Less production of OAs in over-ripe dry pulp were observed along with the whole fermentation time.

Overall, these results showed that fermentation using ripe pulp without sucrose and the inoculation of 7 log cfu ml^{-1} was sufficient for effected fermentation.

In chapter 5, the screened isolates of Y12bR (*Rhodotorula mucilaginosa*) and Y18W (*Wickerhamomyces anomalus*) were used as yeast starter candidates, L4 was used as lactic acid bacteria potential starter for mono- and co-inoculation in green coffee beans fermentation. We kept the same fermentation scale for chemical analysis in fermented green beans which contained 5 g of green

coffee beans and 5 g of dry coffee pulp in a sterile flask containing 20 ml sterile distilled water. The beans were collected from 2 origins, Kenya (SL. 28 var.) and Colombia (Castillo var.) coffee beans. The dry pulp was from Geisha var. and was collected in ripe status. OA, CGA and caffeine were detected at 24 h, 48 h and 72 h by HPLC. The 5 incubations were mono-culture of selected yeast isolates (YR12b for *Rhodotorula mucilaginosa* and Y18W for *Wickerhamomyces anomalus*), labeled as YR and YW; co-culture of selected yeast and lactic acid bacteria (L4 for *Enterococcus mundtii*), labeled as YRcoL and YWcoL, and non-inoculation as control.

pH changes and microbial counting during fermentation were monitored.

The pH of both beans, a significant decrease from 0 to 24 h was observed. For Colombia beans, the co-inoculated beans of YRcoL showed a continuous drop from 24 to 48 h. Generally, at same fermentation time, no significant differences were observed in pH between different inoculations. The dry pulp and coffee beans were naturally acidic, and the pH values before fermentation were 4.55 and 4.75 for the Colombia and Kenya coffee, respectively. The pH decrease from 48 to 72 h may be caused by acids from microbial activities, although the results are only partially explained from the OA measurement. At the end of fermentation, the final pH of 4.9 is similar to the pH generally observed at the end of the commercial fermentation [90]. The moderate pH decrease in the inoculated samples may be because of the relatively weak ability of the *Enterococcus mundtii* strains to lower pH compared to other species of LAB [91].

The microbial population of yeast and LAB at 0, 24, 48, and 72 h of fermentation was monitored for inoculation on Colombia and Kenya coffee beans.

The yeast population generally was observed a first increase from 0 to 48 h and then a decreasing trend from 48 to 72 h. The yeast populations at 24 h of fermentation were much higher for the inoculated groups compared to the control group but no significant difference going along with longer fermentation time. This phenomenon may indicate that first 24 h is the growth booming period, and after it the starters' population dropped and lost the role of predominant community.

LAB population in the inoculated samples showed close to maximum values at 24 h of

fermentation. The 24 h quick boom in LAB population may result from the hydration of dry pulp providing nutrition for bacterial growth. The final population at 72 h reached similar values for all samples (6.97±0.22 log cfu g^{-1} in Colombia beans and 7.22±0.09 log cfu g^{-1} in Kenya).

CGA behaves as indicator of coffee quality and as the important role as an aroma precursor, as well as its biological importance as an antiviral and antioxidant chemical [92-94]. The concentrations of CGA in coffee beans were significantly ($p<0.05$) affected by fermentation time but changed differently between Colombia and Kenya coffee beans. Briefly, the inoculation of *Wickerhamomyces anomalus* in Colombia and the inoculation of *Rhodotorula mucilaginosa* in Kenya beans helped with increase CGA by fermentation time. This increase could be caused by the yeast different metabolisms and showed potential of modifying CGA composition in different varieties of beans. For Colombia beans, concentration of CGA differed greatly with different inoculations. In particular, the fermentation patterns between 2 types of yeasts were significantly different ($p<0.05$), and all inoculations except for YWcoL significantly ($p<0.05$) affected CGA content when compared to the control. For Kenya beans, inoculated groups presented higher CGA than non-inoculation but decreased at 48 and 72 h. Co-inoculation of LAB showed significant difference ($p<0.05$) compared to mono-inoculation of yeast only. Co-inoculation of *Rhodotorula mucilaginosa* and LAB (YRcoL) to the Kenya beans affected CGA content in a similar way to Colombia beans, with a constant increase in CGA during fermentation. While, about contribution of CGA in green coffee beans is controversial, both better sensory were reported in high and low content of CGA.

Caffeine content in Colombia beans, caffeine concentration was significantly ($p<0.05$) different in samples after 24 h of fermentation when compared to 48 h and 72 h, although there was no significant difference among different inoculations. No significant difference was found for inoculation nor fermentation time in Kenya beans. The caffeine concentration generally increased with fermentation time, could be caused by soaking effect instead of microbial activities.

All 7 OAs were detected at each fermentation time. For acetic acid, it was found highest YWcoL at 72 h in both beans. Acetic acid increased with fermentation time in all treatments with the exception

of Colombia beans inoculated with YR in which concentration decreased at 72 h. Highest citric acid was detected in YW fermented Colombia beans at 48 h and YW fermented Kenya beans at 24 h. Citric acid production in Colombia beans was significantly influenced by time and YW inoculation. In Kenya beans, only YWcoL inoculation showed significant difference in citric acid concentration when compared to the control group. Formic and succinic acids were detected in all treatments, but their concentrations were relatively stable during fermentation. The concentration of lactic acid was significantly ($p<0.01$) affected by the co-inoculation of LAB. The highest lactic acid was observed in YWcoL at 72 h in both beans. Lactic acid was also affected by fermentation time and high concentrations were not detected until 72 h, which may indicate that longer fermentation times could be considered in future research with *Enterococcus mundtii* to achieve more lactic acid. Malic acid existed in highest concentrations in beans fermented for 48 h, in YW inoculated Colombia beans and YRcoL inoculated Kenya beans. The concentration of malic acid decreased at 72 h, which may be explained by the conversion of malic acid to lactic acid by bacterial activity [16], an explanation supported by the high concentrations of lactic acid at 72 h.

Volatiles were also analyzed in fermented green coffee beans. In Colombia beans, 104 volatiles were detected, including 55 hydrocarbons which presented mainly, 14 alcohols, 20 esters, 2 lipids, 2 phenols, 1 acid, ketone, ether, pyrazine and aldehyde, and 5 others. In Kenya beans, 98 volatile compounds were detected, including 42 hydrocarbons, 18 alcohols, 20 esters, 3 ketones, 3 lipids, 2 ethers, 1 acid and an aldehyde. The relative concentration is shown in Supplementary file (S-excel 1).

In both fermented Colombia and Kenya beans, hydrocarbons were the most abundant in numbers as well as total concentrations. In fermented Colombia beans, the fermented beans also contained high concentrations of alcohols and esters, with higher concentrations of esters at 72 h of fermentation. In fermented Kenya beans, alcohols and esters showed second and third abundant, in particular multiple esters were detected at 72 h, with mono- and co-inoculation of *Wickerhamomyces anomalus*. Furthermore, alcohol, which is a key metabolite in yeasts, also can be taken as an indicator of growth of yeast, showed an increasing trend from 24 to 48 h but then dropped at 72 h. This trend is in

agreement with the microbial dynamic population. Generally, different inoculations contributed to a variety of chemical compositions both in numbers and in concentration, consequently result in unique flavor and aroma precursors in green beans, which can be transformed into volatile compounds during roasting which finally compose distinctive sensory profiles in the roasted bean [35, 67, 99].

Unfortunately, it's not clear in which mechanism the microbial flora influence aroma composition. Until author published this result, only observation of organic compounds was carried out and recorder. Further correlation of metabolites from yeast or lactic acid bacteria to coffee aroma profile should be done.

Chemical analysis in roasted beans followed chemical analysis in green beans. The complete list of compounds detected are shown in the Supplementary file (S-excel 2). In total, 107 chemicals were identified in roasted Colombia beans, including 7 ketones and lactones, 5 aldehydes, 13 furans, furanone and furfural, 3 acids, 11 pyrazines, pyridines and pyrans, 9 pyrazines 14 alcohols, and 19 esters. 108 chemicals were detected in Kenya beans, including predominant compounds 9 ketones and lactones, 11 furans, furanone and furfural, 5 acids, 2 aldehydes, 17 pyrazines and pyrans, 11 pyrazines, 6 alcohols, and 28 esters. Chemical components that could not be classified into these molecular groups have been grouped as 'Others'. Generally, the increase in abundance and contents of alcohols, furans, and esters may exhibit a positive impact on the aroma and taste [100].

Alcohols are known to provide sweet, clean, and floral flavors to the coffee beverage, specifically using yeast starters to increase volatile alcohol products [53]. In Colombia beans, more than 2 folds of alcohols were found in 24 h co-inoculation with LAB than other time, while at 72 h there is more varieties of alcohols. In Kenya beans, less abundance of alcohols was detected at 72 h. Unique aromatic compounds such as phenylethyl alcohol, 2,3-butanediol and 1-nonanol were only detected in inoculated beans. Phenylethyl alcohol, contributing to rosy honey flavor, was only found after 48 h fermented Colombia beans, furthermore, richer content was observed in YWcoL. 2,3-butanediol, contributing cocoa, fruity and buttery in coffee beverage, was only detected in 72 h fermented Colombia beans with YRcoL and YW; 1-nonanol, characterised by fresh, clean and floral flavor in

coffee was found highest content in 72 h YR inoculated Colombia beans.

Aldehydes could be related to fruity and tropical flavor to coffee beverage. Presentation of furfural and 5-methyl-2-furancarboxaldehyde, described as sweet and caramellike in coffee, could be related with *Wickerhamomyces anomalus* metabolic activity since richest was found in 48 h YW and 24 h YWcoL Colombia beans, respectively. Kenya beans were also observed highest furfural and 5-methyl-2-furancarboxaldehyde in YWcoL inoculation (48 h). 5-methyl-2-furancarboxaldehyde, was reported to be generated by *Saccharomyces cerevisiae* for its aromatic feature, could be novelly correlated to *Wickerhamomyces anomalus*.

Esters are known as fruity and sweet odor in coffee which can be gained by yeast fermentation [102]. In this study, richer esters in Colombia beans was found at 24 h and 48 h. More esters were recorded in the mono-inoculation of *Wickerhamomyces anomalus* than *Rhodotorula mucilaginosa* from 24 to 48 h. Co-inoculated of LAB showed richer and more types of easters during fermentation. Ester only found in inoculated beans, e.g. acetic acid, 2-phenylethyl ester (rosy and flora), a general metabolic product in yeast fermentation was only found in inoculated Colombia beans. In Kenya beans, longer fermentation time gave distinguish more aromatic easters. Such as, hexadecanoic acid, ethyl ester (contributing to fruity and creamy notes) gained more with longer fermentation time in Kenya beans, besides, higher content was detected in *Wickerhamomyces anoma* inoculated Kenya beans.

Furans, another main group which assistance with sweet taste (e.g. furanone described as buttery and caramel-like) were detected in roasted samples. 2-furanmethanol is considered as cooked sugar and burnt taste, was detected in all treatment and highest content at 24 h in YWcoL fermented Colombia beans and YW fermented Kenya beans. 2,5-dimethyl-4-hydroxy-3(2H)-furanone (caramel and honey flavor), was found richer in inoculated Kenya beans than non-incubated beans during whole fermentation process.

Ketones are described as giving buttery, caramel-like, musty, mushroom-like, or fruity notes [103]. Ketones were detected over 2 times high in 24 hours' mono- and co-*Wickerhamomyces anoma* fermented Colombia beans than other time and inoculation. In Colombia beans, aromatic compounds

were only detected in inoculated samples such as 1,2-Cyclopentanedione, 3-methyl- (sweet, caramel and coffee-like).

Nine pyrazines were found in roasted samples. Common detection of 2,6-dimethyl-pyrazine (chocolate, cocoa and roasted nuts), 2-ethyl-6-methyl-pyrazine (flowery, fruity and hazelnut-like), and 3-ethyl-2,5-dimethyl-pyrazine (roasted flavor) in both beans, was richer in 72 hours' fermentation in inoculated Kenya beans. Pyrrole, which gives the odor of caramel, showed relatively higher with shorter fermentation time. Unique detected component, 2,5-dimethyl-pyrazine (nutty, roasty and cocoa-like) were only found in inoculated groups, and 48 h fermentation had higher concentration than other time. 1H-pyrrole, 1-(2-furanylmethyl)-, which is known as hazelnut and coffee-like aroma in coffee beverage, showed richest at 72 h in Kenya beans under mono-inoculation of *Wickerhamomyces anoma*.

Unique compounds were produced only in inoculated beans attracted more attention for its correlation with specific metabolites. Such as, in Kenya beans, 2-furfurylthiol (coffee-like), 2,3-dimethyl-pyrazine (nutty and roasted) and 2(1h)-pyridinone, and esters including acetic acid, 2-phenylethyl ester, butanoic acid-2-methyl-pentyl ester and eicosanoic acid, 2-hydroxyethyl ester were only found in YW, which could be related to activity of additional *Wickerhamomyces anomalus*. Compounds were only detected under inoculation of *Rhodotorula mucilaginosa*, such as, in Colombia beans, (E)-9-Octadecenoic acid ethyl ester (floral odor), which could consist more proof for non-Saccharomyces yeast fermentation. In Kenya beans, 9,12-Octadecadienoic acid, ethyl ester was only found in 48 h YR.

Except aromatic compounds which can help coffee flavor modification, we also found non-flavor related functional chemicals. In particular, co-inoculation of yeast isolates and lactic acid bacteria may help with presentation of those compounds. Such as, in Colombia beans, vitamin E which is famous for its antioxidant activity, was detected at all time in Colombia beans and highest content was found at 24 h in YWcoL. Stigmasterol, known for lowering cholesterol in human intestinal, showed the highest concentration at 48 h in YRcoL, simultaneously, campesterol was also detected most

concentration in same co-icubation. Squalene, relative to immune regulation, antioxidation, anti-inflammatory response and anti-cancerous responses [105], was found to present richest at 24 h in YWcoL. Mono-inoculation of *Wickerhamomyces anomalus* was found to have specific chemicals or contribute higher presentation of some chemicals, for example, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, which reported for its antioxidant and antimicrobial activities [106], was only found in YW fermented beans. 9,12-octadecadienoic acid (Z, Z)-, methyl was found anti-cancer function [54], was detected higher content in YW inoculated groups. Mono-inoculation of *Wickerhamomyces anomalu* also behaved its effects on compounds in Kenya fermented beans, the only observation of phytol characterized as antioxidant and antimicrobial activities [107], furthermore assist the special metabolites with beneficial characteristics from Mono-inoculation of *Wickerhamomyces anomalu*.

In chapter 5, the last session was done with sensory evaluation. With the help of well experienced panel, we cupped the mono- and co-inoculated Kenya and Colombia beans under the SCA protocol. The highest score was given to co-inoculation beans for either Kenya and Colombia beans.

In Colombia beans, YRcoL at 48 h was evaluated as best cup, mainly due to its high score in aroma and flavor. Time gave specific aroma feature, in general, 24 h fermented beans showed lower score (<80); while 48 h, inoculation contributed to richer flavor, aroma and aftertaste; until 72 h, overall rating decreased. Fermentation time significantly ($p<0.05$) affected perceptions of acidity, body, sweetness, aftertaste, flavor, balance, and uniformity, and the beans fermented for 48 h were given a relatively high score in these parameters than other fermentation times. There is no significant difference by inoculation on total score or each cupping characteristic, except aftertaste of YW.

Specific description of each cup was also recorded. At 24 h, all beans presented raw and bad acidity, while at 48 h and 72 h, all inoculated groups behaved good wine/fermented flavor. The best cup (48 h YRcoL) was described as almond and plum flavor, at the same time, other inoculation showed flat and chestnut taste in YR, plum and sweet in YW, flat and almond flavor in YWcoL, and, unpleasant miso acidity control group. At 72 h, YR showed darker and almond flavor but slightly dirty.

The very interesting found in YR inoculated beans is presentation of chocolate flavor.

For Kenya beans, comparing to non-inoculate beans, better aroma, flavor and acidity were achieved in inoculated beans respectively. The best cup was given to YWcoL with the highest score of 85.15±2.58 point. Different from Colombia beans, there is no summarized trend for different fermentation time, but all inoculated groups scored higher than corresponding control groups. At 24 h, higher score in aroma, flavor and acidity was given to 4 fermented beans. At 48 h, YWcoL had highest score in flavor as well as highest score in sweetness, which could be related to the highest concentration of furfural as well as 5-methyl-2-furancarboxaldehyde for their expression of sweet and caramel-like taste. At 72 h, all fermented beans were given higher score in aroma and flavor than control. Higher concentration of aldehyde (benzeneacetaldehyde) in YR and ester (hexadecanoic acid, ethyl ester) in YWcoL may reflect to their better aroma and flavor assessment respectively. Detailed sensory description was recorded as follow. Interesting observation of impacts on flavor by fermentation time was found in Kenya beans, behaving as coffee beans fermented for 24 hours were characterized by tropical fruits; coffee beans fermented for 48 hours were characterized by almond as well as nutty flavor; and coffee beans fermented for 72 hours were characterized by apple like flavor. According to 2-way ANOVA, 24 h was significant different from 72 h on aroma. *Rhodotorula mucilaginosa* inoculation helped modification of flavor by different fermentation time. At 24 h, mono-inoculation of *Rhodotorula mucilaginosa* gave fruity and sweet taste, particularly tropical fruits flavor. At 48 h, mono-inoculation of *Rhodotorula mucilaginosa* showed intense flavor including almond, plum and floral flavor. At 72 h, both mono- and co-inoculation of *Rhodotorula mucilaginosa* were evaluated as green apple flavor. Yeast isolates was found to modify flavor successfully, 24 hours' mono- and co-inoculation of *Wickerhamomyces anomalu* presented raspberry and sweet flavor and plum and nutty flavor, as well as creamy body, respectively.

In chapter 6, the evaluation of self isolates was continued. According to previous content, caffeine and CGA, important indicators for coffee quality as well as healthy impacts and flavor contribution were selected as evaluable targets. *Saccharomyces cerevisiae*, *Candida spp.*, and *Torulaspora*

delbrueckii are the most reported starters using in coffee fermentation, for their contribution in aromatic compounds. By using commercial yeast (*Saccharomyces cerevisiae*) as a comparable stater, we monitored CGA and caffeine concentration change during 24, 48 and 72 h fermentation. Similar observation in chapter 5, the concentration of caffeine stayed stable during fermentation in respective inoculation. At 72 h, caffeine showed lower concentration in all beans, maybe considered by soaking effects from fermentation changed the structure or beans and further influenced on the extraction. CGA decreased by fermentation time, and reached lowest at 72 h. At 24 h, inoculated beans showed significantly high CGA than beans in 48 and 72 h. While at each fermentation time, no significant difference in CGA concentration was observed. Well, more comparison should be carried out on aromatic chemicals and the important aroma precursors to achieve better understanding of fermentation ability from isolates.

Summarized as follows, by utilizing coffee material for spontaneous fermentation, yeasts of and lactic acid bacteria can be taken as potential coffee fermentation starters were successfully isolated and identified. After screening and evaluating by high OAs producing, strains YR12b (*Rhodotorula mucilaginosa*) and Y18W (*Wickerhamomyces anomalu*), L4 (*Enterococcus mundtii*) were chosen for next fermentation evaluation. At the same time, growth factors were also compared. Ripe pulp and inoculum of 7 log cfu ml^{-1} were used as optimized condition. Finally, we used the selected strains and conditions for lab scale fermentation with 2 varieties of green coffee beans, and 4 types of starter combination were used for final fermentation evaluation, including mono-inoculation of *Rhodotorula mucilaginosa*, mono-inoculation of *Wickerhamomyces anomalu*, co-inoculation of *Rhodotorula mucilaginosa* and *Enterococcus mundtii*, and co-inoculation of *Wickerhamomyces anomalu* and *Enterococcus mundtii*. Firstly, pH and microbial counts during fermentation were monitored to check primary evaluation of fermentation ability and growth status. Then evaluated from OAs, CGA, caffeine and volatiles in fermented green beans to volatiles in roasted beans. Apart from chemical analysis, sensory analysis was also carried out to further understand how self isolates impacts on flavor formation. As a result, modification of coffee flavor and chemical composition was successfully

realized by using indigenous isolates in re-fermentation with green coffee beans. Using starters can be a very potential and easy to control method for achieving different flavor and quality of coffee, and further bring unexplored economic and enjoyment flavor value in both coffee belt and non-coffee planting countries and regions.

7.2 Future outlook

Deep analysis should be considered with the chemical data, find the correlation of microbial metabolites and these contribution to coffee aroma formation. Dig more about the mechanisms behind yeast and lactic acid bacteria starters.

Compounds like organic acids, amino acids and sugars in fermented green coffee beans is necessary to monitor also, which can be clear indicator of fermentation products and nutrition utilizing ability of starters. Consequently, be able to have better knowledge of how microbial activity involved with fermentation and metabolites. As well as the volatile analysis in roasted coffee beans, to find out how compounds change in green beans influence on the aromatic profiles and sensory.

Modification of coffee flavor by post-harvest methods can be done in multi-angle and multi-faceted. More trial with pre-treatment of green beans should be performed in close future, such as using acetic, malic, lactic, quininic acids or using various sugars (e.g., fructose, sucrose and glucose) to treat green Robusta beans and improve the blend ratio with Arabica beans [116, 117]. Brazil is one of the biggest coffee producers all around the world, well Brazil beans have less aroma or earthy aroma which is unpleasant for some coffee consumers, may be modified with organic acids pre-treatment.

Besides, pre-experiment was done in filed in Colombia coffee farm, mandarin residue after juice making was used in coffee fermentation during wet process. By cupping, mandarin fermented coffee gave strong fruity note and nice acidity, as well as dense aroma and flavor. Fermentation has been proved to uplevel coffee aroma and flavor [36, 67, 75, 111], combination with fruits residue such as strawberry, mandarin, pineapple may further assistant with flavor formation during fermentation.

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Appendices A

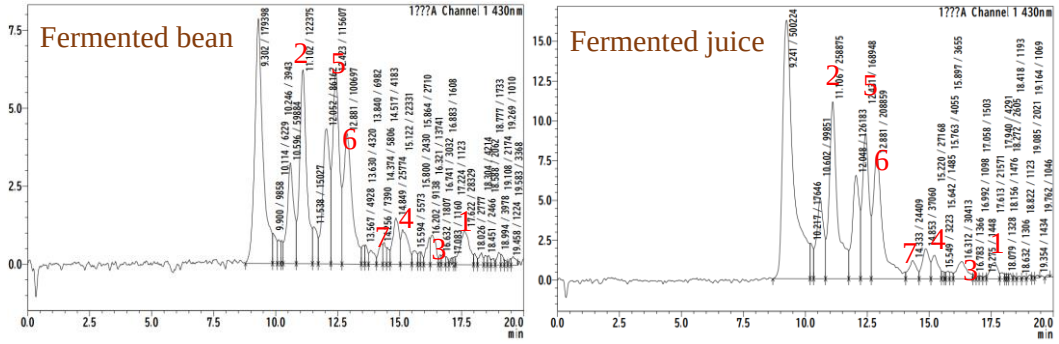
OAs monitoring in liquid fractions with LAB isolates fermentation

Same ratio of green coffee beans (5 g) and dry coffee pulp (5 g) from Colombia (Castillo var.) and sterile water (20 ml) were added in to 250 ml fermentation container in advance. Inoculum of isolates was around 10^7 cfu/ml. Culture yeast and LAB inoculated beans at 28 ◦C and 30 ◦C, respectively. Fermentation liquid was collected at 24, 48 and 72 h. When sampling, 200 μl of juice was transferred to 1.5 ml EB-tube, and vortexed 10 min at room temperature, and followed by centrifuging at 14 000 **g** for 10 min at 4 °C. Then draw 100 μl of upper liquid and added 900 μl of 3mM HClO4 then vortex for another 10 min at room temperature to mix well. Finally, all samples should be filtered by 0.45 μm membrane before testing. All samples were prepared two repeats.

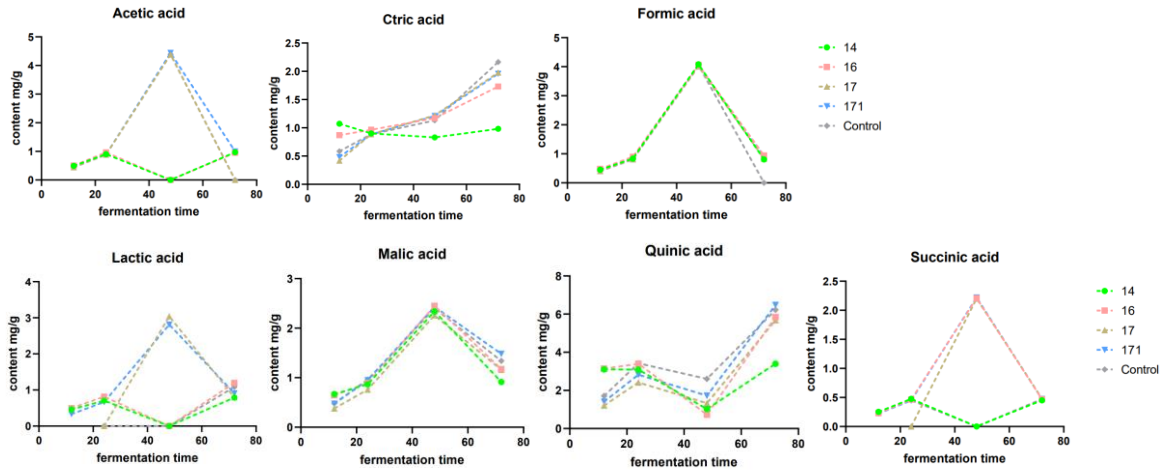
HPLC detection method as chapter 2.1.3 by using KC811 column (8.0 mm I.D.×300 mm) with KC-G 8B guard column (8.0 mm I.D.×35 mm) (Shodex, Japan) was used for OAs measurement.

Both fermentation juice and beans were sampled and qualified for OAs contents. HPLC elution images of juice and beans are shown in A-Fig. 1., eluted order was citric, malic, quinic, succinic, lactic, formic, acetic acid.

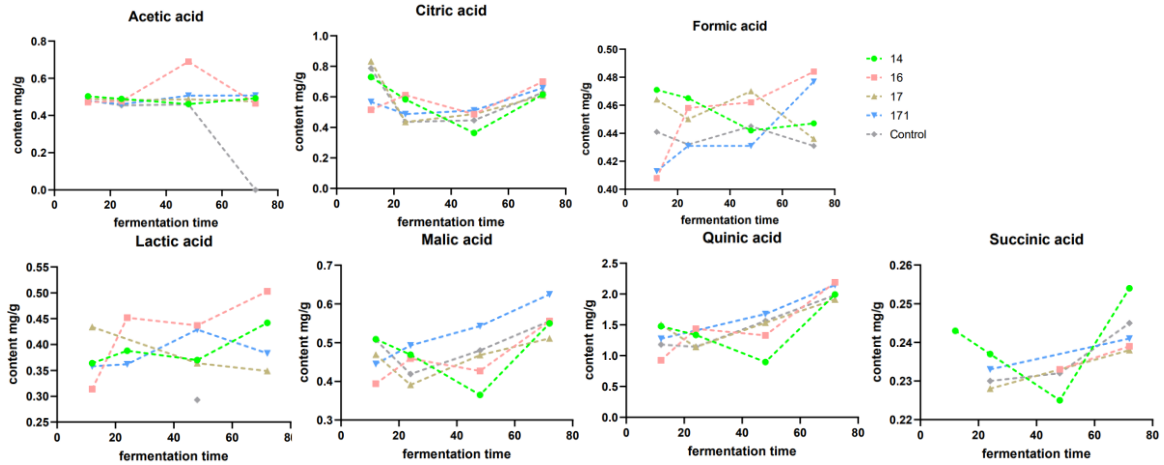
For LAB isolates fermentation with strain L14, L16, L17a and L17b, the juice and beans faction OAs detection results showed in A-Fig. 2. and A-Fig. 3. as the first batch, and strains of L4, L5, L6a, L6b, L8, L10 and L12 as the second fermentation batch, the juice faction and beans OAs detection showed in A-Fig. 4. and A-Fig. 5.



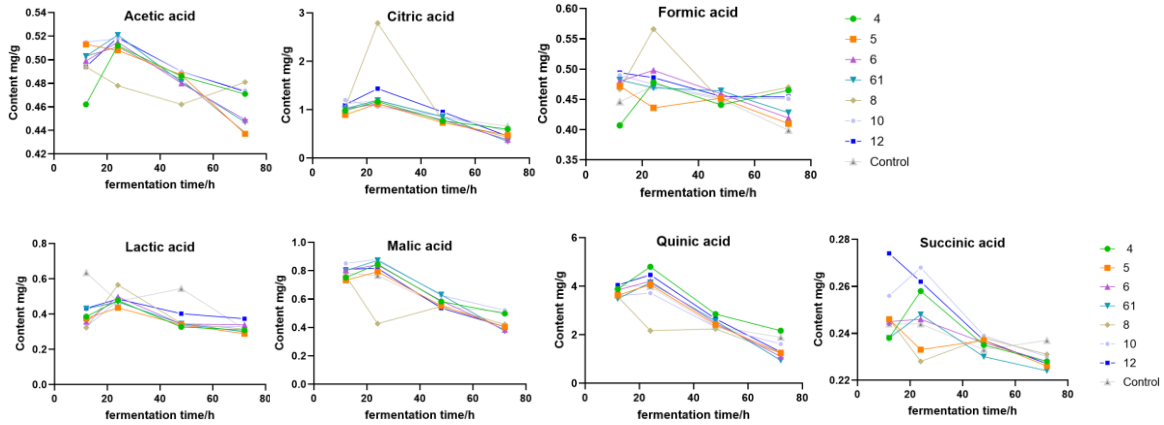
A-Fig. 1. Elution peaks of fermentation juice and beans by post column HPLC,1: Acetic acid, 2: Citric acid, 3: Formic acid, 4: Lactic acid, 5: Malic acid, 6: Quinic acid, 7: Succinic acid.



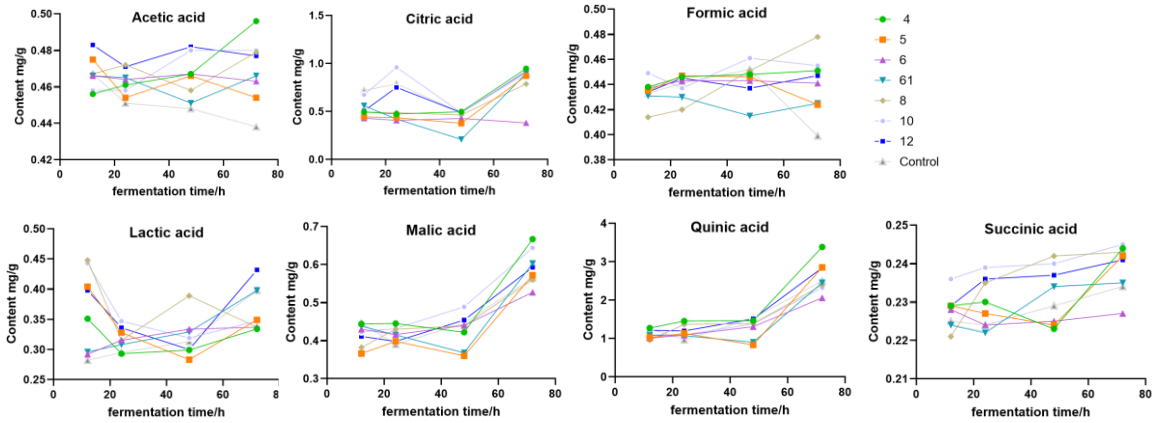
A-Fig. 2. OAs detection results in juice faction when fermented with LAB (batch 1).



A-Fig. 3. OAs detection results in beans when fermented with LAB (batch 1).



A-Fig. 4. OAs detection results in juice faction when fermented with LAB (batch 2).



A-Fig. 5. OAs detection results in beans faction when fermented with LAB (batch 2).

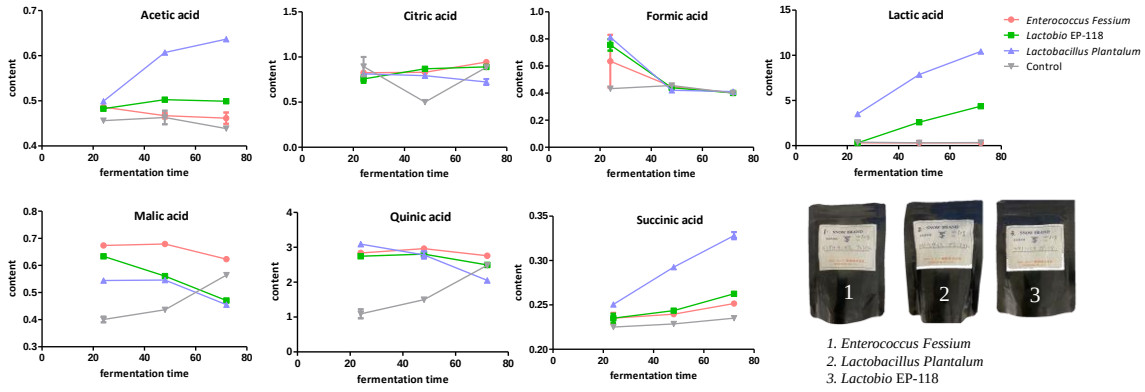
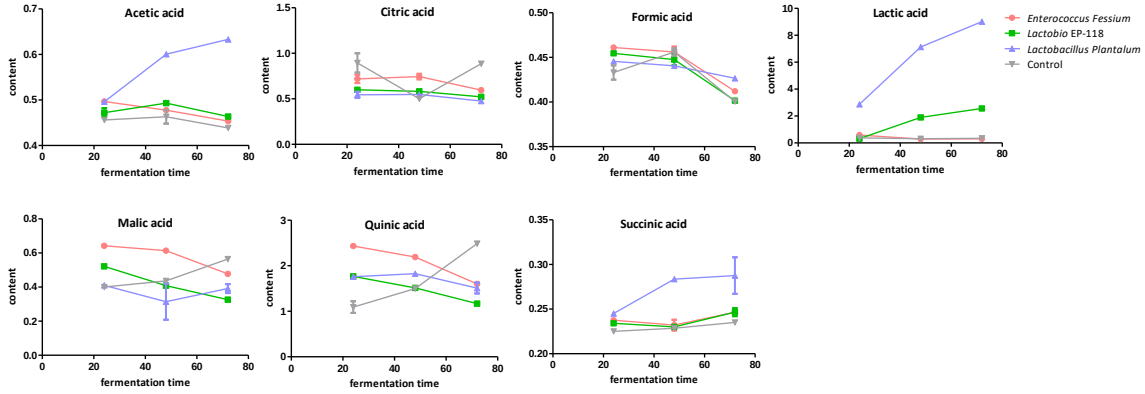
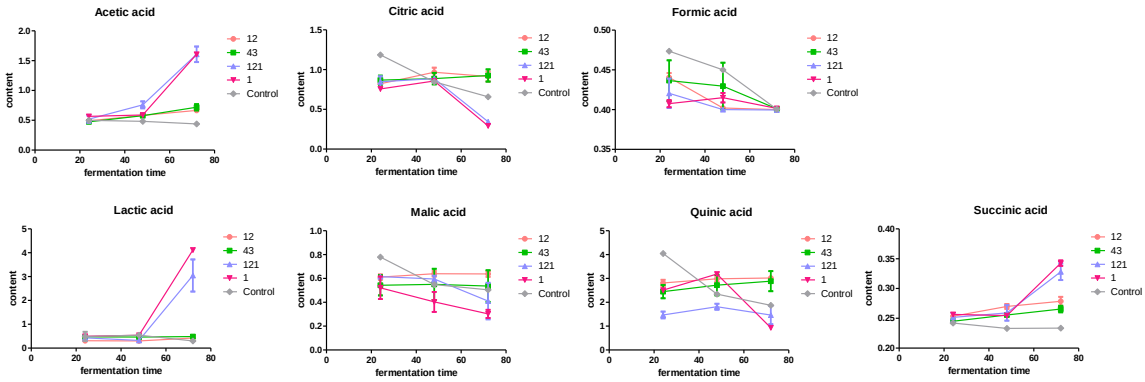


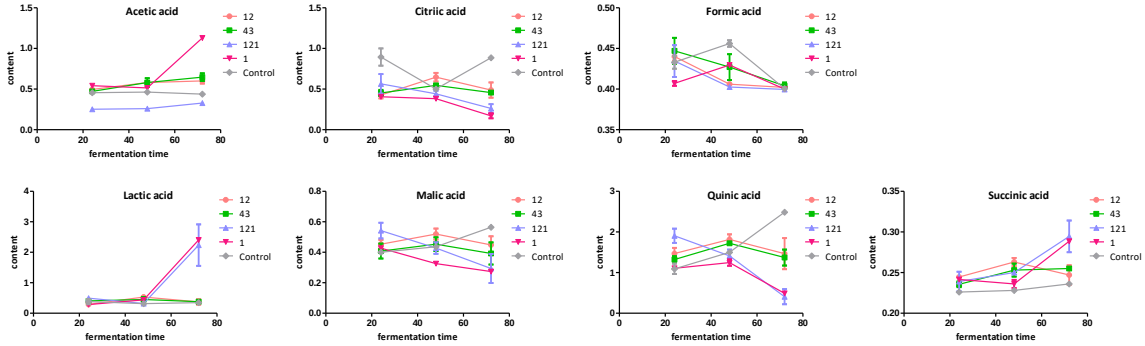
Fig. A. 6. OAs detection results in juice faction when fermented with commercial LAB.



A-Fig. 7. OAs detection results in beans when fermented with commercial LAB.



A-Fig. 8. OAs detection results in juice faction when fermented with yeast.



A-Fig. 9. OAs detection results in beans when fermented with yeast.

Appendix B

Evaluation of fermentation with milled pulp and whole pulp

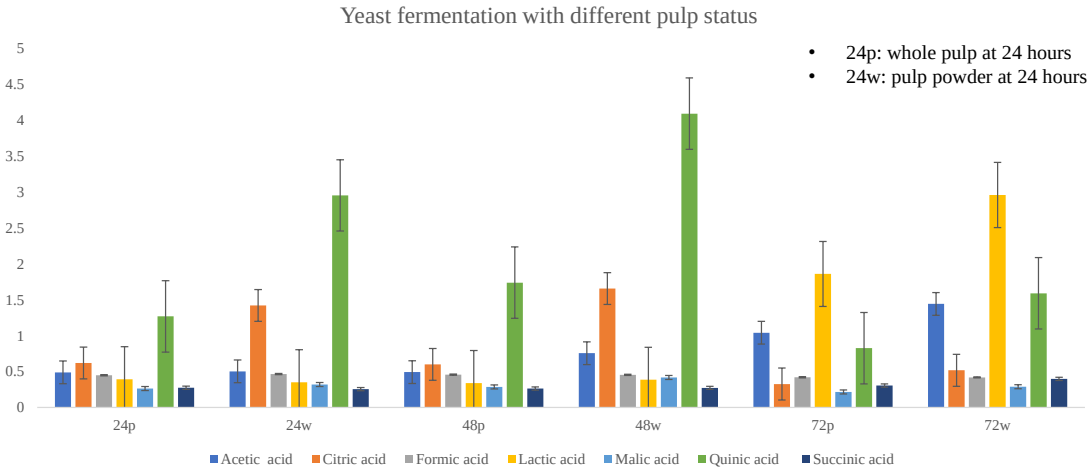
For better evaluation of fermentation ability from yeast isolates, milled pulp and whole pulp were used for proper fermentation substrate. Same ripe pulp of 5 g was milled by for 15 s added to fermentation bottle.

OA production from whole and milled pulp was shown in B Fig. 1. Generally, using powder helped higher OA production. Among which, quinic, citric, acetic and lactic acids showed significant difference compared to whole pulp.

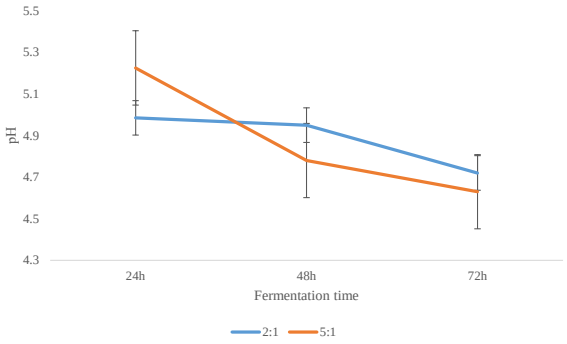
Different ratio of pulp used as fermentation substrate was also compared here. Ratio as 2:1 and 5:1 of pulp to green coffee beans. This evaluation was carried out by monitoring pH from fermentation juice. As results shown in B Fig. 2., from 48 h to 72 h, lower pulp showed larger drop of pH. More pulp as fermentation substrate did help with lowing pH, well, more considers like cost-saving also should be counted in for larger scale of fermentation application.

Higher ratio of powder (B Fig. 3.) was found to be contaminated by microorganisms from environment. Considering food safety, higher ratio of pulp was not used in future fermentation.

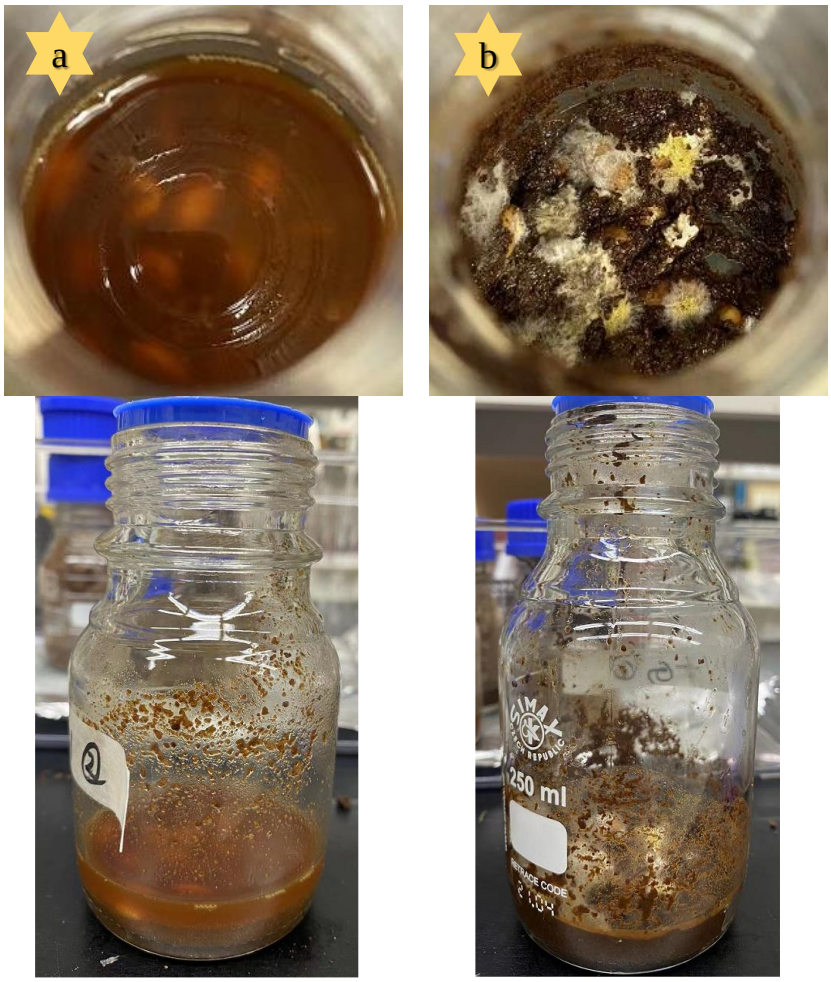
As conclusion, higher ratio of pulp as fermentation substrate may help with maximizing microbial activity, but also may result in contamination which cause risk in food safety. Ratio of 1:1 for green beans and pulp will be used for following fermentation experiments.



B-Fig. 1. Yeast (Y12b) fermentation (Colombia) with different pulp status (beans: pulp=1:1): OAs changes at 24/48/72 h.



B-Fig. 2. pH changes under the beans: pulp ratio of 2:1 and 5:1.



B-Fig. 3. Green coffee beans: pulp powder is 2:1 (a) and 1:1(b)

Appendix C

CGA and caffeine extraction methods comparison

To ensure following measurement is stable and suitable for coffee beans, few extraction methods were compared as re-experiments. Extraction by 1) methanol, 2) distilled water and 3) perchloride acid, at different temperature.

To find a proper extraction method for all material used in this study, in trial experiments, two varieties of beans were used, including SL 28 from Kenya and Castillo from Colombia, considering different structure of these beans.

Firstly, extraction was carried out by adding 25 ml of distilled water into 0.5 g of well milled coffee power. Both green and roasted coffee beans were prepared previously by milling 15 s via Konappy (Labonect, Japan). The temperature was set as boiling at 100 °C for 10 min.

To create time-saving method, whole beans were also considered to be directly extracted form solvent. Thus, powder and whole beans extraction were used as C-Table 1. Furthermore, different ratios of beans and solvent were compared, including 2 g of beans to 10 and 20 ml of solvent at different temperature. Temperature was set to 100 ℃ and 60 °C according to literature.

For methanol extraction (C-Table 2), 2 g of whole beans was added to 50 ml and 20 ml of methanol and stirred at 60 °C for 10 min. And same ratio of beans and solvent was compared at room temperature.

Higher temperature did help with more extraction of CGA and caffeine, but by repeating test, heating up showed not stable for repeating experiments, especially heating up to 100 ℃. 60 °C is stable than boiling or boiling and cool down to room temperature.

Comparing water and methanol for CGA and caffeine extraction, the latter showed better extraction for both and nicer stability.

C-Table 1 CGA and caffeine concentration by different extraction methods with water

Extraction	No.	Targets	Rt/min	Colombia	Kenya
100 °C	1 g *: 50 ml	CGA	7.224	3106.266	1567.311
		Caffeine	7.937	126.742	131.425
100 °C~RT*		CGA	7.309	1221.27	692.596
		Caffeine	7.975	38.432	48.273
100~ 60 °C	2 g: 10 ml	CGA	7.34	/	20.609
		Caffeine	7.993	/	16.682
	2 g: 20 ml	CGA	7.341	/	62.098
		Caffeine	7.978	/	12.83
RT	2 g: 10 ml	CGA	7.326	/	12.265
		Caffeine	7.991	/	3.812
	2 g: 20 ml	CGA	7.333	/	10.209
		Caffeine	7.979	/	2.922
60 °C	2 g: 10 ml	CGA	7.333	3.389	10.08
		Caffeine	7.986	8.163	15.785
	2 g: 20 ml	CGA	7.296	1.216	7.943
		Caffeine	7.951	5.753	6.902

*: milled by Konappy for 15 s; RT: room temperature.

C-Table 1 CGA and caffeine concentration by different extraction methods with methanol

Extraction	No.	Targets	Rt/min	Colombia	Kenya
Methanol 60 °C	2 g: 50 ml	CGA	7.299	1545.788	1587.353
		Caffeine	7.954	53.456	51.643
	2 g: 10 ml	CGA	7.273	14.125	12.867
		Caffeine	7.944	2.783	2.844
Methanol RT	2 g: 20 ml	CGA	7.289	7.581	5.594
		Caffeine	7.955	2.346	1.376
	2 g: 50 ml	CGA	7.293	3.839	2.407
		Caffeine	7.963	1.390	0.286

Appendix D

Field experiment

In this study, dry coffee pulp was used as fermentation substrate, in July 2023, filed study with fresh harvest material was done. In filed, from seedling to blooming, from cherry picking to processing, and, from drying to roasting were studied by following local experienced farmers (D-Fig. 1.). Three trial research were carried out in farm as follows.

1) Fermentation with Koji and yeast on-farm
2) Sugar treated green beans
3) Mandarin treated beans

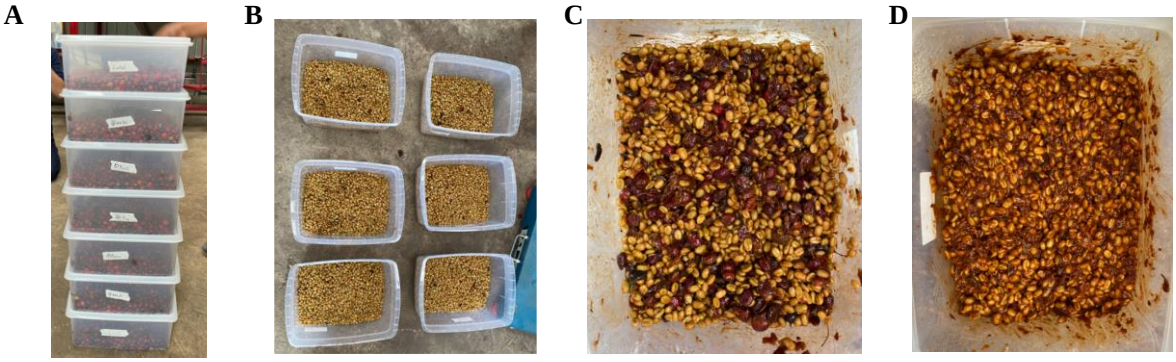
About Koji fermentation, different fermentation conditions were compared with Geisha var. freshly harvest cherries (D-Fig. 2.). A) Whole fresh mature cherries of 3 kg fermented with 10 g of koji powder. B) Puled green beans of 3 kg were used for six types of koji with inoculation of 10 g of dry powder of respective koji. C) Puled green beans of 3 kg were used for six types of koji with inoculation of 10 g of dry powder of respective koji, with whole pulp of 1 kg adding previously. D) Puled green beans of 3 kg were used for six types of koji with inoculation of 10 g of dry powder of respective koji, with milled pulp of 1 kg adding previously.

Fermentation conditions (D-Fig. 3.) for Koji growth were also tested, including heating up equipment and draining for less moist. As well as the way of injection koji, including mixing with beans, spreading the powder on the surface, koji pre-cultured on rice as starter, and adding rice powder as koji extra nutrition.

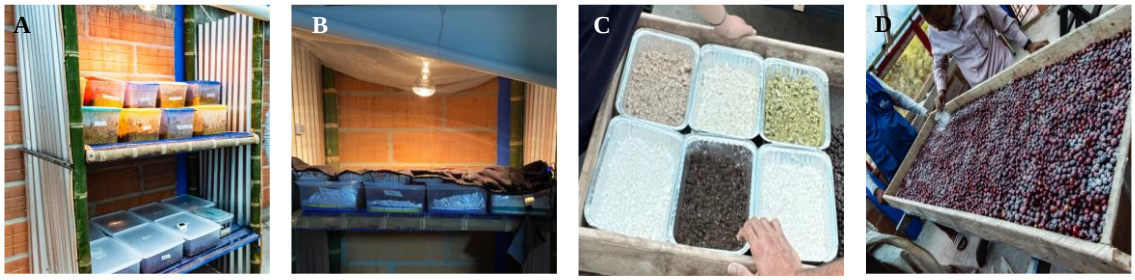
Brown sugar of 150 g and 2 kg of pulped green beans were boiled for 10 min and lay on the ventilation drying house (D-Fig. 4.). Drying took longer time than wet- or dry- process for its sticky surface after boiling with sugar, 5 days were taken before de-parchment.

Mandarin residues were collected after making juice and 1 kg of mandarin was added to 3 kg of pulped beans (D-Fig. 5). Fermentation happened in drying house for its high temperature.

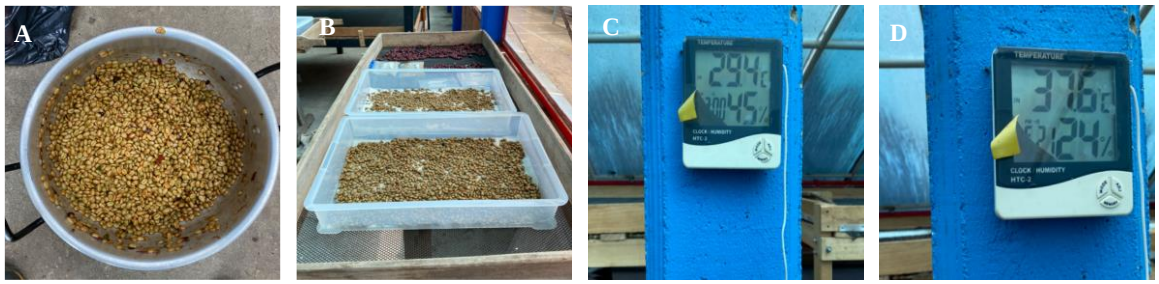
D-Fig. 1. Filed visit in coffee farm, including seedling to harvesting and processing.



D-Fig. 2. Koji fermentation in farm, including whole cherry, pulped beans only, pulped beans with whole pulp, and pulped beans with milled pulp.



D-Fig. 3. Koji fermentation condition in farm, including heating up housing (A), and draining plates (B). Koji pre-cultured on rice (C) and spreading koji powder on the cherry surface (D).



D-Fig. 4. Sugar fermentation with pulped coffee beans. Sugar treatment (A), and fermentation while drying (B), temperature range during fermentation (C& D).

D-Fig. 5. Mandarin fermentation with pulped coffee beans. Fermentation tank (A), and fermentation with mandarin residues (B). Yeast powder used for fermentation (C).