Impact of cocoa (*Theobroma cacao* L.) fermentation on composition and concentration of polyphenols: Development of a fermentation model system and utilization of yeast starter cultures

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Abstract

Consumption of cocoa and dark chocolate products has been associated with positive health outcomes including reduced onset of cardiovascular disease, inflammation, diabetes, obesity, and platelet disorders. Cocoa polyphenols, putatively responsible for these beneficial activities, are highly impacted by cocoa variety, agronomic effects and processing history. However, the difference in polyphenol concentration and composition between cocoa products originating from different hybrid clones (selected for high yield) or from different fermentation conditions is not fully understood. Detailed polyphenol characterization including determination of total polyphenol and total procyanidin concentrations, and qualitative and quantitative analysis of (mean) degree of polymerization was conducted. Significant differences in total polyphenol and procyanidin concentrations were observed between five genetic clones grown by the USDA-ARS Cocoa Germplasm Repository located in Mayagüez, Puerto Rico. To facilitate cocoa fermentation research in laboratories distant from cocoa harvesting sites, a laboratory-scale cocoa fermentation model system was developed in this study. This model system used dried, unfermented, cocoa beans and simulated pulp medium as the starting material. The model system supported growth of the essential succession of cocoa fermenting microorganisms and generated similar chemical changes to those observed in on-farm cocoa fermentation. Using this model system, the impact of inoculation with proprietary yeast strains *Saccharomyces cerevisiae* Lev F and *Saccharomyces cerevisiae* Lev B on cocoa polyphenol concentration and composition was evaluated. Inoculation with both yeast strains resulted in increased fermentation rate and Lev B inoculation resulted in higher total polyphenol and procyandin contents at the end of fermentation. Overall, the present work addressed the influence of cocoa variety selection and fermentation process conditions on the composition and concentration of polyphenols. These findings will contribute to continued efforts to develop cocoa products with optimized bioactivity and maximum disease preventative effects.

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General Audience Abstract

Annual worldwide cocoa production exceeds four million tons and the cocoa market impacts not only cocoa growing regions, but also countries producing cocoa products. Consumption of cocoa and dark chocolate products has been associated with positive health outcomes including reduced onset of cardiovascular disease, inflammation, diabetes, obesity, and platelet disorders. Cocoa polyphenols, compounds increasingly associated with health benefits, are highly impacted by the variety and processing history of cocoa. However, the extent to which cocoa genetic variety and processing conditions impact polyphenol concentration and composition is not fully understood. Polyphenol characterization was conducted for five genetic varieties sourced from the USDA cocoa germplasm repository located in Mayagüez, Puerto Rico and it was found that genetic variance could contribute to differences in polyphenol concentration. The main focus in this thesis was to develop laboratory cocoa fermentation model system to facilitate cocoa research in laboratories distant from the cocoa growing region. This model system used dried, unfermented cocoa beans and simulated pulp medium as the starting material. The model system was effective in that it supported growth of the essential succession of coca fermenting microorganisms and generated similar chemical changes to those observed in on-farm cocoa fermentation. This model system could be further applied to test the impact of fermentation conditions on cocoa bean quality. The potential for using yeast starter cultures to improve control and consistency of cocoa fermentation was evaluated using the model fermentation system. Yeast inoculation improved fermentation rate, and polyphenol concentration in fermented beans was higher when cocoa fermentation was inoculated with the yeast strain *Saccharomyces cerevisiae* Lev B. Overall, the present work addressed the influence of cocoa variety selection and fermentation process conditions on the composition and concentration of polyphenols. These findings will contribute to continued efforts to develop cocoa products optimized for maximum health benefits.

Dedication

This dissertation is dedicated to my beloved wife and my precious daughter for always giving happiness to my life and to my mother and father for your continuous support, trust, and love.

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Chapter 1: Introduction

Defining the problem

While the consumption of cocoa and dark chocolate are associated with positive human health outcomes, the mechanisms behind this phenomenon remain a topic of current investigation. Cocoa intake has been linked to reduced risk of various diseases including cardiovascular disease and metabolic syndrome (Hooper, Kay et al. 2012, Davison et al. 2008). Polyphenols are increasingly believed to be responsible for the health benefits associated with cocoa consumption (Latif 2013, Martin, Goya et al. 2013, Strat, Rowley et al. 2016). Polyphenols contribute 12~18% dry weight of the cocoa bean, making cocoa one of the most concentrated sources of dietary polyphenols. The predominant polyphenols in cocoa are catechins (37%), procyanidins (58%) and anthocyanins (4%), but larger polymers are also present (Nazaruddin, Seng et al. 2006). Cocoa processing steps including fermentation, roasting and alkalization significantly affect the composition and concentration of polyphenols in cocoa (Payne, Hurst et al. 2010, Hurst, Krake et al. 2011), thus likely modifying health benefits. Studies tracking the impact of fermentation on cocoa polyphenols have mainly focused on changes in total polyphenol content analyzed by colorimetric assays or changes in individual flavanol monomers like catechin and epicatechin (Nazaruddin, Seng et al. 2006, Payne, Hurst et al. 2010, Hurst, Krake et al. 2011, Cruz, Leite et al. 2015). While these studies do provide useful information, other facets of polyphenol characterization important to bioactivity, including concentrations of oligomeric and polymeric procyanidins and the overall mean degree of polymerization, were not addressed in previous studies. As emerging evidence suggests that oligomeric and polymeric procyanidins play a role in alleviation of metabolic syndrome and their related diseases (Dorenkott, Griffin et al. 2014, Bitzer, Glisan et al. 2015, Strat, Rowley et al.

2016), it is important to thoroughly characterize the fate of polyphenols through cocoa production and processing in order to better understand how these factors affect bioactivity.

Long term goal

The long-term goal in our research group is to understand the impact of food and beverage fermentation processes on bioactive compounds relevant to human health.

Overall objective

The overall objective of this study is to understand the impact of variety selection and cocoa fermentation on composition and concentration of polyphenols.

Central hypothesis

Our central hypothesis is that cocoa variety and fermentation conditions can influence the qualitative and quantitative profiles of dietary bioactive compounds in fermented cocoa beans.

Specific objectives

We are proposing the following specific aims and hypotheses to test our central hypothesis.

Objective 1: Determine if dry unfermented cocoa beans show differences in polyphenol concentration and composition across different genetic clones.

Hypothesis: Concentration and composition of polyphenols will vary among different genetic clones since each clone exhibits a different bitterness and astringency profile.

Objective 2: Determine if cocoa fermentation model system at the laboratory scale using dry unfermented cocoa bean as a substrate can produce similar quality of fermented cocoa bean

compared to conventional on-farm cocoa fermentation.

Hypothesis: Cocoa fermentation in a model system using dry unfermented cocoa bean and simulated pulp media as starting material in laboratory incubator with temperature control will result in fermented cocoa bean with similar microbial and chemical characteristics.

Objective 3: Determine if inoculation with active dry yeasts affects the concentration and composition of polyphenols in cocoa fermentation

Hypothesis: Controlling microbiota of cocoa bean fermentation in model systems is expected to affect the concentration and composition of polyphenols in fermented cocoa beans.

Chapter 2: Literature Review

Cocoa (*Theobroma cacao* L.)

The annual worldwide production of cocoa is 4 million tons (ICCO 2016), and 95% of dried fermented cocoa beans are produced on small farms. Cocoa thus plays an important economic role in their countries of origin (Anglaaere, Cobbina et al. 2011, Ozturk and Young 2017). The average price of dried fermented cocoa in January, 2017 was US $2,196 per ton, showing a downward trend from November, 2016 (ICCO 2017). The change in global cocoa production over year is depicted in Figure 1. It markedly increased from 2009/2010 to 2010/2011 season by 0.7 million tons and slightly fluctuated thereafter. Figure 2 shows the change in the annual average price of cocoa beans. The price of cocoa bean per ton almost doubled in the past ten years (from 2005 to 2015). However, the price has significantly dropped since from 2015 to 2017 by almost $1,100 per ton due to abundant supply and decrease in consumer demand.

It is believed that cocoa (*Theobroma cacao* L.) originated from the forests of the Amazon and Orinoco due to evidence that wild varieties of the trees exist in that region. Cocoa was distributed and cultivated by Aztecs and Mayans in Central America. In 1528, cocoa was first introduced to Europe by Hernando Cortés (Chatt 1953, Schwan and Wheals 2004). The name *cacao* is thought to be originated from the Olmec word, originally pronounced as "kakawa"(Powis, Cyphers et al. 2011). The genus *Theobroma*, a Latin name meaning food of the gods, reflects the longstanding special status of cocoa products in human culture (Schwan and Wheals 2004). The major varieties of cacao include Criollo, Forastero and a hybrid between the two varieties, Trinitario.

Criollo, renowned as the highest quality cocoa variety, is rich in flavor and has smooth bitterness compared to other groups, although only 5-10% of the world's chocolate is made from this variety (Rusconi and Conti 2010). Mexico and Venezuela are the two major producers of Criollo, while Madagascar, Java, Colombia, and Dominican Republic also produce significant quantities (Oracz, Zyzelewicz et al. 2015). Forastero constitutes 80 % of chocolate production globally due to its superior agronomic performance and high disease resistance. Forastero varieties generally exhibit less intense and distinctive aroma than Criollo (Rusconi and Conti 2010). Forastero is grown primarily in Ghana, Ivory Coast, Brazil, Indonesia, and Malaysia (Oracz, Zyzelewicz et al. 2015).

Trinitario variety derived from hybridization of Criollo and Forastero varieties and was first introduced in the 18th century in Trinidad and Tobago (Bekele 2004). They are mainly planted in Trinidad, Venezuela, Ecuador and Cameroon (Oracz, Zyzelewicz et al. 2015). Trinitario offers an attractive alternative to Criollo and Forastero, possessing beneficial traits of both groups such as disease resistance and desirable aromas.

Cocoa pods have 30-40 beans covered with pulp and attached to the placenta (Figure 3). The shell of cocoa bean comprises 10-14% cocoa bean (dry weight) and the internal cotyledon or kernel contributes to 86-90% weight (Afoakwa 2011). Each bean contains two storage cells: polyphenolic cells (14-20% dry bean weight) and lipid-protein cell. The cytoplasm is composed of protein, lipid, and starch. All of these components contribute to the flavor/aroma of cocoa (Kim and Keeney 1984, Ramli, Yatim et al. 2001, Osman, Nasarudin et al. 2004). The relative composition of cocoa bean to pulp by weight is approximately 60:40 (Schwan and Wheals 2004). To make a chocolate product, fresh cocoa beans need to undergo fermentation and further

processing steps to reduce astringency, bitterness and produce flavor compounds to give the expected chocolate-like characteristics.

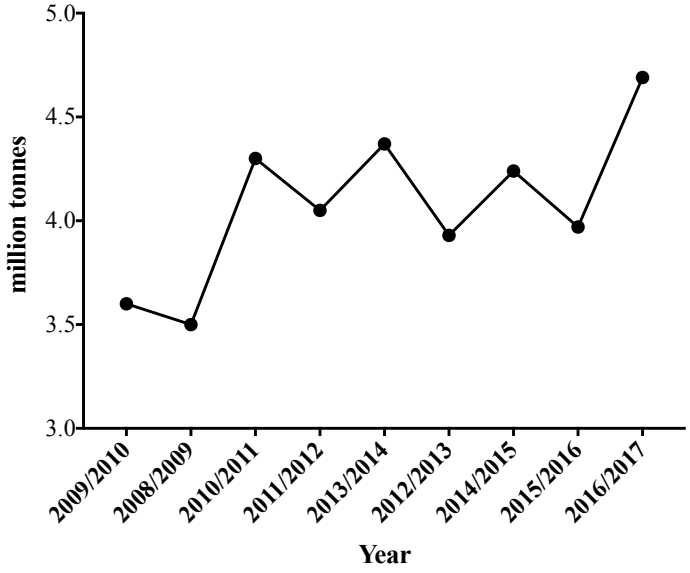

Figure 1. Annual production of cocoa beans (ICCO 2009-2017).

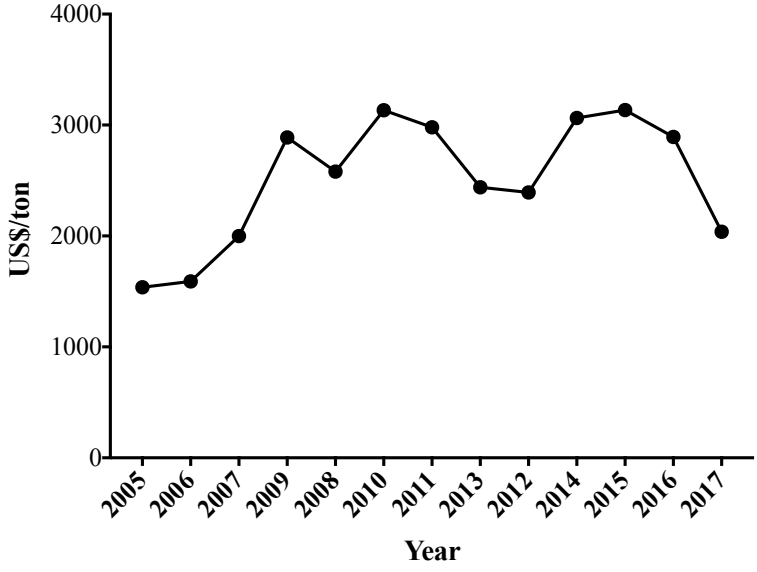

Figure 2. Annual price of cocoa beans (ICCO 2005-2017).

Figure 3. Opened cocoa pod

Cocoa fermentation and processing

A schematic outlining the overall chocolate production process is depicted in Figure 4. The formation of flavor precursors essential to cocoa quality is initiated through microbial fermentation and drying. After pods are harvested, cocoa beans and the surrounding pulp are moved to heaps or wooden boxes to undergo fermentation. Banana leaves are often used to limit heat loss, thus maintaining fermentation temperature (Afoakwa 2011). Cocoa pulp is composed of water (80-90%), sugars (10-13%), pentosans (2-3%), citric acid (1-3%) and pectin (1-1.5%) (Pettipher 1986, Schwan and Wheals 2004). Spontaneous microbial fermentation occurs where yeast, lactic and acetic acid bacteria metabolize the mucilaginous pulp, producing lactic and acetic acid, ethanol and heat as well as flavor compounds. Both acids penetrate into the cotyledons and combine with heat, enzymatic degradation of protein and carbohydrates to yield peptides, free amino acids and reducing sugars, also some contributing to formation of flavor precursors essential to chocolate quality. However, polyphenol, theobromine and caffeine diffuse out of the bean and are lost with the pulp draining. Polyphenols also undergo oxidation, mainly by polyphenol oxidase, to form the typical brown color of cocoa. Astringency and bitterness also decrease through polyphenol oxidation (Schwan and Wheals 2004, Afoakwa, Paterson et al.

2008, Kadow, Niemenak et al. 2015). Fermented cocoa then undergoes 7-8 days of drying, until the moisture content reaches 6-8%. Without sufficient drying, mold growth will take place (Afoakwa 2011). A common method of drying is sun drying with regular turning. Artificial drying, using convection dryers, heat exchangers, or conduction, can also be done with the temperature not exceeding 60°C (to avoid caking or crusting). During drying, some oxidation is expected, and excess acids are volatilized, which is considered a beneficial step (Schwan and Wheals 2004). Roasting is an important processing step due to the formation of the characteristic brown color, mild aroma and texture of roasted beans expected in chocolate (Krysiak 2006, Ioannone, Di Mattia et al. 2015). Roasting is conducted for 5 to 120 minutes at temperature ranges from 120°C to 150°C, depending on desired characteristics of the product. After cracking the roasted cocoa bean, winnowing process removes the shells. The remaining nibs are then ground to make fluid cocoa liquor. By cooling the fluid, a cocoa mass with dark, bitter and astringent character is formed. Cocoa mass is then pressed to separate cocoa cake and a pale yellow, fatty liquid cocoa butter. Cocoa powder is obtained by grinding the cocoa cake. Chocolate manufacturing processes vary depending on the required end products but generally include mixing sugar, sweeteners, milk products, emulsifiers and cocoa butters. After mixing, refining and conching are required to develop the desired viscosity, final texture and flavor of cocoa. Refining is a size reduction of cocoa solids and sugar crystals in chocolate and conching involves the agitation of chocolate at more than 50°C for a variable duration (15 min to 3 days), depending on desired product characteristics (Schwan and Wheals 2004, Afoakwa 2011).

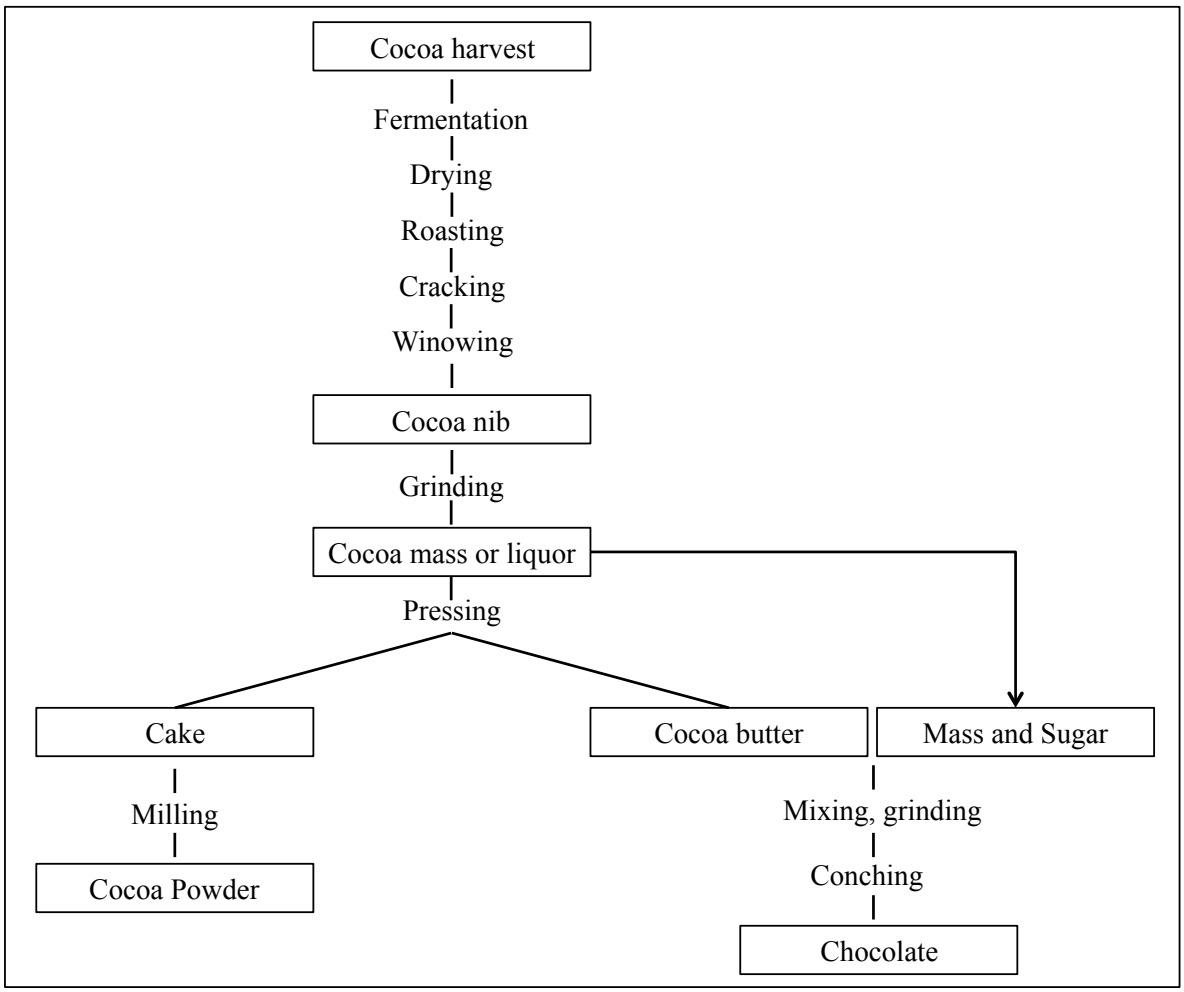

Figure 4. Summary of chocolate production process (Chocolate 2005).

The role of microorganisms in cocoa fermentation

Cocoa bean fermentation is generally conducted by naturally present environmental microorganisms on the farm where the pods are grown. Inoculation with starter cultures is not typical in cocoa fermentation. A comprehensive review listing yeast, acetic acid bacteria and lactic acid bacteria isolated from cocoa fermentations conducted in different countries is presented in Table 1. Microorganisms isolated from these cocoa fermentations include *Saccharomyces* spp., *Lactobacillus* spp., *Acetobacter* spp., and *Gluconobacter* spp. The microbiota involved in cocoa fermentation is complex, involving successive growth of yeast,

lactic acid bacteria and acetic acid bacteria. Spore-forming bacteria and filamentous fungi exist during fermentation at a relatively small number (around 10^3-10^4 CFU/g of pulp for spore forming bacteria and less than 10 CFU/g of pulp for filamentous fungi) however, their number increases in the later stage of fermentation and they are often found in over-fermented mass giving unpleasant flavor to the bean (De Vuyst, Lefeber et al. 2010, Ho, Zhao et al. 2014). Table 2 summarizes the most frequently found species of yeast, lactic acid and acetic acid bacteria observed over the course of cocoa fermentation (Carr, Davies et al. 1981, Passos, Silva et al. 1984, Schwan, Rose et al. 1995, Ardhana and Fleet 2003, Schwan and Wheals 2004, Jespersen, Nielsen et al. 2005, Ozturk and Young 2017). Before fermentation, pulp is typically around pH 3.6 due to the presence of citric acid. Due to the low pH and oxygen environment, yeast preferentially dominated the first stage of fermentation (0 to 12 hours) and increase up to 10^8 CFU/g at 12 hour-time point (Schwan and Wheals 2004). Yeasts undergo anaerobic fermentation and ethanol could be produced from 6 to 8% (v/v) (Schwan 1998, Schwan and Wheals 2004, Ho, Zhao et al. 2014). This fermentation process generates heat, increasing the bean temperature sufficiently for successful fermentation and flavor precursor formation. Pectinolytic yeasts are able to degrade pulp to liquid form, which drains away. Increased airflow by pulp drainage and turning the beans imparts a favorable environment for acetic acid bacterial growth. Acetic acid bacteria undergo exothermic reactions by which ethanol is converted to acetic and heap temperature increases to around 45-50°C (Ozturk and Young 2017). Lactic acid bacteria ferment pulp sugars and produce lactic acid. Lactic acid bacteria populations can reach 10^7 CFU/g of pulp to 10^8 CFU/g of pulp (Schwan and Wheals 2004). Although generally considered important in cocoa fermentation processes, lactic acid bacteria may not be necessary for development of good

quality cocoa(Zhao and Fleet 2015), and production of lactic acid could even result in excessively acidic cocoa.

Ethanol, acetic acid, and lactic acid penetrate the bean and decompartmentalize the storage cells to initiate endogenous enzyme reactions (Afoakwa 2011). Peptides, amino acids, and reducing sugar are formed by peptidase and invertase and undergo Maillard reaction during the roasting step. Various flavor compounds are generated by this reaction (Fowler 2009). Polyphenols are also degraded or condensed by endogenous polyphenol oxidase and non-enzymatic oxidation. The internal pH of unfermented cocoa bean is around 7.0 and should be reduced to pH 5.0 to 5.5 to ensure that endogenous protease can degrade the protein and produce the desired flavor precursors (Biehl, Brunner et al. 1985, Hansen, del Olmo et al. 1998, Ho, Zhao et al. 2014).

Yeasts including *Kloeckera apiculata* and *Saccharomyces cerevisiae* var. *chevalieri* produce desirable flavor compounds such as isopropyl acetate, ethyl acetate, 1-propanol, isoamyl alcohol, 2,3-butanediol, diethyl succinate, and phenylethanol (Rodriguez-Campos, Escalona-Buendía et al. 2011). Fig. 5 is a schematic of a microbial succession during cocoa bean fermentation.

Table. 1 Microorganisms isolated from cocoa fermentations conducted in diverse geographic regions.

	Brazil (Schwan, Rose et al. 1995) (Passos, Silva et al. 1984)	**Ghana (Jespersen, Nielsen et al. 2005) (Carr, Davies et al. 1981, Jespersen, Nielsen et al. 2005, Nielsen, Teniola et al. 2007)**	**Malaysia (Carr, Davies et al. 1981) (Meersman, Steensels et al. 2013)**	**Indonesia (Ardhana and Fleet 2003)**
Yeast, Fungi	*Candida bombi* *C. pelliculosa* *C.rugopelliculosa,* *C. rugosa* *Kloeckera apiculata* *Kluyveromyces marxianus* *Kluyveromyces thermotolerans* *Lodderomyces elongisporus* *Pichia fermentans* *Saccharomyces cerevisiae* var. *chevalieri* *S. cerevisiae* *Torulaspora pretoriensis*	*C. krusei.* *Hansenula* spp. *Kloeckera* spp. *P. membrenifaciens* *P. kluyveri* *S. cerevisiae* *Saccharomycopsis* spp. *Schizosaccharomyces* spp. *Torulopsis* spp. *Trichosporon asahii* *Hanseniaspora guilliermondii*	*C. asiatica* *C. fructus* *Debaryomyces* spp. *H. thailandica* *H. opuntiae* *Hansenula* spp. *Kloeckera* spp. *Rhodotorula* spp. *S. cerevisae* *Torulopspora globosa* *P. kudriavzevii*	*Penicillium citrinum* *Kloeckera apis* *S.cerevisiae* *C.tropicalis*
LAB[1)]	*Lactobacillus. acidophilus* *Lb. brevis, Lb. casei* *Lb. delbrueckii* *Lb. fermentum* *Lb. lactis, Lb. plantarum* *Lactococcus lactis,* *Leuconostoc mesenteroides* *Pediococcus acidilactici* *P. dextrinicus, P. cerevisiaae* *Streptococcus lactis*	*Lb. collinoides* *Lb. fermentum* *Lb. mali* *Lb. plantarum* *P. acidilactici* *Leu. pseudomesenteroides*	*Lb. collinoides* *Lb. plantarum* *Lb. fermentum*	*Lb. cellobiosus,* *Lb. plantarum*
AAB[2)]	*Acetobacter aceti subsp. liquefaciens* *A. pasteurianus* *A. peroxydans* *Gluconobacter oxydans subsp. suboxydans*	*A. ascendens* *A. rancens* *A. xylinum* *G.oxydans* *A. tropicalis*	*A. lovaniensis* *A. rancens* *A. xylinum A. pasteurianus G. oxydans*	*A. pasteurianus*

1) LAB: Lactic acid bacteria, 2) AAB: Acetic acid baceria

Microbial ecology study of cocoa fermentation

Due to the important role of microbial activity in the development of fermented bean quality, many studies have investigated microbial population dynamics during cocoa fermentation. Nielsen, Hønholt et al. (2005) used a PCR-denaturing gradient gel electrophoresis (DGGE) to investigate yeast populations in Ghanaian cocoa fermentations. Eukaryotic universal primers amplifying the 26S rRNA gene were used. By comparison with traditional culture dependent isolation, the method developed in this study was determined to be effective in amplification of most of the yeasts present, except *Trichosporon asahii.* Identified yeast species include *Hanseniaspora guilliermondii*, *Candida krusei*, *Pichia membranifaciens*, *Saccharomyces cerevisiae*, and *Candida zemplinina*.

Molecular tools offer increasingly effective means by which to follow the yeast and bacterial dynamics over the course of fermentation. Crafack, Mikkelsen et al. (2013) conducted a defined starter culture inoculation study to assess the influence of the yeast and bacteria on flavor characteristics. PCR-DGGE was performed on the fermenting pulps and *Hanseniaspora opuntiae* and *Hanseniaspora thailandica* were found to be the dominant species when the overall yeast population reached a peak at the 12-hour time point. Further strain level characterization with pulse field gel electrophoresis confirmed that up to 88% of *Picha kluyveri* and 100% of *Kluyveromyces marxianus* were identical to the inoculated starter culture strains used in that particular study. The quality difference observed between the starter inoculated fermentation and the control fermentation in the study by Crafack, Mikkelsen et al. is summarized in the next paragraph of this literature review (**Cocoa fermentation using starter cultures**). Recently, the development of next generation sequencing (NGS) has enabled microbial ecologists to characterize the microbial diversity of cocoa fermentation more precisely and deeply. To be

specific, the minor communities of microorganisms (below 10^3 CFU/ml) that could not be detected by previously available molecular methods such as PCR-DGGE (Denaturing Gradient Gel Electrophoresis) are detectable by NGS techniques. Moreover, the use of high-throughput sequencing on metagenomic DNA enabled researchers to overcome the limitation of PCR-DGGE which required amplification of relatively limited regions (16S or 26S rRNA gene), and ineffective for identification of certain species (ex. aforementioned *Trichosporon asahii*). Illeghems, De Vuyst et al. (2012) attempted to identify both dominant and rare yeast and bacterial communities by using 454 pyrosequencing of metagenomic DNA at the 30 hour-time point of cocoa fermentation conducted in Brazil. While Sanger sequencing uses dideoxynucleotides, which terminates the chain amplification reaction, 454 pyrosequencing detects the pyrophosphate released during incorporation of nucleotides. It has a fast reading time compared to other NGS systems but cost and high error rate is a disadvantage of this technique in its current form (Liu, Li et al. 2012). Dominant species identified were *Hanseniaspora uvarum, Hanseniaspora opuntiae, Saccharomyces cerevisiae, Lactobacillus fermentum,* and *Acetobacter pasteurianus*. In addition, viral communities including *Myoviridae* and *Siphoviridae* were also identified by this sequencing technique. Although further characterization is needed on how the microbiota revealed in this study may impact fermentation performance and bean quality, the major benefit of using NGS was obtaining information on a wider diversity of microbial population including bacteria, fungi, and bacteriophage. Metabolomics approaches could be coupled with microbial ecology studies to lead to a better understanding on how population dynamics affect flavor and quality of the fermentation. While in-depth information on fungal, bacterial, and viral communities at one time point during the fermentation process was obtained, the dynamics of these communities over the time course of fermentation was not

identified in this study. Future studies could employ barcode pyrosequencing, which attaches 4-8 nucleotides to PCR primers in multiple samples (in this case, different time points of cocoa bean fermentation) (Park, Chun et al. 2012). After sequencing multiple samples (in this case the bar-coded samples from each time point sampled) within a single run, each barcode can be sorted by post-analysis software thus giving sequence information by each time point of sample. Due to the high cost per run in NGS, this approach will be practical to use in the food fermentation field where information on microbial dynamics are needed.

Table. 2 Summary of most frequently found microorganisms observed during fermentation of cocoa

Microorganisms[1)]	**Species**
Yeasts (Initial stage)	*Hanseniaspora guilliermondii,* *Hanseniaspora opuntia*
Yeasts (Dominant)	*Saccharomyces cerevisiae* *Kluyveromyces marxianus* *Pichia membranifaciens* *Pichia kudriavzevii* *Candida spp.*
Lactic acid bacteria	*Lactobacillus plantarum* *Lactobacillus fermentum*
Acetic acid bacteria	*Acetobacter pasteurianus*

1) Microorganisms summarized in this table are found from the following literatures: (Carr, Davies et al. 1981, Passos, Silva et al. 1984, Schwan, Rose et al. 1995, Ardhana and Fleet 2003, Schwan and Wheals 2004, Jespersen, Nielsen et al. 2005, Nielsen, Teniola et al. 2007, De Vuyst, Lefeber et al. 2010, Lefeber, Gobert et al. 2011, Meersman, Steensels et al. 2013, Ho, Zhao et al. 2014, Ozturk and Young 2017)

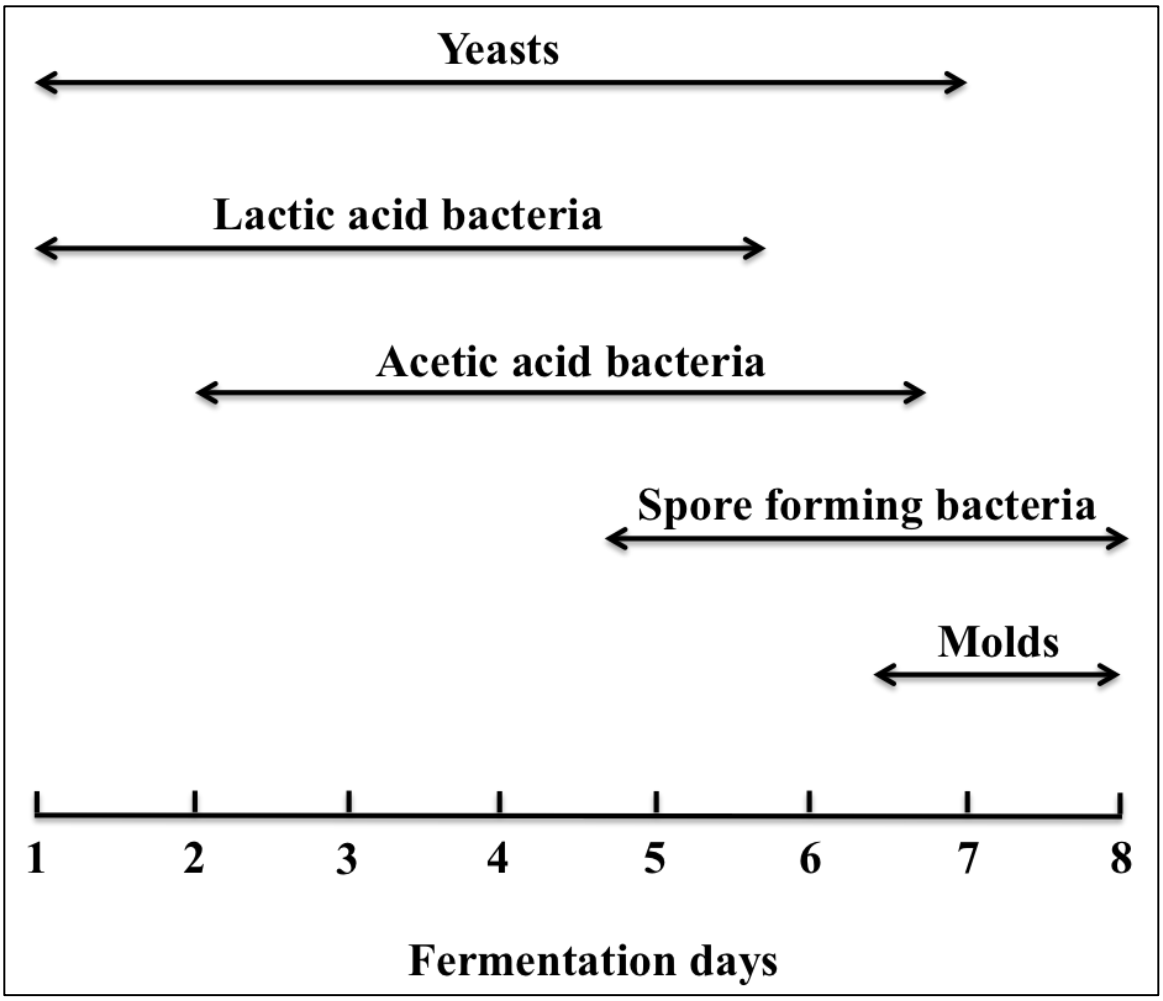

Figure 5. Typical microbial succession during cocoa fermentation (adapted from (Schwan and Wheals 2004)). It should be noted that the pattern of microbial succession varies between different fermentation sites.

Cocoa fermentation using starter cultures

Food and beverage fermentation using starter cultures has been widely applied in the wine, brewing and baking industries to obtain consistent product quality. However, cocoa fermentation is generally conducted in a relatively uncontrolled manner. The beans are usually fermented on or near farms immediately following harvest, with microbial populations derived directly from the surrounding environment. The development of effective starter cultures for cocoa fermentation is of interest due to the potential to reduce the variation in quality such as excess acid production, lack of flavor and production of off-flavors, all of which result in low crop value (Schwan and Wheals 2004). Utilization of a starter culture could promote more reliable fermentation outcomes, and inoculated fermentations may be completed relatively faster compared to spontaneous fermentation of cocoa. Some research has been conducted to this end in recent decades, although the practice is not widely adopted in commercial cocoa production. Schwan (1998) used a defined microbial cocktail to inoculate fresh unfermented cocoa. The microorganisms used were one pectinolytic yeast species, two lactic acid bacteria and two acetic acid bacterial species: *Saccharomyces cerevisiae* var. *chevalieri* is a pectinolytic and ethanol tolerant yeast capable of fermenting the glucose, fructose and sucrose present in the pulp at pH 3.5 to 4.0. *Lactobacillus lactis* and *L. plantarum* are lactic acid-producing bacteria also tolerant of acidic conditions (pH 3.5). *Acetobacter aceti* and *Gluconobacter oxydans* produce acetic acid from ethanol and are tolerant of temperatures up to 45°C. The results of this study were promising since the cut test and sensory analysis showed similar results between naturally fermented cocoa (without inoculation) and starter culture-fermented cocoa. The rate of fermentation was faster when the starter culture was used. However, a more in-depth chemical analysis evaluating any changes in metabolites and volatile compounds would be necessary to

support the practical relevance of this outcome. Moreover, it would be interesting to monitor any changes in polyphenol profile imparted by fermentation with starter cultures, since the starter culture may possess different characteristics than environmental microbiota such as enzyme activity or acid degradation, thus changing the pH and biochemistry of the fermentation matrix.

A recent study published by Magalhães da Veiga Moreira, de Figueiredo Vilela et al. (2017) inoculated *S. cerevisiae* UFLA CCMA 0200, *L. plantarum* CCMA 0238, and *A. pasteurianus* CCMA 0241 at the beginning of cocoa fermentation. The impact of these starter cultures on chocolate flavor was assessed. Cocoa fermentation inoculated with starter cultures were rich in bitter, sweet and cocoa tastes while un-inoculated cocoa dominated with only bitterness. Characterization of volatile compounds revealed that the fermented cocoa bean resulting from the inoculated fermentation contained increased concentrations of 2,3-butanediol and 2,3-dimethylpyrazine, which are desirable volatile compounds in cocoa. The influence of inoculation with yeast starter cultures, *Pichia Kluyveri* and *Kluyveromyces marxianus* on sensory attributes of un-conched chocolate made with the fermented cocoa beans were studied by Crafack, Mikkelsen et al. (2013). Inoculation with *P. kluyveri*/*Lb. fermentum*/*A. pasteurianus* resulted in a higher "general liking" descriptor compared to un-inoculated control. Also, the control had the lowest intensity of cocoa aroma and fruitiness, confirming that the starter culture inoculation had an impact on sensory characteristics. Taken together, the results of these studies suggest that inoculation of cocoa fermentation with starter cultures has the potential to lead to increase the value and production efficiency of fermented cocoa beans.

Chemical changes during cocoa fermentation

Microbial growth during cocoa fermentation results in the production of metabolites including ethanol and acetic acid that penetrate into the bean cotyledon causing bean death.

Changing conditions inside the bean, such as decreasing pH, increasing temperature and the structural damage of cell walls and membranes all favor endogenous enzyme activity including that of invertase, glycosidase, proteases and polyphenol oxidase. Activity of these enzymes change the chemical composition of the cocoa bean, result in the evolution of various chocolate flavor precursors including reducing sugars, peptides and amino acids (Biehl, Brunner et al. 1985, Kadow, Niemenak et al. 2015) and degrade pigment (typically anthocyanin). Some flavor precursors or flavor compounds such as esters and pyrazines can be directly produced by microorganisms and penetrate in to the bean cotyledons (Camu, De Winter et al. 2008). *Kloeckera apiculata* and *Saccharomyces cerevisiae* Var. *Chevalieri* produce various flavor compounds such as isopropyl acetate, ethyl acetate, 1-propanol, isoamyl alcohol, 2,3-butanediol, diethyl succinate and phenylethanol, all considered desirable contributors to good quality cocoa products (Schwan and Wheals 2004).

A fermentation-like incubation model was developed by Kadow, Niemenak et al. (2015) to assess the effects of acid, ethanol and heat on chemical changes of fresh raw cocoa bean. To eliminate the intervention of microbial effects, cocoa beans were pre-sterilized prior to incubation with the added chemical constituents. It was determined that this fermentation-like incubation could effectively develop free amino acids, reducing sugars, and decrease astringency. The authors claimed that the metabolites (acids and ethanol) were the exclusive key to development of desirable cocoa bean quality, rather than microbial metabolism, per se. While this is partially true, the relevance of volatile compounds produced directly by microorganisms such as higher alcohols, aldehydes, ketones, and carboxylic acids to cocoa quality was not considered in this study (Magalhães da Veiga Moreira, de Figueiredo Vilela et al. 2017). However, the development of a fermentation-like incubation model is valuable. This model could

allow investigation of the relationship between the concentration of particular microbial metabolites and the chemical changes moderated by endogenous enzymes in cocoa beans during fermentation.

Polyphenols in cocoa

Polyphenols are a class of bioactive compounds present in plant foods. More than 8,000 different phenolic structures have been identified in nature. In terms of chemical structure, polyphenols are defined as having more than one phenolic unit. Composition and concentration of polyphenols in a given plant tissue are dependent on the plant genetic factors and environmental conditions (Kris-Etherton, Hecker et al. 2002, Martins, Mussatto et al. 2011). Polyphenols are present in plants as secondary metabolites. Secondary metabolites, different from primary metabolites required for survival and growth, serve no essential function in growth, but instead facilitate a plant's resistance to environmental stressors, thus increasing the chance of its survival (Azmir, Zaidul et al. 2013). Plant phenols are synthesized as carbohydrates go through the shikimate pathway and acetate pathway (Bravo 1998). The shikimate pathway, present in certain microorganisms and plants, produces aromatic amino acids (Robards 2003), which serve as precursors to polyphenol catabolism. Polyphenol structure impacts bioactivity. Depending on their structure, polyphenols can be subdivided into various classes (Wollgast and Anklam 2000) (Figure 6). Among those, flavonoids are generally considered to be one of the important groups with regards to bioactive compounds.

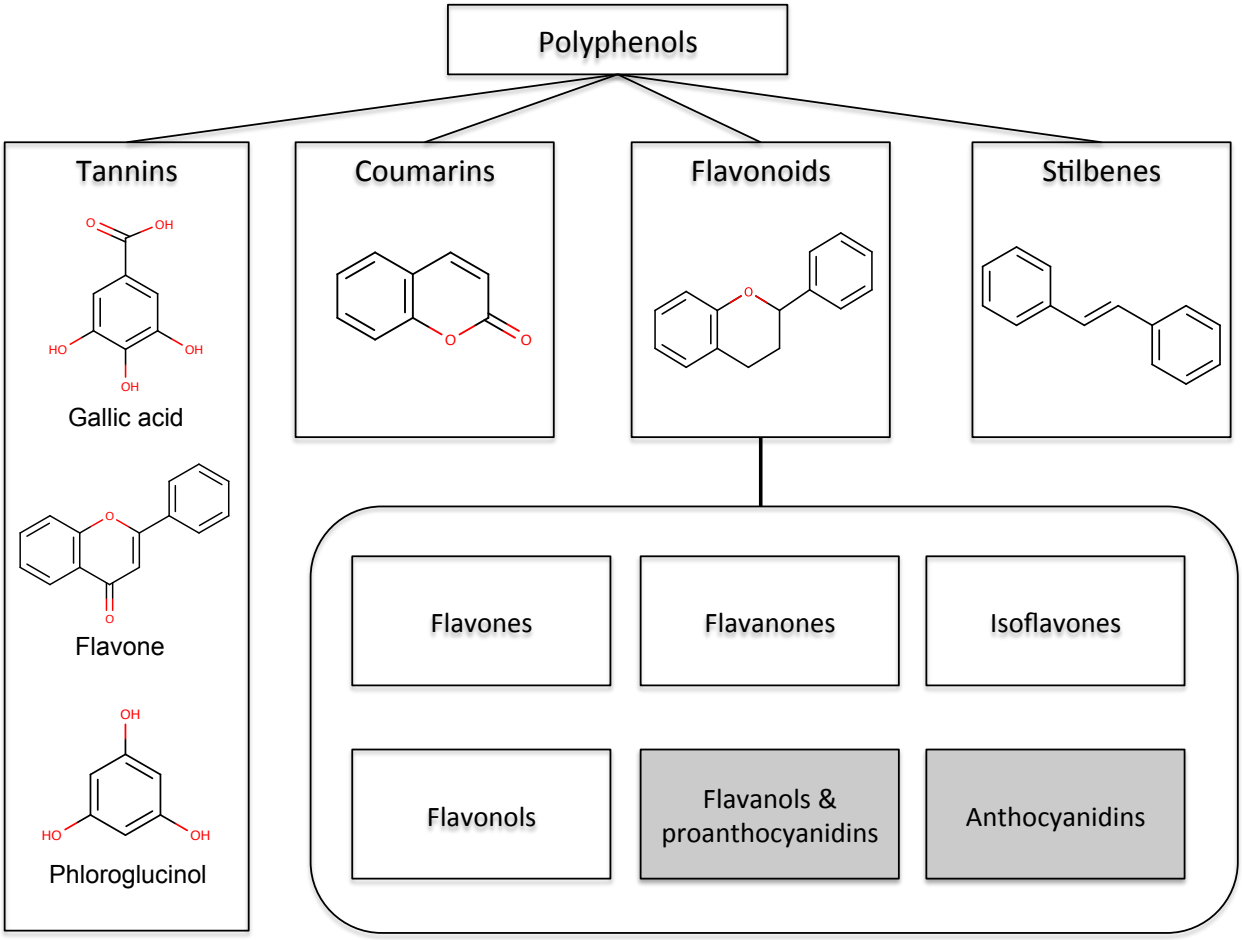

Figure 6. Classification of polyphenols with basic structural units of 4 subclasses of polyphenols. Compounds in grey colored boxes are major polyphenols found in cocoa.

Flavonoids

Flavonoids are a class of polyphenols that have a general chemical structure including a C6-C3-C6 carbon frame (Figure 7). This C15 skeleton is joined to a benzopyran moiety with an aromatic ring at the C-2, C-3 or C-4 position (Grotewold 2006, Aron and Kennedy 2008). Based on the degree of hydroxylation and oxidation of the aromatic rings, flavonoids can be subdivided into 13 classes (Steinberg, Bearden et al. 2003). Main groups of flavonoids (Figure 8) include flavonols, flavanones, flavanols (same as flavan-3-ols), flavones, isoflavones, and anthocyanidins (Nijveldt, van Nood et al. 2001).

Flavonols are extensively found in plants and plant-based foods and the representative flavonols include quercetin, myricetin, kaempferol. Foods rich in flavonols include onion, cherries, apples, broccoli, kale, berries, red wine, and tea (Yao, Jiang et al. 2004). Flavanones are found mainly in citrus fruits and often exist with flavones. Cumin, oranges, grapefruits, and peppermints are rich source of flavanones. Representative flavanones include hesperidin, naringenin, eriodictyol, and neohesperidin. Flavones includes apigenin, chrysin, luteolin are often found in herbs, cereals, fruits, flowers, and parsley. Soybeans, chickpeas, pistachios, fruits, and nuts are rich in isoflavones. Daidzein, genistein, glycitein, formononetin fall into this group. Anthocyanins are derived from glycosidation of anthocyanidins and are water-soluble pigments giving various colors to plant materials (Clifford 2000, Wollgast and Anklam 2000). Major anthocyanins are cyanidin delphinidin, malvidin and are found in berries, grapes, cherries, and red cabbages (Yao, Jiang et al. 2004). Chun, Chung et al. (2007) estimated the daily consumption of dietary flavonoids (189.7 mg/day) by US adults and reported that flavanols were the main form (83.5%) of flavonoid in the US diet. Flavanols are also the primary component of cocoa polyphenols, and will be reviewed in further detail in the following section.

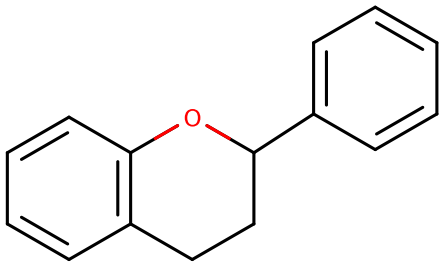

Figure 7. Basic flavan ring structure

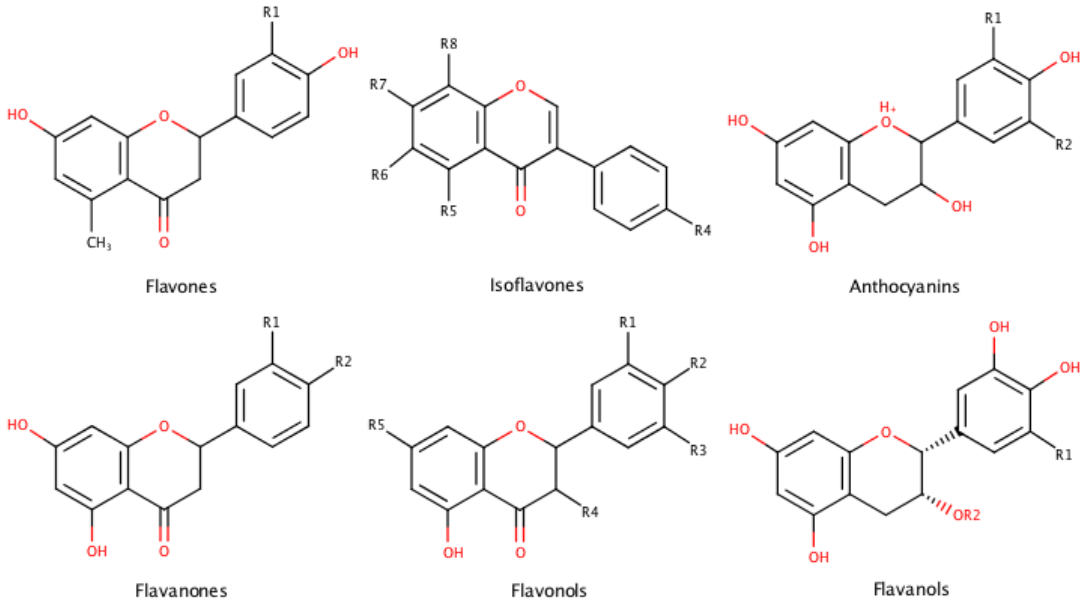

Figure 8. Structure of representative flavonoids

Flavan-3-ols (flavanols)

Flavan-3-ols are one of the major subclasses of flavonoids. They are functional compounds existing in fruits, vegetables and plant materials (Aron and Kennedy 2008). Flavan-3-ols contain monomeric catechins and procyanidins that are polymeric compounds consists of monomeric catechins (Bitzer, Glisan et al. 2015). Structures of representative flavanols are depicted in Figure 9.

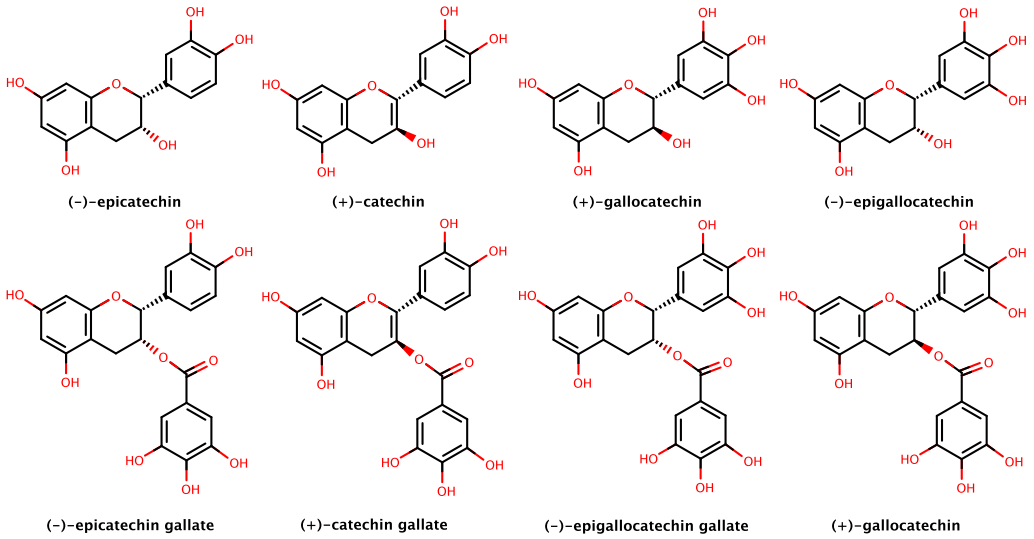

Figure 9. Structures of representative flavan-3-ols (flavanols).

Cocoa polyphenols

Cocoa contains the highest polyphenol concentration of any commonly consumed food item, even greater than wine and tea (Latif 2013). Polyphenols make up 12-18% of the dry weight of cocoa beans (Gu, Hurst et al. 2011). The main polyphenols in cocoa (by weight) are catechins (37%), procyanidins (58%) and anthocyanins (4%) (Nazaruddin, Seng et al. 2006). In the catechin group, the major constituent is (−)-epicatechin, representing up to 35% of total polyphenol content. The (−)-epicatechin concentration ranged from 34.65 to 43.27 mg/g in defatted fresh cocoa beans of Catongo and Forastero varieties (Kim and Keeney 1984). The compounds (+)-catechin, (+)-gallocatechin and (−)-epigallocatechin have been found in smaller concentrations in the cocoa bean. Cyanidin-3-α-L-arabinoside and cyanidin-3-β-D-galactoside are the primary constituents of the anthocyanin group. Procyanidins with varying of degrees of polymerization exist in cocoa (Neilson and Ferruzzi 2011). Procyandins are mainly composed of monomeric catechin and epicatechin units linked by C4β→C8 and/or C4β→C6 bond, and are

categorized as B-type procyanidins (Oracz, Zyzelewicz et al. 2015). A-type procyanidins have an additional ether bond between C2→O7 (Miller, Stuart et al. 2006). Procyanidin C1 and C2 are both B-type procyanidins but the former is an epicatechin trimer while the latter is a catechin trimer. The most abundant procyanidins in cocoa are dimers B2 and B5, trimer C1 and cinnamtannin A2 with epicatechin as the main constituents in these procyanidins (Cooper, Campos-Gimenez et al. 2008). Other cocoa procyanidins have a varying degree of polymerization generally around 2-10 or higher (Hammerstone, Lazarus et al. 2000).

Variation in cocoa polyphenol composition and concentration is attributed to genetic variety, region and agricultural practices including utilization of soil fertilizer and pod storage (Tomas-Barberan, Cienfuegos-Jovellanos et al. 2007, Elwers, Zambrano et al. 2009, Oracz, Zyzelewicz et al. 2015). Genetics and variety of the beans (Forastero, Criollo, and Trinitario) are likely the greatest factor determining the polyphenol profile of cocoa beans (Niemenak, Rohsius et al. 2006, Othman, Jalil et al. 2010). Different varieties of cocoa beans can have up to 4-fold difference in concentration of an individual polyphenol compound as well as significant differences in total polyphenol concentration. For example, Criollo cocoa beans contain two-thirds of the total polyphenol concentration observed in Forastero beans (Nazaruddin, Seng et al. 2006). Anthocyanins are absent or present in very low concentrations in Criollo beans whereas they are much more concentrated in the Forastero variety (Oracz, Zyzelewicz et al. 2015). Geographic growing region can influence catechin and epicatechin concentration as well. One study showed that cocoa beans originating from Ecuador had the highest concentration of catechin and epicatechin, while beans from Ghana and Trinidad had a lower concentration (Paoletti, Poli et al. 2012). Another study by Caligiani, Acquotti et al. (2010) also confirmed that a Forastero variety originated from Ecuador contained a higher concentration of (−)-epicatechin

and derivatives of caffeic acid when compared to Ghanaian Forastero cocoa beans. A comparative study of polyphenol concentration between cocoa beans grown in different regions of Colombia was conducted by (Carrillo, Londoño-Londoño et al. 2014). They suggested that the altitude at which cacao is grown could significantly impact the concentration of flavanols including (−)-epicatechin, where cacao grown in lower altitude contained a higher concentration.

Cocoa polyphenols and human health

The presence of polyphenols in cocoa and the recent discovery of their positive effects on human health has begun to change the common perception of chocolate as a "sin rather than a remedy" (Latif 2013). There is abundant evidence that the moderate consumption of cocoa and dark chocolate may promote human health. Compared to milk chocolate, dark chocolate has considerably higher concentrations of flavonoids (Vinson, Proch et al. 1999). Previously, it was believed that milk chocolate possessed decreased biological activity due to the inhibitory effect of milk on intestinal epicatechin absorption (Serafini, Bugianesi et al. 2003). While this study focused on confections, other researchers conducted related experiments with cocoa beverages and found no significant difference in cocoa flavanol bioavailability between beverages containing milk and controls (Schroeter et al. 2003, Roura et al. 2007). When absorption of epicatechin from beverages and confections were compared, there was only a minor decrease in epicatechin absorption from confections, not being significantly different from beverages (Neilson et al. 2009). In summary, the presence of milk may not reduce intestinal epicatechin absorption, as was previously believed.

Epidemiological studies revealed that elderly men, post-menopausal women and survivors of a first heart attack who consumed chocolate had lower risk of cardiovascular disease (Zomer, Owen et al. 2012), heart attack, or death compared to non-consumers. *In vitro* studies

showed that cocoa possesses high antioxidant activity (Vinson, Proch et al. 2006), anti-inflammatory and anti-atherosclerotic activity (Vinson, Proch et al. 2006), lowering blood pressure (Buijsse, Feskens et al. 2006), immune function modulation and platelet activation (Pearson, Paglieroni et al. 2002). The potential for cocoa flavanols to prevent cardiovascular disease (CVD) has been the subject of tremendous research interest. Hooper, Kay et al. (2012) conducted a systematic review to look at the effects of chocolate or cocoa on CVD. By searching randomized controlled trials (RCT) through available databases (a total of 42 acute or short-term chronic RCT were included in this review), it was concluded that dietary interventions of chocolate or cocoa provide both acute and chronic improvement of flow-mediated dilatation (FMD) and reduction in fasting serum insulin concentrations. Diastolic blood pressure was also significantly reduced after chronic intake of chocolate or cocoa.

Metabolic syndrome increases the risk of development of CVD and Type 2 diabetes mellitus (T2DM) (Eckel, Grundy et al. 2005). Metabolic syndrome is caused mainly by excess central adiposity (Cameron, Boyko et al. 2008). Glucose intolerance (and/or insulin resistance), elevated blood pressure, dyslipidemia, pro-inflammatory state, and pro-thrombotic state are 6 major symptoms of metabolic syndrome (Grundy, Hansen et al. 2004). There is emerging evidence that bioactive cocoa flavanols can prevent or alleviate metabolic syndrome. A systematic review of current literature by Strat, Rowley et al. (2016) suggests potential primary mechanisms of action by which cocoa flavanols prevent the onset of metabolic syndrome. Based on their review of both animal and human studies, in many cases cocoa or chocolate products were found to improve glucose control and insulin resistance (Brand-Miller, Holt et al. 2003, Grassi, Lippi et al. 2005, Yamashita, Okabe et al. 2013, Dorenkott, Griffin et al. 2014). However,

they also pointed out that dosing methods were inconsistent between different animal studies and long-term clinical trials on prediabetes were not evaluated yet (Strat, Rowley et al. 2016).

There is increasing evidence that structural differences in cocoa flavanols impart differences in biological activity, particularly due to the degree of polymerization (Dorenkott, Griffin et al. 2014). In cell and animal model studies, the degree of polymerization determined the efficacy of procyanidins in alleviation of inflammation and cancer (Mao, Powell et al. 2000, Gosse, Guyot et al. 2005). Although past studies have focused mainly on the effects of cocoa flavanol monomers due to their higher bioavailability compared to oligomers and polymers as well as technical difficulties in analyzing higher polymers, recent studies revealed that despite their limited bioavailability, dietary oligomeric cocoa procyanidins also prevent obesity and insulin resistance (Yamashita, Okabe et al. 2013, Dorenkott, Griffin et al. 2014). Another study testing the impact of cocoa procyanidin degree of polymerization on alleviating colonic inflammation discovered that high molecular weight polymeric procyanidins possessed the highest anti-inflammatory activities (Bitzer, Glisan et al. 2015). Although larger polymers of procyanidins have very low to no bioavailability, they may still be able to exert bioactivity via delivery to the intestinal lumen. At the intestinal epithelial surface and also in the lumen, polymeric procyanidins may interact with gut microbiota to improve gut barrier function or inhibit digestive enzymes (Strat, Rowley et al. 2016). Therefore, bioavailability may not be a prerequisite for bioactivity.

Recent findings regarding the relationship between degree of polymerization of cocoa flavanols and bioactivity suggest that fermentation or other processing studies should not just focus on total polyphenol content or monomer contents but also monitor the overall degree of

polymerization to assess the potential impact of cocoa fermentation and processing on human health (Neilson et al 2015).

Impact of fermentation on cocoa polyphenols

Despite the high concentration of polyphenols in cocoa, processing unit operations for cocoa including fermentation, roasting, conching and other steps significantly affects the composition and concentration of polyphenols. As a result, cocoa powder and chocolate substantially differ in polyphenol concentration and composition as compared to raw cocoa beans (Tomas-Barberan, Cienfuegos-Jovellanos et al. 2007). During fermentation, cocoa polyphenols diffuse out from the storage cells and undergo oxidation and condensation into insoluble tannins (condensed tannins formed by polymerization of flavans). Anthocyanins are hydrolyzed by glycosidases to form anthocyanidins and sugar, imparting a purple to white color change in cotyledons. Anthocyanin content is often considered as a marker for quality of fermented cocoa beans. For commercial assessment, lower anthocyanin content is regarded as "well fermented" or "good quality cocoa" (Camu, De Winter et al. 2008). Degradation of polyphenols is caused by either enzymatic (polyphenol oxidase) or non-enzymatic oxidation during the manufacturing processes resulting in the formation of large, insoluble tannins (Wollgast and Anklam 2000). During fermentation, (-)-epicatechin diffuses from the storage cell and oxidation and polymerization occur to form tannins. (-)-epicatechin is also subjected to oxidation during the drying stage, resulting in formation of melanin and melanoproteins which give the characteristic brown color of fermented, processed cocoa beans. Fermentation of cocoa beans resulted in a decrease of average (-)-epicatechin concentration from 21.9-43.3 mg/g to 2.2-10.0 mg/g in dry defatted samples (Kim and Keeney 1984). Evina, De Taeye et al. (2016) used a fermentation-like incubation model developed by (Kadow, Niemenak et al. 2015) to study the

impact of acetic and lactic acid on flavanol degradation. It was found that acetic acid was the key to reduce the concentration of flavanol monomers and oligomers (higher polymers were not analyzed in this study). Lactic acid however, showed a somewhat protective effect on flavanol degradation since co-incubation of acetic acid and lactic acid had a lower decrease in (+)-catechin and (-)-epicatechin concentration. Since microbial metabolites including ethanol and acids have an effect on flavanol composition, it could be hypothesized that conditions affecting microbial growth such as temperature, aeration, or starter culture inoculation could impact the concentration and composition of polyphenols.

Conclusion

Cocoa polyphenols undergo significant changes in concentration and composition during fermentation and processing. Recently, some of the chemical compounds and enzymes responsible for the mechanisms underlying these changes have been characterized. However, a gap in knowledge remains regarding the extent to which the microbiota present during fermentation could impact the concentration and composition of polyphenols. Also, while it has been shown that the concentration of total polyphenol and flavanol monomers in cocoa beans differ among cacao varieties and environmental conditions during cultivation, no detailed information is available as to the extent to which these factors may influence polyphenol mDP and concentration of procyanidins having higher degree of polymerization (oligomer to decamer). Understanding the impact of processing conditions on concentration and structure of polyphenols will lead us to better design cocoa processing steps to achieve quality cocoa in regards to its targeted biological activity.

The purpose of the work reported in this dissertation was to understand the extent to which cocoa cultivar selection and fermentation process affect the concentration and composition

of polyphenols. The first step was to assess the difference in polyphenol concentration and composition among cocoa genetic clones developed through controlled-pollinated hybridization for improved agronomic performance, specifically high yield (Goenaga, Irizarry et al. 2009). This is intended as a first step to provide insight into the extent to which plant genetics and agronomic factors influence polyphenol composition in raw cocoa beans and further, their chocolate products. The next step was to develop a laboratory cocoa fermentation model system to allow us to conduct cocoa fermentation experiments in a laboratory setting. Then, using this model, the impact of yeast starter culture on polyphenol concentration and composition was evaluated. The work reported in this dissertation fills a prior gap in knowledge of the extent to which cacao genetics and the cocoa fermentation process affect polyphenol concentration and composition. Understanding how production and processing of cocoa affects polyphenol concentration and composition will lead to a better understanding of how agricultural and food processing factors can be targeted to enhance the bioactivity of dietary cocoa polyphenols.

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Chapter 3:

Comparison of polyphenol concentration and composition between genetically diverse cocoa (*Theobroma cacao L.*) accessions selected for high yield

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Abstract

There is abundant evidence that moderate consumption of cocoa or dark chocolate is associated with positive health outcomes, and that polyphenols likely play a role in this association. Variation in cocoa polyphenol composition and concentration can result from differences in genetic background, agro-environment and cultural practices and can translate to differences in bioactivity. It is therefore important to consider several factors when selecting cocoa seed (beans) for research investigating the mechanisms underlying the health benefits of this popular component of the human diet. In this study, dried unfermented seeds of five different cocoa clones (TARS-9, TARS-14, TARS-27, TARS-30, TARS-31) were obtained from USDA-ARS Tropical Agriculture Research Station (TARS) located in Mayaguez, Puerto Rico. These clones were developed through controlled-pollinated hybridization for high yield. Prior sensory evaluation of 65% chocolate made from these clones revealed that each conferred distinct profiles in bitterness. We thus hypothesized that differences in polyphenol profiles would exist between these clones. To test this hypothesis, total polyphenol, total procyanidin, polyphenol extraction yield, and qualitative flavanol profile were compared between the five cocoa clones. TARS-27 had the lowest total polyphenol concentration (9.75 ± 0.18 mg gallic acid equivalents/g cocoa bean), whereas TARS-9 showed highest concentration (16.5 ± 0.74 mg gallic acid equivalents/g cocoa bean). For total procyanidin, TARS-27 showed the lowest concentration (42.3 ± 1.08 mg procyanidin B2 equivalents/g cocoa bean) while other clones did not show significant differences. Polyphenol extraction yield (cocoa extract weight/cocoa bean weight) differed among clones ranging from 0.1-0.16, contributing to the difference in total polyphenol and procyanidin concentration.

Qualitative flavanol profiles obtained using normal-phase high-performance liquid chromatography (HPLC) indicated that mean degree of polymerization (mDP) of polyphenols is likely similar between these clones. This trend was further confirmed by calculating the mDP value using thiolysis method. In conclusion, these insights into the variability of polyphenol composition among genetically diverse cocoa will help to inform targeted selection of cocoa clones for future studies investigating the impact of cocoa consumption on human health.

Introduction

Associations of positive health outcomes with chocolate consumption have motivated recent research into the mechanisms underlying the relationship between cocoa polyphenols and human health. Cocoa consumption has been associated with health-promoting effects including but not limited to: reduced onset of cardiovascular disease (1-3), inflammation (4, 5), diabetes and obesity (6, 7), and platelet disorder (8, 9).

Polyphenols are the bioactive compounds in cocoa believed to exert these beneficial effects (10). Sensory characteristics of cocoa, especially bitterness and astringency, are also highly influenced by polyphenols. Cocoa is one of the richest available dietary sources of polyphenols. It contains even greater concentrations of polyphenols than wine or tea, beverages often associated with similar positive health outcomes (11). The polyphenol in cocoa bean contribute to 12-18% dry weight (12) of the whole bean and flavanols are the largest subgroup possessing high bioactivities (10). Flavanols are composed of monomeric (−)-epicatechin, (+)-catechin, (−)-catechin, (+)-gallocatechin, (−)-epigallocatechin and procyanidin (oligomer and polymers linked by mainly epicatechin and/or catechin units) (13, 14). The chemical structures of main flavanols are depicted in Figure 1.

In addition to the total concentration of polyphenols, there is also increasing evidence that degree of polymerization impacts biological activity of cocoa flavanols (6). Recent studies revealed that oligomers or more highly polymerized procyanidins confer protective effects against obesity, insulin resistance, and colonic inflammation (4, 6, 15).

The concentration and composition of polyphenols in cocoa beans is expected to vary depending on the genetic variety, growing region, usage of soil fertilizer, and pod storage time (13, 16), which could highly affect the polyphenol profile of various cocoa products. Among

these factors, genetics and variety of the beans (Forastero, Criollo, and Trinitario) is the greatest contributor to differences in polyphenol concentration (13, 17, 18). A. Cambrai, et al. (19) discriminated the varietal origin of dark chocolates based on the polyphenol profile. Compounds including (−)-epicatechin, (+)-catechin, and oligomeric procyanidins (DP2-DP4) were analyzed and principal component analysis (PCA) was performed based on the concentration of these compounds and total polyphenol contents. As a result dark chocolates from different geographic origins were separated and grouped by their varietal origin (Forastero, Criollo, and Trinitario). However, there have been no reports of comparison of overall mDP among cocoa beans having different varietal origin.

Improved understanding of the relationship between cocoa polyphenol structure and bioactivity could allow the development of cocoa products with polyphenol composition designed for targeted health promoting functions. Recently, unfermented cocoa beans have become commercially available as readily ingestible raw cocoa nibs or powders. Unfermented cocoa beans from different plant germplasms are expected to exhibit differences in polyphenol concentration and composition (13, 17).

Characterization of polyphenols in raw unfermented cocoa beans is the first step in understanding the extent to which plant genetics and agronomic factors influence polyphenol composition.

For this study, we assessed the extent to which polyphenol concentration and composition differed among cocoa genetic clones developed through controlled-pollinated hybridization for improved agronomic performance, specifically high yield. Based on sensory evaluation of 65% cocoa containing chocolate made from these clones, each possessed a different chocolate flavor profile including bitterness and astringency (20). As sensory

differences were observed, we hypothesized that the raw cocoa beans would also exhibit different polyphenol profiles. The objective of this study was to compare the polyphenol profiles including total polyphenol and procyanidin concentration, mean degree of polymerization, and detailed individual flavanol concentrations among five genetic clones of cocoa.

Materials and methods

Theobroma cacao clones

Dried unfermented cocoa beans for each clone (TARS-9, TARS-14, TARS-27, TARS-30, and TARS-31) were obtained from USDA-ARS Cocoa Germplasm Repository, Tropical Agriculture Research Station (TARS) located in Mayagüez, Puerto Rico. This repository consists of over 200 accessions, planted to three randomized blocks with six trees per clonal accession. All the germplasm held in the collection at USDA-ARS TARS were grafted into seedling rootstock Amelonado, which is a lower Amazon Forastero type of cocoa commonly grown in Brazil and West Africa. Trees were grown irrigated three times a week for three hours during the dry season (December – March), and managed for height by annual mechanical pruning to a height of approximately 2 m. Distance between rows of trees is two meters with two meters separating trees within a row. Cocoa pods (10 pods for each clone) were harvested from up to six trees per clone. Pods were harvested when ripe, as determined by the onset of color change. For each genetic clone, 300~400 beans were received and 40 g of beans from each clone were used for cocoa flavanol extraction. Environmental variability among clones including climatic condition, planting area, soil, and use of fertilizer were minimum since they were grown under identical period and culture condition. Once cocoa pods were harvested, they were immediately washed and dried and shipped to the laboratory. Cocoa beans received were stored at −80°C until the time of analysis.

Cocoa flavanol extraction

Flavanol-rich cocoa extract (CE) was obtained using the method developed by M. R. Dorenkott, et al. (6), with minor modifications. Cocoa beans were frozen using liquid nitrogen and then ground in a Waring laboratory blender (Waring Products, New Hartford, USA) for 2 min to obtain a powder. To de-fat the cocoa powder, ground powder (40 g) was extracted with 150 mL of hexane and sonicated for 10 min. The mixture was centrifuged at 5000 × *g* for 5 min and the supernatant was discarded. After repeating the de-fatting step, the residual hexane was evaporated. Extraction solution (150 mL) and dried defatted cocoa powder were mixed and sonicated (10 min). Flavanol extraction solution consisted of acetone, ultrapure water (EMD Millipore, Darmstadt, Germany), and glacial acetic acid with 70:28:2 ratio (v/v/v). Supernatant was collected after centrifuging the mixture at 5000 × *g* for 5 min. This extraction procedure was repeated until colorless supernatant was obtained (3 or 4 times) and acetone was evaporated by using rotary evaporator RV 10 basic (IKA®, Wilmington, NC). The remaining extract was frozen (−80 °C) and freeze-dried. Freeze dried CE was then crushed into powder form and stored at −80 °C until further analysis. Extraction yield for each clone was calculated by dividing the weight of finial CE by the initial weight of the ground cocoa powder prior to defatting step (w/w).

Quantification of total polyphenols and procyanidins

Total polyphenol content (antioxidant/reducing activity) was quantified by the Folin-Ciocalteu method (21). CE was diluted with aqueous ethanol (40%) to the concentration of 0.2 mg/mL. Fifty μL of diluent was combined with 450 μL or ultrapure water, 1.25 mL 0.2 N of Folin-Ciocalteau reagent (Sigma, USA), and 7.5% saturated sodium carbonate (Na_2CO_3) and vortex mixed. Absorbance at 765 nm was measured after 2 hours of reaction (25 °C) (

GENESYS^{TM} 10S UV-Vis Spectrophotometer, Thermo Scientific, USA). Total polyphenol content was expressed as mg gallic acid equivalents/g cocoa bean ± SD.

Total procyanidin content was measured using the 4-dimethylaminocinnemaldehyde (DMAC) method, as described by M. J. Payne, et al. (22). HCl (3 mL) and ethanol (27 mL) were combined with DMAC (0.03 g) to make a DMAC solution. CE was diluted with ethanol to 100 ppm. EtOH blank (50 µL) and CE diluent (50 µL) were mixed with DMAC solution (250 µL) in a 96-well plate and the absorbance was read at 640 nm. Total procyanidin content was expressed as mg procyanidin B2 equivalents/g cocoa bean ± SD.

Qualitative measurement of the degree of polymerization

Normal-phase HPLC was performed to qualitatively compare the profiles of cocoa procyanidin DPs between different genetic clones (6). CE were diluted with acetone: water: acetic acid (70:28:2, v/v/v) to the concentration of 10 mg/mL immediately prior to analysis and held at 5 °C in an auto-sampler. Agilent Technologies (Santa Clara, CA) 1260 Infinity HPLC equipped with a fluorescence detector (FLD, λ_{ex} = 230 nm, λ_{em} = 321 nm) was used to analyze samples. A Develosil Diol Column (100Å, 250 x 4.6 mm, 5 µm particle size) (Phenomenex, Torrance, CA) was used to carry out separations at 35°C. Binary gradient elution was performed with 2 % acetic acid (v/v) in ACN (phase A) and 2 % acetic acid (v/v) and 3 % ddH2O (v/v) in MeOH (phase B) at a flow rate of 1 mL/min. The gradient was applied as follows: 93% A at 0 min, 93% A at 3 min, 62.4% A at 60 min, 0.0% A at 63 min, 0.0% A at 70 min, 93.0% A at 76 min, 7.0% B at 0 min, 7.0% B at 3 min, 37.6% B at 60 min, 100.0% B at 63 min, 100.0% B at 70 min, and 7.0% B at 76 min. Injection volume was 5 µL and analytical duplicates were run for each genetic clone.

Thiolysis

Thiolysis was performed to estimate mean degree of polymerization (mDP) of genetic clones according to the methods of M. R. Dorenkott, et al. (6), S. Guyot, et al. (23) with modifications. Cocoa exracts were diluted with methanol (0.5 mg/mL) and 50 µL of the diluted solution, 50 µL of HCl solution (3.30 % HCl in methanol), and 100 µL of benzyl mercaptan solution (5 % benzyl mercaptan in methanol) were mixed. Unthiolyzed controls were prepared by adding 150 µL of methanol instead of HCl and benzyl mercaptan solution. Both sample solutions and controls were incubated (90 °C) for 5 min and transferred into ice for 10 min to stop the reaction. For LC/MS analysis samples and controls were prepared by mixing 100 µL of reaction product with 900 µl of 0.1 % formic acid in water (phase A)/ 0.1 % formic acid in ACN (phase B) solution (95:5 A:B).

UPLC-MS/MS analysis of Thiolysis products

Thiolysis reaction products (released flavanol and benzylthioether derivatives) were immediately quantified by UPLC-MS/MS. A Waters Acquity H-class separation module equipped with Acquity UPLC HSS T3 Column (2.1 mm × 100 mm, 1.8 µm particle size, 40 °C) was used to perform the analysis. Binary gradient elution was performed with 0.1% (v/v) formic acid in water (phase A) and 0.1% formic acid in ACN (phase B). Solvent flow rate was 0.6 mL/min and the linear gradient elution was carried out as follows: 95% A (0-0.5 min), 65% A (6.5 min), 20% A (7.5-8.6 min), 95% A (8.7–10.5 min). (−)-Electrospray ionization (ESI) coupled to tandem mass spectrometry (MS/MS) on a Waters Acquity triple quadrupole (TQD) MS was used to analyze the UPLC effluent. Ionization settings were as follows: (−) mode, capillary voltage of −4.25 kV, cone voltage of 30.0 V, extractor voltage of 3.0 V, source temperature of 150 °C and desolvation temperature of 400 °C. N_2 was used for cone and desolvation gas with flow rates of 75 and 900 L/h respectively. For MS/MS, Ar was used as a collision gas with 0.25 ml/min flow

rate. Parent ions and signature daughter ions followed by collision-induced dissociation (CID) were subjected to multi-reaction monitoring (MRM) with a mass span of 0.2 Da and 1.0 sec of inter-channel delays and inter-scan times. MRM settings for each compound are listed in Table 1. MassLynx software (version 5.1, Waters) was used to acquire the data. mDP of total flavanols were calculated based on the following equations, where total monomers and net thiolytic derivatives were quantified by UPLC. Due to the unavailability of flavanol benzylthioether standard, thiolysis products were quantified using monomer standards.

$$Calculated\ mDP\ (total\ flavanols) = \frac{total\ monomers + net\ thiolytic\ derivatives}{total\ monomers}$$

Statistical Analysis

Data were expressed as mean ± SD for n=3 analytical replicate sets of five cocoa clones for all analyses. Data for total polyphenol, total flavanol and mDP were analyzed by one-way ANOVA followed by Tukey's HSD posthoc test for all possible comparisons. Significance was defined as $p < 0.05$. All statistical analyses were performed using Prism v6.0e (GraphPad Software, Inc., La Jolla, CA).

Results and discussion

Total polyphenols and procyanidins

Among the five genetic clones, TARS-27 had the lowest polyphenol concentration per gram bean (dry weight basis) (9.75 ± 0.18 mg/g bean) (Figure 2A). When polyphenol concentration per gram CE was considered, TARS-27 also possessed the lowest polyphenol concentration (97.6 ± 1.74 mg/g CE), but TARS-14 had the highest concentration of 116.8 ± 0.0 mg/g CE (Figure 2B). TARS-27 also had the lowest total procyanidin concentration per gram cocoa bean (42.3 ± 1.08 mg/g bean), whereas the concentrations of total procyanidins did not

significantly differ for the other four clones (Figure 2C). There was no significant difference in total procyanidin concentration per gram CE among the five clones (Figure 2D). TARS-27 likely had the lowest concentration per gram cocoa bean in both analyses. One reason could be due to its low polyphenol extraction yield from the bean (Table 2). Extraction yield for TARS-27 was 10% while the extraction yield for other clones ranged from 14% to 16%. The amount of total polyphenol per gram cocoa bean in TARS-27 could be lower compared to other genetic clones, or other factors influencing the extraction efficiency. Other factors such as having lower response by colorimetric assays from the higher polymeric compounds extracted could also contribute to the difference in total polyphenol and procyanidin concentration. This phenomenon warrants further investigation, as factors affecting extraction efficiency may also have the potential to impact bioavailability.

Both total polyphenol and total procyanidin concentration of cocoa beans are linearly correlated with antioxidant activity. Total polyphenol content showed a moderate positive correlation with ferric reducing activity ($r = 0.76$) (24), while total procyanidin content possessed a strong positive correlation with oxygen radical absorbance capacity (ORAC) ($r \geq 0.92$) in vitro (25, 26). Therefore, the total polyphenol and total procyanidin concentration in cocoa beans could be reasonably expected to provide useful information where physiological function impacted by antioxidant activity is of interest. However, it should be noted that total polyphenol concentration is not always correlated with bioactivity or health outcomes. For example, the reduction of polyphenol concentration during fermentation was not correlated with the inhibition of digestive enzymes and dipeptidyl peptidase-IV (14, 27). Detailed characterization of individual cocoa polyphenols or other potential cocoa bioactive compounds such as condensed

polymers is needed to improve the understanding of the mechanisms underlying the health promoting properties of cocoa.

Qualitative measurement of degrees of polymerization and calculated mDP

Qualitative fingerprints representing the degrees of polymerization (DP) of polyphenols present in the five genetic clones are depicted in Figure 3. Cocoa flavanols from monomer to polymers are separated proportionally by elution time, with larger compounds eluting later (6). Overall, HPLC chromatograms for the five genetic clones showed a wide distribution of distinct peaks, suggesting flavanols with various DP (monomeric flavanols to larger polymers) were present in each clone. It should be noted that qualitative comparison of DP pattern between the genetic clones was the main purpose of this experiment. Therefore, quantitative comparison between peaks with different elution times was not conducted in this uncalibrated chromatogram, where relative response factor decreases with increasing DP (88% decrease from DP1 to DP10) (28). There were no major differences observed in DP pattern among the five genetic clones, which indicates that the same compounds were likely present in each clone, but does not give any information regarding the relative concentrations of these compounds among the genetic clones.

Calculated mDP for five genetic clones ranged from 2.19 ± 1.06 (TARS-14) to 3.22± 0.15 (TARS-31)(Figure 4.). However no significant difference was observed between samples. One possible explanation for the consistency of polyphenol mDP among the clones evaluated in this study could be that all five clones were hybrids derived from the same variety, Forastero (20). Future work should compare polyphenol mDP among cacao accessions with even greater biodiversity, and the potential for plant genetics and agricultural production practices to impact cocoa bioactivity.

Conclusions

Recent discoveries on the relationship between polyphenol profiles and biological activities warrant thorough characterization of concentration and composition (structure) of polyphenols in cocoa bean. It was found in this study that genetic variability could be associated with differences in total polyphenol and procyanidin concentration (TARS-27 having the lowest concentration). Therefore, it is reasonable to expect that differences in bioactivities including antioxidant activity may be observed between these clones, and further research is warranted. Structural differences regarding degree of polymerization were not evident between the five genetic clones suggesting that structural differences might be not significant with cocoa beans of different genetic clones belonging to the same variety (Forastero). Further study should be conducted to determine if structural differences exist between different varieties of cocoa including Forastero, Criollo, and Trinitario.

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Figure legends

Figure 1. Chemical structure of flavanols found in cocoa.

Figure 2. Total polyphenol (A: per gram cocoa bean, B: per g cocoa extract) and procyanidin (C: per gram cocoa bean, D: per gram cocoa extract) concentration of five cacao accessions. Values are expressed as mean ± SD. Treatments with different letter are significantly different when compared with one-way ANOVA followed by Tukey's HSD post hoc test for all possible comparisons ($p < 0.05$).

Figure 3. (A): profiles of normal-phase HPLC chromatograms of five cacao accessions. (B): zoomed in version from 24 min retention time in order to see small differenced in the high DP region. Scale for each chromatogram in fluorescence units is identical (900 LU).

Figure 4. Calculated mDP for five cacao accessions. (mDP for procyanidin only will be re-analyzed)

Figure 1.

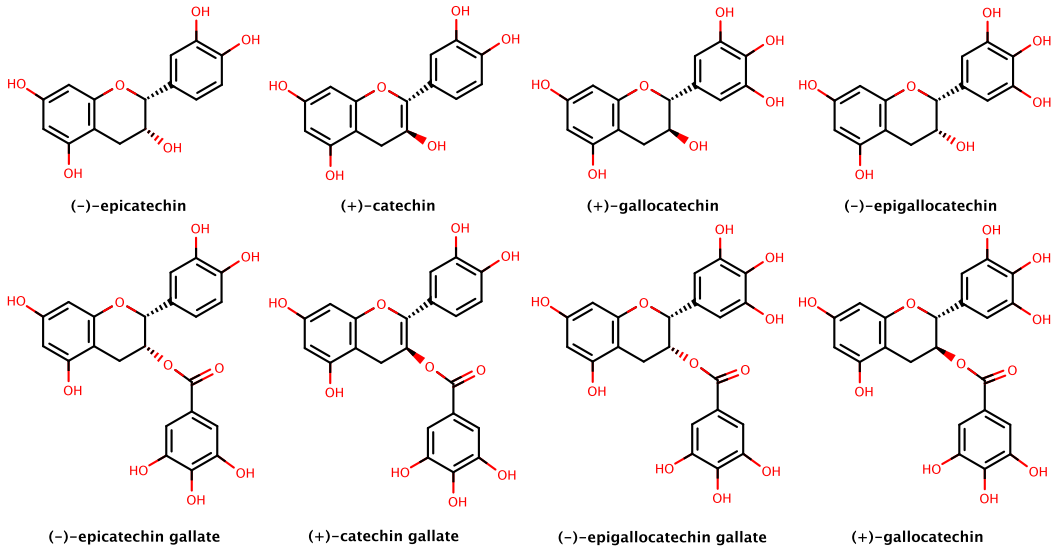
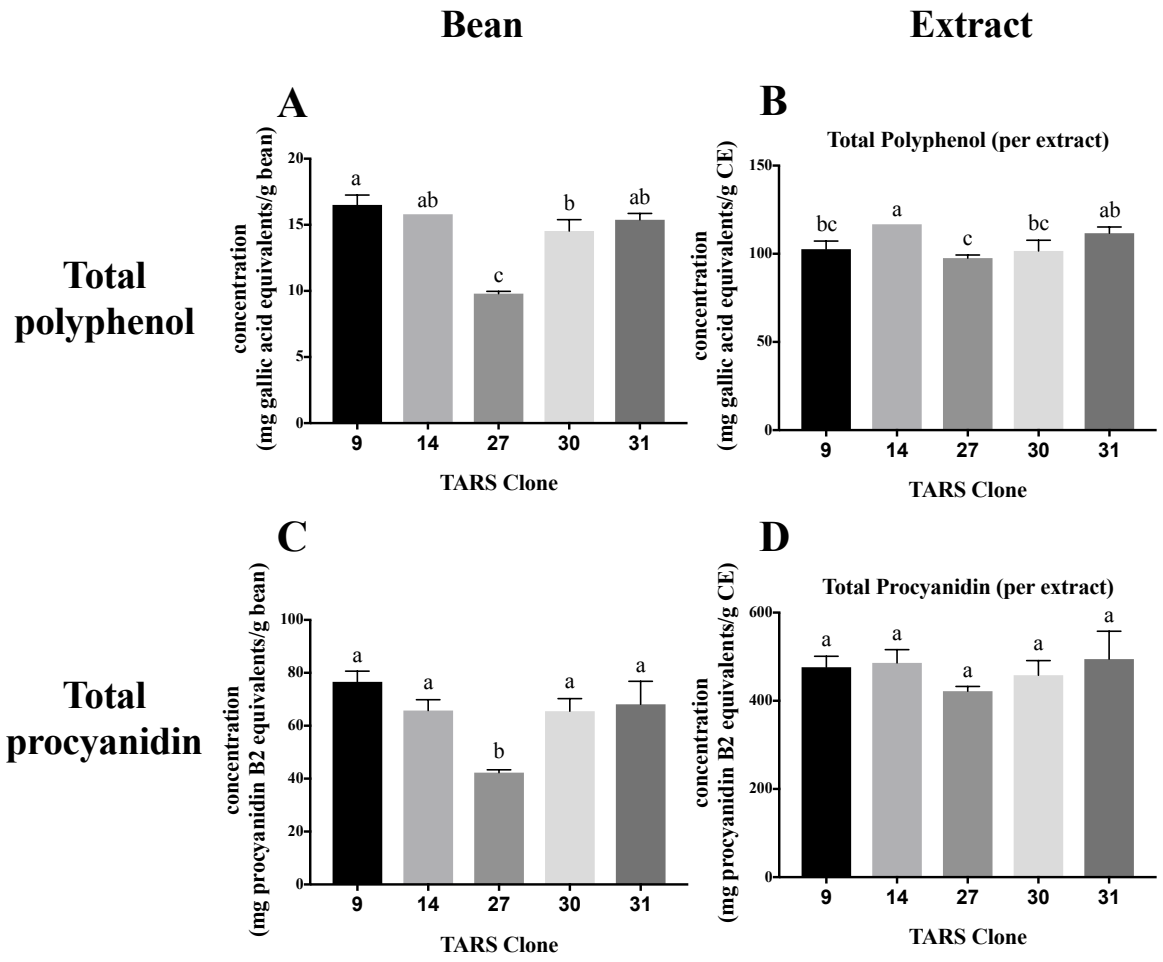

(−)-epicatechin
(+)-catechin
(+)-gallocatechin
(−)-epigallocatechin
(−)-epicatechin gallate
(+)-catechin gallate
(−)-epigallocatechin gallate
(+)-gallocatechin

Figure 2.


Bean
Extract
Total polyphenol
Total procyanidin
A
B
C
D
concentration (mg gallic acid equivalents/g bean)
Total Polyphenol (per extract)
concentration (mg gallic acid equivalents/g CE)
concentration (mg procyanidin B2 equivalents/g bean)
Total Procyanidin (per extract)
concentration (mg procyanidin B2 equivalents/g CE)
TARS Clone
9
14
27
30
31
a
ab
c
b
ab
bc
a
c
bc
ab
a
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b
a
a
a
a
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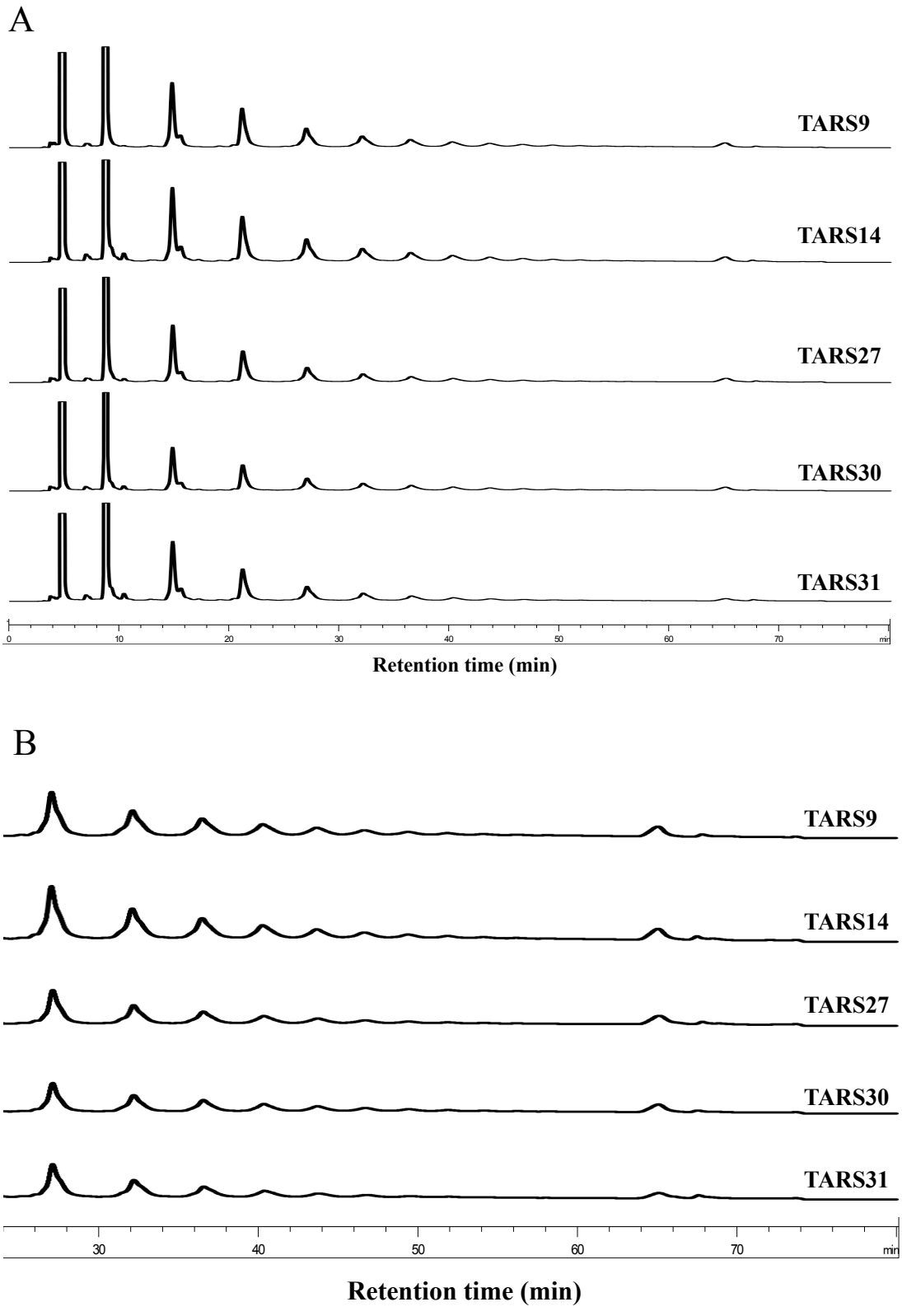
a
a

Figure 3.


A
TARS9
TARS14
TARS27
TARS30
TARS31
0
10
20
30
40
50
60
70
min
Retention time (min)
B
TARS9
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TARS27
TARS30
TARS31
30
40
50
60
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Retention time (min)

Figure 4.

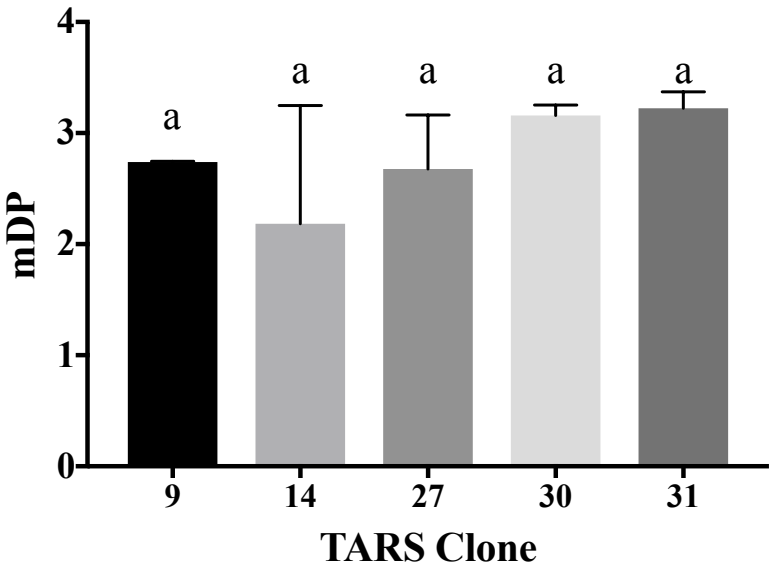

Table 1. MS/MS Settings for MRM Detection of monomers and thiolyzed product.

Compound	Retention time (t_R) (min)	MW (g mol-^{1})	$[M-H]^-$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
C/EG	3.17-4.38	290.142	288.98	245.05	40	14
ECG	4.55-5.06	441.952	440.92	169.00	38	16
C/EC benzylthioether derivative	7.35-8.38	412.031	410.94	124.97	30	18
ECG benzylthioether derivative	7.36-7.99	563.824	563.05	287.06	38	16

Table 2. Polyphenol extraction yields of five genetic clones.

Genetic clone	Polyphenol extraction yield
TARS9	0.16
TARS14	0.14
TARS27	0.10
TARS30	0.14
TARS31	0.14

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Chapter 4:

laboratory-scale model cocoa fermentation using dried, unfermented beans and artificial pulp can simulate the microbial and chemical changes of on-farm cocoa fermentation

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Abstract

Cocoa bean fermentation is an essential step for chocolate production whereby flavor potential is generated and bitterness and astringency are moderated. Microbial dynamics and interactions during fermentation largely determine fermented bean quality. Cocoa beans are generally fermented *in situ* on or near the farm where they are grown. To facilitate fermentation research in laboratories geographically distant from cacao production and fermentation sites, and to simplify and control the fermentation system, a laboratory fermentation model system was developed. The model system consisted of dried unfermented beans, artificial pulp medium, and laboratory incubator to control temperature and agitation. No starter cultures were added in order to determine whether the selective conditions of the model system including media composition, pH, mixing and temperature were sufficient to encourage the growth of essential microorganisms present in the environment. Microbial and chemical changes were monitored over a period of 144 hours of cocoa bean fermentation. Without inoculation, the model system developed in this study showed a succession of key microorganisms including yeast, lactic acid bacteria, and acetic acid bacteria. Identified microorganisms included *Saccharomyces cerevisiae*, *Latobacillus plantarum*, *Lactobacillus fermentum*, *Acetobacter pasterurianus*, *Acetobacter, tropicalis,* which have been previously identified in various *in situ* on-farm cocoa bean fermentation systems. The cocoa bean pH decreased from 5.7 to 4.8, an acceptable final pH for quality beans. Sugars were well-consumed in the beans, and acetic acid and ethanol were produced during fermentation. Desirable volatile compounds for chocolate including phenylethyl alcohol, benzyl alcohol, acetoin, 2-phenylethyl acetate, and 3-methylbutyl acetate increased during fermentation. In addition, the concentration of total polyphenols and procyanidins were decreased by fermentation. These results lead to the conclusion that the model system developed in this study

is able to simulate the microbial and chemical changes occurring in *in situ* cocoa fermentation. This laboratory-scale model system will enable systematic examination of the effects of process parameters such as bean variety, nutrients for microbiota, inoculation cultures (single or mixed starter), and fermentation conditions including temperature and aeration, on fermented bean composition. A better understanding of the impacts of these process parameters will lead to the development of practical cocoa fermentation management strategies to achieve more consistent, targeted fermented bean composition parameters in the field.

Key words:

Cocoa fermentation, laboratory model, Spontaneous fermentation, Microbial identification, metabolite analysis (HPLC), Volatile compound analysis (GC-MS), Total polyphenol assay

1. Introduction

Cocoa beans are a significant fermented food product globally, with an annual production of 4 million tons in 2016 (ICCO, 2016). Approximately 95% of fermented dried cocoa beans are produced by small businesses in equatorial states, then shipped from their agronomic origins to processors around the world, where they are further processed into a range of value-added food products (Ozturk and Young, 2017; Saltini et al., 2013). The cocoa pod has a thick wall and contains 30-40 fresh beans. Cocoa beans contain 40-60% cocoa butter, 10-15% proteins, 6% starch, 0.9-1.4% theobromine, 0.2% caffeine and 5-9% flavanols (expressed as dried matter) and the internal pH of fresh cocoa bean is around 7.0 (Bernaert et al., 2012). Each bean contains two cotyledons and an embryo that are surrounded by a seed coat and covered by a sugar-rich, mucilaginous pulp. Once harvested, beans are removed from the pods and piled on banana leaves or transferred into wooden boxes with holes on the bottom to enable drainage of the pulp. Cocoa pulp is a rich substrate for microbial growth consisting of 80-90% water, 10-13% simple sugar (mainly glucose, fructose and sucrose), 2-3% pentosans, 1-3% citric acid, and 1-1.5% pectin (Pettipher, 1986).

Cocoa bean fermentation is generally conducted immediately following harvest, on the farm where the cacao is grown. The fermentation process is initiated spontaneously by naturally present environmental microorganisms. A comprehensive review of fermented cocoa conducted in four different countries identified a succession of yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) as the dominant species in cocoa fermentation (Schwan and Wheals, 2004). These microorganisms are generally considered to be the essential microorganisms for successful cocoa fermentation (Camu et al., 2007).

Microorganisms isolated from cocoa fermentation in various countries include *Saccharomyces* spp., *Pichia* spp., *Lactobacillus* spp., *Acetobacter* spp., *Gluconobacter* spp. (Bortolini et al., 2016; Maura et al., 2016; Ozturk and Young, 2017). Occasionally, undesired growth of *Bacillus* or filamentous fungi occurs in over-fermented product giving unpleasant flavor to the cocoa (De Vuyst et al., 2010; Ho et al., 2014). A typical pH of the pulp before fermentation is 3.6, due to the presence of citric acid. The acidic environment combined with low oxygen concentration provides a competitive advantage for yeast growth during the initial stage. During the first 12 hours of fermentation, yeasts utilize pulp sugar and the population increases to 7-8 log CFU/g of pulp. Under low-oxygen conditions, alcoholic fermentation generates ethanol up to 6-8% (v/v) (Ho et al., 2014; Schwan, 1998; Schwan and Wheals, 2004). The heat generated by this fermentation process increases internal bean temperature. Some yeasts produce pectinase to degrade the mucilaginous pulp facilitating aeration and thus promoting acetic acid bacterial growth. AAB growth is promoted by aeration of the fermentation mass (often achieved practically by turning the cocoa bean piles or mixing contents in a wooden box), and by temperature above 37°C. AAB reach a population peak of around 7 log CFU/g of pulp. AAB oxidize ethanol and produce acetic acid exothermically, increasing the temperature even higher to 45-50°C (Afoakwa, 2011). Along with ethanol, acetic acid penetrates into the bean cotyledons and kills the embryo, inhibiting germination (Thompson et al., 2007). In this step, endogenous enzyme activity is triggered due to the breakdown of cell walls and membranes and chocolate flavor precursors are formed (Thompson et al., 2007). These important precursors undergo Maillard reactions during the roasting stage to form cocoa flavor compounds (Fowler, 2009). Volatile compounds including isopropyl acetate, ethyl acetate, 1-propanol, isoamyl alcohol, 2,3-butanediol, diethyl succinate and phenylethanol are produced by microorganisms

(mainly yeasts) during cocoa fermentation and also contribute to flavor formation of cocoa during subsequent processing steps (Rodriguez-Campos et al., 2011). LAB generally increase in population to 7-9 log CFU/g and produce mainly lactic acid during fermentation (De Vuyst et al., 2010). Endogenous biochemical reactions also result in oxidation of polyphenols, mainly by polyphenol oxidase, forming the typical brown color of cocoa, and reducing astringency and bitterness (Afoakwa et al., 2008; Kadow et al., 2015; Schwan and Wheals, 2004). The internal pH of unfermented cocoa bean is around 7.0, and the pH of good quality cocoa bean should be brought by fermentation to 5.0-5.5 to ensure optimal activity of endogenous proteases and production of desirable flavor precursors (Biehl et al., 1985; Hansen et al., 1998; Ho et al., 2014).

Historically, obtaining fermented beans of consistent high quality has been difficult because the cocoa fermentation process is relatively uncontrolled and relies on an empirical approach (Gálvez et al., 2007; Lefeber et al., 2011). Therefore, understanding the impact of fermentation conditions on microbial and chemical quality of beans and the interactions between microbial species during cocoa fermentation has the potential to improve control of on-farm cocoa fermentation.

Study of cocoa fermentation in laboratories geographically distant from cocoa production sites is challenging for several reasons. On-farm cocoa fermentation in piles or boxes generally requires at least 25 kg of fresh wet beans to achieve the proper conditions for fermentation. Smaller piles with greater surface area to volume ratios allow greater heat loss during early stages of fermentation, and thus do not reach appropriate temperatures to encourage the desired succession of microbial populations. Study of 25 kg piles of cocoa beans in sites geographically distant from farms is not feasible due to shipping and storage considerations. Depending on the

growing region, cacao is harvested once or twice per year, and storage of large quantities of pods and/or unfermented wet beans and pulp for later fermentation studies is not feasible.

To overcome these problems, a model fermentation system was developed to facilitate experimentation with cocoa fermentation using commercially available dried unfermented cocoa bean, artificial pulp media and a laboratory incubator. The use of dried beans has several advantages. First, it significantly reduces shipping costs for raw material since the weight of dried bean is much lighter than that of fresh cocoa pods. The average weight of a fresh cocoa pod is 400 g and one pod yields 40 g of dried beans (Abenyega and Gockowski, 2001). Second, drying beans prevents over-ripening and mold growth, which are challenges to shipping and/or storage of fresh cocoa pods or wet beans. The changes in the population of microorganisms, and physico-chemical properties of the medium were monitored over the course of the fermentation, and compared to published data to test the validity of our model fermentation system.

2. Materials and methods

2.1. Dried unfermented cocoa beans

Commercially available dried unfermented cocoa beans were purchased from an on-line retailer (Feeling Better Every Day, Fennimore, Wisconsin, USA). These beans had been removed from pods, washed and dried immediately before packaging and distribution. The moisture content of the dried beans was 3.5%. The beans were of the Criollo variety and originated from Ecuador.

2.2. Laboratory scale simulated cocoa fermentation

Dried beans were rehydrated with ultrapure water, prepared using a Direct-Q® Water Purification System (EMD Millipore, Darmstadt, Germany), until the moisture content reached equilibrium, which occurred at 35% moisture. Rehydrated beans (1.2 kg) were mixed with

simulated cocoa pulp medium (2L), which was formulated based on previous work by Lefeber et al. (2010) and Pettipher (1986) with modifications mainly affecting sugar composition and concentration (Table 1). The simulated pulp medium was autoclaved prior to fermentation experiments. Magnesium and manganese were added after autoclaving to support microbial growth and the pH was adjusted to 3.6 with sodium hydroxide. Mixture of rehydrated beans and the simulated cocoa pulp medium were then transferred into 30 cm × 44.2 cm × 16.9 cm plastic fermentation boxes (Polypropylene, IRIS USA, Inc., WI). The box was loosely covered to simulate covering cocoa heaps with banana leaves in the field. A temperature gradient was employed over the 144 hour fermentation period and was controlled using a standard laboratory incubator (New Brunswick^{TM} Innova 42, Eppendorf, Hauppauge, New York, USA) as follows: 25°C (0-24 hr), 35°C (24-48 hr), 40°C (48-72 hr), 45°C (72-96 hr), 48°C (96-144 hr). This cycle mimics the temperature condition observed at the center of the pile during on-farm fermentation (Schwan and Wheals, 2004). Constant agitation was provided as follows: 0 rpm (0-24 hr), 120 rpm (24-36 hr), 150 rpm (36-144 hr).

The fermentation lasted for 144 hours and samples of both pulp and bean were taken every 24 hours over the course of fermentation for microbial and chemical analysis. Cocoa beans were thoroughly mixed at each sampling time point using gloved hands, to simulate pile mixing in on-farm cocoa fermentation, which provides aeration. Moisture content was measured using a Moisture Analyzer IR-120 (Denver Instrument, New York). Microbial analysis was performed immediately after sampling while samples for chemical analysis were stored at −80°C until the time of analysis. All analyses were conducted in triplicate.

2.3. Microbial enumeration

For microbial cell count, 5 mL of the liquid portion of the fermentation medium was mixed with 45 mL of 0.1% peptone in water in a 50 mL centrifuge tube and vortexed vigorously for 2 min to obtain a uniform homogenate. A 1 mL sample was then removed and subjected to 10-fold serial dilution. The appropriate diluates were spread on three different selective media to select for yeast, LAB and AAB. For yeast culture, samples were spread on YM medium (3 g/L yeast extract, 3 g/L malt extract, 3 g/L peptone, 10 g/L glucose, 20 g/L agar) with 100 mg/L of oxytetracycline and incubated at 37°C. Lactic acid bacteria were cultured (37°C) on de Man-Rogosa-Sharpe (MRS) agar with 400 mg/L of cycloheximide in an anaerobic condition generated by GasPAK^{TM} EZ gas generating system (BD, USA). GYC medium (50 g/L glucose, 10 g/L yeast extract, 30 g/L calcium carbonate, 20 g/L agar, pH adjusted to 5.6) with 400 mg/L of cycloheximide were used to select acetic acid bacteria by incubating at 25°C. Colonies were classified based on their cell morphology and representative colonies were subjected to genomic DNA (gDNA) extraction and sequence analysis after successive transfers on the appropriate selective media (MRS for LAB, GYC for AAB, YM for yeast) to obtain pure cultures.

2.4. DNA extraction and identification of microorganisms

Yeast gDNA was extracted by using YeaStar Genomic DNA Kit^{TM} (Zymo Research, CA, USA). Briefly, Yeast cells were enriched in a liquid media (YM) to OD_{600}=1, corresponding to 2-4 × 10^7 CFU/mL. Cultures (1 mL) were then collected by centrifugation at 500 × *g* for 2 min and the supernatants were discarded. For cell lysis, cells were resuspended by 120 µL of YD Digestion Buffer and 5 µL of R-Zymolase^{TM} (RNaseA+Zymolase) was added and the mixtures were incubated at 37°C for 40 min. After incubation, 120 µL of YD Lysis Buffer was added followed by 250 µL of chloroform. Supernatants obtained by centrifugation at 10,000 × *g* were

loaded to the Zymo-spin III column and washed twice with DNA wash buffer. DNA was eluted by adding 60 μL of water.

Bacterial gDNA was obtained by using Quick-gDNA^{TM} MicroPrep (Zymo Research, CA, USA). A 200 μL aliquot of cells cultured in MRS liquid media was mixed with 800 μL of Genomic Lysis Buffer and allowed to stand at room temperature for 5 min. The mixtures were transferred to a Zymo-SpinTM IC Column and centrifuged at 10,000 × *g* for one minute. Next washing was performed by adding 200 μL DNA Pre-wash Buffer followed by addition of 500 μL of gDNA wash buffer. Elution buffer was added (10 uL) in the final step to obtain the gDNA. Both bacterial and yeast gDNA were stored at −20°C until further analysis.

The identification of yeast species was achieved by amplifying 5.8S-Internally Transcribed Spacer (5.8S-ITS) rDNA region by PCR using the primers ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS4 (5-TCCTCCGCTTATTGATATGC-3) (Esteve-Zarzoso et al., 1999). After the initial pre-denaturation step at 95°C for 5 min, 35 cycles of PCR amplification were performed using the following conditions: 94°C for 1 min, 55.5°C for 2 min, 72°C for 2 min, followed by a final extension at 72°C for 10 min.

The 16S rDNA of bacteria were amplified by PCR using the primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1495R (5-CTACGGCTACCTTGTTACGA-3) for LAB and the primers 16Sd (5-GCTGGCGGCATGCTTAACACAT-3) and 16Sr (5-GGAGGTGATCCAGCCGCAGGT-3) for AAB (Ho et al., 2014). PCR condition for both LAB and AAB was as follows: 94°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 2 min; and finally, 72°C for 10 min. Purity of PCR products were verified by electrophoresis with 1% agarose gel containing ethidium bromide (Bio-Rad, California, USA).

PCR products were purified by using DNA Clean & Concentrator™ (Zymo Research, CA, USA) and sent to Eton Bioscience (Durham, North Carolina, USA) for sequencing analysis. The 5.8S-ITS region and 16S rDNA sequence data were subjected to BLASTn (nucleotide BLAST) search (http://blast.ncbi.nlm.nih.gov) to identify microorganisms.

2.5. pH analysis

The standard method for cocoa nib pH determination was employed. Briefly, the shell of the beans was peeled and the resulting nibs (flesh of the cocoa bean) were crushed by using mortar and pestle. Cocoa nibs (5 g) were then homogenized for 30 s in 100 mL of hot distilled water (90°C) and then filtered through Whatman #4 filter paper. A 25 mL aliquot of the filtrate was transferred into a beaker and the pH was measured using a bench top pH meter equipped with ROSS Ultra pH/ATC Triode Electrodes (Orion Versastar, Thermo Scientific, USA) (Nazaruddin et al., 2006). The pulp pH was determined in a solution made by mixing 10 g of the liquid portion of the fermentation medium and 90 mL of ultrapure water for 5 min (Ho et al., 2014).

2.6. Sugars, ethanol, glycerol, and organic acids analysis

The cocoa pulp and the nib preparation for analyses of sugars, ethanol, glycerol, mannitol and organic acids concentrations were performed according to the methods described by Ho et al. (2014) with appropriate modifications for this study. Briefly, the liquid portion of the fermentation medium and cocoa nibs were separated. Each sample fraction (10 g) was mixed with 90 mL of ultrapure water and homogenized in a Waring laboratory blender (Waring Products, New Hartford, USA) for 3 min. After centrifuging the homogenate at 2,500 × *g* for 10 min at 5°C, the supernatant was collected. The sediment was washed with 20 mL of the previously collected supernatant and the new supernatant was combined with the first

supernatant. These extracts were then filtered through 0.45 uM PVDF membrane filter (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of sugars, ethanol, glycerol and organic acids (Sigma-Aldrich, St. Louis, MO, USA) were analyzed by Agilent HPLC 1200 Infinity Series (Agilent Technologies, California, USA) equipped with Aminex HPX-87H column (300 mm × 7.8 mm, 50°C, Bio-Rad, California, USA) and Refractive Index (RI) detector. The isocratic mobile phase (0.005 M H_2SO_4) flowed at 0.6 mL/min and the injection volume was 5 µl.

2.7. Volatile compound analysis

The volatile compounds in cocoa nib were extracted by head-space solid phase microextraction (HS-SPME) using a 50/30 µm divinylbenzene/carboxene/ polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). Cocoa nibs (1 g) were ground and put into a 4 mL headspace vial and sealed with septum cap. The extraction condition was 15 min in order to reach equilibrium, with a fiber exposition of 30 min at 60°C to the headspace of cocoa samples.

To analyze cocoa volatiles, a SHIMADZU GCMS-QP 2010 Ultra equipped with a Phenomenex Zebron capillary GC column (ZB-WAX plus, 60 m length × 0.25 mm ID × 0.25 µm film thickness) was used. The GC oven temperature was initially set at 30°C for 5 min and increased to 200°C by 3°C/min, then to 250°C by 5°C/min maintaining 250°C for 5 min. Helium was the carrier gas and injection mode was set to splitting for 5 min at 250 °C. The ion-source temperature for mass spectrometer was 280°C. Identification of volatile compounds was done by 1) comparing the mass spectra of each peak with the National Institute of Standards and Technology (NIST) mass spectral library (2014 version) and 2) comparing the Kovats retention index with the literature data.

2.8. Polyphenol extraction

Polyphenols were extracted from the cocoa bean samples according to the method of Dorenkott et al. (2014) with minor modifications. Methodological details are included in the supplementary file.

2.9. Total polyphenol and procyanidin assays

Quantification of total antioxidant/reducing activity (interpreted as polyphenol content) of cocoa bean extract was based on the Folin-Ciocalteu method reported by Waterhouse (2002). Total polyphenol concentration was expressed as mg gallic acid equivalents/g cocoa bean ± SD. Total procyanidin of cocoa bean extract was quantified by using 4-dimethylaminocinnemaldehyde (DMAC) method, described by Payne et al. (2010), with appropriate modifications for this study. Total procyanidin concentration was expressed as mg procyanidin B2 equivalents/g cocoa bean ± SD. Methodological details for total poplyphenol and procyanidin assays could be found in the Supplementary file. Both analyses were conducted in triplicate.

2.10. Statistical analysis

Data were expressed as mean ± SD for n=3 samples obtained at each time point. Data were compared by 1-way ANOVA to determine if significant differences were observed along the time course of fermentation for each compound of interest, with differences being considered significant when $p < 0.05$. For the polyphenol and procyanidin data, ANOVA was followed by Tukey's HSD post-hoc test to determine at which time point during fermentation changes in concentration occurred, with differences being considered significant when $p < 0.05$. Statistical analyses were conducted using Prism v6.0e (GraphPad Software, Inc., La Jolla, CA).

3. Results and Discussion

3.1. Moisture content and pH

Moisture content of rehydrated dried unfermented cocoa beans started at 35%, increased to a peak (40%) after 48 hours of fermentation, and remained level until 96 hours and then decreased to 30% until the end of the fermentation (Figure 1A). The model fermentation reproduced similar evolution of moisture content of wet beans during on-farm fermentation which started at 35% upon harvest and reached 40% after bean death (Mozzi and Vignolo, 2010). The decrease in moisture content from 96 hours may be attributable to the high incubation temperature and airflow inside the incubator which evaporated the water from the bean.

The initial pH of nib was 5.7 and decreased to 4.8 after 144 hours of fermentation, while the pH of the simulated pulp medium increased from pH 3.6 to pH 4.9 over the course of fermentation (Figure 1B).

Bean and pulp pH can serve as an indicator of proper cocoa fermentation. Generally, high final bean pH (5.5-5.8) after fermentation is considered an indicator of poor fermentation, and associated with lower quality fermented beans. Lower final pH (4.75-5.19) is associated with high quality fermented beans (Afoakwa et al., 2008).

The pH changes of pulp media and nib in our model system showed a similar trend to that of cocoa fermentation conducted at three estates in Indonesia by traditional wooden box fermentation, where the pH of the pulp increased from 3.7-3.9 to 4.8-4.9 and bean pH decreased from 6.3-6.5 to 5.0-5.1 (Ardhana and Fleet, 2003). A similar trend was also observed in heap fermentation (750 kg of cocoa bean) conducted in Ghana where the pH of the pulp increased from 4.1 to 4.55 during fermentation (Nielsen et al., 2007), although nib pH was not evaluated in that study.

3.2. Microbial identification

The simulated pulp media developed in this study allowed for selective growth of yeast in the initial stage of fermentation, as in typical on-farm cocoa fermentation. Selective pressure is imparted by high sugar concentration (25 g/L sucrose, 40 g/L glucose, and 45 g/L fructose) and low pH (3.6) mainly due to citric acid in the media. Cocoa-fermenting yeasts are able to tolerate low pH and grow well in these conditions.

Microbial counts using three different selective media were performed to characterize the changes in population during fermentation. Yeast populations increased rapidly from 2.6 log CFU/mL initially to a peak of 8.7 log CFU/mL after 96 hours, however by 120 hours yeast were below the limit of detection (Figure 2A). AAB and LAB were detected beginning at 24 hours (1.6-1.8 log CFU/mL) increasing exponentially until 96 hours (9.4-9.5 log CFU/mL). The decrease at 144 hours was larger for AAB compared to LAB (Figure 2 B and C). This pattern of microbial succession of yeasts, LAB and AAB are characteristic of microbial populations within *in situ* cocoa fermentation piles (Schwan and Wheals, 2004). The cocoa fermentation model developed in this study was thus able to support the growth of a succession of natural microbiota including yeast, LAB and AAB without inoculation of a starter culture in the initial stage.

Yeast plays an important role in the early stage of cocoa fermentation. First, certain yeasts, e.g. *S. cerevisiae* identified in this model system, secrete pectinolytic enzymes during fermentation, which degrade pectin, decreasing pulp viscosity. The decrease in viscosity increases oxygen transport in the fermentation environment and promotes subsequent AAB growth (Gálvez et al., 2007). Second, other yeast species degrade citric acid within the pulp, which increases the pH of the pulp and allows LAB and AAB to grow (Ardhana and Fleet, 2003). Although *S. cerevisiae* is typically the most abundant yeast species observed in on-farm cocoa fermentation, other yeast species are generally also detected. In our model system, *S. cerevisiae*

was the only detected yeast species over the course of fermentation. It is likely that *S. cerevisiae* exclusive presence comes from the laboratory environment where the fermentation was conducted since hard cider fermentation research utilizing *S. cerevisiae* strains is conducted there. The dominance of *S. cerevisiae* in the initial stages of fermentation could also be due to the strong growth of a particular strain out-competing other yeast species. From total microbial count, yeast species (mainly *S. cerevisiae*) showed exponential growth from 0-24 hours and their growth was detected until the 96 hour-time point. The rapid disappearance of yeasts after 96 hours might be due to the inhibitory effect of ethanol and high acetic acid concentration in the fermentation matrix (Deak, 2006). The growth of yeast during cocoa fermentation has been extensively studied by others, and maximum yeast populations were detected at time points ranging from 2 to 96 hours.

As yeasts liberate simple hexose sugars from the cocoa pod pectin, populations of hetero-fermentative LAB including *L. plantarum* and *L. fermentum* are able to grow. Acetic acid produced by these bacteria, results in death of the bean embryo, one of the primary goals of cocoa fermentation. In this model system, *L. plantarum* and *L. fermentum* were identified at various stages of fermentation (Table 2). These two species are the most commonly found LAB in on-farm cocoa fermentation throughout the world (Ardhana and Fleet, 2003; Camu et al., 2007; Kostinek et al., 2008; Zhao and Fleet, 2015). The maximum population achieved by LAB during fermentation varied across different fermentation studies, ranging from 18 hours (Camu, González, et al., 2008) to 96 hours (Nielsen et al., 2007). The maximum LAB growth detected in our model fermentation system was at 96 hours.

The growth of AAB in cocoa fermentation is essential to ensure good quality product. AAB use ethanol as a carbon source and produces acetic acid (Camu et al., 2007), which in turn

penetrates into the bean, and along with ethanol, causes structural damage. Similar to yeast metabolism, AAB metabolism is an exothermic process and increases the temperature of the fermentation matrix (Lima et al., 2011).

In our model system, *A. pasteurianus*, *A. tropicalis*, and *L. plantarum* were identified using GYC media. Growth of *L. plantarum* (LAB) in GYC media at our incubation condition (aerobic, 25°C; intended to select for AAB) makes the selective isolation of only AAB difficult, and could result in inflated counts for AAB (Cleenwerck et al., 2008). Better strategies should be developed for accurate enumeration of this genus. *A. pasteurianus* is a typical species found in cocoa fermentation and its ability to oxidize ethanol and lactic acid into acetic acid and acetoin, an essential reaction during fermentation, was revealed by Moens et al. (2014). *A. tropicalis* in not frequently found in on-farm cocoa fermentation but their presence has been reported in Ghanaian cocoa bean fermentation (Cleenwerck et al., 2007). The time point during fermentation at which AAB were first detected varied among different regions ranging from 0-48 hours and AAB persistence also differed. In some cases, AAB were detected until the end of the fermentation (Camu et al., 2007) while one fermentation only detected AAB until 24 hours of fermentation (Ostovar and Keeney, 1973). While this could be attributable to differences in detection strategy, it is also very possible that actual AAB population dynamics differ to this extent, highlighting the variation in growth patterns among successful industrial cocoa fermentations.

3.3. Sugars, glycerol, ethanol, and organic acids

The concentration of sugars, glycerol, ethanol and organic acids in the system changed over the time course of fermentation ($p < 0.05$, one-way ANOVA for each compound over the time course of fermentation). In the liquid portion of the medium, all sugar sources were

effectively utilized during fermentation. Sucrose was rapidly consumed or hydrolyzed into glucose and fructose for the first 24 hours and thereafter slowly decreased until the end of the fermentation. Glucose and fructose remained at fairly constant concentrations for the first 24 hours. It is likely that glucose and fructose were being consumed during this period, but the conversion of sucrose to glucose and fructose resulted in a fairly constant concentration of these two sugars within the system, despite consumption thereof. From 24-48 hours, glucose and fructose were co-fermented and showed a sharp decrease in concentration (Figure 3A). Ethanol production began from the 24 hour time point, reached a maximum concentration at 48 hours (20 ± 0.02 mg/mL), then decreased. It is reasonable to conclude that these changes were driven largely by yeast metabolism during initial stages of fermentation. Glycerol was also detected at a low concentration, less than 2 mg/mL (Figure 3C).

The consumption pattern of sugars reported previously varied across different studies. One study showed a similar trend to that of our model system in that sucrose was initially consumed while glucose and fructose began to decrease from 12-24 hours during small-scale cocoa fermentation (de Melo Pereira et al., 2012). However, a cocoa fermentation done by Ho et al. (2014) showed an opposite trend in that the consumption of sucrose lagged until 24 hours whereas glucose and fructose were fermented from the initiation of fermentation, or 0 hours.

Sugars, ethanol and glycerol were also analyzed in the fermenting cocoa nibs. Initial sucrose concentration was the highest (5.74 ± 1.65 mg/g) compared to glucose (1.04 ± 0.48 mg/g) and fructose (1.36 ± 0.29 mg/g). All sugars were mostly consumed at 72 hours and remained at low concentrations until the end of the fermentation (Figure 3B). Glycerol concentration remained low (less than 0.64 ± 0.10 mg/g) throughout the process, whereas ethanol concentration increased from 24 hours and reached the highest concentration (6.57 ± 0.28 mg/g) at 48 hours

(Figure 3D). In our model system, the change in the level of sugars and ethanol in the nib showed a similar trend to the pulp after 24 hours, except that the level of all sugars increased from 0-24 hours. This difference might be due to the high level of sugars in the pulp diffusing in to the cocoa bean.

The concentrations of citric acid, acetic acid, lactic acid, and succinic acid in the liquid portion of the fermentation medium are reported in figure 3E. Citric acid concentration slightly increased during 0-48 hours but decreased thereafter and the lowest concentration of citric acid (2.46 ± 0.03 mg/mL) was detected at 96 hours. The concentration of acetic acid and lactic acid increased significantly from 48 hours to 144 hours. Acetic acid concentration (24.6 ± 0.09 mg/mL) was higher than the concentration of lactic acid (11.9 ± 0.04 mg/mL) at 144 hours. Succinic concentration slightly increased during fermentation and the final concentration was 1.83 ± 0.02 mg/mL. Notably, the concentration of acetic acid in the cocoa nibs increased dramatically from 48 hours (1.47 ± 0.41 mg/g) to 144 hours (9.83 ± 1.09 mg/g). The concentration of citric acid slightly increased to 48 hours (4.03 ± 0.51 mg/g) and decreased until 96 hours when it reached a lower concentration (1.45 ± 0.16 mg/g). Lactic acid concentration increased from 48 hours (0.60 ± 0.05 mg/g) to 144 hours (3.90 ± 0.59 mg/g). Succinic acid concentration increased from 0 to 144 hours but remained a minor constituent at less than 0.63 ± 0.07 mg/g.

An essential outcome of yeast metabolism during cocoa fermentation is the decrease of citric acid concentration in the pulp to leading to pH increase and allowing LAB and AAB growth. Moreover, ethanol and acetic acid must reach sufficient concentration in the pulp to diffuse into the bean, and along with heat, cause bean death. The level of acetic acid remained

higher than lactic acid in our model system, which is in agreement with conditions observed in on-farm cocoa fermentation studies (Lima et al., 2011).

3.4. Volatile compounds

In addition to yielding flavor precursors such as amino acids and reducing sugars, the fermentation process itself is able to produce volatile compounds contributing directly to chocolate sensory characteristics (Afoakwa et al., 2008). In total, 34 volatile compounds were identified in the cocoa beans fermented in our model system, including alcohols, acids, aldehydes and ketones, and esters. The relative amount (expressed as area unit/g bean) of major volatile compounds is depicted in Figure 4.

In the alcohol group (Figure 4A), phenylethyl alcohol showed the highest increase during fermentation. 3-methyl-1-butanol, also called isoamyl alcohol increased and reached the second highest amount at 48 hours and gradually decreased thereafter. Linalool showed a similar trend to isoamyl alcohol, but its change in concentration over the time course of fermentation was not significant. Benzyl alcohol increased from 48 hours to the end of the fermentation (Figure 4A). 2-heptanol and 2-nonanol maintained a similar concentration over the course of fermentation (Supplementary Figure S1A). In the acids group (Figure 4D), isovaleric acid and isobutyric acid increased during fermentation. On the other hand, butyric acid and octanoic acid maintained a fairly constant concentration throughout the fermentation.

The volatile compound showing notable change in the aldehydes and ketones group was acetoin increasing by 13-fold between the 48 hour- and 72 hour-time points. Diacetyl (or 2,3-butanedione), 2-heptanone, 2-nonanone, 3-methylbutanal, and phenylacetaldehyde did not change over the course of fermentation (Figure 4B). Isobutyraldehyde and nonanaldehyde were detected but maintained very low compared to other compounds. Acetaldehyde was produced

and increased to 24 hours (9×10^5 area unit/g nib) but decreased thereafter (Supplementary Figure S1B).

Among the esters group (Figure 4C), the relative amount of 2-phenylethyl acetate sharply increased. Isoamyl acetate and ethyl acetate were the major esters in unfermented beans but decreased sharply during the first 24 hours of fermentation. Isoamyl acetate levels recovered, increasing from 24 to 96 hours (Figure 4C). Other minor compounds maintained fairly constant levels Supplementary Figure S1C).

It has been reported that yeasts produce a wide range of different volatile compounds including alcohols, fatty acids and esters. The relative concentrations of these compounds produced by yeast depend on the species and strains and fermentation conditions such as temperature and aeration (Mateo et al., 1991; Suomalainen and Lehtonen, 1979). *Kloeckera apiculata, S. cerevisiae, S. cerevisiae* var. *chevalieri, Candida sp., and Kluyveromyces marxianus* have been identified as major contributors of volatile compounds during fermentation (Schwan and Wheals, 2004). The above study also suggested that *Kloeckera apiculata* and *S. cerevisiae* var. *chevalieri* are major producers of isopropyl acetate, ethyl acetate, 1-propanol, isoamyl alcohol, 2,3-butanediol, diethyl succinate, and 2-phenyl ethanol, aroma compounds associated with high-quality cocoa. Among these compounds, ethyl acetate, isoamyl alcohol (3-methyl-1-butanol), and 2-phenyl ethanol were also produced during fermentation in our model system. Higher alcohols can contribute flowery and candy notes (Frauendorfer and Schieberle, 2006) while esters give fruity flavor notes to chocolate (Jinap et al., 1998).

One group monitored the change of volatile profiles during on-farm Ghanaian cocoa bean fermentation and compared the amount of volatile compounds generated before 24 hours and after 72 hours (Aculey et al., 2010). Interestingly, several results were in agreement with the

model fermentation system developed in this study, strengthening the conclusion that our model system is able to properly reproduce natural fermentation of the bean and generate key volatile compounds. In the model fermentation, ethyl acetate was highest in the unfermented bean and decreased during fermentation. This compound was also reported to have relatively high concentrations before 24 hours of fermentation in a prior study (Aculey et al., 2010). On the other hand, acetoin and 2-phenylethyl acetate showed increases during fermentation in our model system. These compounds were also reported by others to reach high concentrations after 72 hours (Aculey et al., 2010; Rodriguez-Campos et al., 2011). Acetoin itself has a butter and creamy odor but also can act as a precursor of tetramethylpyrazine, which is an important flavor component of chocolate. Other than acetic acid, isovaleric and isobutyric acid showed increases during fermentation. Others have reported that acetic and isovaleric acid were dominant in the unroasted Criollo cocoa bean (Frauendorfer and Schieberle, 2008). However, excessive concentration of these acid compounds could cause off aromas such as hammy flavor (Rodriguez-Campos et al., 2011).

3.5. Total polyphenol and procyanidin

Polyphenol and procyanidin concentration in cocoa beans decreased over the time course of fermentation ($p < 0.05$). The initial total polyphenol concentration was 20.6 ± 0.96 mg/g. By the end of fermentation this concentration had significantly decreased ($p < 0.05$) to 16.8 ± 0.24 mg/g (Figure 5A). The greatest decrease in total polyphenol concentration occurred between the 96-hour (20.8 ± 0.28 mg/g) and 120-hour (17.7 ± 0.41 mg/g) time points. The initial total procyanidin concentration was 11.4 ± 0.40 mg/g, and the final concentration was 4.55 ± 0.06 mg/g (Figure 5B). The greatest decrease in total procyanidin concentration was observed between the 72-hour (11.3 ± 0.83 mg/g) and 96-hour (5.83 ± 0.24 mg/g) time points, occurring

slightly earlier in the fermentation than the greatest decrease in total polyphenols. The increase in both total polyphenol and procyanidin concentration at 48 hours was unexpected. This increase corresponds with the timing of peak ethanol concentration (Figure 3D), which may have contributed to higher polyphenol extraction efficiency at that time point. Increase in ethanol concentration was reported to show higher polyphenol extraction yield in ground grape seed meal in a previous study (Shi et al., 2003). Visual observation of the color of polyphenol extracts during this procedure revealed that while the color of the extract was purple until 72 hours, it changed to a brown color in the 96 hours and subsequent samples (Supplementary Figure S2).

During cocoa bean fermentation, polyphenol concentration, especially that of procyanidin, decreases due to oxidation, condensation, and diffusion out of the beans (Nazaruddin et al., 2006). In a report where on-farm spontaneous cocoa bean fermentation was conducted in Ghana in two different seasons at two different locations, total polyphenol decreased by approximately 10-50%, while more than 70% of epicatechin content was lost (Camu, De Winter, et al., 2008). To determine whether a similar trend occured in our model system, total polyphenol and total procyanidin concentration were analyzed and found to decrease by 19% and 60%, respectively. The sharper decrease (60%) in procyanidin concentration compared to that of total polyphenols is in agreement with the findings of Ryan et al. (2016), and demonstrates a higher sensitivity of procyanidin to fermentation compared to total polyphenols. The decrease in total procyanidin content occurred mainly during the 72-96 hours time period. One possible reason for this observation is that the incubation temperature of 45°C during this time period promoted degradation since the optimum temperature of polyphenol oxidase is known to be around 45°C (Lee et al., 1991)**.** It is especially important to understand the effects of cocoa fermentation on polyphenols. Both polyphenol concentration and composition have been demonstrated to impact

bioactivity relevant to human health (Bitzer et al., 2015; Dorenkott et al., 2014; Ryan et al., 2017).

4. Conclusion

Fermentation process conditions are important determining factors of cocoa quality and value. Even though similar type of yeast, LAB, and AAB are observed in cocoa fermentations in different environments and geographic regions, microbial growth patterns are very different. Flavor and polyphenol composition of fermented cocoa are likely influenced by growth patterns, since microbial metabolic activity results in formation of flavor precursors and the oxidation of polyphenol compounds inside the bean. The major microorganisms identified in our model system, including *S. cerevisiae*, *L. plantarum*, and *A. pasteurianus* were recently used as starter cultures for cocoa fermentation in a study demonstrating the potential for inoculation of cocoa heaps with a microbial cocktail to impact flavor volatiles (Magalhães da Veiga Moreira et al., 2017). Use of our model system for similar evaluations in the laboratory setting could streamline the process of developing and testing starter cultures for cocoa fermentation. In conclusion, the model fermentation system developed in this study can facilitate evaluation of the relationship between microbial activity and fermented bean quality. Practical strategies for better control of on-farm cocoa fermentation will lead to more consistent fermented cocoa of higher quality and value.

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Figure legends

Figure 1. Moisture content and pH profiles during 6 days of cocoa model fermentation. (A): Moisture content of cocoa bean (●). (B): pH of pulp medium (■), pH of nib (▲).

Figure 2. Microbial count from three different selective medium during 6 days of cocoa model fermentation. (A): Yeast (YM, ▼), (B): LAB (MRS, ◆), and (C): AAB (GYC, ⬣).

Figure 3. Concentrations of sucrose (●), glucose (▲), and fructose (■) in cocoa pulp (A) and nib (B). Concentration of ethanol (●) and glycerol (▲) in pulp (C) and nib (D). Concentration of citric acid (●), succinic acid (▲), lactic acid (■), and acetic acid (▼) of cocoa pulp, (E) and nib (F). Values represent mean ± SD for each time point (n=3 replicate samples from different areas of the fermenting mixture).

Figure 4. Concentrations of alcohols (A), acids (B), aldehydes and ketones (C), and esters (D) during fermentation analyzed by GC-MS. Values represent mean ± SD for each time point (n=3 replicate samples from different areas of the fermenting mixture).

Figure 5. Concentration of total polyphenol (A) and total procyanidin (B) during 144 hours of cocoa model fermentation. Values represent mean ± SD for each time point (n=3 replicate samples from different areas of the fermenting mixture).

Figure 1.

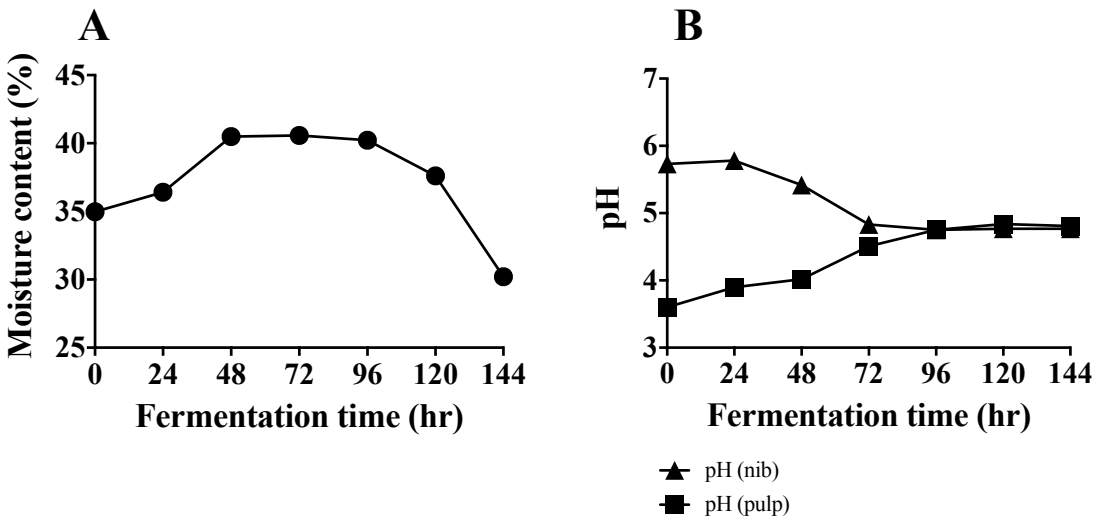
A
Moisture content (%)
45
40
35
30
25
0
24
48
72
96
120
144
Fermentation time (hr)
B
pH
7
6
5
4
3
0
24
48
72
96
120
144
Fermentation time (hr)
pH (nib)
pH (pulp)

Figure 2.

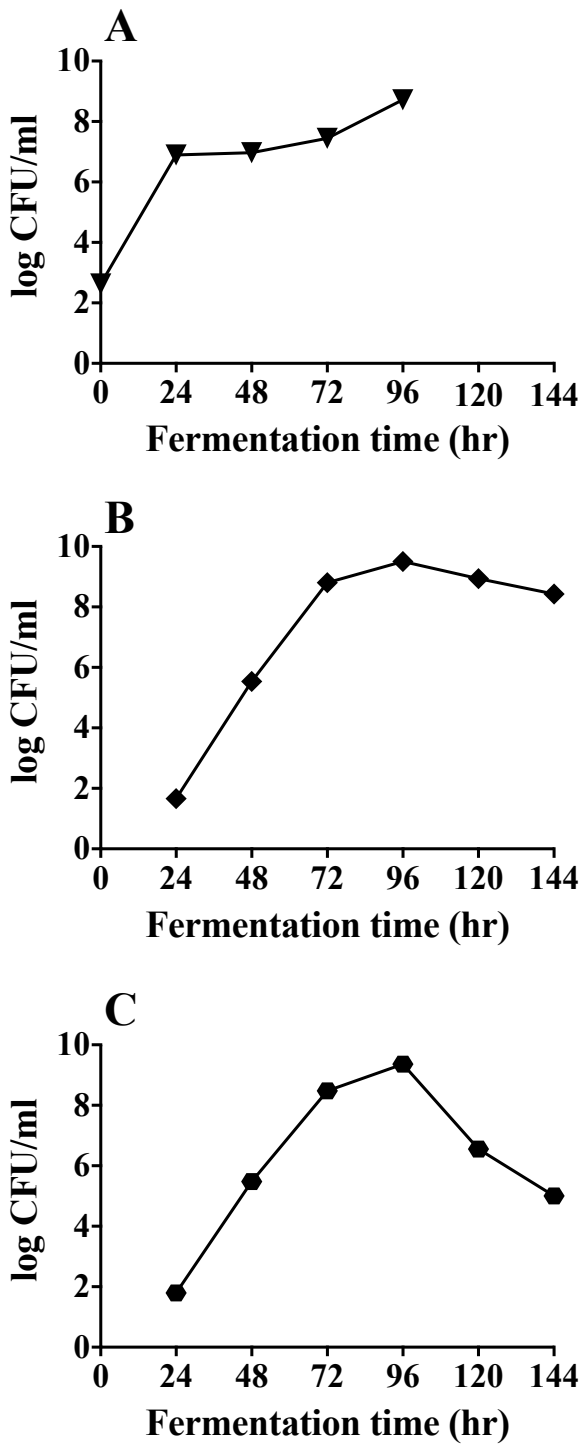
A
log CFU/ml
0
2
4
6
8
10
0
24
48
72
96
120
144
Fermentation time (hr)
B
log CFU/ml
0
2
4
6
8
10
0
24
48
72
96
120
144
Fermentation time (hr)
C
log CFU/ml
0
2
4
6
8
10
0
24
48
72
96
120
144
Fermentation time (hr)

Figure 3.

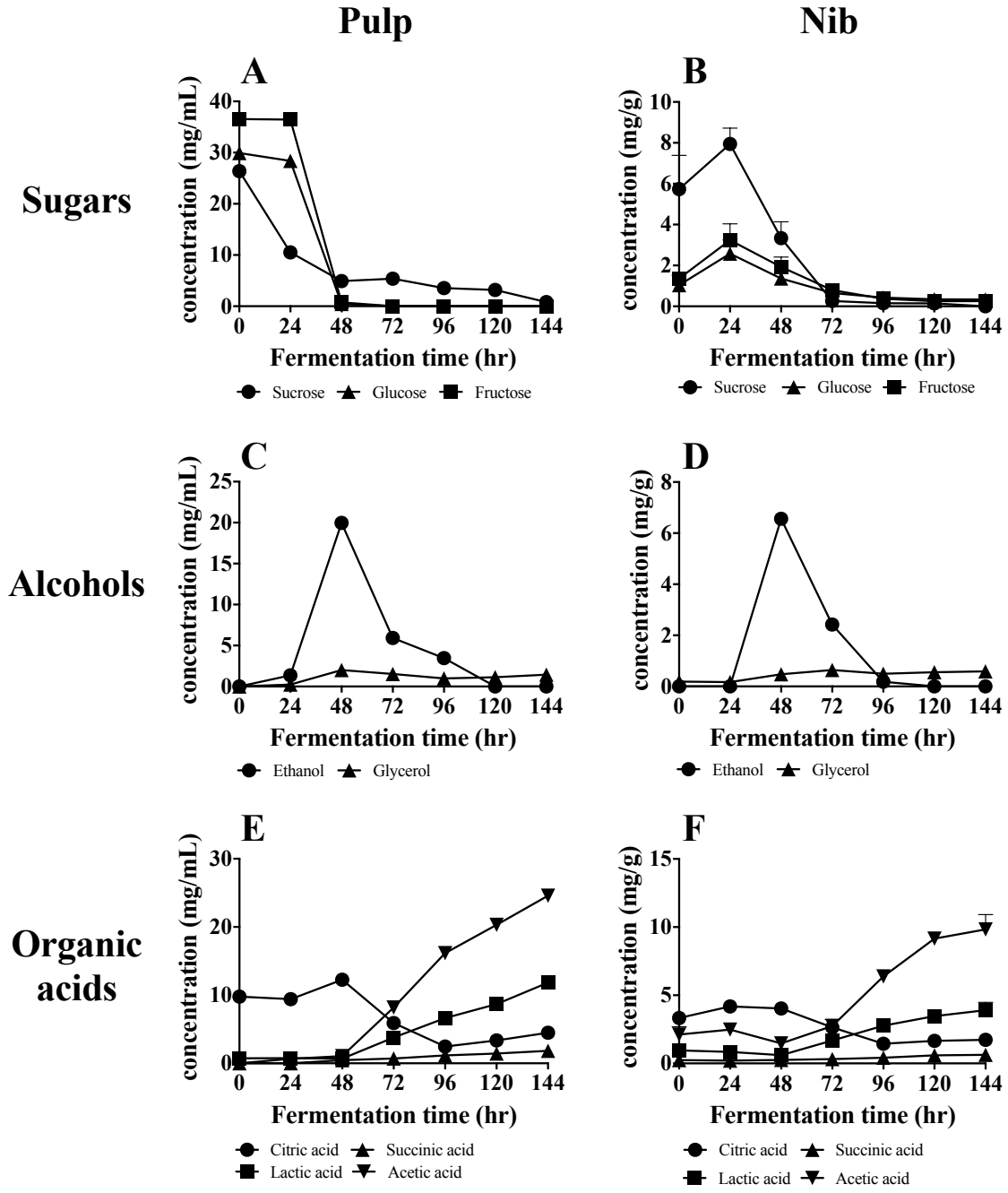
Pulp
Nib
Sugars
Alcohols
Organic acids
A
B
C
D
E
F
concentration (mg/mL)
concentration (mg/g)
Fermentation time (hr)
Sucrose
Glucose
Fructose
Ethanol
Glycerol
Citric acid
Succinic acid
Lactic acid
Acetic acid

Figure 4.

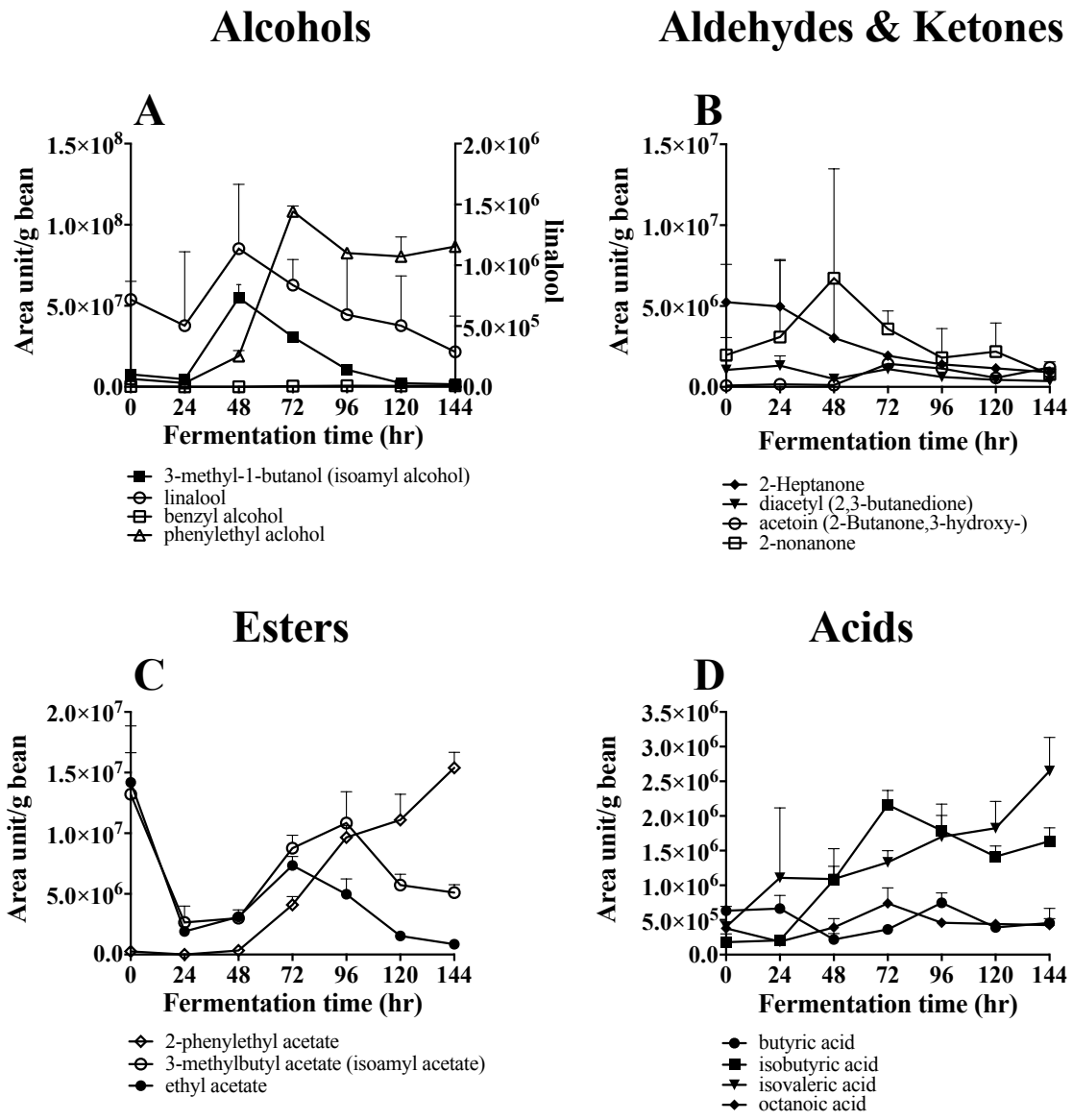
Alcohols
Aldehydes & Ketones
A
B
Area unit/g bean
linalool
Fermentation time (hr)
3-methyl-1-butanol (isoamyl alcohol)
linalool
benzyl alcohol
phenylethyl aclohol
2-Heptanone
diacetyl (2,3-butanedione)
acetoin (2-Butanone,3-hydroxy-)
2-nonanone
Esters
Acids
C
D
2-phenylethyl acetate
3-methylbutyl acetate (isoamyl acetate)
ethyl acetate
butyric acid
isobutyric acid
isovaleric acid
octanoic acid

Figure 5.

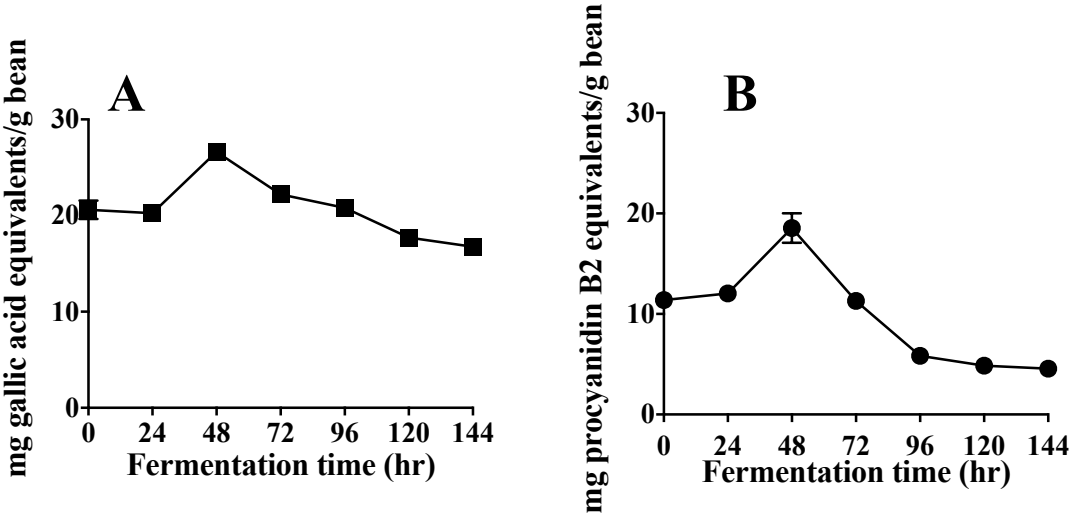
A
mg gallic acid equivalents/g bean
30
20
10
0
0 24 48 72 96 120 144
Fermentation time (hr)
B
mg procyanidin B2 equivalents/g bean
30
20
10
0
0 24 48 72 96 120 144
Fermentation time (hr)

Table 1. Composition of simulated cocoa pulp medium

Component	Mass	
Sucrose	25g	
Glucose	40g	
Fructose	45g	
Citric acid	10g	(a)
Yeast extract	5g	
Peptone	5g	
Calcium lactate-pentahydrate	1g	
Tween 80	1g	
Magnesium sulfate-heptahydrate	0.5g	
Manganese sulfate-monohydrate	0.2g	(b)

[(a)] Compounds in this section was filled up with distilled water to give final volume 980mL and pH was adjust to 3.6 with NaOH

[(b)] These two components were mixed with 20mL of distilled water, filter sterilized and added to the autoclaved solution to give final volume 1L

Table 2. Microorganisms isolated by different selective medium during 6 days of fermentation identified by sequence of 16S rDNA or ITS1 amplicon sequencing.

Fermentation (hours)	YM isolated	MRS isolated	GYC isolated
0	*S. cerevisiae* (5)[b]	N.D.	N.D.
24	*S. cerevisiae* (10)	*L. plantarum* (12)	*A. pasteurianus* (7) *L. plantarum* (4)
48	*S. cerevisiae* (9)	*L. plantarum* (8)	*A. pasteurianus* (3) *L. plantarum* (9) *A. tropicalis* (1)
72	*S. cerevisiae* (10)	*L. plantarum* (9)	*L. plantarum* (11)
96	*S. cerevisiae* (11)	*L. plantarum* (6) *L. fermentum* (3)	*L. plantarum* (13)
120	N.D.[a]	*L. plantarum* (3) *L. fermentum* (6)	*A. pasteurianus* (1) *L. plantarum* (8)
144	N.D.	*L. plantarum* (1) *L. fermentum* (9)	N.D.

[a] not detected

[b] number of identified colonies per plate

Target Journal: Applied and Environmental Microbiology

Chapter 5:

The impact of yeast starter cultures (*Saccharomyces cerevesiae* Lev F and Lev B) on cocoa fermentation biochemistry including polyphenol composition and concentration

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Running title: Yeast starter culture could impact cocoa polyphenol

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Abstract

The use of starter cultures to improve fermentation and quality of cocoa beans is of increasing interest to cocoa producers. Inoculation with yeast starter cultures can enhance flavor characteristics of cocoa bean and hasten the fermentation process. Despite the recent interest in this technique, little is known regarding the influence of yeast starter cultures and their metabolites on cocoa polyphenol concentration and composition. The purpose of this study was to determine the impact of two yeast strains *Saccharomyces cerevisiae* Lev F and *Saccharomyces cerevisiae* Lev B on metabolite production in the fermentation system and on polyphenol profiles of cocoa beans. A model fermentation system using dried unfermented beans, simulated pulp medium, and a laboratory incubator was used to ferment cocoa beans. Starter culture inoculation resulted in more consistent fermentation performance including microbial succession, pH and metabolite production. Decreases in the concentrations of total polyphenols and procyanidins occurred one day earlier in both inoculated fermentations, compared to un-inoculated control. The post-fermentation total polyphenol and procyanidin concentrations remained higher in fermentation inoculated with *Saccharomyces cerevisiae* Lev B. Along with these results, further characterization of the concentrations of individual polyphenols will help to further efforts to link the relationship between cocoa fermentation management and bioactivity.

Introduction

The annual global production of cocoa is 4 million tons (1) with an average price of US $2,196 per ton in January, 2017 (2). The majority of dried fermented cocoa beans (95 % of total production) are produced by small farms (3, 4). Immediately after cocoa pods are harvested, the beans are removed from the pods and subsequently undergo fermentation. Through fermentation, important flavor precursors are produced (peptides, free amino acids, and reducing sugars) (5, 6), bitterness and astringency decrease (mainly through polyphenol oxidation) (7-9). Poorly fermented cocoa beans may lack characteristic cocoa flavor or exhibit off-flavors and strong bitterness, all resulting in the loss of value and reduced revenue to cocoa farms (10).

Fermentation is initiated when the harvested cocoa bean and the surrounding pulp are piled into heaps or loaded into fermentation boxes (11). Despite the fact that the microbial populations present in cocoa fermentation include many different microorganisms present in the surrounding environment, core groups of microbiota are generally consistent among various cocoa fermentation sites (2). Yeast, lactic acid bacteria, and acetic acid bacteria are considered to be the essential microorganisms present during cocoa fermentation (12). Yeast predominates the first stage of fermentation because the initial conditions of cocoa pulp (high sugar concentration and acidity) and anaerobic fermentation environment are favorable for yeast growth. *Saccharomyces*, *Candida*, *Hanseniaspora*, *Pichia*, and *Torula* species are common yeast species found in cocoa fermentation, with *Saccharomyces cerevisiae* being the most common yeast species identified across geographic sites *(2, 13-15)*. The role of yeasts in cocoa fermentation is well characterized by V. T. T. Ho, et al. (12). Yeasts are responsible for pulp degradation, breakdown of citric acid, production of ethanol, and generation of important flavor compounds including higher alcohols and esters. It has been proposed that yeasts promote acetic acid

bacterial growth by increasing the pH (from pH 3.5 to 4.2-5.0) by citric acid consumption (16, 17) and providing ethanol which in turn is consumed by acetic acid bacteria (15, 16). In addition, degradation of pulp allowing more air ingress, thus increasing the oxygen concentration and allowing subsequent growth of bacteria which require oxygen (7).

Lactic acid bacteria (LAB) metabolize pulp sugars and citric acid to produce mainly lactic acid as well as small amounts of acetic acid, ethanol, CO_2. The most abundant species of lactic acid bacteria found in cocoa fermentations worldwide are *Lactobacillus plantarum* and *Lactobacillus fermentum (18)*.

Acetic acid bacteria produce acetic acid, a key metabolite for cocoa fermentation, via oxidation of ethanol (2). The exothermic reactions of acetic acid bacterial metabolism during cocoa fermentation increase heap temperature to 45-50°C, thus facilitating the penetration of pulp metabolites in to the beans. Acetobacter and Gluconobacter spp. are the most commonly found species during cocoa fermentation (18).

The combined reactions occurring during metabolism by these essential microorganisms has the potential to significantly affect the polyphenol profiles of cocoa beans (19). Decrease in flavanol concentration could be attributed to increased internal bean temperature increasing the rate of degradation reactions, and decrease in pH coupled with penetration of acids (microbial metabolites) into the bean resulting in the disintegration of cell compartments, allowing internal polyphenol oxidase to become active (20). Studies have been shown that decrease in total polyphenol concentration and flavanol monomers including catechin and epicatechin were observed during fermentation. (21-23).

Polyphenol compounds in cocoa bean have been the subject of increasing research interest not only for their impact on sensory characteristics (bitterness and astringency), but also

due to their potential for improving human health. Cocoa intake is associated with reduced risk of various diseases including cardiovascular disease and metabolic syndrome (24, 25). Studies investigating the impact of fermentation on cocoa polyphenols have mainly focused on change in total polyphenolic compounds analyzed by colorimetric assays or changes in individual flavanol monomers like catechin and epicatechin (21, 23, 26, 27). These studies provide useful information, however, other polyphenolic constituents such as oligomeric and polymeric procyanidins may also impart physiologically relevant impacts. As emerging evidence suggests that oligomeric and polymeric procyanidins play a role in alleviation of metabolic syndrome and their related diseases (24, 28, 29), it is important to thoroughly characterize the fate of polyphenols during cocoa fermentation.

The purpose of this study was to determine the impact of yeast starter culture inoculation on polyphenol concentration and composition during cocoa fermentation. While effects on flavor characteristics and quality of beans have been widely studied (10, 30, 31), no study has focused on the microbial effects on detailed polyphenol profiles. Using the model system described in Chapter 3 of this dissertation, two yeast starter cultures *Saccharomyces cerevisiae* Lev F (LF) and *Saccharomyces cerevisiae* Lev B (LB) were evaluated. The impact of inoculation of each starter culture on polyphenol profiles as well as production of metabolites in the system was assessed and compared to an uninoculated control.

Results

pH

Control fermentation (no starter culture added) resulted in inconsistent pH of the pulp between the two replicated batches (Figure 1A). Although the two fermentations were initiated in

the same day and incubated in the same condition, the pulp pH of the first control fermentation (control-1) reached a peak of pH 4.5 (144 hour-time point) while the maximum pH of control-2 fermentation was 5.0 (72 hour-time point). However, the pH of cocoa nib for both controls showed similar decreasing trends in that the final value was pH 5 for control 1 and pH 4.9 for control 2 (Figure 1B).

The mean pH values were reported for both LF and LB inoculated fermentations since the trends in pH change were almost identical between the replicated batches. The pulp pH of LF and LB fermentation reached a peak of pH 4.5 at the 72 hour-time point (Figure 1C and 1E). The nib pH of LF and LB fermentations decreased to a final pH of 4.5 and 4.6 respectively at the end of fermentation, which was slightly lower than control fermentations (Figure 1D and 1F).

Microbial counts

Control-1 and control-2 fermentation batches again showed inconsistent results in microbial counts. The difference in yeast population was observed from 120 to 144 hour-time points. The final count of yeast in control-1 fermentation was 6.8 log CFU/mL while control-2 fermentation dropped to 5.3 log CFU/mL (Figure 2A). Differences were even greater in LAB and AAB populations for the control (un-inoculated) batches. The final LAB counts for control-1 and control-2 fermentations were 3.7 and 8.0 log CFU/mL, respectively (Figure 2B) and the final AAB counts were 2.9 log CFU/mL for control-1 fermentation and 7.2 log CFU/mL for control-2 fermentation (Figure 2C). By comparing these results with the model fermentation developed in chapter 3, control-1 fermentation in this study was considered as a failed cocoa fermentation.

Due to the consistency of the microbial count results shown between LF and LB fermentation replicates, mean values were reported for these treatments. Yeast count in the LF fermentation was 7.0 log CFU/mL at the initial time point (due to inoculation) and reached a

maximum population of 8.4 log CFU/mL (48 hours) then rapidly decreased from 96 hour (6.7 log CFU/mL) to the 120 hour (1.6 log CFU/mL)-time point. Yeast population was below the detection limit at 144 hour-time point and was thus not plotted (Figure 2D). Yeast count in the LB fermentation was initially log 6.8 CFU/mL, reached a peak at the 72 hour-time point, then decreased sharply from the 96 hour (7.4 log CFU/mL) to the 120 hour (3.3 log CFU/mL) time point. Yeast count was below the detection limit at the end of the fermentation and was thus not plotted at the 144 hour-time point (Figure 1G).

LAB counts for both LF and LB fermentations reached their maximum (LF: 8.9 log CFU/mL and LB: 9.1 log CFU/mL) at the 72 hour-time point. However, LAB in LF fermentation remained higher at the end of fermentation (7.5 log CFU/mL) compared to LAB in LB fermentation (6.0 log CFU/mL) (Figure 2E and 2H). AAB in LF fermentation reached the highest population of 8.7 log CFU/mL (72 hours) and AAB in LB fermentation reached 8.3 log CFU/mL (96 hours). AAB in LF fermentation remained higher at the end of fermentation (4.5 log CFU/mL) than AAB in LB fermentation (3.9 log CFU/mL) (Figure 2F and 2I).

Sugars, alcohols, and organic acids

Control-1 and Control-2 fermentation did not show differences in sugar consumption patterns in the pulp even though microbial growth pattern differed (Figure 3A and 3C). Likewise, sugar consumption patterns were similar in the cocoa nibs for both control batches (Figure 3B and 3D). The biggest difference in cocoa pulp sugar concentrations between control and starter culture inoculation (both LB and LF) was that there was no lag phase for glucose or fructose consumption at the 0 to 24 hour-time points in the inoculated fermentation (Figure 3E, 3G, 3I, and 3K). This in turn affected the sugar concentration of cocoa nibs. The glucose and sucrose concentration in cocoa nibs in the control fermentation increased from the initial stage to 48

hour-time point (Figure 3B, 3D). However, the glucose and fructose concentration of the cocoa nibs from LF fermentation decreased from 24 hour-time point (Figure 3F and 3H) and for LB fermentation, the concentration decreased from the 0 hour-time point (Figure 3J and 3L).

Ethanol and glycerol concentration in cocoa pulp and nibs during fermentation showed similar patterns between the control-1 and the control-2 fermentation (Figure 4A, 4B, 4C and 4D). Ethanol concentration in both pulp and nibs reached the highest concentration at the 48 hour-time point and decreased constantly until the end of the fermentation. Glycerol was produced from 24 hours in pulp and increased in the nibs from that time point as well. Yeast inoculation resulted in different trends regarding ethanol and glycerol production compared to the control fermentation (Figure 4E through 4L). No lag phase was reflected in the production of these two compounds in the inoculated batches, (unlike the control fermentation, which did not see production of these compounds between 0-24 hours) and their concentrations were already high at the 24 hour-time point. However, differences in maximum concentrations of ethanol and glycerol were observed between LF, LB, and the control fermentation. Due to the consistency of the results shown between LF and LB fermentation replicates, mean values of the two biological replicates were reported. LF fermentation reached a lower maximum ethanol concentration in the pulp (27.0 g/L) compared to LB (41.2 g/L) and to the control fermentation (41.2 g/L). However, LF fermentation produced the highest glycerol fermentation in the pulp (9.60 g/L) compared to LB (5.21 g/L) and control fermentation (4.25 g/L). While this trend in glycerol production reflected the concentration in the nibs as well (LF bean having the highest glycerol concentration), ethanol concentrations in the nibs were similar between the treatments (12-13 g/L).

As mentioned previously, control-1 fermentation was regarded as a "failed" fermentation since the bacterial population did not increase to the expected level, as observed in the work reported in Chapter 3 of this dissertation. This was also confirmed by comparing acid concentrations in the pulp from control-1 and control-2 fermentation. First, citric acid was not consumed at all in the pulp of control-1 fermentation (Figure 5A) while the concentration of citric acid decreased from the 48 hour-time point in control-2 fermentation (Figure 5C). Second, acetic acid and lactic acid concentration remain very low in the pulp and nibs from control-1 fermentation. LF and LB inoculation resulted in more consistent pattern in acid concentration between replicates and therefore, their concentrations were reported in average value (Figure 5E through 5L). While citric acid consumption, and acetic acid production was similar in the pulp between the two inoculated fermentation, LB fermentation produced more lactic acid in both pulp (4.2 g/L) and nibs (3.7 g/L). Citric acid concentration in the cocoa nib constantly decreased until the end of fermentation when inoculated with starter cultures whereas for the control fermentation, the nib citric acid concentration decreased to a lesser extent and even increased from 96- to 144-hour time points.

Total polyphenol and procyanidin

Total polyphenol concentration of the cocoa beans did not show a decreasing trend in control-1 fermentation over the time course of fermentation except that a lower concentration was observed at the 72 hour-time point than other time points (Figure 6A). Control-2 fermentation showed a decreasing trend in total polyphenol concentration but no significant difference ($p < 0.05$) was observed through the time course of fermentation (Figure 6B). Total polyphenol concentration of the cocoa beans in yeast inoculated fermentations showed differences during fermentation (mean value reported for LF and LB fermentations). A

significant difference was first observed in LB fermentation between 24 hour- (29.6 ± 3.23) and 48 hour- (24.0 ± 3.82) time points and in LF fermentation between 0 hour- (36.3 ± 0.60) and 24 hour- (27.8 ± 1.09) time points. The final total polyphenol concentration was the lowest (19.7 ± 0.95) in LF fermentation compared to LB fermentation (25.3 ± 1.62) and control-2 fermentation (25.3 ± 3.06) (Figure 6).

The trend in the decrease of total procyanidin concentration in the cocoa beans differed between the two control fermentations (Figure 7A and 7B). For control-1 fermentation, total procyanidin was decreased between 48 hour- (20.3 ± 3.98) to 72 hour- (11.0 ± 1.06) time points while total procyanidin in control-2 fermentation decreased between 24 hour- (25.9 ± 2.13) to 48 hour- (16.5 ± 0.75) time points. Both yeast starter culture inoculations (LB and LF) resulted in a faster decrease of total procyanidin concentration in the cocoa beans compared to control fermentations. Significant decrease was observed from the 0 hour- (27.0 ± 2.52) to the 24 hour- (20.6 ± 0.15) time points for LB fermentation and from the 0 hour- (23.0 ± 1.30) to the 24 hour- (17.9 ± 1.77) time points for LF fermentation (Figure 7C, 7D, 7E, and 7F). The final total procyanidin concentration was lower (11.6 ± 0.80) in LF fermentation compared to LB (15.9 ± 2.25) fermentation, which was a similar trend observed in the total polyphenol concentration analysis.

Discussion

Impact of starter culture inoculation

Cocoa is one of the few remaining fermented food products for which spontaneous fermentation, which employs the natural microbiota from the surrounding environment, is performed on an industrial scale. Due to this processing approach, the quality of fermented cocoa

bean is often variable between different regions or even from batch to batch within the same fermentation site (16, 32, 33).

Although the two control fermentations conducted in this study were initiated in the same day with the same fermentation conditions, different outcomes including pH of the pulp, microbial dynamics, and their resulting metabolite production were observed. Citric acid was not consumed in control-1 pulp and the essential metabolites such as lactic acid and acetic acid were produced at very low levels. Absence of these metabolites could lead to unsuccessful fermentation resulting in cocoa bean with excess bitterness/astringency, and insufficient production of desirable flavor precursors (8). On the other hand, the use of starter culture in this study resulted in more consistent fermentation outcomes. Even though yeast was the only species inoculated, LAB and AAB were both well generated in the pulp media (even reached a slightly higher population) in the inoculated treatments. Possible explanations are: 1) LAB could either use sugar or citric acid as a carbon source, thus not competing with yeast for sugar for their growth (30). This is observed in Figure 5E and 5G where citric acid concentrations were not decreasing from the initial time point (high yeast population) but rapidly decreasing from the 48 hour- to the 72 hour-time points. During this period, exponential growth of LAB population was observed (Figure 2E and 2H). 2) AAB have a commensal relationship with yeasts in the cocoa fermentation environment in that AAB consumes ethanol, which is produced by yeasts (33, 34), thus growing well in the environment.

A beneficial effect of employing a starter culture in cocoa fermentation was observed in this study. Due to the high population (>6 log CFU/ml) of yeasts present from the initial stage of fermentation, ethanol and glycerol were produced one day earlier in both pulp and nibs compared to the control fermentation. It is known that ethanol could accelerate the de-

compartmentalization (bean death) of the internal bean, facilitating endogenous enzymes to initiate various biochemical processes, and resulting in a shorter time required to complete fermentation (35). Indeed, while no significant reduction in total polyphenol concentration was observed in either of the control fermentation batches, total polyphenol concentration decreased during fermentation in both the LF and LB inoculated cocoa beans. Moreover, total procyanidin concentration significantly decreased one day earlier in LF and LB inoculated beans compared to the control-2 fermented beans.

Difference between LF and LB inoculated fermentations.

An interesting difference in fermentation activity between LF inoculated and LB inoculated fermentation was observed. It should be noted that initial sucrose concentration was not identical for these two fermentations, likely due to insufficient sucrose being added in the pulp for LB treatment, and initial sugar concentration of the cocoa nibs was higher compared to the nibs in the other treatment, therefore making direct comparison difficult. Also initial citric acid concentration of nibs in LF fermentation was lower compared to the nibs in control and LB fermentation. However, even though the pulp from LF fermentation had a higher total sugar concentration, production of ethanol in the pulp was 66% lower compared to LB fermentation while glycerol production was 84% higher in the LF fermented pulp. The *in vitro* fermentation activities between yeast starter cultures observed by the manufacturer of these ADY strains were in accordance with our results. It was reported that the glycerol production yield of LF from high sugar content medium was 7% while LB was only 3%. On the other hand, ethanol was produced at a lower concentration by LF compared to LB (unpublished results, Lallemand, Inc.). Inoculation with yeast starters cultures could therefore be an effective tool for control or modification of such properties (ethanol and glycerol production) during cocoa fermentation.

Although the decreasing trend of total polyphenol and procyanidin concentration was similar between LF and LB fermentation, the final concentrations of total polyphenol and procyanidin were higher in LB fermentation. This was interesting since the final ethanol and acetic acid concentrations were similar in the bean. One possible explanation for this is mentioned in the study by V. J. E. Evina, et al. (20) suggested that lactic acid can inhibit the degradation of flavanol monomers during cocoa bean fermentation. Since lactic acid was produced 44% higher in LB fermentation, it could be possible that total polyphenol and procyanidin were degraded to a lesser extent. However, the impact of lactic acid not only on the monomers but on the total polyphenol and procyanidin concentration should be further examined.

Conclusions

Currently, starter cultures are used to produce consistent and reliable fermented food and beverage products and even to improve their flavor and quality. Although commercial cocoa fermentation currently relies on spontaneous fermentation, a number of studies have demonstrated the potential for starter culture inoculation to improve flavor characteristics and reduce fermentation time in cocoa fermentation systems (30, 33, 35-38). The model system utilized in this study effectively tested the impact of yeast start culture addition on cocoa fermentation. Yeast starter inoculation resulted in more consistent microbial and chemical qualities and accelerated the fermentation process (early degradation of total polyphenol and procyanidins) compared to the un-inoculated control groups. The model system also tested the difference between the LF and LB fermentation outcomes and confirmed that different strains of yeast starters could result in different polyphenol and procyanidin concentrations. To best of our knowledge, this is the first study to test the impact of starter cultures on total polyphenol and procyanidin contents. Detailed profiling of individual polyphenol compounds should be

conducted as this information is of interest to researchers investigating the mechanisms underlying the impact of cocoa polyphenols on human health. Additionally, studies applying defined mixed starter cocktails (yeast, AAB and LAB) merit further investigation for potential to improve the control and outcome of cocoa fermentation.

Materials and methods

Dried unfermented bean and simulated pulp medium

The model fermentation system developed in Chapter 3 of this dissertation was used to conduct the fermentation experiments described in this chapter. Dried unfermented beans were obtained from an on-line retailer (Feeling Better Every Day, Fennimore, Wisconsin, USA). Beans were Criollo variety and grown in Ecuador. After being removed from the pods, beans were washed to remove the pulp and dried before packaging.

Simulated pulp media described in Chapter 3 Table 1 was utilized in the model system. Briefly, the medium contained sugars, citric acid, yeast extract, peptone, calcium lactate, tween 80, magnesium, and manganese, and the pH was adjusted to 3.6. The pulp conditions were optimized to support growth of the typical microorganisms previously observed in on-farm cocoa fermentation and considered essential for successful cocoa fermentation.

Active dried yeast starter culture and rehydration

Active dried yeast *S. cerevisiae* Lev F and Lev B were obtained from Lallemand, Inc. (Blagnac, France). For rehydration, 7 g of active dried yeast was mixed with 70 mL of pre-heated (40°C) ultrapure water (Direct-Q® Water Purification System, EMD Millipore, Darmstadt, Germany). The mixture was incubated at 40 °C for 20 min for yeast rehydration prior to inoculation.

Model fermentation of cocoa bean and experimental treatment

Dried unfermented beans were rehydrated with ultrapure water, as previously described. When the bean moisture content reached equilibrium, which occurred at 35% moisture, 2 kg of rehydrated beans, 3.5 L of simulated pulp medium and 70 mL of rehydrated active yeast starter culture were mixed into a 30 cm × 44.2 cm × 16.9 cm fermentation box (Polypropylene, IRIS USA, Inc., WI). Temperature and agitation of the box and its contents were controlled using a standard laboratory incubator (New Brunswick^{TM} Innova 42, Eppendorf, Hauppauge, New York, USA). Temperature conditions for the incubator over the time course of fermentation were as follows: 25°C (0-24 hr), 35°C (24-48 hr), 40°C (48-72 hr), 45°C (72-96 hr), 48°C (96-144 hr) and agitation was as follows: 0 rpm (0-24 hr), 120 rpm (24-36 hr), 150 rpm (36-144 hr).

Fermentation experiments were conducted in duplicate, and included three different treatments: (1) control fermentation with no starter culture addition, (2) inoculation with *S. cerevisiae* Lev B, and (3) inoculation with *S. cerevisiae* Lev F. Every 24 hours during the fermentation, samples of cocoa beans and the liquid portion of the medium were collected (separately) for chemical and microbial analyses. Microbial plate counts were performed immediately after sampling and samples for chemical and molecular assays were stored at −80 °C until the time of analysis. Prior to each sampling step, cocoa beans and pulp media were thoroughly mixed with gloved hands.

Microbial enumeration

The detailed methods used for microbial enumeration are provided in Chapter 3 of this dissertation. Briefly, plate counts were performed on the liquid portion of the fermenting media, sampled every 24 hours during fermentation, to enumerate yeasts, LAB and AAB. YM agar medium containing 100 mg/L of oxytetracycline was used to selectively grow yeasts at 30°C. MRS and GYC agar medium with 400 mg/L of cycloheximide were used to culture LAB in an

anaerobic condition (37°C) and AAB in an aerobic condition (30°C) respectively. Anaerobic conditions were generated using GasPAK^{TM} EZ gas generating system (BD, USA). Total plate counts were performed in duplicate.

pH

The detailed method used for pH analysis is described in Chapter 3 of this dissertation. Briefly, pH of the cocoa nib was measured using a standard approach. Shell of the cocoa beans was pealed and the resulting nibs (5 g) were crushed and thoroughly mixed with 100 mL of hot ultrapure water and filtered through Whatman #4 filter paper and the pH of the filtrate was measured. The liquid portion of fermentation media (10 mL) was combined with 9 mL of ultrapure water and homogenized before measuring the pH of this homogenate.

Sugars, alcohols and organic acids analysis

Sugars (glucose, fructose, and sucrose), alcohols (ethanol and glycerol) and organic acids (citric acid, lactic acid, and acetic acid) were analyzed in cocoa pulp and nib over the time course of fermentation, with samples taken every 24 hrs. The liquid portion of the media was diluted 10 times by mixing 5 mL of sample with 45 mL of ultrapure water and vortex mixing for 2 min. Cocoa nibs (10 g) were mixed with 90 mL of ultrapure water and homogenized in a Waring laboratory blender (Waring Products, New Hartford, USA) for 3 min. Both samples were centrifuged at 2,500 × *g* for 10 min at 5°C and the supernatants were collected. Supernatants were filtered through 0.45 uM PVDF membrane filter (Thermo Fisher Scientific, Waltham, MA, USA) and HPLC analysis was performed. Agilent HPLC 1200 Infinity Series (Agilent Technologies, California, USA) equipped with Aminex HPX-87H column (300 mm × 7.8 mm, 50°C, Bio-Rad, California, USA) and Refractive Index (RI) detector were used for the analysis. Mobile phase used was 0.005 M H_2SO_4 aqueous solution and the flow rate was 0.6 mL/min.

Injection volume was 5 µl for each sample and standard compounds (Sigma-Aldrich, St. Louis, MO, USA). Authentic analytical standards were used in quantification of all compounds evaluated in this study. All analyses were performed in triplicate for statistical analysis.

Polyphenol extraction

Extraction of polyphenols prior to analysis was performed using the method described by M. R. Dorenkott, et al. (28) with minor modifications. The method is described in detail in Chapter 3 of this dissertation.

Total polyphenol and procyanidin assays

Total polyphenol concentration (total antioxidant/reducing activity) was quantified from the cocoa polyphenol extract using the Folin-Ciocalteu method described by A. L. Waterhouse (39) with modification. Total polyphenol concentration was expressed as mg gallic acid equivalents/g cocoa bean ± SD. Total procyanidin concentration was analyzed using the 4-Dimethylaminocinnemaldehyde (DMAC) method described by M. J. Payne, et al. (21) with minor modifications. Results were expressed as mg procyanidin B2 equivalents/g cocoa bean ± SD. Detailed methods for both assays are provided in Chapter 3 of this dissertation. Analyses were performed in triplicate.

Statistical analysis

Data for control-1 and control-2 fermentation (one batch for each control) were expressed as mean ± SD for n=3 samples analyzed at each time point, and for *Saccharomyces cerevisiae* LB and LF treated fermentation (two batches for each treatment), data were expressed as mean ± SD for n=6 total samples obtained at each time point. This was due to the need to clearly illustrate the inconsistent results observed between the two batches of control fermentation. To determine the significant differences for each compound of interest between treatments or within

the same treatment, 1-way ANOVA followed by Tukey's HSD post-hoc test for all possible comparisons was used with differences being considered significant when $p < 0.05$. Statistical analyses were conducted using Prism v6.0e (GraphPad Software, Inc., La Jolla, CA).

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Figure titles and legends.

Figure 1. pH of control-1, control-2, LF, and LB treated cocoa nibs during 144 hours of cocoa model fermentation: control 1 (■), control 2 (▲), LF1 (●), LF2 (◆), LB1 (⬣), and LB2 (▼). Error bars represent standard deviation of analytical replicates.

Figure 2. Microbial count from three different selective media during 6 days of cocoa model fermentation: control 1 (■), control 2 (▲), LF1 (●), LF2 (◆), LB1 (⬣), and LB2 (▼). YM is selective for yeast, MRS is selective for LAB, and GYC is selective for AAB. Error bars represent standard deviation of analytical replicates.

Figure 3. Concentration of sugars in control-1, control-2, LF-1, LF-2, LB-1, and LB-2 treated cocoa pulp and nib: sucrose (●), glucose (▲), and fructose (■). Error bars represent standard deviation of analytical replicates.

Figure 4. Concentration of alcohols in control-1, control-2, LF-1, LF-2, LB-1, and LB-2 treated cocoa pulp and nib: ethanol (●) and glycerol (▲). Error bars represent standard deviation of analytical replicates.

Figure 5. Concentration of organic acids in control-1, control-2, LF-1, LF-2, LB-1, and LB-2 treated cocoa pulp and nib: citric acid (●), succinic acid (▲), lactic acid (■), and acetic acid (▼). Error bars represent standard deviation of analytical replicates.

Figure 6. Total polyphenol concentrations in cocoa beans over the course of fermentation. Error bars represent standard deviation of analytical replicates. Different letter refers to significant difference when $p < 0.005$

Figure 7. Total procyanidin concentrations in cocoa beans over the course of fermentation. Error bars represent standard deviation of analytical replicates.

Figure 1.

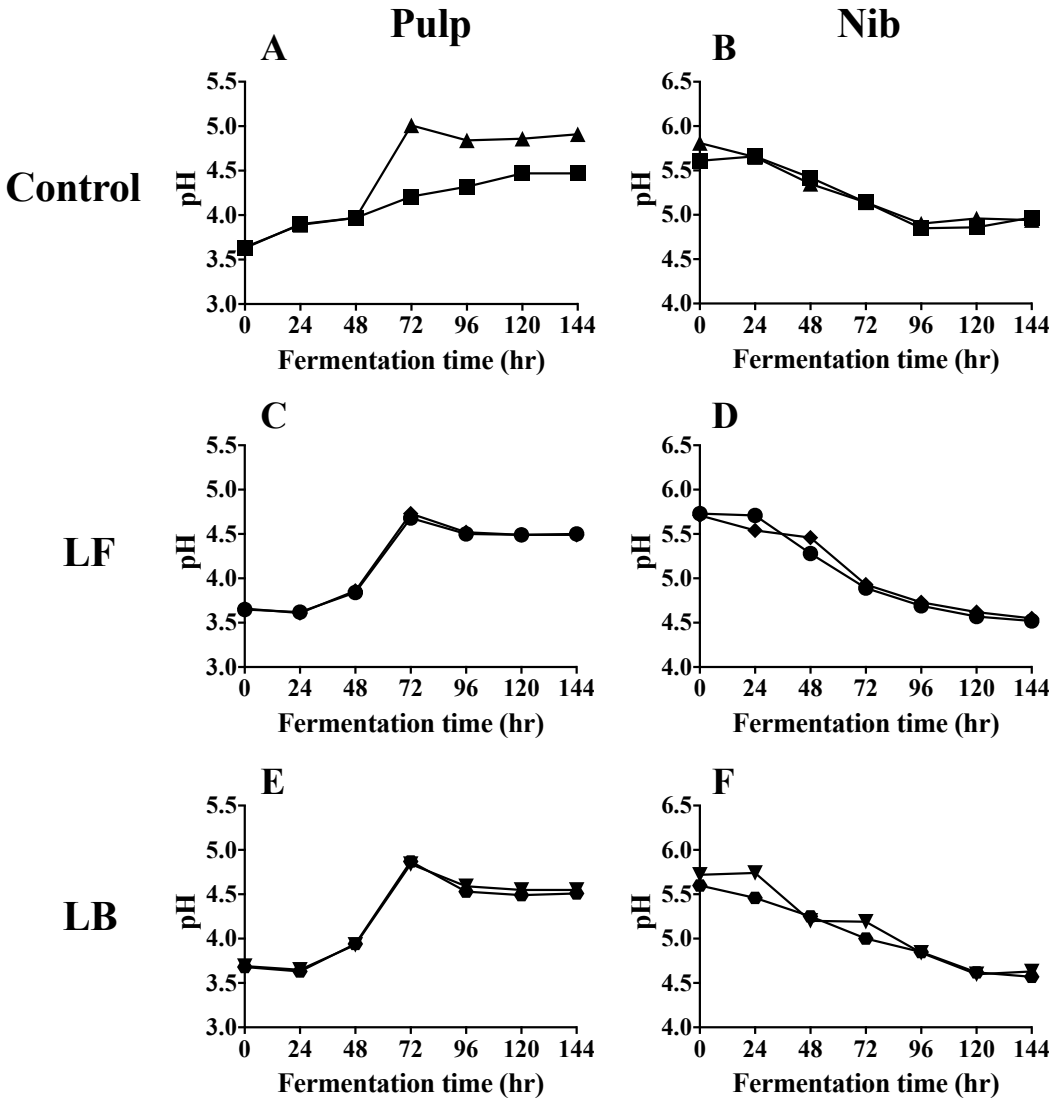

Pulp
Nib
A
B
C
D
E
F
Control
LF
LB
pH
Fermentation time (hr)
0
24
48
72
96
120
144

Figure 2.

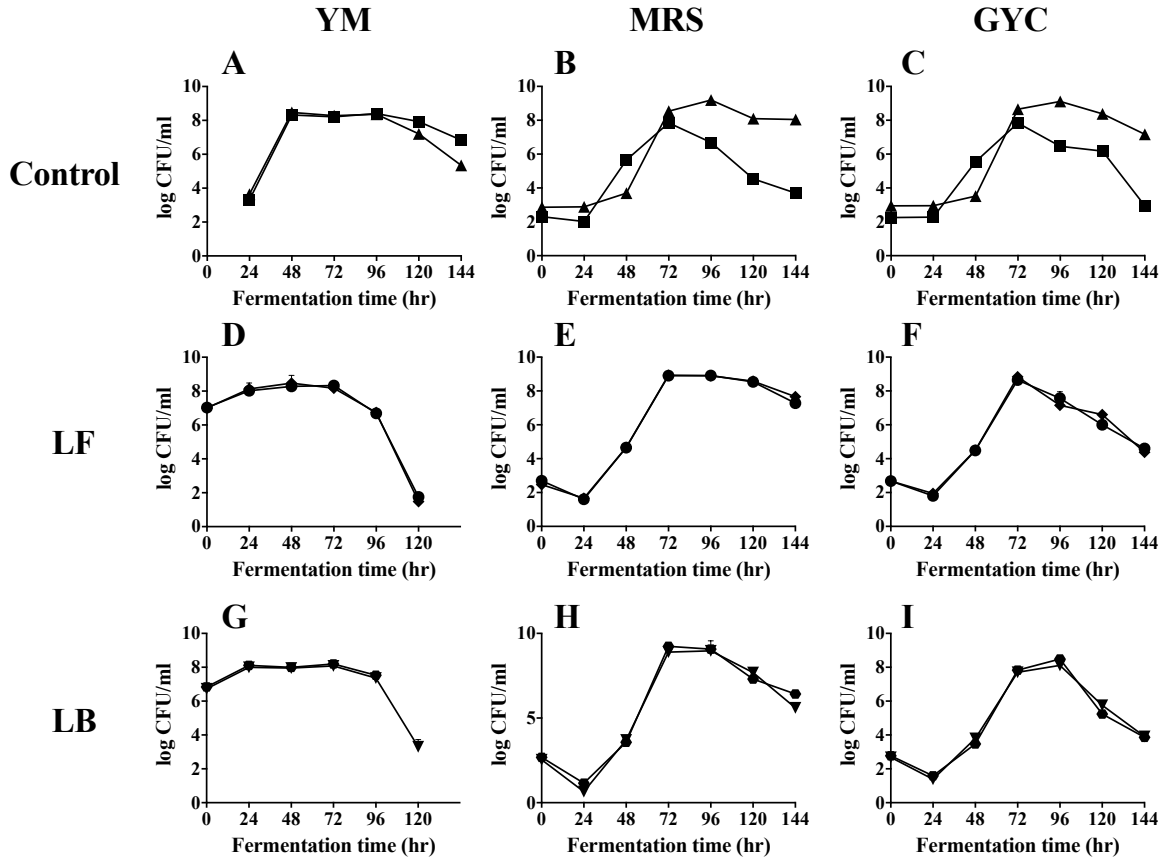

YM
MRS
GYC
Control
LF
LB
A
B
C
D
E
F
G
H
I
log CFU/ml
Fermentation time (hr)

Figure 3.

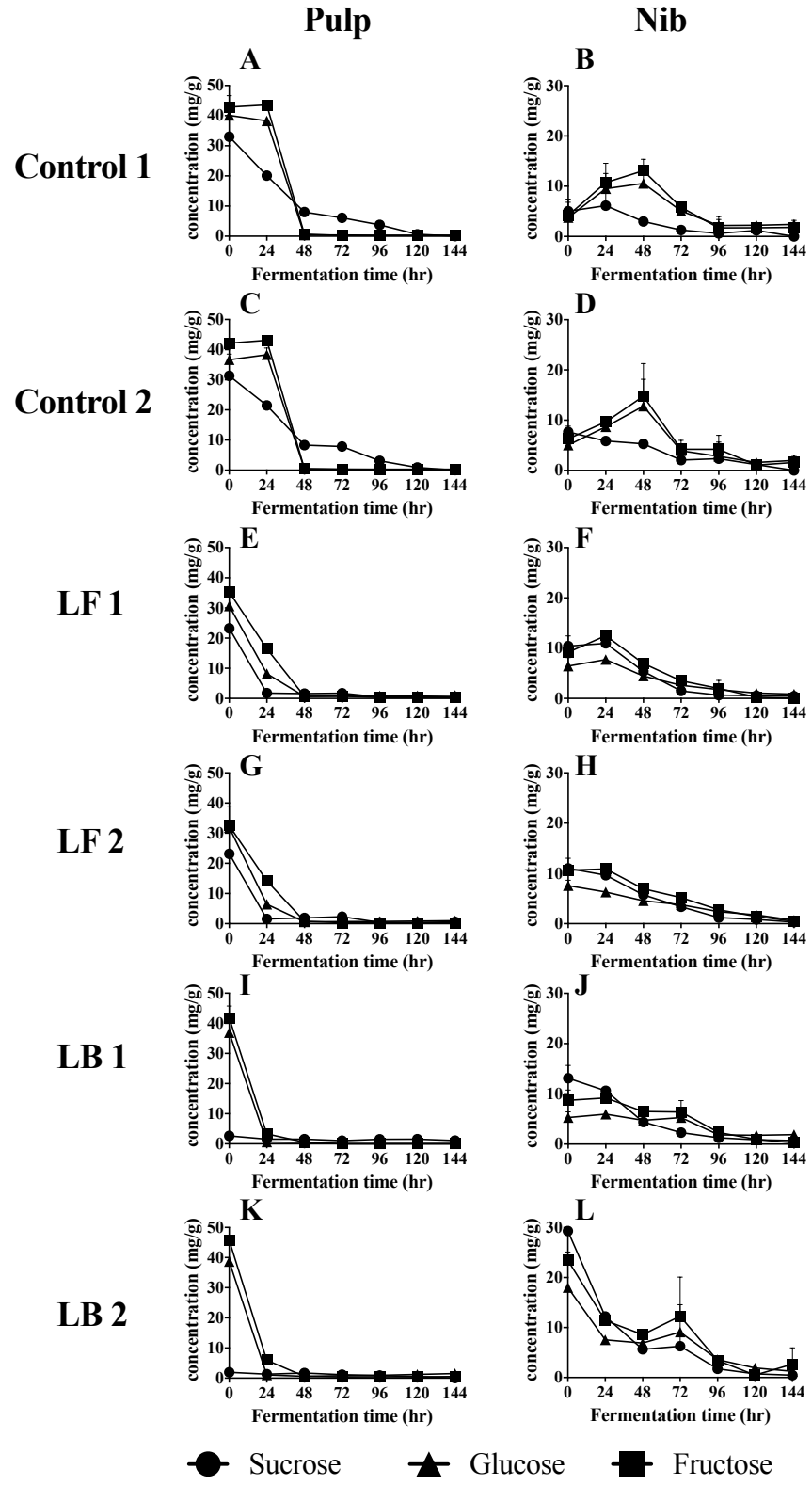

Pulp
Nib
A
B
C
D
E
F
G
H
I
J
K
L
Control 1
Control 2
LF 1
LF 2
LB 1
LB 2
concentration (mg/g)
Fermentation time (hr)
0 24 48 72 96 120 144
Sucrose
Glucose
Fructose

Figure 4.

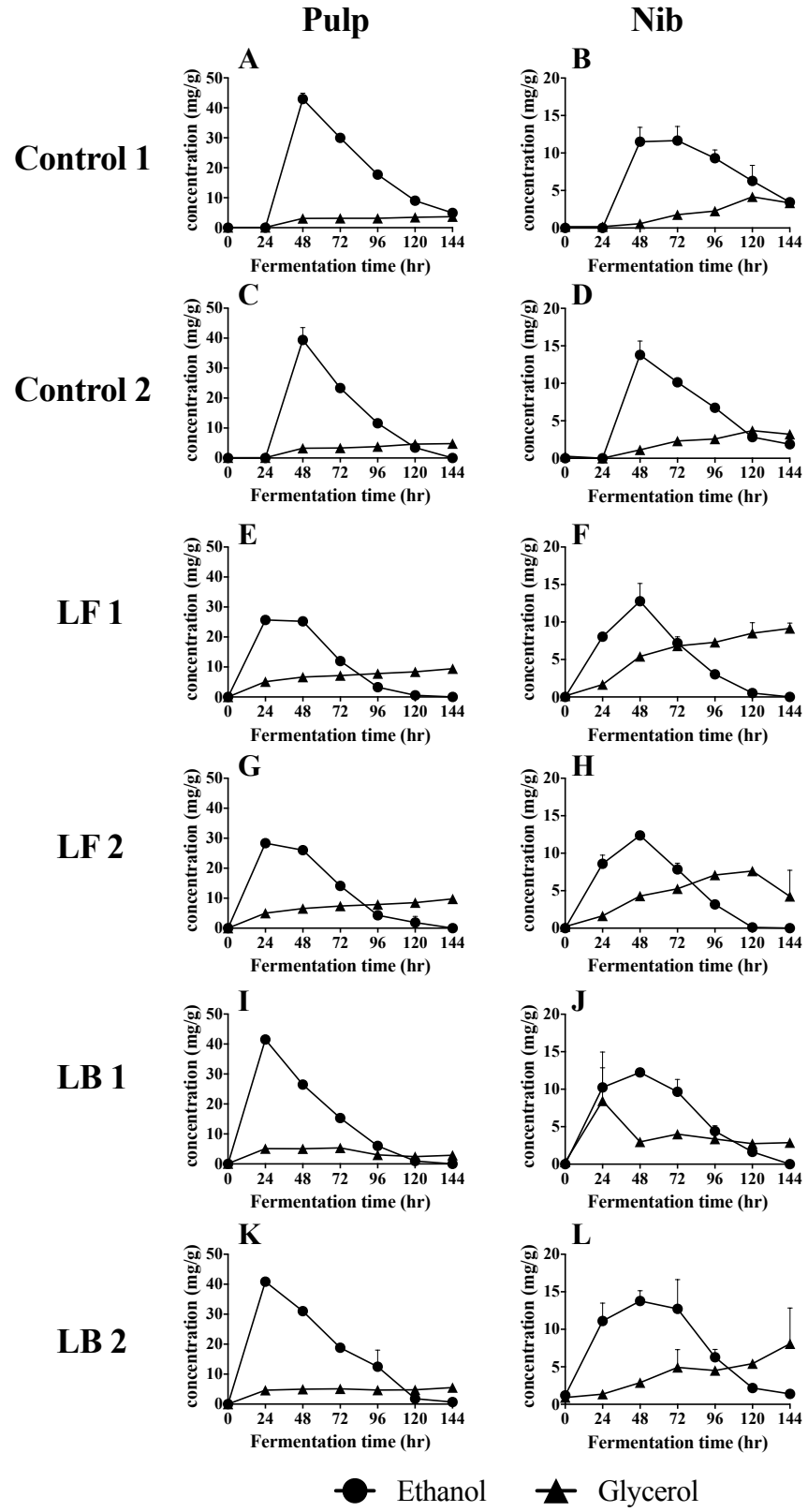

Pulp
Nib
Control 1
Control 2
LF 1
LF 2
LB 1
LB 2
A
B
C
D
E
F
G
H
I
J
K
L
concentration (mg/g)
Fermentation time (hr)
0 24 48 72 96 120 144
Ethanol
Glycerol

Figure 5.

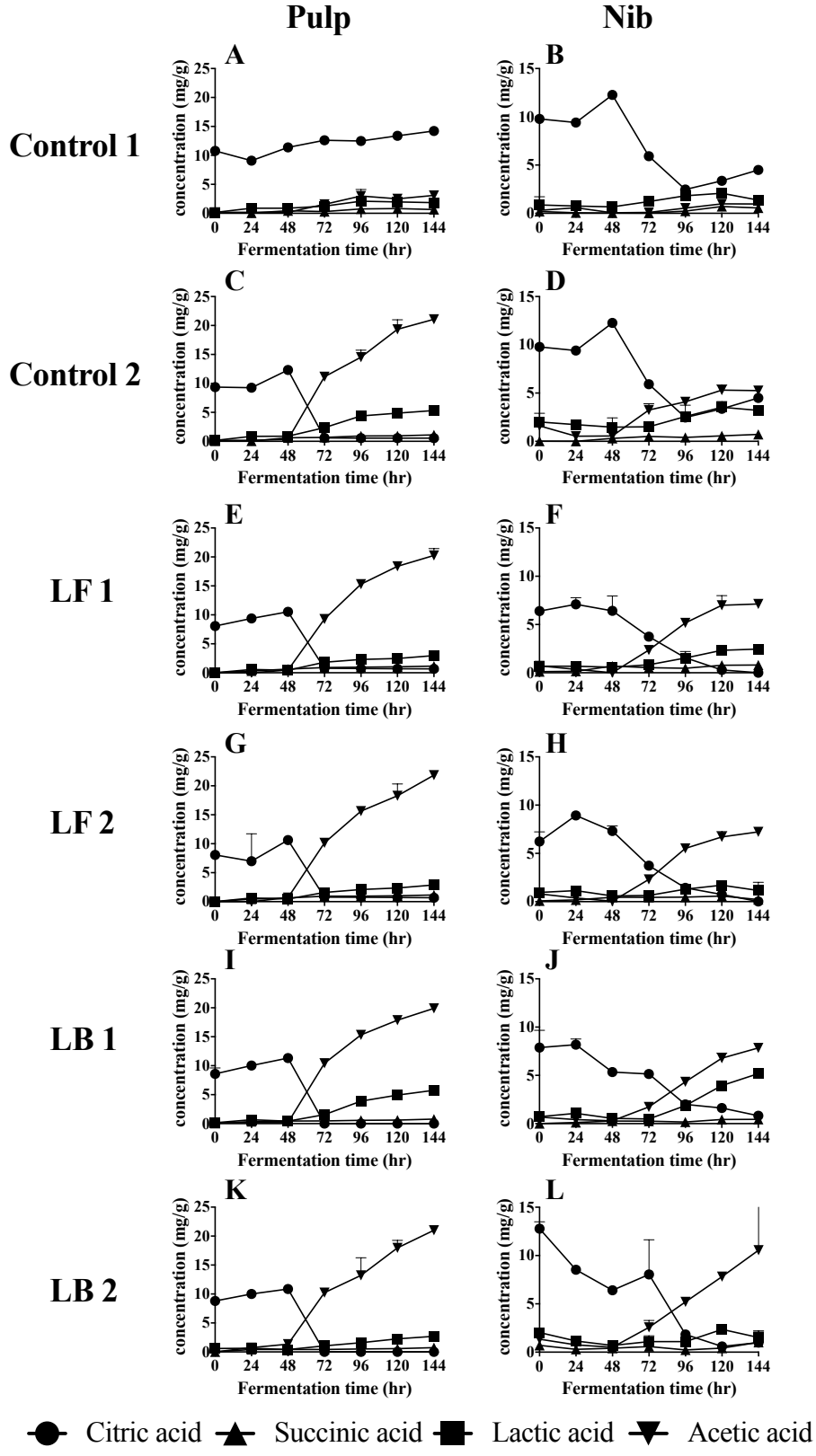

Pulp
Nib
Control 1
Control 2
LF 1
LF 2
LB 1
LB 2
A
B
C
D
E
F
G
H
I
J
K
L
concentration (mg/g)
Fermentation time (hr)
0 24 48 72 96 120 144
Citric acid
Succinic acid
Lactic acid
Acetic acid

Figure 6.

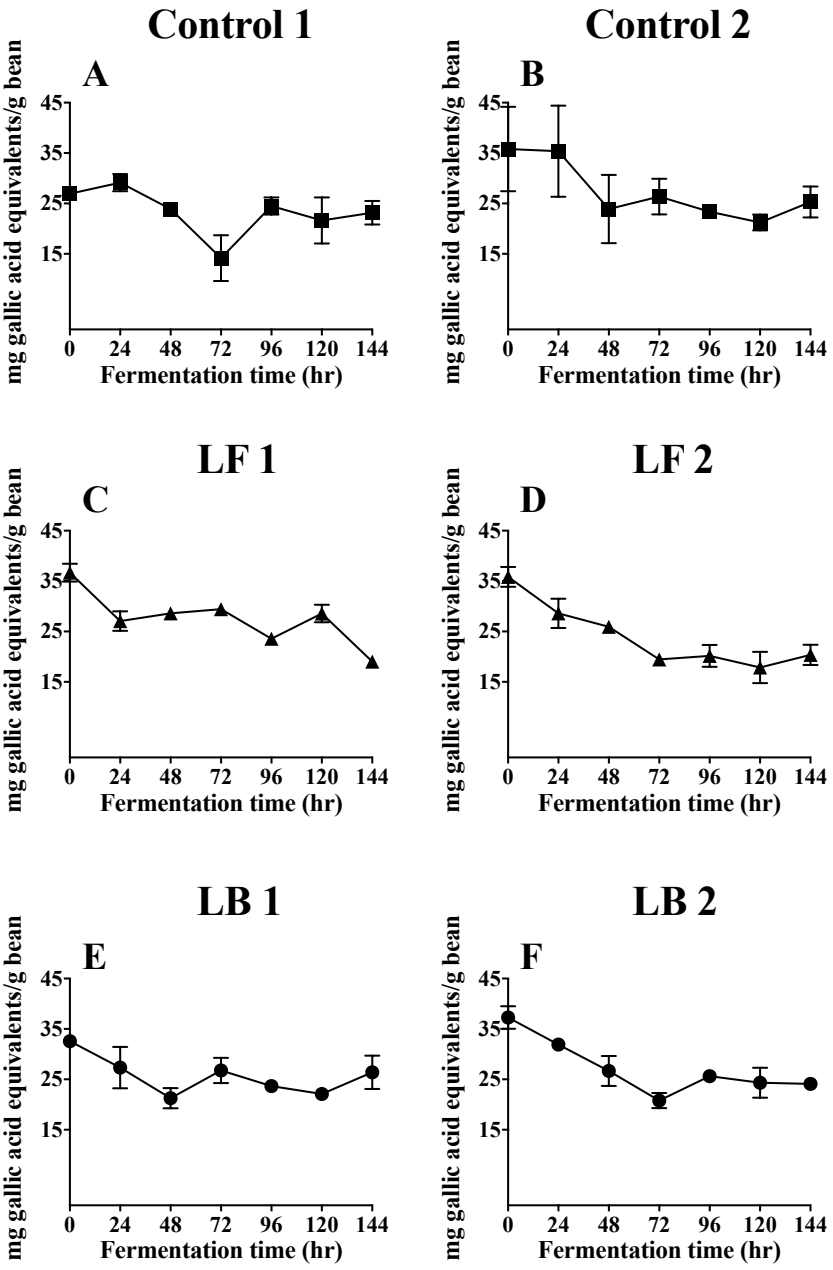
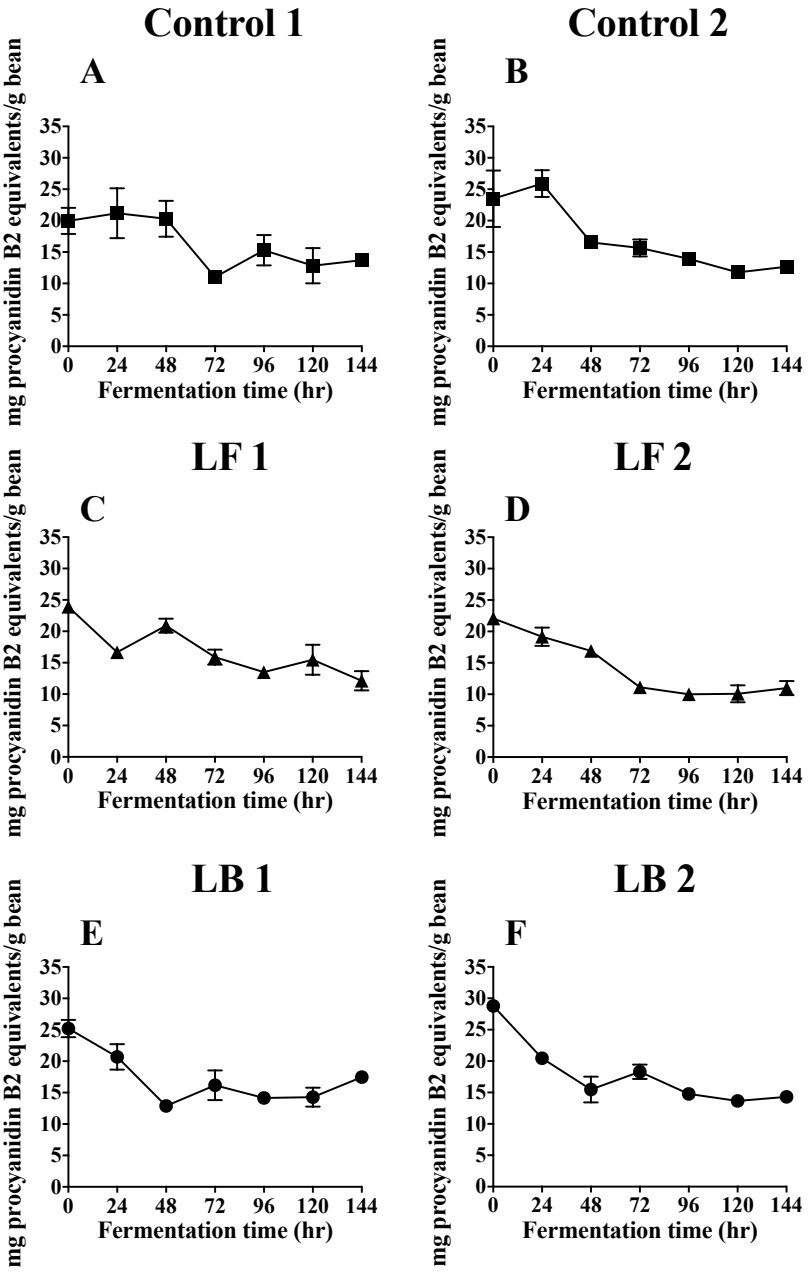

Control 1
A
Control 2
B
LF 1
C
LF 2
D
LB 1
E
LB 2
F
mg gallic acid equivalents/g bean
45
35
25
15
0
24
48
72
96
120
144
Fermentation time (hr)

Figure 7.


Control 1
A
Control 2
B
LF 1
C
LF 2
D
LB 1
E
LB 2
F
mg procyanidin B2 equivalents/g bean
Fermentation time (hr)
0
24
48
72
96
120
144
0
5
10
15
20
25
30
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Chapter 6: Conclusions and future work

There is an emerging interest in the beneficial effects of cocoa polyphenols on human health. Recent studies have revealed that not only the total concentration of polyphenols, but also the identities and concentrations of the individual polyphenol compounds present influence bioactivity. The regional and genetic origin of cocoa, and its fermentation are critical steps that influence the concentration and composition of polyphenols in cocoa products. Therefore, as a first step toward optimization of the potential health benefits of cocoa, we sought to understand the extent to which cocoa genetic variety and fermentation conditions influence polyphenol profiles of cocoa.

The first step of this study was to characterize the polyphenol profiles of five genetic clones of cocoa selected for high yield. Although genetic clones evaluated in this study were derived from the Forastero variety, sensory differences of bitterness and astringency (mainly due to polyphenols) had been ascribed to these varieties, leading us to hypothesize that polyphenol profiles would also differ among these clones. Of the five genetic clones tested, one had a significantly lower concentration of polyphenols compared to other clones. Although the differences in polyphenol profiles among these clones were not as distinct as we had expected, the fact that sensory differences have been reported remains as evidence of their different physiological impacts. Taste is, after all a physiological response. One limitation of this study was that bioactivity was not evaluated directly. Future studies should test how the concentration and compositional difference between the varieties may impact bioactivity including inhibition of digestive enzymes, inflammation, and oxidative stress, in addition to sensory (taste) characteristics.

Fermentation contributes to major loss and/or compositional change of cocoa polyphenols, and is thus a processing step with high potential to impact bioactivity. A fermentation model system was developed in this study in order to facilitate cocoa fermentation research at the laboratory scale. Our model system effectively modeled on-farm cocoa fermentation. Essential microorganisms for cocoa fermentation were populated well and desirable chemical changes including production of ethanol, acetic acid, lactic acid, and volatile compounds as well as degradation of polyphenols were observed. From the perspective of fermentation research, this model has several advantages over on-farm fermentation system: 1) It uses the dried unfermented cocoa beans as a starting material, greatly reducing the risk of spoilage and the expense involved in the storage and shipping of cocoa pods or wet unfermented beans, a significant advantage when conducting fermentation research in countries distant from the cocoa harvesting region. Taking advantage of this system, laboratories could also obtain cocoa varieties from multiple countries and test how their polyphenol profiles will be affected when the same microbiota are employed. 2) Model system allows for easy control of fermentation parameters including microbiota, pulp composition, temperature, and aeration. Moreover, it limits risks associated with conducting fermentation trials outdoors, such as climatic conditions, contamination, or animal predation. Researchers interested in the effect of fermentation conditions on volatile compounds and sensory characteristics could use this model system to facilitate their research programs, benefiting from the convenience of small batch size and easily controlled fermentation parameters.

The last chapter in this study utilized the model system to determine the impact of yeast starter culture inoculation on microbial and chemical aspects of cocoa fermentation. This study is thought to be the first attempt to test the influence of starter culture inoculation on polyphenol

and procyanidin concentrations throughout cocoa fermentation. Using yeast starter cultures in the model fermentation system yielded consistent results between replications in all of the aspects compared to the fermentation with no starters added (one advantage of using starter is consistency). Due to the initial high population of yeasts in the inoculated condition, ethanol, an important metabolite to start the biochemical reaction inside the cocoa bean was produced earlier and thus accelerated the fermentation process, resulting in less time for degradation of polyphenols. Decreased time to process cocoa and more consistent fermentation results could result in economic benefits to producers.

Total polyphenol and procyanidin concentrations were also compared between fermentation inoculated with different yeast strains. It was found that the final concentration of total polyphenol and procyanidin were higher in fermentation inoculated with *S. cerevisiae* Lev B possibly due to the higher lactic acid concentration that could inhibit the degradation of flavanol monomers. To better control the system, future work should test the impact of mixed starter cultures including lactic acid bacteria and acetic acid bacteria in addition to yeasts since the two bacteria could directly change the acid level in the fermentation system thus affecting the polyphenol and procyanidin concentrations. Moreover, detailed profile of individual polyphenols should be analyzed in the future to see whether structural differences (degree of polymerization) resulted from the use of starter cultures.

One limitation of this study was the use of Criollo variety cocoa beans. We obtained small lots of commercially available dried unfermented cocoa beans at the outset of this project. Criollo variety cocoa beans have only 2/3 or even less the concentration of polyphenols compared to Forastero varieties, which is the most popularly used variety in the chocolate industry. Using Forastero beans in the future will be a better experimental approach since it is the

most consumed cocoa variety and the change in polyphenol concentration and composition by fermentation could potentially be more significant, due to the high initial level of polyphenols. Collaborations should be established with government agencies interested in cocoa cultivation and processing, such as the USDA or cocoa processing industries to obtain unfermented dried Forastero beans to improve the relevance and applicability of future research in this area.

Overall, the present work advanced the understanding of the impact of upstream processes including variety selection and fermentation on cocoa polyphenol profiles. It also provided perspective on how yeast starter culture inoculation could affect the overall fermentation characteristics and the polyphenol profile during cocoa fermentation. Evaluation of the impact of starter cultures was possible due to the development of laboratory scale model system of cocoa fermentation. Finally, this study dealt with mixed microbial fermentation. Due to the complexity and variability of mixed microbial fermentations in food and beverage systems, the effects of microbes on the quality and characteristics of fermented foods are not well understood, and the fermentation processes used to produce these foods and beverages are often not well-controlled. In addition to cocoa, sourdough, kimchi, sauerkraut, cheese and other fermented foods and beverages are fermented by mixed microbiota. The knowledge obtained in this study on the interaction of yeast, lactic acid bacteria, and acetic acid bacteria and their influence on the cocoa quality will contribute to the overall understanding of the mixed microbial system in the field of food and beverage fermentation.

Appendix: Supplementary materials for Chapter 4

Model cocoa fermentation using artificial pulp can simulate the microbial and chemical changes of on-farm cocoa fermentation

Polyphenol extraction

Polyphenols were extracted from the unfermented cocoa beans according to the method of Dorenkott et al. (2014) with some modifications. Cocoa bean samples were frozen using liquid nitrogen and ground to obtain a powder. The powder was defatted by mixing with hexane in a 1:4 ratio and sonicated for 10 min. The mixture was centrifuged for 5 min at 5000 × *g* and the supernatant was discarded. This defatting step was repeated once more and the hexane was then evaporated from the mixture to obtain the final dried defatted cocoa. Polyphenol extraction solutions were made by mixing acetone, ultrapure water and glacial acetic acid with 70:28:2 ratio. Dried defatted cocoa and the polyphenol extraction solution were mixed in a 1:4 ratio and sonicated for 10 minutes. The mixture was centrifuged for 5 min at 5000 × *g* to obtain the supernatant. Extraction steps were repeated 3-4 times until the supernatant obtained was colorless. The acetone in the supernatant solution was evaporated at 40°C under vacuum using a rotary evaporator RV 10 Basic (IKA®, Wilmington, NC). The remaining extract was frozen in a −80 °C freezer for 20 min and then freeze-dried for two days using a FreeZone 1 Liter Benchtop Freeze Dry System (Labconco, Kansas City, MO). After obtaining water free cocoa extract, the extract was crushed to a powder form and stored at −80 °C until the time of analysis.

Total polyphenol and procyanidin assays

Quantification of total polyphenol content of cocoa bean extract was based on the Folin-Ciocalteu method as reported by (Waterhouse, 2002). 40% EtOH was used to dilute cocoa extract to the concentration of 0.2 mg/mL extract. 100 uL of the diluted sample was then mixed with 900 uL ultrapure water and 2.5 mL of 0.2 N Folin-Ciocalteau reagent (Sigma, USA) and vortexed. Samples were left at room temperature for 2 hours in the dark and the absorbance was then measured at 765 nm using GENESYS^{TM} 10S UV-Vis Spectrophotometer (Thermo Scientific, USA). Total polyphenol concentration was expressed as mg gallic acid equivalents/g cocoa bean ± SD.

Total procyanidin of cocoa bean extract was quantified by using 4-Dimethylaminocinnemaldehyde (DMAC) method, described by Payne et al. (2010), with appropriate modifications for this study. To make DMAC solution, 3 ml of HCl and 27 mL of EtOH were combined with 0.03 g of DMAC. The cocoa extract samples were diluted with EtOH to 100 ppm. Using a 96-well plate, 50 µL of EtOH (blank), and 50 µL of diluted cocoa extract or 50 µL of standard solution were mixed with 250 µL of DMAC solution and the absorbance was read at 640 nm. Total procyanidin concentration was expressed as mg procyanidin B2 equivalents/g cocoa bean ± SD. Both total polyphenol and procyanidin analyses were conducted in triplicate.

Figure S1. Volatile compounds detected but not showing significant changes during fermentation

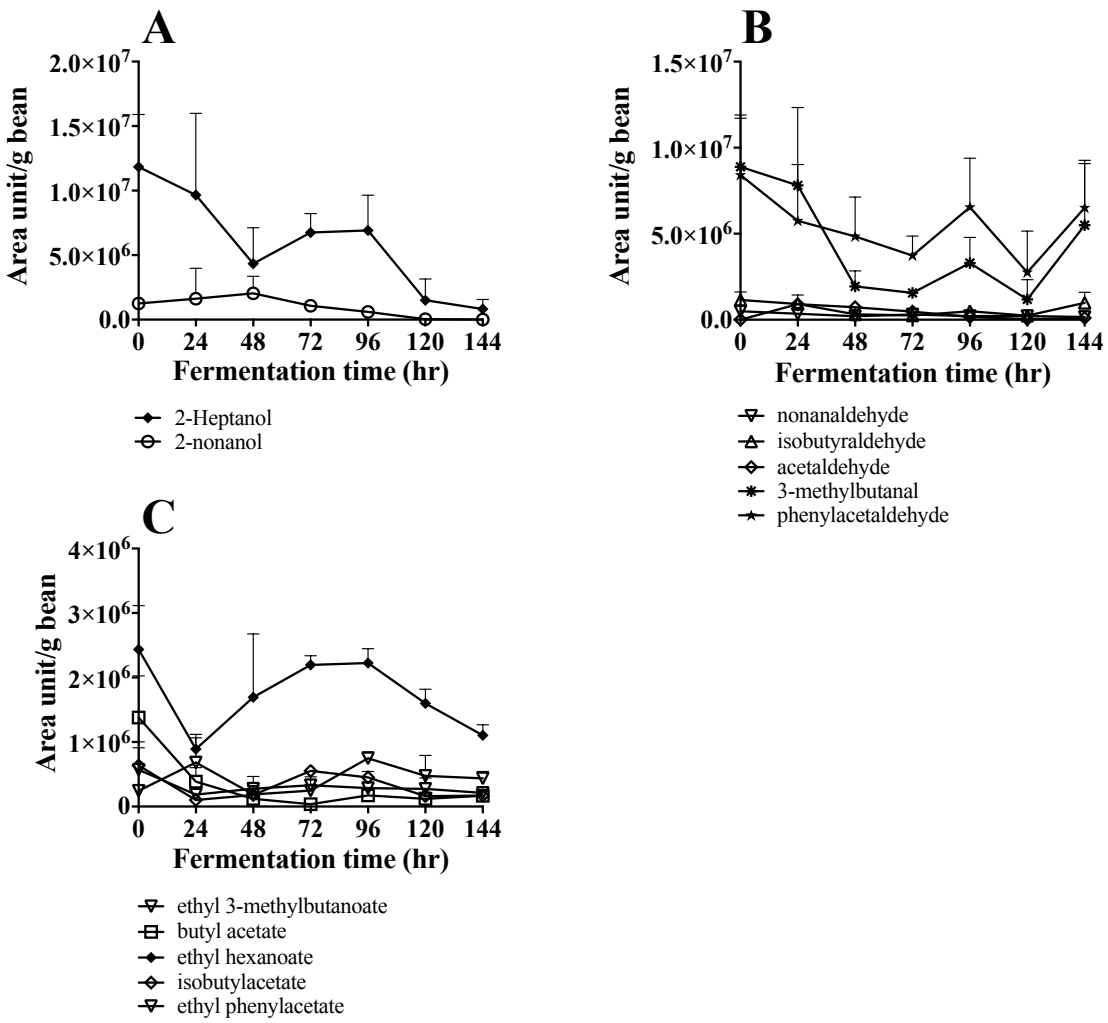

Figure S2. Change in the color of PPO extract during fermentation

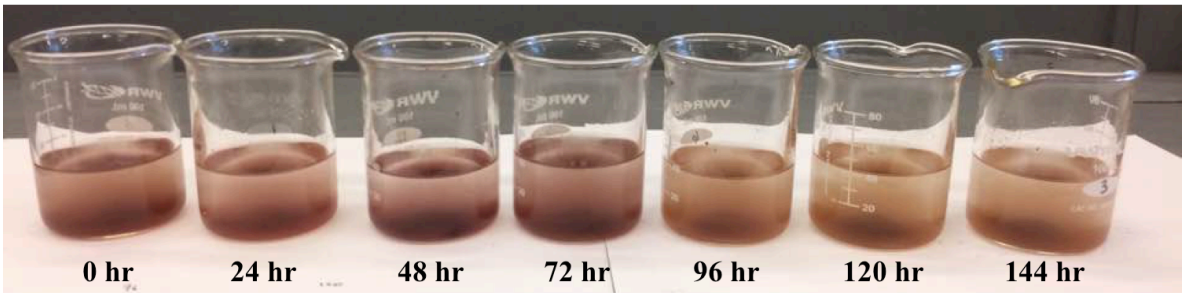

Table S1. Microorganisms typically found in on-farm cocoa fermentation

Microorganisms	Species found in cocoa fermentation
Yeast (most frequently found)	***S. cerevisiae***[(a)] *Kluyveromyces marxianus* *Candida spp.*
Yeast (not frequently found but contributions reported)	*Pichia kudriavzevii* *Hanseniaspora guilliermondii* *Hanseniaspora opuntia*
LAB (most frequently found)	***Lactobacillus plantarum*** ***Lactobacillus fermentum***
LAB (not frequently found but contributions reported)	*Pediococcus spp.* *Leuconostoc spp.*
AAB (most frequently found)	***Acetobacter pasteurianus***
AAB (not frequently found but contributions reported)	*Gluconobacter oxydans* ***Acetobacter tropicallis*** *Acetobacter lovaniensis* *Acetobacter syzygii*

[(a)]Species written in bold character are found in our model system.

References

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