The Pennsylvania State University

The Graduate School

EFFECT OF THE ADDITION OF COCOA SWEATINGS AND TIME OF FERMENTATION ON FLAVOR COMPOUNDS AND SENSORY PERCEPTION OF COCOA

A Thesis in

Food Science

by

Nixon Andres Meneses-Marentes

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

December 2022

The thesis of Nixon Andres Meneses-Marentes was reviewed and approved by the following:

Helene Hopfer
Associate Professor of Food Science
Thesis Advisor

Gregory R. Ziegler
Distinguished Professor of Food Science

Misha Kwasniewski
Assistant Research Professor of Food Science

Robert F. Roberts
Professor and Head of Food Science

ABSTRACT

The fruit seeds of the *Theobroma cacao* L. plant are the raw material for chocolate products, and their processing contributes significantly to the characteristic cocoa flavor. Cocoa processing includes fermentation, drying, and roasting, and depending on the final product also grinding, mixing, tempering and molding. During certain processing steps, such as fermentation, drying, and roasting, a chain of numerous chemical reactions transforms bean constituents into flavor compounds in the final cocoa product. Flavor compounds are classified as volatile and non-volatile compounds that are able to elicit basic taste, smell, and/or mouthfeel sensations. Monitoring their changes along the processing steps provides key information about cocoa flavor, which is one of the main drivers of consumption and purchase of cocoa products.

During fermentation, the fruit flesh, the so-called cocoa pulp, serves as a substrate for biochemical transformation, including the production of ethanol, lactic acid and acetic acid, alongside other important flavor compounds and precursors. The kinetics of these compounds, and correspondingly, final flavor, are affected by the microbial communities at play, including yeasts, acetic and lactic acid bacteria. Despite some strategies for altering the pulp and thus, shifting cocoa fermentation kinetics, have been explored (such as cocoa pod storage, pre-drying of cocoa seeds, de-pulping of cocoa seeds, etc.), the collection and back-addition of the cocoa pulp draining, the so called cocoa sweatings (CS) during fermentation have not been studied. CS are the lixiviate produced mainly during early stages of cocoa bean fermentation and result from de-pectination of the cocoa pulp, which releases water and other components such as fermentable sugars, pectin, and organic acid from the pulp. CS are typically removed from the fermentation system through drainage, but CS could provide additional substrates during cocoa bean fermentation if not drained away. The addition of CS thus could enhance flavor precursor formation, and consequently, change final flavor composition in cocoa, however, this has not been studied so far. This thesis therefore aims to determine the effect of the addition of CS to cocoa fermentation compared to conventional cocoa fermentation (i.e., without the addition of CS) on cocoa flavor by measuring volatile and non-volatile compounds in unroasted cocoa powder and roasted cocoa liquor. A second aim investigates the effect of the addition of CS (vs. no addition) on fermentation kinetics at later stages, comparing 5, 6 and 7 days of fermentation, by measuring volatile and non-volatile compounds in unroasted cocoa powder and roasted cocoa liquor. Last, this study also assessed the effect of the addition of CS during fermentation on sensory perception of roasted cocoa liquor samples using regular chocolate consumers.

This study showed that the addition of CS during fermentation stabilized the content of 7 compounds in unroasted cocoa powder (Benzyl alcohol, 2-acetylfuran, pantolactone, transclovamide, (-)-epicatechin, procyanidin B2, and isoquercetin) and 17 compounds in roasted cocoa liquor (2-methyl-1-butanol, octanal, benzaldehyce, α-ethylidene-benzeneacetaldehyde, ethyl 2-methyl butanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, furfural, 4-butyrolactone, 3-Furanmethanol, pantolactone, 4-methyl-2-hexanone, acetophenone, (-)-epicatechin, Procyanidin B2, Cyclo(Pro-Val), Cyclo(L-Leu-L-Pro)) over time of fermentation. Additionally, not differences in sensory perception over fermentation time were identified only in cocoa liquor fermented with the addition of CS. Therefore, this study suggests that CS have a protective effect in stabilizing the content of some flavor compounds and consequently generate cocoa with similar sensory perception in shorter times of fermentation. On the opposite, cocoa samples without addition of CS may develop similar sensory perception but in longer times of fermentation. Lastly, most of the compounds quantified shower a smaller coefficient of variation in the

samples with the addition of CS. Therefore, this study also suggest that the addition of CS may have the potential for providing more consistent batches of cocoa with similar flavor composition. The above may be highly appreciated for the market of mainstream cocoa products, but further studies are required to evaluate which mechanisms are affected to provide such consistency.

This exploratory study showed that the addition of CS and the time of fermentation both affect flavor composition of unroasted and roasted cocoa and sensory perception of roasted cocoa. Further studies are needed, such as sampling a broader range of time of fermentation (e.g., 3 days), and screening of flavor precursors to gain insights into the mechanisms how the addition of CS affects cocoa flavor. Lastly, although this study potentially identified changes in perceived bitterness as the attribute that differentiated cocoa liquors with and without the addition of CS over time of fermentation, more targeted sensory methodologies such as descriptive analysis are necessary to determine the qualitative and quantitative effects of adding back CS to the fermentation mas on cocoa flavor attributes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

First, I want to recognize Fulbright Colombia and ICETEX for allowing me to represent Colombia as an international student in the United States. Not only was I honored to do my best every day, but also, I really enjoyed the path towards achieving the completion of these studies by making friends and discovering the US culture. I still remember when I decided to be fluent in English and check the requirements for studying abroad back in 2017. Thankfully, that dream came true due to the opportunity that Fulbright Colombia and ICETEX granted to me. With that in mind I proudly say: I did it as a Fulbrighter!

Dr. Helene Hopfer, thank you so very much for your impeccable and genuine mentorship during these years. I will never forget your kindness and energy since the first meeting we had over zoom when I was reaching out faculty in 2019. During my time as a master student, I enjoyed every single one-o-one meeting we had. You let me take the leadership in every meeting, and you always were open to talk about everything I had in my list. From NMR, volatile compounds, LCMS, sensory analysis, R, statistics, classes, professional development, life, news, trips, music, podcast in Spanish etc. Not only did you give me the space for intellectually challenge myself as student and researcher, but also to discover me in this whole experience that has been to be abroad. I am deeply grateful for your support even since day zero, when you picked me up in the State College airport that cold night in January 2021. A pandemic, that stopped the world for almost a year (and my studies for one semester), did not stop you for welcoming me in the middle of that winter. From home supplies to early access to COVID-19 booster, and even a “buenos días” every day… All the above were just a couple of things that let me feel how fortunate was to be your student. As your mentee I say these two things: (1) you gave me an excellent example of how to be an excellent mentor, and I hope to reflect that when I provide guidance to people on my own in the future. (2) I expect to continue working with you and keep building a nice friendship along the way!

Thank you, Dr. Gregory Ziegler, and Dr Misha Kwasniewski for being part of my evaluation committee. Dr. Gregory Ziegler, thank you for allowing me to participate, help and even burn with the microwave a chocolate sample in the chocolate short course in 2022. Despite I did not process too much cocoa for my thesis, I learned a couple of things about processing during the lectures of the chocolate short course. Also, helping with the short course gave me enough training for sample preparation of my sensory test (with almost 2000 similar chocolate dots made by hand). Dr. Misha Kwasniewski, thank you for your guidance with the LCMS. I am still impressed on the way you can fix and figure out all these very expensive and delicate equipment. From my perspective, a person that hardly ever puts together a manual puzzle, seeing how to fix the nitrogen generator was an achievement. Thank you as well for providing extra solvents during my non-volatile quantification of my cocoa samples, for the name of science!

Sarah Bharath, I am very grateful for the opportunity I got to work with your samples and let me get involved in this project. All this cocoa went all the way from Trinidad and Tobago, and because of the pandemic, they were just sitting in a fridge at the SEC. These children (you know that was how I called them) went through tons of work, but they needed a parent for finishing the duty of characterize them. Thank you for allowing me being the adoptive dad of these children and tell their story in this study. Thank you also for sharing your passion for cocoa through zoom and in person during the chocolate

short course in 2022. Talking to you was stimulating learning from the real farmer context outside of academia.

The correct use of the lab equipment and resources was possible due to the training and support provided by Dr. Bethany Lam, Aaron Wiedemer, and Laura Taraboletti. Dr. Bethany Lam, thank you for the multiple afternoons you spent next to me in front of the LCMS. Some of these days were exhausting, but I had a lot of fun figuring out MRM transitions whereas we were talking about random things such as tv shows, our cats, weekend plans, etc. Aaron, thank you for being the first person from the Hopfer Lab on introducing me to the NMR and GCMS methods. Cleaning fragile NMR tubes whereas listening to Madonna and Jazz was the first cleaning challenge in my thesis and a good memory with you. Laura, thank you for your help with the organization of the plethora of standards that constantly were coming to the lab, as well as the additional assistance with label making and sample weighing. I hope the small introduction in lab work with me during these months gave you some idea about how to be methodic and have fun whereas carrying out experiments.

This work was also achieved with the support of the Kwasniewski Lab and the SEC. Thank you people from the Kwasniewski lab for allowing me to use the lab equipment and tolerate the awful smell I left when I was measuring the standards for my GCMS analysis. It was my first time dealing with these compounds. The good thing is that now I know I must work in the fume hood whereas dealing with these stinky compounds. To the SEC, thank you for helping me to set an incredible organized sensory analysis from the design of the forms in Compusense till the execution of the 2-day taste of cocoa liquor. The guidance of Alicia Leitch in Compusense was fundamental, as well as the help of Olive Djonou Komou and my amazing colleagues from the cubicle farm during the sample prep and the tasting. To all of them, thank you.

I am incredibly fortunate to live this graduate experience along with all the friends I have found along the way during these years, but especially to my beautiful Food Science Cohort 2020-2021 (here included my beautiful queen: Bridget Reed). Not even in my dreams, I expected to meet such smart, funny, adventurous, and capable people. Every single one of them gave me the best energy at work and outside of it. From numberless lunch times, RuPaul nights, parties, workouts, movies, weekend brunches, rides, and trips, to even a space in their houses during my homeless experience in August 2022. All the above gave me the motivation to go hard and be consistent in everything. They said I irradiated happiness and optimism in the building, but I feel it was only a reflection of the beautiful emotions they caused in me because of their constant support. I did not expect to love this experience abroad as much as I love it because of all of them, and I cannot wait to show Colombia with the same affection they showed me their culture.

Last but not least, I want to acknowledge my family for being the biggest support ever during my whole life. The example and approach to deal all kinds of challenges that my mom, Ligia Marentes Diaz, and my dad, Marco Fidel Meneses faced have been the major tool for my success as a professional and as a graduate student. I always have in my mind all their stories about how their entrepreneurship and persistence gave them wings to rise me and my brother, and provide us with the best education possible. All the long shifts making tamales tolimenses with them showed me that is necessary, like Madonna says, to feel the blood and sweat in my fingertips, to have good things in life. For that and for the ever-present support regardless of the distance, thank you. My success is always their success too.

This work was supported by the USDA National Institute of Food and Agriculture Federal Appropriations under projects PEN04624 (Accession No. 1013412) and PEN04792 (Accession No. 7002577). The findings and conclusions of this work do not necessarily reflect the view of the funding agency.

This work was supported by the program Pasaporte a la Ciencia, a Colombia Científica component from ICETEX, in alliance with Fulbright Colombia. This project aligns to the food research focus, more specifically associated with harvest, post-harvest, transformation and commercialization management for the use of agrobiodiversity in productive systems. The findings and conclusions of this work do not necessarily reflect the view of the funding agency.

INTRODUCTION

The fruit seeds of the *Theobroma cacao* L. plant are the raw material for chocolate products. *Theobroma* cacao originates in South and Central America and is grown between 20° north and south of the equator, including the current cacao growing areas in West Africa, Southeast Asia, and South and Central America (Fowler & Coutel, 2017). According to the Food and Agriculture Organization of the United Nations (FAO, 2021), the top ten cocoa bean producers since 2000 are Ivory Coast, Ghana, Indonesia, Nigeria, Brazil, Cameroon, Ecuador, Peru, Dominican Republic, and Colombia. Cacao beans undergo numerous post-harvest processing steps to turn them into cocoa liquor or cocoa mass, cocoa butter, and cocoa powder as ingredients for other food and non-food products.

The cocoa value chain: from tree to cocoa liquor

The cocoa value chain starts in the southern hemisphere, where *T. cacao* trees are typically planted in plantations with some form of shade that covers the cacao trees during their establishment and development, often accompanied by inter-cropping with other food crops. Cacao trees require between 2-3 years to start producing fruits, and once production starts, can last for 25-30 years or more, as long as trees are properly maintained (e.g., pruning, weed and pest control) (Fowler & Coutel, 2017). Once cacao pods are ripe, depending on a change in peel color from green to yellow, orange, or red, depending on the variety, they are harvested (Gutiérrez, 2017). The selection of the cacao pods for harvesting is made visually, and they are harvested using a penknife, knife, or pruning shears. Sometimes the harvested pods are stored for a couple of days prior to opening to trigger the development of flavor precursors and accelerate the subsequent processing steps (Koné et al., 2021); however, post-harvest pod storage is not universally carried out as the benefits are not fully clear. The cacao pods are then husked, and the beans are removed by hand. Once the cacao pods are opened, the beans are exposed to the environment, equipment, and operators. Thus, good manufacturing practices need to be applied to not spoil the seed mass (Suh et al., 2020), and ensure adequate spontaneous fermentation.

Fermentation is one of the most important steps in cocoa bean processing because it transforms the native bean chemical composition into flavor precursors that are needed to develop the characteristic flavor of cocoa products upon later processing, such as roasting (Aprotosoaie et al., 2016; Fowler & Coutel, 2017; Gutiérrez, 2017). There are different fermentation methods, with the two most widely used being the box and heap fermentation (Gutiérrez, 2017). In heap fermentation, the fermenting mass is placed on a solid surface such as the ground in a heap and covered with banana leaves. In contrast, for box fermentation, the main fermentation container material is made of wood, and sometimes, depending on the level of technique in the plantation, may have small holes at the bottom to drain off the lixiviates, the so called cocoa sweatings (CS). The biotransformation of cocoa beans during fermentation is caused by a cocktail of yeast and bacteria from the environment that act upon the cocoa beans and cacao seed mucilage. Mainly, the microorganisms degrade the pulp that covers the beans, cause the death of the beans by lowering the pH and increasing the temperature, and change the bean composition through further enzymatic and chemical reactions. This process allows the production of flavor compounds and precursors. Cocoa box fermentation can take between five and seven days and usually includes bean-mass turning after the first 48 hours and then every 24 hours to ventilate and oxygenate the system (Gutiérrez, 2017).

Once fermented, a drying step is necessary to prevent mold growth and inactivate microbes. This process is carried out until the cocoa beans reach moisture levels between 7-8% (Fowler & Coutel, 2017). The widest technique used is solar drying, but this is highly dependent on the weather conditions.

Alternative techniques such as artificial drying, can cause off-flavors due to the source of heat; for example, heat sources that produce smoke have been reported to induce ham-like and bacon off-flavors in the beans, affecting final quality of the cocoa beans (Gutiérrez, 2017). Once the cocoa beans are dried, they are packed in bulk to be sold and transported.

Once cocoa beans arrive in processing facilities (mainly located in the northern hemisphere) they are cleaned, potentially blended, thermally pre-treated, their shells are removed and they are roasted (Aprotosoaie et al., 2016). Cocoa beans may be roasted as whole beans with the shell or without it (so called cocoa nib roasting), but higher inputs of energy are necessary for whole-bean roasting. Additionally, some fat may remain in the shell after roasting and cause fat loss in the roasted bean (Gutiérrez, 2017). Regardless of the type, roasting is a fundamental step in the development of the characteristic chocolate flavor and color (Afoakwa et al., 2008). During roasting, the moisture content is further reduced from 7% to 1-5%, and chemical transformation of flavor precursors and other bioactive compounds such as phenols take place due to the high temperatures which are normally between 120-140°C (Ziegleder, 2017) and prolonged times of operation, typically between 12 and 30 minutes, depending on roasting equipment and desired roasting degree (Afoakwa et al., 2008; McClure et al., 2021; Santander et al., 2020). Once the cocoa beans are roasted and the shell is removed, the cocoa is ground into so called cocoa liquor, a suspension of cocoa solids in a cocoa butter/cocoa fat matrix. There are different mills that are used for grinding cocoa such as impact, bar, and disc mills, and their objective is to reduce the particle size of cocoa solids to a level that can subsequently be mixed with other ingredients to make chocolate (Gutiérrez, 2017). Industry standards often aim for a d90 particle size of around 20 μm, however, it might be more important how homogenous the distribution is as very sensitive un-trained chocolate consumers were able to perceive a difference in particle diameter of 4.44 and 5.04 μm in chocolate (Breen et al., 2019).

As briefly described, the cocoa value chain involves many actors and technological steps from different parts of the world. Since the quality of the cocoa products depends on how well all the steps are pursued, international trading rewards cocoa beans that are produced with good post-harvest practices. Therefore, studies regarding how to characterize cocoa quality are fundamental to guarantee that the cocoa value chain provides the best products, and thus the best chocolate.

Cocoa bean quality

The monetary value of cocoa beans is driven by the offer and demand of the markets, and the physical trading is mostly done through the standard terms designed by associations such as The Federation of Cocoa Commerce (FCC) and The Cocoa Merchant's Association of America (CMAA) (Dand, 2011). Within the contract terms, the quality and weight specifications are key in establishing the prices of the cocoa beans. Moreover, there are some quality requirements that affect the trade, and these are divided into food safety, economic, and qualitative aspects (Fowler & Coutel, 2017). In terms of food safety, high concentrations of cadmium, lead, mineral oil hydrocarbons, mycotoxins, and pesticides, as well as the high presence of moldy or infested beans are not desirable (CAOBISCO/ECA/FCC, 2015). Regarding economic aspects, the bean size and uniformity, shell content, fat, and moisture percentage, along with the presence of foreign matter and cocoa residues are critical (CAOBISCO/ECA/FCC, 2015). For the qualitative aspects, hardness and flavor are of primary importance (CAOBISCO/ECA/FCC, 2015). Despite the multiple quality requirements that describe good cocoa bean production, a detailed evaluation of all the parameters is not always done during trading. However, the most critical characteristics, such as fermentation parameters (presence of moldy, slaty, and infested cocoa beans, which are highly related to good agricultural/manufacture practices during post-harvesting and transportation), as well as the bean size are assessed for the shiploads. The sampling for quality test is

done for typically 20% and one-third of the shipment (Fowler & Coutel, 2017), using the cut test and the bean count test. The cut test is a visual observation of the surface of the cocoa bean after a longitudinal cut to determine the degree of well-fermented beans, whereas the count test consists of determining the average number of cocoa beans that weigh 100 g.

Qualitative aspects such as the cocoa flavor is one of the most important quality properties of cocoa beans (Mohamadi Alasti et al., 2019). According to ICCO (2021), the world cocoa market distinguishes between two broad categories of cocoa beans: bulk and fine or flavor cocoa beans. Most of the cocoa produced worldwide is considered bulk, whereas fine or flavor cocoa beans have their own supply chain, which differs by preserving the identity of the individual lots and testing bean quality before delivery (Fowler & Coutel, 2017). The definition of fine or flavor cacao is controversial, and there are no agreed and objectively measured specific standards for that denomination, other than a classification by the ICCO what percentage of a country's production is fine or flavor cacao. Fine or flavor cacao is estimated to account for about 12% of the total cocoa bean production, with Latin America being the main producer (ICCO, 2021). The production of fine or flavor cocoa beans has been encouraged in recent years due to a growing demand for premium cocoa products. The assumption that this higher quality cacao also commands a premium over the price of the London and New York cocoa stock exchanges, however, is not guaranteed, therefore, premiums over bulk cocoa prices are not always paid. Nevertheless, the certification by the ICCO for countries to be designated fine or flavor cacao producing countries is still highly desirable. Flavor is an important characteristic in the cocoa market. Studies regarding the monitoring of flavor during cocoa processing are fundamental to define what are the main drivers for flavor and how to improve the current production towards higher cocoa quality. It is therefore necessary to define what is flavor and summarize the current knowledge how cocoa post-harvest processes affect cocoa flavor.

Development of cocoa flavor during processing

Flavor is defined as the integration of smell, taste, and oral touch sensations (Bartoshuk et al., 2019), and is driven by the chemical composition of the food. Flavor compounds are divided into volatile and non-volatile compounds. In cocoa manufacture, more than 600 volatile compounds have been identified in cocoa and chocolate products (Afoakwa et al., 2008), whereas at least 66 non-volatile compounds (Kauz et al., 2021) have been found in the non-fat cocoa portion to elicit taste and/or mouthfeel sensations. The variability of chemical composition of the cocoa beans has many sources, starting with the geographical origin (Marseglia et al., 2020), the cacao variety (Tuenter, Delbaere, de Winne, et al., 2020), the ripeness stage of harvested pods (Dang & Nguyen, 2019), as well as various postharvest operations (Hamdouche et al., 2019; Hinneh et al., 2018; Rodriguez-Campos et al., 2012). Therefore, the same good agricultural and manufacturing practices in cocoa bean processing may still lead to different flavor profiles. The above is a challenge for the consistency of mainstream standardized products. Therefore, procedures that generate consistency in cocoa bean production are highly desirable in industry.However, new approaches for cocoa processing may take advantage of that variation to provide unique products with high quality standards.

Flavor development of cocoa during processing starts right after the cocoa pods are opened once harvested. With the environment that cocoa pulp provides which contains 10-15% sugars, 1-3% citric acid and the presence of protein and minerals (Afoakwa et al., 2013; Schwan & Wheals, 2004), microorganisms such as yeast, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) inoculate the cocoa beans and participate in the fermentation process in succession. This succession is ruled by the products of metabolism of one type of microorganisms that encourage or represses the growth and activity of the subsequent group.

Cocoa fermentation can be divided into three main phases: A first anaerobic phase is dominated by yeast during the first 24-48 hours of fermentation (de Vuyst & Weckx, 2016), and leads to de-pectination of the pulp, which liquifies it and generates the CS, and the production of ethanol. *Sacharomyces cerevisiae* is the dominant yeast in the cocoa beans taken from boxes immediately after filling (Schwan & Wheals, 2004), but other genera such as *Pichia* and *Kluyveromyces* have also been reported (Crafack et al., 2014; Schwan & Wheals, 2004). Ethanol production in cocoa fermentation increases the temperature, and volatile compounds such as esters and alcohols are also generated (Crafack et al., 2014; Koné et al., 2021; Owusu et al., 2012). The effect of yeast strains with high aromatic and pectinolytic activities on volatile and sensory profiles of chocolate was studied by Crafack et al., (2014), who showed that compared to spontaneously fermented cocoa, chocolate made from beans that have been inoculated with *P. kluyveri* (a highly aromatic strain) had a higher concentration of phenylacetaldehyde, whereas chocolate made from beans fermented with *K. marxianus* (a highly pectinolytic strain) had higher contents of benzyl alcohol, phenethyl alcohol, benzyl acetate and phenethyl acetate. Despite these significant chemical changes semi-trained judges were not able to perceive a significant difference in triangle tests of 66% chocolate made of cocoa liquor fermented by *P. kluyveri* or *K. marxianus* and cocoa liquor with spontaneous fermentation. It could be that the observed chemical chantes were too small to lead to perceivable sensory differences in the 66% finished chocolates. Therefore, more studies are needed to better understand the effect of specific yeast strains and yeast combinations during fermentation on final cocoa and chocolate flavor.

At the end of the anaerobic phase in cocoa bean fermentation after 24 to 72 hours, the pulp is completely liquified and drained off, and more air enters the fermentation mass. These conditions favor the growth of lactic acid bacteria (LAB) (de Vuyst & Weckx, 2016), which metabolize sugars into lactic acid that imparts sourness (Ardhana & Fleet, 2003). Despite their presence in spontaneous cocoa bean fermentation, Ho et al., (2015) reported that LAB may not be necessary for successful cocoa fermentation: in fermentations where LAB growth was restricted, similar levels of ethanol and acetic acid were formed, and the volatile profiles were similar to the control fermentations where LAB were present. More studies are needed to confirm this finding (de Vuyst & Weckx, 2016).

Following the LAB phase, cocoa fermentation enters an aerobic phase after 48 hours, where acetic acid bacteria (AAB) become predominant. During this stage, the ethanol and the lactic acid produced previously is metabolized into acetic acid and acetoin (de Vuyst & Weckx, 2016; Schwan & Wheals, 2004). An additional transformation of the acetic acid into carbonic acid and water is also facilitated by AAB, which leads to fluctuating pH due to the production and degradation of organic acids. All these chemical reactions are exothermic, and the temperature of the fermenting mass may reach 45-50°C (Aprotosoaie et al., 2016; de Vuyst & Weckx, 2016). These changes lead to evaporation of volatile compounds and favor the formation of additional flavor precursors in the cocoa beans, such as peptides due proteases activity (Santander et al., 2021). In advanced stages of fermentation, aerobic spore-forming bacteria and filamentous fungi may become dominant and generate off-flavors (Schwan & Wheals, 2004) and pyrazines (Reineccius et al., 1972; Schwan & Wheals, 2004).

During fermentation, microbial activity causes changes in temperature, pH, and oxygen in the fermentation mass. These changes trigger the death of the bean, the release of its constituents and their consequently interaction and transformation into flavor precursors (de Vuyst & Weckx, 2016; Gutiérrez, 2017). The main constituents that experience changes during cocoa fermentation are proteins, carbohydrates, and polyphenols (Santander et al., 2020). Storage proteins like vicilin-class (7S) globulin are hydrolyzed due to action of proteases such as aspartic endoprotease and carboxypeptidase, generating peptides and free amino acids (de Vuyst & Weckx, 2016; Santander et al., 2020). Since the catalytic activity of cocoa enzymes is affected by pH, Santander et al. (2020) described that moderate

acidification (pH 5.5-5.0) results in high levels of hydrophobic amino acids and hydrophilic peptides, which are related to high flavor potential in cocoa. Therefore, despite microorganisms do not directly degrading bean storage proteins, their activity encourages conditions under which proteases form these flavor precursors. Similarly, carbohydrates in the cocoa bean are also hydrolyzed by endogenous bean invertases to produce reducing sugars during fermentation (Santander et al., 2020). Because peptides, free amino acids, and reducing sugars are considered important cocoa flavor precursors, understanding which conditions impact enzymatic activities in cocoa bean is key for linking cocoa fermentation kinetics to final cocoa flavor. Additionally, designing strategies for affecting the media physicochemical conditions may be a way to control the flavor compounds produced in cocoa.

Polyphenols are compounds related to bitterness and astringency in cocoa (Kauz et al., 2021), and are also affected during fermentation. After 3-4 days of fermentation these compounds typically decrease in concentration (Santander et al., 2020). In fact, Albertini et al. (2015) reported epicatechin and total polyphenol content (measured by the Folin-Ciocalteu method) to decrease by up to 80 and 67%, respectively, in fermented beans compared to unfermented dried cocoa beans. The mechanisms of transformation of polyphenols in cocoa beans varied during processing. For instance, oxidases such as polyphenol oxidase or glucosidases may degrade polyphenols during fermentation (Santander et al., 2020), in a pH-dependent manner. Besides measuring total polyphenol content and/or individual monomeric polyphenols, mean degree of polymerization or Maillard reaction products have been suggested for tracking polyphenol transformations occurring due cocoa processing (Racine et al., 2019a).

After fermentation, the cocoa beans are dried until reaching a moisture of approximately 7-8% (Fowler & Coutel, 2017). Volatile compounds such acetic acid evaporates, and some flavor development continues due to Maillard reactions between the flavor precursors produced during fermentation. For instance, low concentrations of aldehydes such as 2-methylbutanal and 3-methylbutanal that have been reported to be important to cocoa flavor (Frauendorfer & Schieberle, 2006) are already found in unroasted cocoa beans (Rodriguez-Campos et al., 2012).

Roasting is the subsequent operation in cocoa bean processing after drying. It is considered an important step in flavor formation as volatile compounds are evaporated, while new compounds are formed via Maillard and other thermal reactions (Afoakwa et al., 2008; Aprotosoaie et al., 2016; Santander et al., 2020). The group of Maillard reactions involve reduced sugars and other carbonyl compounds reacting with an amino group of a peptide or a free amino acid to form a Schiff base (Afoakwa et al., 2008; Santander et al., 2020). The Schiff base is transformed into Amadori compounds that undergo numerous reactions to generate volatile compounds. Different sequences of reactions take place during roasting, such as Strecker degradation, which generates flavor-active aldehydes and ketones. These flavor compounds can further react through aldol condensation to form pyrazines and other heterocyclic compounds (Afoakwa et al., 2008; Ziegleder, 2017), which are also important for cocoa flavor. According to Afoakwa et al. (2008), pH influences the intermediate formation and the type of flavor compounds that are produced during roasting. Basic pH such as the ones triggered in alkalinization increase the rate of Maillard reactions due to the deprotonation of the amino group, and the consequently facilitation of the formation of the Schiff base. This alteration in pH prior to roasting causes reduction in astringency, bitterness and intensify color in cocoa (Moser, 2015; Ziegleder, 2017)

Non-volatile compounds that are responsible for astringency or bitterness in cocoa are also affected by roasting. For example, the heat during roasting induces the formation of 2,5-diketopyperazines from peptides in cocoa beans (Ziegleder, 2017), epimerize flavanols such as (-)-epicatechin into (-)-catechin (Kothe et al., 2013), reduce the content of procyanidin B2 (McClure et al., 2021), procyanidin B1, and

procyanidin C1 (Żyżelewicz et al., 2016a), and increase the content of hexamers and heptamers of procyanidins (Stanley et al., 2018). Conversely, there is not a consensus about the effect of processing on the alkaloids, caffeine and theobromine. Whereas McClure et al. (2021) reported no quantitative changes during roasting and Ziegleder (2017) indicates no quantitative change during fermentation and roasting, Aprotosoaie et al. (2016) stated that alkaloids decrease with longer periods of fermentation. Thus, understanding how non-volatile compounds change during processing (i.e., fermentation, roasting) will provide knowledge about their chemical stability and potential flavor effect in cocoa products.

Preconditioning operations for flavor development in cocoa – use of Cocoa Sweatings (CS)

Processing induces several changes in the volatile and non-volatile composition of cocoa beans. During fermentation, the media conditions of the cocoa mass, and more specifically, the acidification of the cocoa beans plays an important role in facilitating the enzymatic and non-enzymatic reactions important to cocoa flavor formation (Afoakwa et al., 2008). According to Santander et al. (2020), moderate acidification (pH 5.5-5.0) during fermentation leads to high levels of hydrophobic amino acids and hydrophilic peptides, while strong acidification (pH 4.5-4.0) generates a nonspecific proteolysis of the cocoa seed proteins, and consequently nonspecific flavor compounds. Because cocoa pulp is the substrate for ethanol, lactic and acetic acid production (compounds directly related to acidification), the following strategies to limit acidification of the pulp have been explored: cocoa pod storage, pre-drying of cocoa seeds, de-pulping of cocoa seeds, and addition of pectinolytic enzymes (Santander et al., 2020; Schwan & Wheals, 2004). These operations are meant to increase de-pectination of the pulp, reduce its volume/weight, decrease the acidification of the fermentation mass, and subsequently, generate optimal conditions for desirable flavor compound production. For instance, short periods (2-3 days) of pod storage have been found to be beneficial for the generation of esters (Hamdouche et al., 2019), aldehydes, and pyrazines (Koné et al., 2021) in unroasted cocoa beans from Ivory Coast. In addition, long times of pod storage (7 days) were recommended for Ghanian cocoa beans to obtain a higher content of free amino acids, and the formation of more volatile compounds upon roasting (Hinneh et al., 2018). Taken together, changes to the cocoa pulp matrix appear to enhance the production of flavor precursors in cocoa beans and potentially affect final cocoa flavor. Even though changing the fermentation media in cocoa processing has been shown to influence the production of flavor precursors positively, the addition of cocoa sweatings during fermentation has not been explored yet. Additionally, despite the effect of the disruption of the cocoa pulp in flavor compounds, none of the studies previously mentioned carried out any sensory evaluation in cocoa products. Therefore, the effect of sensory perception after the application of any of this cocoa postharversing operations in these studies still unknown.

Cocoa sweatings (CS) are the lixiviate produced mainly during early stages of cocoa bean fermentation, resulting from de-pectination of the cocoa pulp, which releases water and other components from the pulp, and are typically drained out of the fermentation system. This liquid is almost transparent and rich in fermentable sugars, pectin, and organic acids (Guirlanda et al., 2021; Schwan & Wheals, 2004; Vásquez et al., 2019). Depending on when during fermentation CS is sampled, physical-chemical parameters reported in the literature include pH (2.76 - 5.25) (Afoakwa et al., 2013; Guirlanda et al., 2021), soluble solids (4.38 – 19.60 %), titratable acidity (0.71 - 0.72 % citric acid), reducing sugars (8.63 – 10.41%), glucose content (2.13 - 21.40%), fructose (1.06 - 4.42%) and sucrose (2.13 - 4.06%) (Adams et al., 1982; Guirlanda et al., 2021). Therefore, CS may affect the physicochemical properties of the cacao

mass and provide additional substrates to cocoa bean fermentation. Subsequently, the addition of CS could affect flavor precursor formation, and consequently, change flavor composition in cocoa. Cocoa pulp products have been investigated for product development such as jellies (Vallejo T. et al., 2010) and wine (Dias et al., 2007), but further studies are necessary to determine how the addition of CS to the cocoa fermentation mass affects cocoa flavor composition.

Chemical composition and sensory perception of cocoa

Monitoring changes in volatile and non-volatile composition of cocoa throughout the processing chain may give insights about how final flavor composition links to intermediate processing steps. Additionally, linking changes in composition to perceptual differences detected with sensory methods may lead to further understanding how compounds affect overall flavor of cocoa. Despite odors such as honey, nutty, rancid, etc. and tastes, such as astringency and bitterness, are perceivable for individual volatile and non-volatile compounds (Aprotosoaie et al., 2016; Kauz et al., 2021), it is important to note that these individual odor and taste notes may not necessarily be reflected in the overall flavor in cocoa. Factors such as individual differences in sensory threshold, differences in psychophysical functions as well as perceptual synergistic and antagonistic interactions between flavor compounds need to be considered (Miyazawa et al., 2008). Therefore, these attributes were not used in this study exclusively by the presence of flavor compounds that were reported in literature with those sensory notes.

To address which volatile compounds have the greatest effect on overall flavor perception in cocoa, Odor Activity Values (OAV) have been used. The OAV is defined as the ratio of the compound concentration in the food divided by the threshold concentration in the same matrix (Audouin et al., 2001). Volatiles with OAV above 1 have been deemed important for overall flavor perception in cocoa (Escobar et al., 2021; Frauendorfer & Schieberle, 2019; Tuenter, Delbaere, de Winne, et al., 2020). However, Audouin et al. (2001) demonstrated that the odor intensity function depends on the odorant, and that two components having the same OAV do not necessarily exhibit the same perceived intensity, and subsequent contribution to overall flavor. Compounds can differ not only in thresholds but also in the response along the psychophysical range curve, that is, the same change in concentration can lead to vastly different intensities depending on the slope of the curve (Ferreira, 2010). Furthermore, sub-threshold enhancing and suppressing compounds to overall flavor in volatile mixtures have been determined (Ferreira, 2010; Grosch, 2001) Thus, selection of volatile compounds based exclusively on OAV is in almost all cases not a true representation of their contribution to overall cocoa flavor. In a similar manner, sensory threshold concentrations of bitter and astringent cocoa non-volatile compounds were determined by Stark et al. (2006). Using fractionation of cocoa, individual compounds were isolated and reconstituted in aqueous solutions, then ranked in importance for their contribution to cocoa bitterness and astringency based on their threshold values. Most critically, threshold values are also a function of the matrix – reconstituting compounds in water are not reflective of the low water and high fat matrix of cocoa. Past work has shown that even small changes to the matrix strongly affect threshold values (Perry & Hayes, 2016). For cocoa, the reported non-volatile compounds are found in a wide variety of cocoa products (Kauz et al., 2021), however, their actual contribution to bitterness and astringency of cocoa requires further studies. This study did not used AOV determination in the screened flavor compounds due their lack of reliability in the contribution of flavor/taste in mixtures.

Alternative approaches to elucidate the relationship between chemical composition and sensory perception have been attempted by pairing instrumental analysis of volatile and/or non-volatile compounds with descriptive analysis (DA). Crafack et al. (2014) reported significant concentration differences in some alcohols (benzyl alcohol, phenethyl alcohol), esters (benzyl acetate, phenethyl acetate) and aldehydes (phenylacetaldehyde) in chocolate fermented with *K. marxianus* and *P. kluyvery*

compared to spontaneous fermentation, which resulted in higher fruity, berry, yogurt, and balsamic flavor profiles. However, these differences were too small to significantly change sensory perception based on triangle tests when compared to chocolate from spontaneous fermentation. Using DA, Escobar et al. (2021) concluded that a 96 hour (4 day) fermentation produced chocolate with fruity, floral, spicy, and nutty (fine flavor) sensory attributes compared to chocolate fermented for 72 hours and 122/144 hours. They also determined 20 volatiles and 48 non-volatile metabolites as potential quality biomarkers in these samples by untargeted volatile/nonvolatile analysis. The selection of these compounds was based on regression models with time of fermentation as dependent variable. The identified biomarker compounds showed the strongest association in the model that classified cocoa flavor quality (basic, fine, off flavor) according to descriptors used in DA. Chocolate produced for 96 hours of fermentation showed the highest levels of the identified selected metabolites and strongest correlation to the fine flavor category.

Sensory analysis with regular chocolate consumers has been used to determine the effect of roasting and cocoa origin on cocoa flavor. McClure et al. (2022) showed that longer time and high temperature during roasting in general decreased the bitterness of 100% chocolate, and consequently, increased consumer liking. Therefore, despite consumers may not be able to consistently provide or notice subtle changes in specific and more difficult to define sensory attributes, they are still able to perceive sensory differences in products. In fact, chocolate flavor is one of the main drivers for purchase. Therefore, linking consumer perception with chemical analysis provides a better understanding how processing changes cocoa flavor and acceptability, and through correlation analysis could indicate potentially responsible compounds. More studies are required to link chemical data of cocoa with sensory analysis, and thus, describing flavor changes of cocoa products due to processing.

Although difference testing with regular chocolate consumers does not provide information about how and by how much the samples differ from each other as DA does, It would provide information whether consumers are able to perceive differences between cocoa samples. These results open the door for further DA studies to determine which are the drivers of difference. On the opposite, if DA with a trained panel is done first, the differences found between samples may be too subtle for consumers to notice. Additionally, the limited amount of sample available did not allow for conducting a trained DA panel Therefore, this study aimed to identify if the difference in fermentation time and fermentation type would lead to sensory difference perceivable by regular chocolate consumers.

Hypothesis and Objectives

Cocoa flavor is an important characteristic in defining cocoa quality, and it is driven by volatile and non-volatile composition. Volatile and non-volatile compounds in cocoa are generated from the transformation of the native cocoa bean constituents during processing. Despite numerous studies examined volatile and non-volatile composition of cocoa during processing, including fermentation and roasting, no studies thus far have studied the effect of the addition of CS during fermentation on cocoa flavor formation throughout processing or their effect on sensory perception of the final cocoa product. Since CS contains sugars and other substrates that drive fermentation, their addition may stimulate the production of flavor precursors, and subsequent flavor composition. Based on prior literature, I hypothesize the following:

(1) **The addition of CS** produced in the first 18 hours of fermentation **will increase the production of cocoa flavor compounds** in unroasted cocoa powder and roasted liquors.

(2) **Fermentation time** (5, 6, and 7 days) **significantly affects cocoa flavor compounds in unroasted cocoa, either by increasing or decreasing in concentration**. I further hypothesize that **this fermentation time effect remains detectable after roasting**

(3) **Cocoa liquors fermented with the addition of CS will be perceptual similar** after 5 days of fermentation **to cocoa liquors fermented without the addition of CS** after 7 days of fermentation.

To test the proposed hypotheses, the following objectives were established:

(1) To determine the effect of the addition of CS on flavor compounds during late stages of fermentation time (5, 6, and 7 days) by measurement of volatile and non-volatile compounds in unroasted cocoa beans and roasted cocoa liquor.

(2) To assess the effect of the addition of CS during fermentation on sensory perception of roasted cocoa liquor using regular chocolate consumers in a sensory triangle test.

MATERIALS AND METHODS

Chemical and reagents

Naphthalene-D8 (Sigma Aldrich, St. Louis, MO, USA) (131 mg/L in methanol) was used as internal standard for volatile semi-quantitation, and an alkane standard solution (C8-C20; 40 mg/L in n-hexane; Sigma Aldrich, St. Louis, MO, USA), was used for retention index (RI) determination. Pure reference standards (at least 97 % purity) were obtained from Alfa Aesar (Tewksbury, MA, USA or Heysham, GB): heptanal, 2,3-dimethylpyrazine, octanal, 1,2-propanediol monomethyl ether acetate, 2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-(-)- (pantolactone), 2-methyl-1-butanol, 2,3-Butanedione, 2-pentylfuran. The following compounds (at least 90% purity) were obtained from Sigma Alrich (St. Louis, MO, USA): 2,3-Butanediol, phenethyl alcohol, 3-methylbutanal (isovaleraldehyde), ethyl phenylacetate, benzaldehyde, acetophenone, methyl phenylacetate, phenethyl acetate, 1-Penten-3-one, ethyl acetate, 3-Methylbutyl acetate (Isoamyl acetate), 3-methylbutanoic acid, acetoin, ethyl hexanoate, (+/-) catechin hydrate, procyanidin B2, caffeine and (-)-epicatechin. The standards (at least 95% purity) 2-methylbutanal, furfural, 2-heptanol, ethyl 2-methylpropanoate (ethyl isobutyrate), 2-methylpropanoic acid (isobutyric acid), hexanal, 2-methylpropyl acetate, methyl 2-methylpropanoate, methyl 3-methylbutanoate, ethyl-2-methylbutyrate, 2-acetylfuran, and pentanal were sourced from TCI (Portland, OR, USA). Benzyl alcohol and 2-methylbutyric acid (98%) were acquired from Fisher Thermo Scientific (Millersburg, PA, USA). Anhydrous ethanol (200 proof) was sourced from Koptec (Philadelphia, PA, USA). Cyclo(Proline-Valine) was acquired from Bachem (Bubendorf, Switzerland). Cyclo(L-Leucine-L-Proline), Quercetin-3-O-glucoside (isoquercitin) and trans-clovamide were obtained from Cayman Chemicals (Ann Arbor, MI, USA). Quercetin 3-galactoside (Hyperoside) was sourced from Indofine Chemical (Hillsborough, NJ, USA). Theobromine was purchased from MP Biomedicals (Solon, Ohio, USA). HPLC and GC-grade solvents were sourced from Fisher Scientific, Sigma-Aldrich, and Alfa Aesar

Cacao samples processing

Harvesting and production of dried cocoa beans

A mixture of ripe cocoa pods from different TSH varietals were harvested from a farm in Wallerfield, Trinidad and Tobago (DD: 10.645364, -61.213983) in June 2019. The pods were opened, the seeds collected directly into a clean bucket, and 144 kg of cocoa seeds were thoroughly mixed in a big container. Six sub-samples of 24 kg each were then placed into polystyrene fermentation boxes (44.5 cm x 20 cm x 30.5 cm) with holes at the bottom. The top of the cacao seed mass inside the boxes was covered with a layer of banana leaves, a polypropylene bag, and the polystyrene box lid. All the boxes went under spontaneous styro-cooler fermentation based on the recommendations stipulated by CAOBISCO/ECA/FCC (2015).

The six fermentation boxes were allocated to two different fermentation treatments with three biological replicates each (**Figure 1**):

(1) Three boxes were fermented conventionally where the beans were turned and re-mixed every two days – fermentation without addition of cocoa sweatings (CS)
(2) The other three boxes were fermented in a novel way, where the cocoa pulp run-off from the first 18 hours, so-called cocoa sweatings (CS), was collected from the bottom holes of the three boxes and poured over the top of the mass once at 18 hours – fermentation with addition of CS. The same turning procedure and times as for the control fermentation treatment were applied.

All the boxes were allowed to ferment up to 7 days. On days 5, 6, and 7 of fermentation, 4 kg of wet fermented beans were taken from each fermentation box (**Figure 1**). Temperature of fermentation was measured every 12 hours at the corners and the center of each fermentation box, reaching a maximum average temperature of 39.6 ± 0.2 ˚C and 40.07 ± 1.81 ˚C in the treatments with and without addition of CS, respectively. The complete temperature profiles for both types of fermentation and all 6 boxes are shown in **Supplemental figure 1**.

All fermented beans were solar dried until the moisture level was around 7%. Moisture content of the beans was measured in triplicate with a calibrated digital moisture analyzer (MC-7825KK, AMTAST, Lakeland, FL, USA). Once dried, samples were packed in press-seal bags and shipped to The Pennsylvania State University. Upon arrival at the Food Science department at The Pennsylvania State University, samples were transferred to food-grade plastic containers, stored in the dark at room temperature and controlled humidity until further processing.

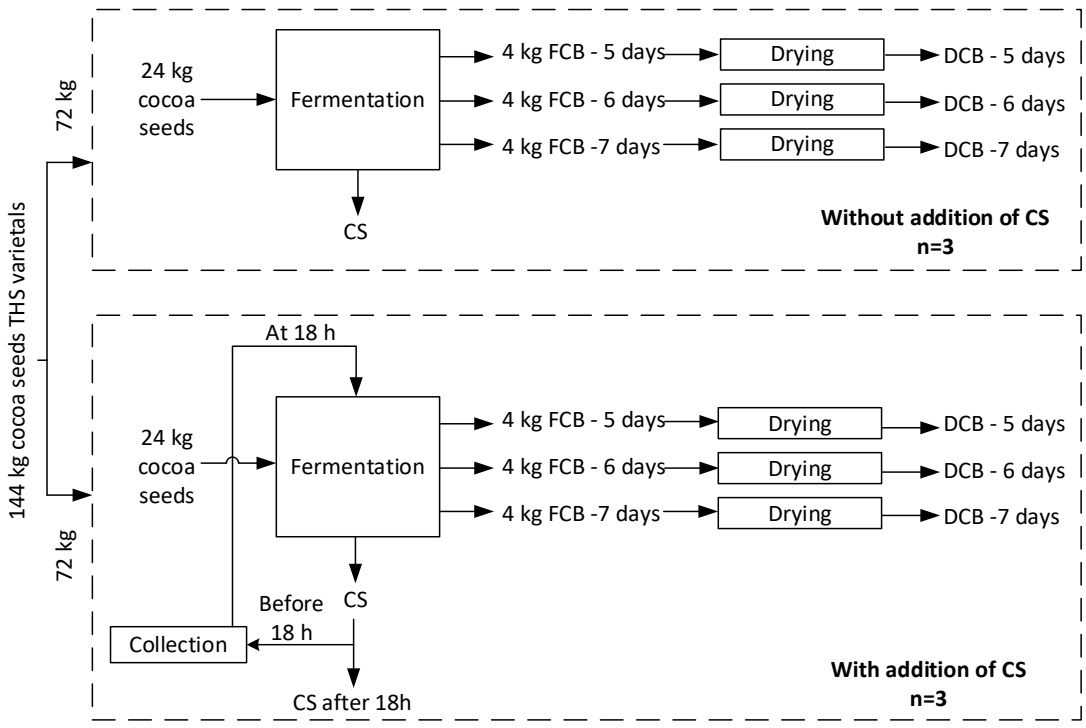


Figure 1 Production of fermented and dried cocoa beans. CS = Cocoa sweating, FCB = Fermented cocoa beans, DCB = Dried and fermented cocoa beans

Unroasted cocoa powder preparation

Fifty dried beans from each sample were manually deshelled, and the deshelled unroasted cocoa beans were ground with liquid nitrogen (Praxair, Bellefonte, PA, USA) in a A11 basic mill (IKA, Wilmington NC, USA) for 30 seconds to obtain a fine powder. The unroasted cocoa powder was stored in amber glass vials at -80˚C until analysis. Prior to analysis, sample were thawed overnight at room temperature.

Due to logistic reasons, one biological replicate of one of the fermentation treatments was lost: the cocoa powder from 6 days of fermentation with the addition of CS only had two biological replicates, whereas the rest of the conditions had three.

Cocoa liquor preparation

Roasting of 1 kg of cocoa beans from each sample was done in a forced-air laboratory oven (Model no. FED 56UL, Binder, Tuttlingen, Germany) at 125°C for 30 minutes, following the procedures described in Brown (2021). The roasted product was winnowed (Cacao Cucina, Clearwater, Florida, USA) and the nibs were collected. The nibs were preheated in the oven at 45°C and then placed in 8-pound capacity grinders (Premier Wonder Grinder, Diamond Trading, Hillsborough, New Jersey, USA) and ground for 24 hours to ensure a particle size distribution (d90) below 19 µm (Brown, 2021). The resulted cocoa liquor was placed in glass jars with caps covered with aluminum foil, and stored at 5˚C until analysis. Prior to analysis, the samples were preheated at 40°C overnight in a forced-air laboratory oven (Model no. FED 56UL, Binder, Tuttlingen, Germany).

Instrumental analysis

Total fat content by low resolution Nuclear Magnetic Resonance (NMR) Spectroscopy

Unroasted cocoa powder or cocoa liquor were weighed (1.54 ± 0.04 g) into glass NMR tubes (180 mm x 17.75 mm x 0.6 mm; Bruker, Billerica, MA, USA) and preheated at 40˚C for 2 h in a forced-air laboratory oven (Model no. FED 56UL, Binder, Tuttlingen, Germany). For analysis, the preheated tube was removed from the oven and immediately placed in the NMR for analysis (minispec mq20, Bruker, Billerica, MA, USA), following method as described in Told et al. (2006). The fat content was expressed as grams of fat per 100 g of sample, and each cocoa powder and liquor sample was measured in triplicate. Accuracy of the instrument was checked daily at the beginning of analysis using vegetable oil.

Untargeted volatile analysis by headspace solid phase-microextraction coupled to gas chromatography-mass spectrometry (HS-SPME-GC-MS)

Unroasted cocoa powder or molten cocoa liquor (0.54 ± 0.02 g) were placed in an amber headspace screw cap vial (20 mL, 23 x 75 mm; Restek, Bellefonte, PA, USA), together with a magnetic stir bar and 10 μL of internal standard (131 mg/L D8-napthalene in methanol), deposited onto a small circle of filter paper (0.5 cm; Whatman filter paper grade 597). Each analytical replicate of samples was bracketed by QC samples, containing 0.5 g of cocoa butter (Blommer Chocolate company, East Greenville, PA, USA) in lieu of sample. Alongside each set of samples, a vial with a mix of n-alkanes (C8-C20; Sigma-Aldrich, St. Louis, MO, USA) was analyzed for retention index (RI) determination. All vials were capped with magnetic screw caps with a PTFE/silicon septum (Restek) and equilibrated at room temperature for 24 h prior to analysis by HS-SPME-GC-MS. For analysis, a MPS robotic autosampler (Gerstel US, Linthicum Heights, MD, USA) was used. Samples were equilibrated at 37°C and agitation of 500 rpm for 5 min prior to volatile extraction for 45 min and 250 rpm with a 2 cm SPME fiber (divinylbenzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS) (Supelco, Bellefonte, PA, USA). Extracted volatiles were thermally desorbed at 250°C for 10 min in a 0.7 mm inner diameter SPME inlet liner (Supelco) using a 7890B GC system with a 5977B single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Volatiles were transferred in splitless mode (purge time 1.2 min) using a constant carrier gas flow of 1 mL/min ultrapure helium (Linde, Bellefonte, PA, USA) and separated on a Rtx-WAX column (Restek; 30 m x 250 μm x 0.25 μm) with the following temperature gradient: 30°C for 3 min, 3˚C/min to 150˚C, 50˚C/min to 250˚C, with a final hold at 250˚C for 10 min.

Volatiles were detected in electron ionization (EI) scanning mode (m/z 33-350; 8.1 scans/sec). Automatic compound deconvolution and peak area extraction was carried out using PARADISE software (Version 3.9) (Johnsen et al., 2017), and compounds were tentatively identified by mass spectral matching with the NIST Mass Spectral Library (version 17; (NIST, 2014a)), after applying a minimum abundance cut-off limit of 10^5 units. The peak areas of each tentatively identified compound were expressed as internal standard equivalent (ISE) concentrations in μg D8-Napthalene per g sample. For compound verification, retention indices (RIs) according to van Den Dool & Dec. Kratz (1963) were calculated and compared to literature RI reported in the NIST spectral library. In addition, compounds were verified with authentic standards (where available), otherwise peaks were classified as unknown.

Targeted non-fat non-volatile analysis by Ultra-High-Performance - Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS)

Unroasted cocoa powder or cocoa liquor were weighed (0.506 ± 0.004 g) into 50 mL centrifuge tubes (Celltreat, Pepperell, MA, USA) and kept at 40˚C overnight prior to extraction. Each sample was extracted twice. For extraction, 3.75 mL of acetonitrile:water (80:20, v/v) were added to the tubes and shaken vigorously by hand for 5 sec, then vortexed for two minutes. Then, samples were shaken by hand again and placed in an ultrasonic water bath held at 40˚C for 10 min and centrifuged for 5 min at 20˚C and 3184 *g*. The supernatant was transferred onto a SPE cartridge (600 mg, 6 mL, Captiva EMR-Lipid, Agilent Technologies, California, USA) and separated using a VacElut manifold and a slight vacuum of -5 inHg (~ -16.9 kPa). The pellet from the first extraction was extracted a second time with another 3.75 mL of acetonitrile:water (80:20, v/v) as described above. The supernatant from the second extraction was transferred to the same SPE cartridge as the first extraction, and similarly eluted. The combined defattened extract from both extractions was stored at 5˚C for 24 hours. For analysis, 150 µL of defattened extract was diluted 1:10 with 0.1% formic acid in HPLC-grade water, filtered through a 0.2 µm PTFE filter (VWR International, Philadelphia, PA, USA), and stored at 5˚C until analysis within 3 days.

Reversed-phase ultra-high performance liquid chromatography (RP-UHPLC) analyses were performed on a 1290 Infinity II LC system (Agilent Technologies) adapted from methods described by Ortega et al., (2010) and Kauz et al., 2021: A C-18 column (2.1 mm x 100 mm, 1.8 µm; Acquity UPLC HSS, Waters, Milford, MA, USA) was used at a flow rate of 0.4 mL/min and a column temperature of 35˚C. The mobile phase A was 0.1% formic acid in water, whereas mobile phase B was 0.1% formic acid in acetonitrile. The volume of injected sample was 5 µL. The elution gradient was 100% A, 0 – 0.5 min; 100 - 84.47% A, 0.5 – 3.2 min; isocratic 84.47% A, 3.2 – 7.5 min; 84.47 – 5 % A, 7.5 – 8.0 min; isocratic 5 % A, 8.0 – 11.1 min; 5 – 100% A, 11.1 min – 12.50min; and isocratic 100% A, 12.5 min – 13.0 min. The UHPLC was coupled to a triple quadrupole mass spectrometer (6460 Triple Quad LC/MS, Agilent Technologies, California, USA). The MS/MS was using electrospray ionization (ESI), operated in positive and negative mode, with a capillary voltage of 3500 V, a nitrogen sheath gas flow of 4 mL/min held at 350˚C, and the nebulizer pressure set to 20 psi. Compounds of interest (**Table 1**) were detected and quantified using multiple reaction monitoring (MRM). The collision energies were optimized to obtain two transitions per compound, one qualitative and one quantitative, and the obtained peak areas were converted into concentrations per g sample using external calibration curves using authentic standards. For all the compounds, the fragmentor voltage was set at 135 V and the cell accelerator voltage was set at 4 V (**Table 1**). The compounds of interest were chosen from a list reported by Kauz et al. (2021) and studied by McClure et al. (2021), focusing on cocoa compounds involved in the perception of astringency and bitterness, including one amino acid amide (trans-clovamide), two polyphenol glucosides (quercetin-3-O-β-D-glucopyranoside and quercetin-3-O-β-D-galactopyranoside), three flavan-3-ols ((-)-epicatechin, (+)-catechin, and procyanidin B2), and two alkaloids (caffeine and theobromine) (**Table 1**). MassHunter Workstation LC/MS Data Acquisition (Version 10.1, Agilent Technologies) and Quantitative Analysis for QQQ (Version 10.1, Agilent Technologies, California, USA) were used for data collection and quantitation.

Table 1. Non-volatile compounds calibration curves and MRM settings for identification and quantification of analytes in cocoa samples.

Compound	MRM mode	RT (min)	Precursor Ion (m/z)	Product Ion (m/z)		Calibration curve*	R^2	LOD	LLOQ	Range
				Product Ion	Collision Energy			(ng/mL)		(ng/mL)
Caffeine CAS [58-08-2]	+	4.1	195	83 138	38 32	y = 5.68x + 44165	0.980	0.80	2.4	[852; 10016]
Cyclo (Pro-Val) CAS [2854-40-2]		4.0	197	72 169	29 10	y = 38.9x + 65.9	0.999	0.12	0.36	[74; 1143]
Theobromine CAS [83-67-0]		3.0	181	138 163	10 10	y = 2.66x + 423876	0.960	1.2	3.8	[$5.5x10^5$; $1.1x10^6$]
Cyclo (L-Leu-L-Pro) CAS [2873-36-1]		5.6	211	70 86	1 1	y = 4.67x + 118	0.995	1.1	3.2	[108; 4229]
(±)-Catechin CAS [7295-85-4]	-	3.9	289	245 109	10 40	y = 8.68x + 159	0.997	0.56	1.71	[108; 4229]
(-)-Epicatechin CAS [490-46-0]		4.5	289	245 123	10 38	y = 4.69x + 7425	0.993	0.81	2.45	[852; 10016] [601; 6830]
Procyanidin B2 CAS [29106-49-8]		4.2	577	425 289	13 20	y = 8.479x - 218.510	0.998	0.50	1.51	[100; 1087] [22, 216]
Isoquercitin (Quercetin-3-β-D-glucoside) CAS [482-35-9]		6.8	463	300 271	20 56	y = 26.75x + 24.9847	0.990	0.18	0.55	[57; 1646] [852; 10016]
Hyperoside (Quercetin 3-D-galactoside) CAS [482-36-0]		6.5	463	300 271	20 56	y = 47.20x + 93.8515	0.996	0.09	0.27	[601; 6830] [100; 1087]
Trans-clovamide (N-[(2E)-3-(3,4-dihydroxyphenyl)-1-oxo-2-propen-1-yl]-3-hydroxy-L-tyrosine) CAS [53755-02-5]		5.1	358	178 135	20 50	y = 11.46x – 313.406	0.993	0.36	1.09	[22, 216]

* y = Area under the peak, x = concentration of standard in dilution (ng/mL)

Sensory Analysis

To determine whether the experimental treatments led to perceivable differences between samples and/or if sensory similarity could be concluded, five non-specified triangle tests were conducted. The tests were designed to evaluate the effect of fermentation time (5 days vs. 7 days for both fermentation types, i.e., with and without the addition of CS), the addition of CS (with vs. without the addition of CS after 5 days and 7 days of fermentation), and the interaction between these two factors in the cocoa liquor samples (5 days of fermentation with the addition of CS vs. 7 days of fermentation without the addition of CS).

For the evaluation, a screener questionnaire (**Appendix**) was sent to the participant database of the Sensory Evaluation Center (SEC) at Penn State. The selection criteria for this study were: over 18 years in age; no allergies or insensitivities to dairy, peanuts, tree nuts, wheat, soy; no smell or taste disorders; not smoking; not pregnant or breastfeeding; not taking medications that alter smell or taste function; no history of choking or trouble swallowing; consuming dark or baking chocolate at least once per month.

Based on the screener and selection criteria, 120 participants were invited to taste the cocoa liquor samples in two sessions on subsequent days at the same hour. The first session was composed of three triangle tests whereas the second had two. All tests were presented in a counter-balanced order with regards to triangle test and regarding sample presentation within a test. In between each triangle test a 2-min break was enforced where participants were encouraged to cleanse their palate with room-temperatured water. For each triangle test, samples were presented in 1 fl.oz. plastic medicine cups labelled with a unique three-digit code. One piece of sample (0.30 ± 0.02 g of cocoa liquor) was provided, and participants were instructed to place the whole sample in their mouth to make a judgement. The test was performed in individual tasting booths in the Sensory Evaluation Center (SEC) at Penn State under red lights to mask small color differences between samples. After selecting the “odd or different” sample from the triad, participants were asked why they thought the selected sample was different using an open-comment box. Of the 120 participants, 92 participants completed all five triangle tests, while 16 participants attended only to the first session. All data was collected electronically using Compusense 20 sensory software (Compusense Inc., Guelph, ONT, Canada), and all screening and study procedures were exempt from institutional oversight based on exemption category 6, as determined by professional staff in the Institutional Review Board (IRB) at Penn State (protocol 33164).

Statistical analysis

Statistical analyses for the chemical measurements were conducted using R studio (Version 2022.02.2, Boston, MA, USA). Fermentation type (with and without the addition of CS), fermentation time (5, 6 and 7 days), type of sample (cocoa powder and cocoa liquor, only used in fat analysis) and the interaction between fermentation type and fermentation time were all treated as fixed effects, while the biological replicate (boxes 1, 2 and 3) was set as a random effect. The level of significance was set at 0.05 for all analyses.

For total fat content analysis, discriminating volatiles, and non-fat non-volatiles, a mixed effects analysis of variance (ANOVA) was carried out. Outliers were identified by the interquartile range (IQR) criterion (Stats and R, 2020), and identified outliers were removed from the analysis. For significant fixed effects

identified by ANOVA, estimated marginal means were compared using Tukey's honestly significant differences (HSD) test. Coefficients of variation (%CV) were determined for volatile and non-fat-non-volatile compounds to determine which type of fermentation (i.e. with/without addition of CS) has the biggest variability in terms of flavor composition based on the biological replicates. The determination of %CV was done regardless time of fermentation, and within time of fermentation (5, 6, 7 days), separately for unroasted cocoa powder and roasted cocoa liquor. Partial Least-Squares Discriminant Analysis (PLS-DA) as implemented in the pls (Liland et al., 2021) and mdatools (Kucheryavskiy, 2020) packages was performed to identify those volatile compounds that discriminate between samples with regards to fermentation time and fermentation type, separately for unroasted cocoa powder and roasted cocoa liquor. The internal standard equivalent concentrations of volatiles were treated as predictor values whereas the type of fermentation (with and without the addition of CS) or time of fermentation (5, 6 or 7 days) were set as the response variable. Wold's rule was applied for the selection of number of components in the model (Kucheryavskiy, 2020). From the resulting model, Variable Importance in Projection (VIP) values above 1 were used to identify the discriminating compounds. The lmerTest package was used for all mixed-effects ANOVA modeling (Kuznetsova et al., 2017), while the emmeans package was used for the post-hoc tests (Lenth et al., 2022).

Analysis of the triangle test results was performed in Compusense, setting the α =0.05 for a difference test, while testing for sensory similarity was done using β = 0.10 and Pd (the maximum proportion of distinguishers that can be tolerated being able to detect differences between products) was set at 20 % (Sinkinson, 2017).

RESULTS AND DISCUSSION

Temperature was measured throughout fermentation (**Supplemental figure 1**), and all the biological replicates reached the maximum temperature after 75-87 h of fermentation. On average, the highest temperature in the fermentation samples without the addition of CS was 40.07 ± 1.81˚C, whereas in fermentations with the addition of CS the max. temperature was 39.6 ± 0.2˚C. Except for the measurement at 75 h, the average temperatures did not differ significantly between the fermentations with and without the addition of CS ($p > 0.05$) during the first 100 h of fermentation. After 100 h, the fermentations with the addition of CS exhibited significantly lower temperatures compared to the fermentations without the addition of CS ($p < 0.05$). The fermentations without the addition of CS (**Supplemental figure 1a**) showed bigger differences between biological replicates (of about 6.2˚C after 187 h of fermentation) compared to fermentations with addition of CS (3.4˚C after 187 h of fermentation) (**Supplemental figure 1b**). The above may imply that the addition of CS may generate more consistent temperature profiles between batches. All fermentations followed a similar temperature profile throughout, and thus, it is assumed that the subsequent differences in composition are attributed mainly to the factors evaluated in this study (addition of CS and fermentation time) than the effect of the biological replicate. None of the samples exceeded temperatures higher than 45˚C, which is what is commonly reported during the aerobic phase of cocoa fermentation (Aprotosoaie et al., 2016; de Vuyst & Weckx, 2016).

Instrumental Analysis

Total fat content by low resolution Nuclear Magnetic Resonance Spectroscopy (NMR) Spectroscopy

Fat represents almost half of cocoa beans chemical constituents (Sirbu et al., 2018), and its increase is typically associated with a decrease in drying/roughing and puckering sensory sensations, which are subqualities that define astringency (Hamada et al., 2020). Additionally, although cocoa fat is not usually categorized as a flavor precursor during cocoa processing; lipids, and their oxidation products can interact with compounds created from those precursors during roasting reactions, and influence the final flavor (Ziegleder, 2017). Based on the role of fat in cocoa flavor, this study assessed how the addition of CS and the fermentation time affect fat content in unroasted cocoa powder and roasted cocoa liquor.

Fat content in unroasted cocoa powder varied between 54.8 ± 0.18% to 55.9 ± 0.18% in this study (**Supplemental table 1**). These results are slightly higher compared to dried cocoa beans of the MCC02 varietal from Indonesia (51.9%) (Assa et al., 2019) and lower than was reported for upper Amazonian Forastero cocoa beans from Ghana (59.3%) (Oracz et al., 2014). For the roasted cocoa liquor samples, the total fat content ranged from 56.4 ± 0.23 % to 57.6 ± 0.23 % (**Supplemental table 1**), which was slightly higher to what was determined for unroasted cocoa powder. The cocoa liquor of this study did not show losses in fat after whole roasting as reported by Gutiérrez (2017). Conversely, the increase of fat in the cocoa liquor may be explained by the evaporation of water from the unroasted bean (with a moisture content of around 7%), to cocoa liquor after roasting (typically about 3% (Gutiérrez, 2017)).

Regarding the effect of the experimental factors, addition of CS and fermentation time, the unroasted cocoa powder with addition of CS exhibited a significantly higher total fat content (55.7 ± 0.12%) than

the samples without the addition of CS (55.4 ± 0.11%), regardless of fermentation time, based on mixed-effects ANOVA ($p < 0.05$). The same trend was also observed in cocoa liquor. Cocoa liquor with the addition of CS had a significantly higher fat content of 57.1 ± 0.20 %, compared to the cocoa liquor without the addition of CS (56.7 ± 0.20 %) ($p < 0.05$). In terms of fermentation time, no clear trend was identified regarding fat content, with highest fat contents found in cocoa liquor samples after six days of fermentation (57.27 ± 0.20 %). Since the main molecules that are transformed during cocoa processing are proteins, carbohydrates, and polyphenols (Santander et al., 2020), fat is not directly involved in the chemical transformations of cocoa during processing, and minimal changes were expected.

Although statistically significant, the difference in total fat content between the two fermentation treatments is not meaningful relevant. Ziegler et al. (2001) reported that a difference in fat of 0.28% and 0.62% did not affect the maximum sweetness and chocolate intensity perception in milk chocolate made of coarse ($d_{4,3}$ = 17 µm) and fine ($d_{4,3}$ = 8.5 µm) sugar, respectively. Moreover, Hamada et al. (2020) found a significant linear decrease in drying/roughing, mouth-coating and puckering sensations in cocoa samples with increasing fat content, varying between 30 to 40%. However, the studied differences in fat varied between 5 to 10%, and a 10% reduction on these attributes were less than 10 intensity units. Therefore, despite the role of fat in astringency in cocoa, the changes in fat related to the addition of CS in unroasted cocoa powder in the present study are very small and thus are not able to make a significant difference in sensory perception.

Volatile compound analysis by untargeted HS-SPME-GC-MS

1.1.1.1 Unroasted cocoa powder

A total of 49 volatiles were detected in the unroasted cocoa powders by HS-SPME-GC-MS. From the screened peaks, 48 compounds matched mass spectra and retention indices (RIs) reported in the NIST/EPA/NIH Mass Spectral Library database (NIST, 2014b). In total, 4 organic acids, 7 alcohols, 5 aldehydes, 17 esters, 4 furans, 1 hydrocarbon, 4 ketones, 3 pyrazines, 1 pyrrole, and 2 terpenes were tentatively identified in the samples, whereas one peak did not match any of the mass spectra suggested by the database (**Supplemental table 2**). This peak is listed as unknown.

Partial Least Squares – Discriminant Analysis (PLS-DA) was performed on the detected compounds to determine which volatile compounds are key to differentiate unroasted cocoa powders fermented with and without the addition of CS. Based on Wold's rule and full cross-validation (automatically set in the mdatools package) (Kucheryavskiy, 2022), eight model components were required to discriminate between cocoa powders with and without addition of CS (**Figure 2A**). With the resultant model, the distance plot shows that the model successfully classified all samples with no outliers (no crossing over the upper right dotted line, the limit for outliers; **Figure 2B**). Additionally, regression model coefficients for each volatile compound ranged between -0.55 and 0.37, which indicate that depending on the compound, the addition of CS either increases or decreases its content.

With a similar approach, PLS-DA was performed to classify cocoa powders based on fermentation time, to determine which volatile compounds showed the largest changes due to the length of fermentation. Using Wold's rule and a full cross-validation, a model with one component was determined. However, only 33% of the samples were correctly classified as shown in **Figure 2C**. Moving to a model with a higher number of components was not possible, likely due to the one missing biological fermentation

replicates for 6 days of fermentation with the addition of CS. Applying the one-component model, no outliers were identified (**Figure 2D**), and the regression coefficients ranged from -0.035 to 0.055. The low values of the regression coefficients may mean that fermentation type (i.e., with vs. without the addition of CS) has a larger impact on volatile changes (by having higher regression coefficients in the PLS-DA model) and/or that the PLS-DA model for fermentation time is not sufficiently capturing the effect of fermentation time on the studied volatile compounds.

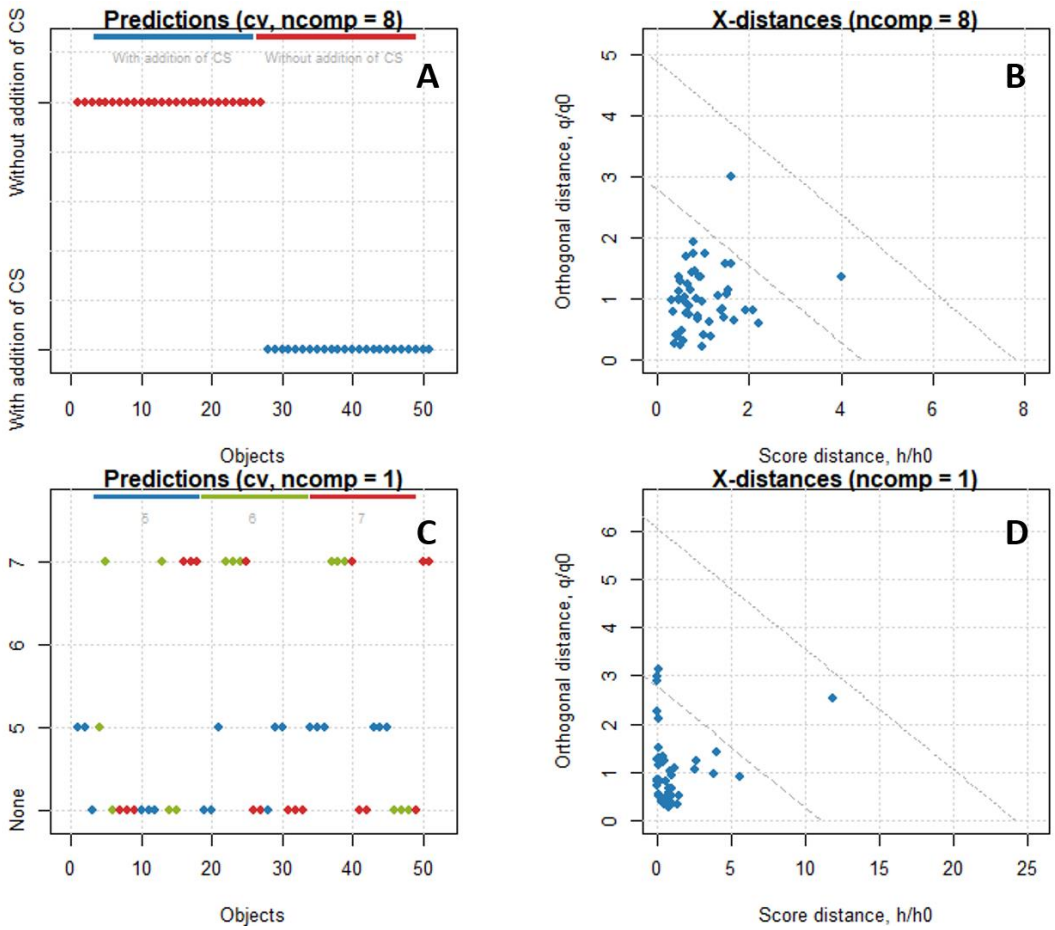


Figure 2. PLS-DA model diagnostic plots and x-distances in unroasted cocoa beans for **(A-B)** the fermentation type model (with or without the addition of CS) and **(C-D)** the fermentation time model (5,6 or 7 days).

The Variable Importance in Projection (VIP) value measures how much a variable contributes to explaining the response and predictors in a PLS-DA model (Andersen & Bro, 2010). Commonly, VIP values over 1 may indicate a compound of high importance for discrimination. Volatile compounds with VIP values smaller than 1 may have a big impact on sensory perception. . Volatile compounds with VIP values smaller than 1 may have a big impact on sensory perception. However, in a first attempt to identify important volatiles, it is assumed that compounds categorize with high VIP values would approximate large effects in sensory perception. Future work is needed to verify this assumption. In **Table 2** the 27 compounds with VIP values above 1 in the two PLS-DA models (fermentation type, fermentation time)

are listed. For 21 out of these 27 compounds, authentic standards were analyzed in the same way as the samples and thus confirmed the chemical identities.

In total, 4 organic acids were identified in unroasted cocoa powder (**Supplemental table 2**), with acetic acid the one with the highest content (up to 474.82 ± 52.69 µg naphthalene D8/g unroasted cocoa powder). Acetic acid is produced mainly during aerobic fermentation by acetic acid bacteria (AAB) (de Vuyst & Weckx, 2016). However, its content did not change due to the experimental treatments included in this study. Since the predominance of acetic acid bacteria is between 48 and 112 h (de Vuyst & Weckx, 2016; Schwan & Wheals, 2004), it is possible that acetic acid concentration stabilized after the fifth day of fermentation. Additionally, due to the low temperatures during fermentation (less than 41˚C for all samples; **supplemental figure 1**) potential evaporation of this volatile acid is most likely not a major factor. The results agree with Rodriguez-Campos et al. (2012), who identified a significant increase of acetic acid in unroasted cocoa beans only during early stages of fermentation (first 4 days), with no changes after 6 and 8 days of fermentation. Similarly, acetic acid was not significantly affected by the addition of cocoa sweating (**Supplemental table 2**). Therefore, despite the important role of acetic acid in cocoa fermentation kinetics (Santander et al., 2020; Schwan & Wheals, 2004), in this study it was not classified as a VIP compound in terms of changes in fermentation time or fermentation type.

For the three other organic acids, 2-methylbutanoic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid, VIP values above 1 indicate their importance in explaining differences between samples due to fermentation type and fermentation time. The two butanoic acids decreased over time in unroasted cocoa powder for both fermentation types (i.e., with and without the addition of CS) up to an average of 83 and 48% for 2-methylbutanoic acid and 3-methylbutanoic acid, respectively. These compounds smelled as pure standards are described in the literature as sweaty and rancid (Crafack et al., 2014; Koné et al., 2021) and have been suggested as markers of overfermentation and hammy off-flavor (Ziegleder, 2017). However, as they decrease over time this implies that these compounds are substrates for other fermentation products. The reduction of organic acids throughout fermentation has been reported in unroasted cocoa beans by Hamdouche et al. (2019), which supports the results observed in this study.

The addition of CS increased the concentration of 2-methylpropanoic acid, especially at later stages of fermentation (i.e., 7 days; **Table 2**). Cocoa sweating (CS) have been described as higher in reducing sugars and organic acids (Adams et al., 1982), thus, adding them to the fermenting mass likely increases available substrates and the production of organic acids such as 2-methylpropanoic acid. Koné et al. (2021) reported that 2-methylpropanoic acid was one of the compounds that differentiates unroasted cocoa beans after fermentation with 2 days of pod-opening delay from immediately opened and fermented cocoa beans . Therefore, the kinetics of the production of 2-methylpropanoic acid may be affected due the degradation of the pulp by either pod storage before opening, or microbial action and addition of CS during fermentation.

Table 2. List of volatile compounds detected in unroasted cocoa powder samples expressed as internal standard equivalent (ISE) per sample weight (µg naphthalene D8/g). All listed compounds are reported with retention indices (RI) and showed VIP values above 1 in either the PLS-DA model for fermentation type (i.e., with or without the addition of CS) and/or fermentation time (i.e., 5, 6, or 7 days). Arrows indicate the effect of the addition of CS on compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs ; $p < 0.05$).

Compound	RI		CS effect	With addition of CS (EMM ± standard error)			Without addition of CS (EMM ± standard error)		
	Sample	Standard		5 days (n=3)	6 days (n=2)	7 days (n=3)	5 days (n=3)	6 days (n=3)	7 days (n=3)
Acids									
2-methylpropanoic acid	1392		↑	14.03 ± 2.3B	16.79 ± 2.77AB	23.74 ± 2.3A	11.84 ± 2.3a	11.54 ± 2.3a	17.3 ± 2.3a
2-methylbutanoic acid	1637	1640		10.36 ± 0.84A	1.77 ± 1.05B	2.23 ± 0.84B	7.77 ± 0.84a	1.94 ± 0.84b	2.08 ± 0.84b
3-methylbutanoic acid	1636	1639		15.96 ± 1.52A	8.26 ± 1.97B	10.62 ± 1.52B	15.33 ± 1.52a	8.59 ± 1.52b	9.9 ± 1.52b
Alcohols									
Benzyl alcohol	1824	1840		0.46 ± 0.04A	0.38 ± 0.05A	0.40 ± 0.04A	0.45 ± 0.04a	0.35 ± 0.04b	0.36 ± 0.04b
Aldehydes									
2-methylbutanal	901	906		1.43 ± 0.11A	1.34 ± 0.14A	1.15 ± 0.11A	1.2 ± 0.11a	1.05 ± 0.11a	1.19 ± 0.11a
3-methylbutanal	905	909		1.59 ± 0.14A	1.34 ± 0.18AB	1.09 ± 0.14B	1.57 ± 0.14a	1.15 ± 0.14a	1.17 ± 0.14a
Benzaldehyde	1476	1483	↑	18.9 ± 1.81A	17.51 ± 2.15A	19.49 ± 1.81A	16.86 ± 1.81a	12.89 ± 1.81a	16.64 ± 1.81a
α-ethylidene-benzeneacetaldehyde,	1871	N.A.		0.22 ± 0.03B	0.44 ± 0.03A	0.35 ± 0.03A	0.26 ± 0.03b	0.2 ± 0.03b	0.48 ± 0.03a
Esters									
Ethyl Acetate	873	877		3.84 ± 1.1B	5.64 ± 1.29AB	8.53 ± 1.1A	4.21 ± 1.1a	5.39 ± 1.1a	6.85 ± 1.1a
methyl 2-methylpropanoate	912	916		0.39 ± 0.04A	0.09 ± 0.05B	0.09 ± 0.04B	0.31 ± 0.04a	0.08 ± 0.04b	0.09 ± 0.04b
Ethyl 2-methylpropanoate	952	955	↑	1.03 ± 0.1A	0.29 ± 0.12B	0.26 ± 0.1B	0.63 ± 0.1a	0.27 ± 0.1b	0.28 ± 0.1b
2-methylpropyl acetate	1003	1005		10.8 ± 1.44A	8.34 ± 1.86A	9.7 ± 1.44A	9.75 ± 1.44a	9.36 ± 1.44a	11.84 ± 1.44a
Methyl 3-methylbutanoate	1007	1009		1.02 ± 0.09A	0.31 ± 0.11B	0.37 ± 0.09B	0.95 ± 0.09a	0.34 ± 0.09b	0.34 ± 0.09b
Ethyl 2-methylbutanoate	1037	1040		2.31 ± 0.23A	0.2 ± 0.26B	0.18 ± 0.23B	1.28 ± 0.23a	0.43 ± 0.23b	0.33 ± 0.23b
Ethyl 3-methylbutanoate	1053	1055	↑	1.36 ± 0.11A	0.52 ± 0.13B	0.54 ± 0.11B	0.87 ± 0.11a	0.49 ± 0.11b	0.49 ± 0.11b

Methyl 2-hydroxypropanoate (Methyl lactate)	1291			2.32 ± 0.63A	2.25 ± 0.76A	2.47 ± 0.63A	3.14 ± 0.63a	2.55 ± 0.63a	2.97 ± 0.63a
2,3-Butanediol diacetate	1483			1.67 ± 0.44B	2.09 ± 0.57B	4.72 ± 0.44A	1.77 ± 0.44a	1.79 ± 0.44a	3.19 ± 0.44a
2-Ethoxyethyl butanoate	1583		↑	1.09 ± 0.09A	0.3 ± 0.11C	0.66 ± 0.09B	0.63 ± 0.09a	0.2 ± 0.09b	0.39 ± 0.09ab
Methyl 2-phenylacetate	1715	1726	↓	0.73 ± 0.07A	0.37 ± 0.09B	0.52 ± 0.07AB	1.16 ± 0.07a	0.45 ± 0.07b	0.45 ± 0.07b
Ethyl 2-phenylacetate	1744	1754	↑	1.17 ± 0.08A	1.03 ± 0.1A	1.1 ± 0.08A	0.96 ± 0.08a	0.89 ± 0.08a	1.02 ± 0.08a
Furans									
2-Acetylfuran	1465	1471	↓	0.2 ± 0.04A	0.14 ± 0.04A	0.14 ± 0.04A	0.31 ± 0.04a	0.19 ± 0.04b	0.17 ± 0.04b
4-Butyrolactone	1569	1576	↓	2.6 ± 0.44A	2.3 ± 0.52A	2.28 ± 0.44A	3.26 ± 0.44a	3.04 ± 0.44a	3.2 ± 0.44a
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)- (Pantolactone)	1973	1991		0.26 ± 0.02A	0.22 ± 0.03A	0.24 ± 0.02A	0.32 ± 0.02a	0.23 ± 0.02b	0.25 ± 0.02ab
5-Acetyldihydrofuran-2(3H)-one	1496		↓	0.1 ± 0.02B	0.13 ± 0.02AB	0.15 ± 0.02A	0.16 ± 0.02b	0.17 ± 0.02b	0.23 ± 0.02a
Ketones									
Acetoin	1251	1254		10.37 ± 1.19A	8.4 ± 1.54A	6.34 ± 1.19A	11.62 ± 1.19a	9.27 ± 1.19a	7.81 ± 1.19a
Acetophenone	1598	1607	↑	2.07 ± 0.14A	1.73 ± 0.18A	2.17 ± 0.14A	1.57 ± 0.14a	1.47 ± 0.14a	1.87 ± 0.14a
Pyrazines									
2,3-Dimethylpyrazine	1313	1315	↑	2.16 ± 0.19A	1.93 ± 0.22A	2.02 ± 0.19A	1.72 ± 0.19a	1.31 ± 0.19a	1.51 ± 0.19a

Alcohols are produced in unroasted cocoa beans during the fermentation process (Aprotosoaie et al., 2016; de Vuyst & Weckx, 2016; Owusu et al., 2012). In this study, 7 alcohols were tentatively identified (**Supplemental table 2**). Compounds such as ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, 2,3-butanediol, benzyl alcohol and phenylethyl alcohol were detected in unroasted samples in previous studies (Crafack et al., 2014; Koné et al., 2021; Marseglia et al., 2020). Therefore, the identification of these volatiles in this study was expected. Ethanol is an important compound produced during anaerobic fermentation by yeast, and it is the main substrate for the production of acetic acid in the aerobic phase of cocoa fermentation (de Vuyst & Weckx, 2016; Santander et al., 2020; Schwan & Wheals, 2004). However, similarly to acetic acid, ethanol levels did not change significantly due to fermentation time nor addition of CS. The production of ethanol, as well as its consumption for further reactions occurs in early fermentation stages, causing its content to stabilize in later fermentation stages. Additionally, since the highest reached temperature during fermentation was less than 41˚C (**Supplemental figure 1**), evaporation of ethanol would have been minimal. Despite the presence of multiple alcohols in unroasted cocoa beans, only two showed significant changes in terms of fermentation time or addition of CS in the ANOVA ($p < 0.05$). For 3-methyl-1-butanol a decrease with increasing fermentation time was seen only in samples with the addition of CS (**Supplemental table 2**), however, the VIP score for this compound in both PLS-DA models was below 1. The benzyl alcohol levels also decreased over time but only in the samples without the addition of CS (with an average decrease of 23% from 5 to 7 days of fermentation, **Table 2**), resulting in a VIP value above 1. The results above evidence that the mechanisms of production and consumption of alcohols, such as 3-methyl-1-butanol and benzyl alcohol diverge. The addition of CS may encourage the consumption of or slow the production of 3-methyl-1-butanol, whereas the addition of CS kept benzyl alcohol levels at the same levels throughout fermentation, while it significantly decreased in samples without the addition of CS.

Five aldehydes were tentatively identified in unroasted cocoa powder (**Supplemental table 2**). Apart from butanal, all the aldehydes had VIP scores above 1 (**Table 2**). The content of 3-methylbutanal decreased over time in all samples, however, this decrease was statistically significant only in samples with the addition of CS (with an average decrease of 32% between 5 and 7 days of fermentation). On the opposite, the content of α-ethylidene-benzeneacetaldehyde increased with longer fermentation times in samples both with (60% increase between 5 and 7 days) and without (86% increase between 5 and 7 days) the addition of CS. Even though most of the aldehydes in cocoa are produced during roasting due to Strecker degradation, Rodriguez-Campos et al. (2012) 2-methylbutanal and 3-methylbutanal have arisen even during fermentation and drying (0.75 and 0.56 µg/g respectively). 3-methylbutanal is produced from isoleucine (Hamdouche et al., 2019; Ziegleder, 2017), and longer fermentation time degrades this compound, especially with the addition of CS. In the case of α-ethylidene-benzeneacetaldehyde, which is produced from phenylalanine, tyrosine, and methionine (Afoakwa et al., 2008), its concentration increases with longer fermentation times independent of fermentation type (with or without CS).

Benzaldehyde has been identified as an important aldehyde in cocoa bean fermentation (Hamdouche et al., 2019), which is in agreement with the results of this study (**Table 2**). In addition, benzaldehyde was the only aldehyde that significatively increased in concentration in the unroasted samples fermented with the addition of CS. The composition of the CS and the acidic environment could have encouraged the production of benzaldehyde, whose likely precursor is phenylalanine. Despite no characterization of CS were executed in this study, the pH of CS is reported to vary between 4.0 and 5.25 (Afoakwa et al.,

2013) which could have affected the production of benzaldehyde. The availability of phenylalanine depends on the degree of proteolysis during cocoa fermentation; for example, in cocoa ferments with moderate acidification (pH 5.5 -5.0) higher contents of hydrophobic amino acids such as phenylalanine were reported (Santander et al., 2020). It could be that the addition of CS may increase proteolysis during fermentation, leading to higher levels of phenylalanine, and/or that the CS addition positively affects the conversion of phenylalanine into benzaldehyde.

The volatile compound family that had the largest number of tentatively identified compounds in unroasted cocoa powder were esters. A total of 17 esters were detected (**Supplemental table 2**), of which 12 had VIP values above 1 in the PLS-DA models (**Table 2**). For 7 out of these 12 esters, increasing fermentation time for both with and without addition of CS, decreased their levels (**Table 2**). These were: methyl 2-methylpropanoate (methyl isobutyrate), ethyl 2-methylpropanoate (ethyl isobutyrate), methyl 3-methylbutanoate (methyl isovalerate), ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 2-ethoxy butyrate and methyl 2-phenylacetate. This observed reduction agrees with results reported by Rodriguez-Campos et al. (2012) who reported large ester decreases from 28 to 4 μg/g with longer fermentation time in unroasted cocoa beans. For the most predominant esters (methyl acetate, 3-methylbutyl acetate (isoamyl acetate), 2-methylpropyl acetate (isobutyl acetate) and 2-phenylethyl acetate) neither fermentation time nor the addition of sweating affected their levels in unroasted cocoa beans (**Supplemental table 2**). According to Aculey et al. (2010) and Aprotosoaie et al. (2016), esters are produced by yeasts during early to mid-stages of fermentation. Given that all fermentation times studied here were at advanced stages of fermentation, the slowing down of the yeasts' activity could explain the constant levels of these dominant esters across the experimental treatments.

The addition of CS affected 5 out of the 12 esters with VIP values above 1 in unroasted cocoa powder. Four esters increased with the addition of CS (ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, 2-ethoxyethyl butyrate and ethyl 2-phenylacetate), whereas one decreased (methyl 2-phenylacetate) (**Table 2**). One reason that may explain the increase of esters in unroasted cocoa powder that were fermented with the addition of CS could be the CS' composition. CS is rich in sugars and organic acids (Adams et al., 1982), thus, its addition increases substrates for ester formation by yeast at early stages of fermentation. Further studies characterizing CS may provide insights about how it affects ester formation in unroasted cocoa powder. The conditions provided by the addition of CS significantly increased the content of ethyl acetate and 2,3-butanediol-diacetate only after 7 days of fermentation. This increase is opposite to what was found by Hamdouche et al. (2019) who reported a decrease of ethyl acetate over fermentation time in unroasted cocoa beans.

Furans are products from Maillard reactions that occur during drying and roasting as a result of degradation of monosaccharides (Aprotosoaie et al., 2016; Marseglia et al., 2020). Four furans were tentatively identified in unroasted cocoa powder and all of them showed VIP scores above 1 (**Table 2**). Even though furans formation during fermentation is much lower than during roasting, the addition of CS during this step appears to have significantly affected the content and stability of furans. With the exception of 2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl- (pantolactone), all furans decreased in unroasted cocoa powder fermented with the addition of CS. One could conclude that adding CS lowers the production of furans such as Butyrolactone, which is the furan with the highest concentration in the unroasted cocoa powder (**Table 2**) and has been reported in unroasted cocoa beans previously (Crafack et al., 2014; Marseglia et al., 2020). Longer times of fermentation increased the content of 2(3H)-Furanone-5-acetyldihydro-in unroasted cocoa powder regardless of the addition of CS (**Table 2**).

However, in samples without the addition of CS the content of 2-Acetylfuran and pantolactone decreased over time. According to these results, the addition of CS decreased the content of some furans in unroasted cocoa powders but kept them stable during fermentation time. Furans in unroasted cocoa powders without addition of CS were higher in concentration but tended to degrade significantly over late stages of fermentation.

Ketones are volatile compounds that are produced by enzymatic degradation of the bean during fermentation, and by Strecker reactions during drying and roasting (Afoakwa et al., 2008; Rodriguez-Campos et al., 2012; Santander et al., 2020). In unroasted cocoa powder, 4 ketones were found (**Supplemental table 2**) and two (acetoin and acetophenone) showed VIP values above 1 (**Table 2**). For acetophenone an increase in concentration up to 24% was found when CS was added. In terms of fermentation time, all ketones except for 2-heptanone (**Supplemental table 2**) remained constant throughout fermentation. These results are in agreement with Rodriguez-Campos et al. (2012) who did not see significant differences in ketone content between different fermentation times in unroasted cocoa bean dried at 70 and 80°C. In the case of 2-heptanone, despite the 30% decrease from 5 to 7 days of fermentation for samples fermented with the addition of CS (**Supplemental table 2**), this decrease was not associated with a VIP value higher than 1. Therefore, it was not categorized as a discriminative compound in unroasted cocoa powder.

Pyrazines are important flavor compounds in cocoa products, mainly produced during roasting by Maillard reactions (Afoakwa et al., 2008; Rojas et al., 2021; Ziegleder, 2017). However, pyrazines are also formed during fermentation by aerobic spore-forming bacteria and during drying (Reineccius et al., 1972; Schwan & Wheals, 2004). In this study, 4 pyrazines were tentatively identified in unroasted cocoa powder (**Supplemental table 2**). Tetramethylpyrazine was the pyrazine with the highest content in the samples, ranging from 31.8 to 38.6 µg naphthalene D8/g (**Supplemental table 2**), supporting Ziegleder (2017) observation that only this pyrazine occurs in large amounts (0.2-2.0 µg/g) in fermented unroasted cocoa. Nonetheless, 2,3-dimethylpyrazine was the only pyrazine reaching a VIP value above 1 in the PLS-DA models, with a significant average increase from 1.51 ± 0.21 to 2.04 ± 0.11 µg naphthalene D8/g unroasted cocoa powder in those samples that were fermented with the addition of CS (**Table 2**). Koné et al. (2021) found that cocoa beans stored for 2 days in the pod prior to fermentation showed higher levels of pyrazines. Thus, pulp degradation products caused by pod storage and/or the addition of CS may encourage the production of pyrazines such as 2,3-dimethylpyrazine.

Except for 2,3,5-trimethylpyrazine, pyrazines did not change significantly between the different fermentation times in unroasted cocoa powder. For 2,3,5-trimethylpyrazine, although not showing a VIP value above 1, contents significantly decreased in unroasted cocoa beans fermented without CS (**Supplemental table 2**). These results disagree with what was reported by Rodriguez-Campos et al. (2012), who showed that pyrazine content increases with fermentation time up to 8 days of fermentation in unroasted cocoa beans dried at 60, 70 and 80°C. Additionally, even though aerobic spore-formic bacteria are predominant after 4 days of fermentation (Schwan & Wheals, 2004), their potential presence during fermentation appears to not have changed the pyrazine content in this study.

One hydrocarbon (Styrene), one pyrrole (2-Acetylpyrrole), and two terpenes (β-Myrcene, β-Ocimene) were also tentatively identified in unroasted cocoa powder. These compounds have been reported previously by Marseglia et al. (2020) in unroasted cocoa beans from different geographical origins. However, none of them showed any significant differences due to fermentation time or addition of CS,

nor reached VIP values above 1. Their levels do not appear to be affected by the experimental treatments studied here.

To summarize, volatile compounds in unroasted cocoa powder varied with the addition of CS and time of fermentation, but the change was not systematic across the identified compounds and compound classes. For instance, whereas the addition of CS increased some esters (Ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, 2-ethoxyethyl butanoate and ethyl-2-phenylacetate) benzaldehyde 2-methylpropanoic acid, acetophenone and 2,3-dimethylpyrazine, it reduced the content of furans. In addition, furans did not change over fermentation time compared to unroasted powder without the addition of CS (Except for 5-Acetyldihydrofuran-2(3H)-one, **Table 2**). The length of fermentation (i.e., 5, 6, 7 days) also affected volatile compounds in different ways even within the same functional group. For example, whereas some organic acids decreased over time (e.g., 2-methylbutanoic acid, 3-methylbutanoic acid), others such as acetic acid did not show any changes at all (**Table 2**). Other compounds such as alcohols, pyrazines and ketones were less affected by time of fermentation or addition of CS. This project showed that the production of volatile compounds in unroasted cocoa powder during fermentation and drying follow different patterns, and both the addition of CS and fermentation time affect their levels in the fermented and dried beans. Escobar et al., (2021) and Santander et al. (2020) provide an overview about the physical and biochemical transformation in cocoa, from raw seeds to dried beans. The combination of microbial activity and kinetics, environmental conditions (pH, temperature, available O_2, etc.) and enzymatic reactions in the bean (proteolysis, hydrolysis, and oxidation) transform the raw seed into fermented and dried beans, changing the initial composition dramatically. The addition of CS and the time of fermentation affect these kinetic changes, presumably by changes in environmental conditions, but the mechanisms that would explain the formation and degradation of each detected compound here remains largely elusive. Despite prior studies followed some of the metabolites that serve as substrates in the cocoa transformation process (Santander et al., 2021), more studies are required to fully understand the mechanisms of production/consumption of cocoa volatiles during fermentation and drying.

The determination of volatile compounds in unroasted cocoa powder had less variability in the samples with the addition of CS. Indeed, unroasted cocoa powder fermented with the addition of CS showed that 36 out of 48 volatile compounds had a smaller coefficient of variation regardless the time of fermentation. Therefore, this may indicate that the addition of CS may provide more consistent batches of dried and fermented cocoa beans, than when the beans are fermented traditionally.

1.1.1.2 Roasted cocoa liquor

61 peaks were detected in the roasted cocoa liquor samples using HS-SPME-GC-MS (**Supplemental table 3**). For 57 out of the 61 screened peaks a tentative identity could be assigned by matching with the mass spectral library (NIST, 2014b), and these 57 volatiles were subsequently classified into 3 organic acids, 7 alcohols, 8 aldehydes, 14 esters, 8 furans, 2 hydrocarbons, 7 ketones, 8 pyrazines, and 1 pyrrole. For the remaining 4 compounds that did not match with any entry in the database, it is presumed that one of them is an alcohol, and another one is an ester (based on the mass spectra profiles).

Similarly to the unroasted cocoa powders, PLS-DA was conducted to determine the volatile compounds that separate cocoa liquor samples based on fermentation type (i.e., with vs. without the addition of CS). As shown in **Figure 3A**, 12 components were necessary to successfully categorize the samples, based on Wold's rule and full cross-validation as implemented in the mdatools package (Kucheryavskiy,

2022)). Although one outlier was identified (**Figure 3B**; one sample slightly crossed the latest dotted line), the model was kept for the selection of compounds with VIP values above 1. Regression coefficients, calculated for each volatile, varied between -0.35 and 0.30, which implies that none of the screened volatile compounds have a strong correlation with fermentation type. The regression values were comparable in size to the coefficients for the fermentation type PLS-DA model for the unroasted cocoa beans which ranged from -0.55 to 0.30. In terms of fermentation time, a PLS-DA model with 6 components was necessary to separate liquor samples by days of fermentation (**Figure 3C**). With the selected model no outliers were identified (**Figure 3D**), and regression coefficients for each volatile compound ranged from -0.12 to 0.17. The regression values were about 3-times larger than the coefficients for the unroasted cocoa powder PLS-DA model for fermentation time, but overall were lower which implies that none of the compounds by itself is an important driver separating cocoa liquor samples based on fermentation time.

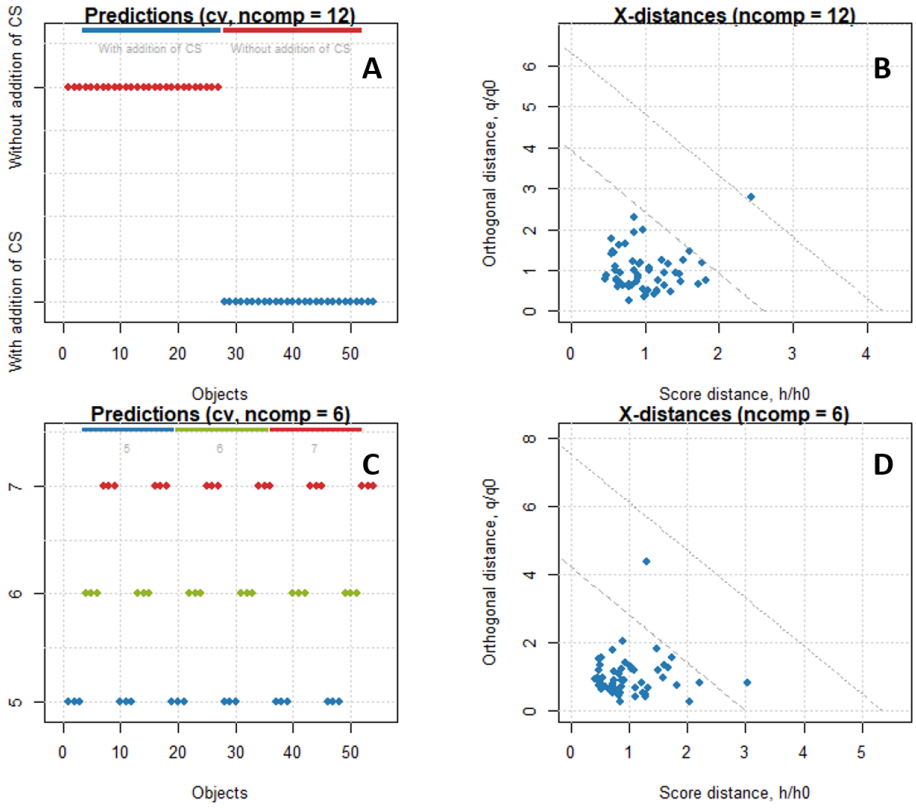


Figure 3. PLS-DA model diagnostic plots and x-distances in roasted cocoa liquor for **(A-B)** the fermentation type model (with or without the addition of CS) and **(C-D)** the fermentation time model (5,6 or 7 days).

VIP values were determined from the two PLS-DA models in order to identify the volatile compounds that contribute the most to the discrimination in the PLS-DA (Andersen & Bro, 2010). **Table 3** lists all volatiles with a VIP value above 1. A total of 37 volatile compounds were retained, where 29 of them were identified with authentic standards on top of matching mass spectra and RI.

Volatile organic acids such as acetic acid typically decrease during roasting due to evaporation (Afoakwa et al., 2008; Santander et al., 2020). However, except for 2-methylpropanoic acid, all the acids identified

in unroasted cocoa powder were also detected in roasted cocoa liquor (**Supplemental table 3**). Acetic acid was the predominant acid in the samples, but despite its significantly elevated levels in the cocoa liquor fermented for 7 days with the addition of CS, its VIP value was below 1, implying changes in acetic acid are not critical for separating samples by fermentation type or time. Both 2-methylbutanoic acid and 3-methylbutanoic acid showed VIP values above 1 in roasted cocoa liquor (**Table 3**), due to their significant decrease (up to 77% and 33% for 2-methylbutanoic and 3-methylbutanoic acid, respectively) with increasing fermentation time, regardless of fermentation type. This trend was similar in unroasted cocoa powder (**Table 2**). Therefore, the time of fermentation decreases the concentration of both 2-methylbutanoic acid and 3-methylbutanoic acid in cocoa before and after roasting. The high boiling points (bp) of 2-methylbutanoic acid and 3-methylbutanoic acid (bp = 175 °C for both (NIST, 2014b)) may be the reason for the retention of both acids during roasting. Furthermore, cocoa liquor fermented with the addition of CS has a lower content of 3-methylbutanoic acid compared to the cocoa liquors fermented without CS, something that was not observed in unroasted cocoa powder. Therefore, one could speculate that the conditions provided by the addition of CS in fermentation may have stimulated the degradation of 3-methylbutanoic acid during roasting more compared to the cocoa liquor without the addition of CS. Despite the variability of the screened volatile acids, and more specifically their reduction at longer times of fermentation, the pH of the resultant cocoa liquors decreased at 7 days of fermentation by 0.1 pH unit (**Supplemental table 1**). The reduction of pH in the cocoa liquors implies that other non-volatile acids may have affected the overall acidic conditions of the cocoa liquor.

Alcohols are mainly produced during fermentation, but can also form from amino acids during roasting due to heat degradation (Aprotosoaie et al., 2016). In roasted cocoa liquor, 8 alcohols were tentatively identified (**Supplemental table 3**), and 6 reached VIP values above 1 in either the time of fermentation or the fermentation type PLS-DA models. All these identified alcohols, including 2-methy-1-butanol, 2-heptanol, benzyl alcohol, and phenylethyl alcohol, have been reported previously in cocoa liquor from different regions around the world (Tuenter, Delbaere, De Winne, et al., 2020). Thus, their presence in the samples was expected. The addition of CS affected the content of five out of the 6 alcohols with VIP values above 1 in cocoa liquor (**Table 3**). Two of them, 2-methyl-1-butanol and phenylethyl alcohol, decreased by 22% and 14%, respectively, in the samples where CS were added during fermentation, whereas the other three increased by 25% (2-heptanol), 12% (benzyl alcohol) and 21% (undefined alcohol) compared to the samples fermented without the addition of CS. The addition of CS did not have any direct effect on alcohols in the unroasted cocoa powder (**Table 2**). Therefore, the addition of CS may play a role in degradation or stimulation of alcohol production in roasted cocoa liquor by affecting their precursor levels. The kinetics of production/degradation of these alcohols during roasting remain largely unknown, but they are assumed to be linked to non-enzymatic browning, Maillard reactions, and other oxidation reactions between peptides, proteins, polyphenols, and lipids, which influence the final flavor of cocoa (Ziegleder, 2017). Except for benzyl alcohol and 2-methyl-1-butanol, alcohol content was not affected by fermentation time. On one hand, benzyl alcohol decreased by 19% over time but only in cocoa liquors fermented with the addition of CS (**Table 3**). On the other hand, 2-methyl-1-butanol (the predominant alcohol in cocoa liquor for this study) was the only alcohol that changed in cocoa liquors fermented without the addition of CS; 2-methyl-1-butanol levels decreased from 2512 ± 236 μg ISE/g at 5 days to 1142 ± 236 μg ISE/g at 7 days of fermentation. These results show that the majority of alcohols detected in this study did not change over fermentation time in roasted cocoa liquor, and that the addition of CS appears to decrease benzyl alcohol levels over time but stabilize 2-methyl-1-butanol levels.

Aldehydes are considered as one of the most important flavor volatile compounds developed during roasting (Aprotosoaie et al., 2016), and represent a major functional group in cocoa flavor (Ziegleder, 2017). Their production is linked to Strecker degradation, which involves amino acids and Maillard intermediary carbonyl compounds (Afoakwa et al., 2008). In the present study, 8 aldehydes were tentatively identified (**Supplemental table 3**), and 6 of them showed VIP values above 1 in the PLS-DA models (**Table 3**). The presence of the aldehydes identified in this study has been reported previously in roasted cocoa (Crafack et al., 2014; Owusu et al., 2012). The addition of CS decreased the content of only one aldehyde in roasted cocoa liquor, namely, α-ethylidene-benzeneacetaldehyde, which showed on average 17% lower levels in samples fermented with the addition of CS (**Table 3**). This result partially agrees with what was found for unroasted cocoa powder, where most of the aldehydes did not change by the addition of CS, except for benzaldehyde, which increased with the addition of CS (**Table 2**).

Fermentation time did not have a clear trend in its effect on aldehyde contents in roasted cocoa liquor, and further, this effect depended on the addition of CS. Whereas hexanal and heptanal tended to decrease over time in cocoa liquors fermented with the addition of CS (from 1.99 ± 0.43 μg Napthalene D8/g at 5 days to 0.84 ± 0.43 μg Napthalene D8/g at 7 days for hexanal, and from 1.00 ± 0.17 μg Napthalene D8/g at 5 days to 0.61 ± 0.17 μg Napthalene D8/g at 7 day for heptanal), their contents increased in cocoa liquors that were fermented without the addition of CS (from 0.62 ± 0.43 μg Napthalene D8/g at 5 days to 2.21 ± 0.43 μg Napthalene D8/g at 7 days for hexanal, and from 0.48 ± 0.17 μg Napthalene D8/g at 5 days to 1.04 ± 0.17μg Napthalene D8/g at 7 day for heptanal) (**Table 3**). The addition of CS appeared to stimulate the degradation of pentanal over time, and does not encourage the production of octanal, benzaldehyde, and α-ethylidene-benzeneacetaldehyde to the same degree as seen in the samples that were fermented without the addition of CS (**Table 3**). Since aldehydes are mainly produced during roasting, there may not be a direct relationship between fermentation time and aldehydes content in roasted cocoa liquor. However, the sugar content and the organic acids present in CS could have altered bean enzymatic activity during fermentation, thus, reducing the production of aldehyde precursors over time, which in turn led to lower levels of aldehydes after roasting.

Esters were the chemical family that similarly to the unroasted cocoa samples, had the highest number of compounds in roasted cocoa liquor samples (**Supplemental table 3**). 15 esters were tentatively identified, and 11 of these reached VIP values above 1 in the PLS-DA models for either time of fermentation or fermentation type (**Table 3**). Esters such as ethyl acetate, 3-methylbutyl acetate, ethyl 2-phenylacetate, and 2-phenylethyl acetate have been previously reported in roasted cocoa products and are described as having fruity aromas (Crafack et al., 2014; Marseglia et al., 2020; Owusu et al., 2012). The addition of CS not only increased the content of five esters (ethyl acetate, ethyl hexanoate, ethyl, 2-phenylacetate, 2-phenylethyl acetate and the undefined ester) in roasted cocoa liquor, but also maintained the amounts of ethyl 2-methylbutanoate and ethyl 3-methylbutanoate over time of fermentation (**Table 3**). Cocoa liquors fermented without the addition of CS had a significantly higher concentration of 3-methylbutyl acetate at 5 days of fermentation (34.58 ± 3.06 μg naphthalene D8/g), which decreased significantly over time to 22.94 ± 3.06 μg ISE/g after 7 days. This was in contrast to cocoa liquors fermented with the addition of CS, where 3-methylbutyl acetate levels remained constant over time. Therefore, the addition of CS appears to stimulate the production and preservation of esters in roasted cocoa liquor at different fermentation times. The time of fermentation by itself also affected ester content in roasted cocoa liquor samples. Three esters (2-phenylethyl acetate, 2,3-Butanediol

diacetate and an unidentified ester) all significantly increased with increasing fermentation time (**Table 3**) whereas methyl heptanoate levels decreased from 0.1 ± 0 µg naphthalene D8/g at 5 days to 0.04 ± 0 µg naphthalene D8/g at 7 days (**Table 3**). The increase of esters over fermentation time is opposite to the trend found in unroasted cocoa powder (**Table 2**). Owusu et al. (2012) stated that the levels of esters in cocoa depend on the differences between the rate of evaporation and the rate of formation, which seems to be affected by time of fermentation in this study.

Roasting cocoa beans degrades monosaccharides that participate in Maillard reactions to produce furans (Aprotosoaie et al., 2016; Marseglia et al., 2020). Eight furans were tentatively identified (**Supplemental table 3**), and 6 of them reached VIP values above the cut-off level of 1 (**Table 3**). Except for 2-pentylfuran, all the furans decreased in roasted cocoa liquor fermented with the addition of CS up to 47%. Additionally, though the content of furans was higher in roasted cocoa liquor fermented without the addition of CS (4.81 ± 0.33 µg naphthalene D8/g vs. 3.92 ± 0.33 µg naphthalene D8/g in samples with CS), levels of four furans decreased over time (furfural, 4-butyrolactone, 3-furanmethanol and pantolactone; **Supplemental table 3**). This observation is similar to what was found for unroasted cocoa beans in this study. Therefore, the addition of CS during fermentation decreases furan content in cocoa before and after roasting, however, appears to stabilize furan concentration throughout fermentation. Since furans are products of Maillard reactions, the addition of CS may discourage the reactions that produce furans, but at the same time also counteract the decrease in furans with increasing fermentation time, leading to stable furan levels independent of the length of fermentation. The presence of 2-pentylfuran only in cocoa liquor agrees with earlier literature (Crafack et al., 2014), who stated that this compound is only found in roasted liquors.

Two hydrocarbons (styrene, octane) were tentatively identified in roasted cocoa liquor, and both showed VIP values above 1 (**Table 3**). Even though styrene had a VIP value above 1, levels did not change significantly due to CS addition nor time of fermentation. For octane, significantly higher levels were found in cocoa liquors with the addition of CS but only in samples fermented for 7 days. Styrene has been reported in roasted cocoa beans from America, Africa, and Southeast Asia (Marseglia et al., 2020), but no studies have reported octane in cocoa samples. Therefore, possible contamination of either the sample or the equipment may explain the presence of octane in roasted cocoa liquor.

Ketones, similar to aldehydes, are produced through Strecker degradation of amino acids during roasting (Aprotosoaie et al., 2016), and high concentrations of these compounds have been associated with high cocoa quality (Rodriguez-Campos et al., 2012). In this study, seven ketones were tentatively identified (**Table 3**), and 4 of those were considered important in the two PLS-DA models based on their VIP values. Most of the ketones decreased with the addition of CS (**Supplemental table 3**). In fact, two of the four VIP compounds (2,3-Butanedione and 4-methyl-2-hexanone) showed significantly higher concentration in cocoa liquors fermented without the addition of CS (up to 30% and 28% for 2,3-Butanedione and 4-methyl-2-hexanone, respectively) (**Table 3**). However, similar to furans, the addition of CS led to lower levels of these ketones but also stabilized them over fermentation time. This is in contrast to liquors made from beans fermented without addition of CS, where ketone levels decreased about 36% from 5 days to 7 days of fermentation. Acetophenone was the only ketone that had elevated levels in roasted samples made with the addition of CS, which was similar to what was observed in unroasted cocoa beans. It seems that the effect of fermentation type (i.e., addition of CS or not) and fermentation time (5, 6 or 7 days) on ketone levels was greater in roasted cocoa liquor than in unroasted cocoa beans. The composition of the CS (Adams et al., 1982) may affect enzymatic activity

during fermentation, for example, suppressing the production of ketone precursors that are transformed during roasting but maintaining a lower level of ketone precursors throughout fermentation, thus, upon roasting leading to constant ketone concentrations at all fermentation times.

Pyrazines are one of the key groups in cocoa flavor and are mainly produced during roasting (Aprotosoaie et al., 2016; Rodriguez-Campos et al., 2012; Ziegleder, 2017). In this study, 8 pyrazines were tentatively identified, but none of them were above the VIP cut-off of 1 in the PLS-DA models. Pyrazines form by dimerization of ketoamines to dihydropyrazines and a subsequent oxidation, which depends, among other factors, on fermentation (Ziegleder, 2017). However, the results of this study evidenced that the addition of CS and the screened time of fermentation did not significantly change pyrazine contents in roasted cocoa liquor.

In general, volatile compounds in roasted cocoa liquor did not show a systematic effect of fermentation type (i.e., with vs. without the addition of CS) or the time of fermentation (i.e., 5 vs. 6 vs. 7 days), somewhat similar what was found for the unroasted cocoa powder. However, the trends of each chemical class of compounds did not necessarily align between unroasted and roasted cocoa samples. Within the volatiles that changed over fermentation time, only organic acids, furans, and acetophenone followed a similar trend in unroasted cocoa powder and roasted cocoa liquor, namely, organic acids decreased over time of fermentation, furans decreased by the addition of CS but their content was stable over time, and acetophenone increased with the addition of CS. In contrast, for alcohols, aldehydes, ketones, and esters different behaviors between unroasted powder and roasted liquor were found: In cocoa liquor (**Table 3**), the addition of CS increased the content of five esters (ethyl acetate, ethyl hexanoate, ethyl, 2-phenylacetate, 2-phenylethyl acetate and the undefined ester), three alcohols (2-heptanol, benzy alcohol, and the undefined alcohol) and acetophenone. On the opposite, 3-methylbutanoic acid, 2-methyl-1-butanol, phenylethyl alcohol, α-ethylidene-benzeneacetaldehyde, 3-methylbutyl acetate, 4-methyl-2-hexanone and all the furans (except for 2-pentylfuran) decreased with the addition of CS. Ketone levels in cocoa liquor had a similar trend to that of furans, where the addition of CS reduced their content but kept it stable over time of fermentation (except for 1-Penten-3-one). Esters also showed stabilization in their content over fermentation time in those cocoa liquors only that were fermented with the addition of CS. Therefore, it seems that the addition of CS had a bigger impact on the stabilization of flavor compounds in cocoa liquor compared to cocoa powder (where only furans were stable over time in unroasted cocoa powder fermented with the addition of CS). Last, compounds such as pyrazines did not show remarkable differences due to fermentation type or fermentation time in unroasted cocoa powder (except for 2,3-dimethylpyrazine (**Table 3**)) and roasted cocoa liquor, implying that the addition of CS and/or changing the duration of fermentation did not affect their concentrations.

Most of the volatile compounds identified in roasted cocoa liquor (36 out of 57) showed an smaller %CV when CS were added to the fermentation. Similar that with unroasted cocoa powder, it seems that the addition of CS causes a more consistent volatile composition in cocoa before and after roasting between replicates. Since consistency in flavor is highly desirable in mainstream standardized cocoa products, the addition of CS may generate batches with less variability in volatile content. Further studies are required to confirm this effect and the mechanisms that cause this consistency.

Table 3. List of volatile compounds detected in roasted cocoa liquor samples expressed as internal standard equivalent (ISE) per sample weight (µg naphthalene D8/g). All listed compounds are reported with retention indices (RI) and showed VIP values above 1 in either the PLS-DA model for fermentation type (i.e., with or without the addition of CS) and/or fermentation time (i.e., 5, 6, or 7 days). Arrows indicate the effect of the addition of CS in the compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs; $p \leq 0.05$).

Compound	Retention Index		CS effect	With addition of CS (mean ± standard error)			Without addition of CS (mean ± standard error)		
	Sample	Standard		5 days (n=3)	6 days (n=3)	7 days (n=3)	5 days (n=3)	6 days (n=3)	7 days (n=3)
Acids									
2-methylbutanoic acid	1637	1640		3.33 ± 0.28A	1.04 ± 0.28B	0.78 ± 0.28B	4.16 ± 0.28a	0.72 ± 0.28b	0.95 ± 0.28b
3-methylbutanoic acid	1636	1639	↓	4.86 ± 0.55A	2.96 ± 0.55B	4.24 ± 0.55A	7.25 ± 0.55a	3.2 ± 0.55C	4.8 ± 0.55b
Alcohols									
Ethanol	921	925		0.21 ± 0.04B	0.45 ± 0.04A	0.25 ± 0.04B	0.28 ± 0.04ab	0.31 ± 0.04a	0.18 ± 0.04b
2-methyl-1-Butanol	1191	1192	↓	1248.83 ± 236.1A	1776.05 ± 236.1A	1218.4 ± 236.1A	2512.8 ± 236.1a	1816.43 ± 236.1ab	1142.62 ± 236.1b
2-Heptanol	1306	1306	↑	0.31 ± 0.05A	0.39 ± 0.05A	0.37 ± 0.05A	0.28 ± 0.05a	0.3 ± 0.05a	0.28 ± 0.05a
Unknown	1510		↑	16.26 ± 2.24B	24.46 ± 2.24A	26.54 ± 2.24A	20.55 ± 2.24a	16.82 ± 2.24a	18.26 ± 2.24a
Benzyl alcohol	1824	1840	↑	0.13 ± 0.01A	0.09 ± 0.01B	0.1 ± 0.01B	0.09 ± 0.01a	0.09 ± 0.01a	0.1 ± 0.01a
Phenylethyl Alcohol	1857	1873	↓	7.36 ± 0.69A	7.07 ± 0.69A	6.52 ± 0.69A	8.61 ± 0.69a	8.32 ± 0.69a	7.47 ± 0.69a
Aldehydes									
Pentanal	959	963		0.59 ± 0.08A	0.29 ± 0.08B	0.2 ± 0.08B	0.35 ± 0.08a	0.28 ± 0.08a	0.55 ± 0.08a
Hexanal	1061	1065		1.99 ± 0.43A	0.6 ± 0.43B	0.84 ± 0.43AB	0.62 ± 0.43b	0.58 ± 0.43b	2.21 ± 0.43a
Heptanal	1164	1166		1 ± 0.17A	0.42 ± 0.17B	0.61 ± 0.17AB	0.48 ± 0.17b	0.42 ± 0.17b	1.04 ± 0.17a
Octanal	1267	1271		0.43 ± 0.08A	0.12 ± 0.08B	0.41 ± 0.08A	0.24 ± 0.08ab	0.14 ± 0.08b	0.45 ± 0.08a
Benzaldehyde	1476	1483		2.83 ± 0.21A	2 ± 0.21B	2.89 ± 0.21A	2.35 ± 0.21b	2.13 ± 0.21b	3.63 ± 0.21a
α-ethylidene-benzeneacetaldehyde	1871		↓	0.47 ± 0.08A	0.45 ± 0.08A	0.56 ± 0.08A	0.51 ± 0.08b	0.59 ± 0.08ab	0.7 ± 0.08a
Esters									
Ethyl Acetate	873	877	↑	0.82 ± 0.15B	1.96 ± 0.15A	1.67 ± 0.15A	0.97 ± 0.15a	1.34 ± 0.15a	0.98 ± 0.15a
Ethyl 2-methylbutanoate	1037	1040		0.36 ± 0.05A	0.29 ± 0.05A	0.23 ± 0.05A	0.63 ± 0.05a	0.28 ± 0.05b	0.22 ± 0.05b

Ethyl 3-methylbutanoate	1053	1055		0.34 ± 0.04A	0.25 ± 0.04A	0.2 ± 0.04A	0.53 ± 0.04a	0.24 ± 0.04b	0.18 ± 0.04b
3-methylbutyl acetate	1110	1110	↓	18.24 ± 3.06A	26.05 ± 3.06A	22.82 ± 3.06A	34.58 ± 3.06a	26.66 ± 3.06ab	22.94 ± 3.06b
Ethyl hexanoate	1218	1220	↑	1.07 ± 0.06B	1.46 ± 0.06A	1.35 ± 0.06A	1.2 ± 0.06a	1.29 ± 0.06a	1.1 ± 0.06a
2-Buten-1-ol, 3-methyl-, acetate	1269	1238		0.73 ± 0.13B	1.18 ± 0.13A	1.17 ± 0.13A	1.31 ± 0.13a	1.11 ± 0.13ab	0.98 ± 0.13b
Methyl heptanoate	1483			0.1 ± 0A	0.04 ± 0B	0.04 ± 0B	0.09 ± 0a	0.04 ± 0b	0.04 ± 0b
2,3-Butanediol diacetate	1483			1.01 ± 0.22C	1.99 ± 0.22B	3.19 ± 0.22A	1.4 ± 0.22b	1.92 ± 0.22b	2.74 ± 0.22a
Unknown	1542		↑	15.15 ± 1.09C	21.51 ± 1.09B	28.28 ± 1.09A	18.8 ± 1.09ab	16.41 ± 1.09b	21.41 ± 1.09a
Ethyl 2-phenylacetate	1744	1754	↑	0.74 ± 0.04A	0.76 ± 0.04A	0.76 ± 0.04A	0.6 ± 0.04b	0.75 ± 0.04a	0.67 ± 0.04ab
2-phenylethyl acetate	1771	1782	↑	8.2 ± 0.34B	9.26 ± 0.34B	10.44 ± 0.34A	9.81 ± 0.34b	10.46 ± 0.34ab	11.59 ± 0.34a
Furans									
2-pentylfuran	1214	1216		0.83 ± 0.2A	0.17 ± 0.2B	0.31 ± 0.2AB	0.16 ± 0.2b	0.19 ± 0.2b	1.02 ± 0.2a
Furfural	1426	1432	↓	0.32 ± 0.06A	0.37 ± 0.06A	0.39 ± 0.06A	0.61 ± 0.06a	0.43 ± 0.06b	0.42 ± 0.06b
4-Butyrolactone	1569	1576	↓	0.56 ± 0.09A	0.77 ± 0.09A	0.76 ± 0.09A	1.23 ± 0.09a	1.05 ± 0.09ab	0.93 ± 0.09b
3-Furanmethanol	1623		↓	0.82 ± 0.15A	0.83 ± 0.15A	0.58 ± 0.15A	1.26 ± 0.15a	0.96 ± 0.15ab	0.8 ± 0.15b
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-(Pantolactone)	1973	1991	↓	0.18 ± 0.01A	0.17 ± 0.01A	0.17 ± 0.01A	0.22 ± 0.01a	0.19 ± 0.01b	0.19 ± 0.01b
2(3H)-Furanone-5-acetyldihydro-	1988		↓	0.06 ± 0.01B	0.07 ± 0.01AB	0.08 ± 0.01A	0.09 ± 0.01a	0.1 ± 0.01a	0.11 ± 0.01a
Hydrocarbons									
Octane	801	800	↑	1.22 ± 0.23B	1.22 ± 0.23B	2.71 ± 0.23A	1.56 ± 0.23a	0.99 ± 0.23a	1.34 ± 0.23a
Styrene	1227	1231		2.18 ± 0.33A	2.62 ± 0.33A	2.53 ± 0.33A	2.55 ± 0.33a	2.25 ± 0.33a	2 ± 0.33a
Ketones									
2,3-Butanedione	961		↓	0.54 ± 0.09AB	0.61 ± 0.09A	0.37 ± 0.09B	0.78 ± 0.09a	0.65 ± 0.09ab	0.49 ± 0.09b
1-Penten-3-one	1003	1005		0.13 ± 0.02A	0.05 ± 0.02B	0.05 ± 0.02B	0.05 ± 0.02a	0.05 ± 0.02a	0.12 ± 0.02a
4-methyl-2-hexanone	1161		↓	0.72 ± 0.06A	0.77 ± 0.06A	0.6 ± 0.06A	1 ± 0.06a	0.8 ± 0.06ab	0.62 ± 0.06b
Acetophenone	1598	1607	↑	0.48 ± 0.1A	0.52 ± 0.1A	0.47 ± 0.1A	0.18 ± 0.1b	0.44 ± 0.1a	0.48 ± 0.1a

Targeted Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS) analysis of non-fat non-volatile compounds

Non-fat non-volatile compounds play a key role in cocoa flavor because they are responsible for the characteristic bitterness and astringency in cocoa. Based on the classification provided by Kauz et al. (2021), cocoa astringency is driven by acid amides, and polyphenol glycosides whereas bitterness is elicited by 2,5-diketopiperazines and alkaloids. Both sensations (bitterness and astringency) are also induced by flavan-3-ols (Kauz et al., 2021). Since the composition of these compounds in cocoa is affected by processing (Aprotosoaie et al., 2016; Santander et al., 2020; Ziegleder, 2017), this research focused on determining the effects of the addition of CS and the time of fermentation on non-fat non-volatiles in unroasted and roasted cocoa.

1.1.1.3 Unroasted cocoa powder

The non-volatile compounds quantified in unroasted cocoa powder in this study are listed in **Table 4**. The two measured alkaloids, theobromine and caffeine, were not affected by the time of fermentation (**Table 4**, $p > 0.05$). This agrees with Ziegleder (2017) who stated that alkaloids remain largely unaltered during fermentation and roasting but disagrees with Ho et al. (2014) who found alkaloids to decrease gradually after 72 h of fermentation. Here, the addition of CS increased only the content of caffeine in unroasted cocoa powder significantly by approximately 6%. The content of theobromine, ranging from 14796 ± 627.88 to 17447 ± 627.88 µg/g unroasted cocoa powder, reported in this study is higher than what was shown by Kauz et al., (2021) in cacao samples from around the world, ranging from 1500 to 9000 µg/g sample), whereas caffeine levels in the samples studied here were within the reported range of 1000 to 5800 µg/g. It appears that differences in terms of growing conditions and genetics may be a larger source of variation for alkaloid levels in cocoa compared to processing. This study found a slight increase of 6% in caffeine in samples that were fermented with the addition of CS. This small increase in content of caffeine may affect sensory perception but sensory studies are required.

Acid amides were termed astringent metabolites by Kauz et al. (2021) and Stark et al. (2006). For the acid amide trans-clovamide, which was quantified here (**Table 4**), only the cocoa powder samples fermented without the addition of CS showed a significant decrease up to 29.7% of trans-clovamide with increasing fermentation time, whereas the samples fermented with the addition of CS did not show any significant changes throughout fermentation. These results indicate that the addition of CS during fermentation may have preserved the content of this astringent compound in unroasted cocoa powder. One could assume that the composition of the CS could have modified the performance of the enzymes that degrade polyphenols such as trans-clovamide. According to Santander Muñoz et al. (2020), polyphenols are oxidized during fermentation due to enzymes like polyphenol oxidases and glucosidases. Thus, it is possible that the addition of CS could have slowed down these enzymatic reactions at advanced stages of fermentation. More studies are needed to confirm if CS affect the kinetics of these enzymes and explain the preservation of trans-clovamide in unroasted cocoa powder fermented with the addition of CS.

2,5-Diketopiperazines are bitter metabolites generated by heat-induced intramolecular breakdown of peptides (Ziegleder, 2017). Even though 2,5-diketopiperazines are produced mainly during roasting, Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) were detected and quantified in unroasted cocoa powder samples (**Table 4**). Peptidases activity during fermentation could have produced the peptides that subsequently

generated the 2,5-diketopiperazines in the unroasted cocoa powder. However, the determined levels of Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) in the samples was neither affected by fermentation type nor the time of fermentation. The concentrations reported in this study were on the lower end of the range reported by Kauz et al. (2021) for Cyclo(L-Leu-L-Pro) (21-63 μg/g vs. 19.42-40.97 μg/g found here, **Table 4**) and below the lower limit for Cyclo(Pro-Val) (39-236 μg/g vs. 23.02-45.95 μg/g here, **Table 4**). The effect of the addition of CS and time of fermentation on 2,5-diketopiperazines may become apparent after roasting.

Flavan-3-ols are non-volatile compounds related to both bitterness and astringency in cocoa. During fermentation and drying, flavan-3-ols interact with enzymes and microorganisms and their content is generally reduced (Albertini et al., 2015). The mechanisms for transformation of polyphenols vary from enzymatic oxidation (Santander et al., 2020), hydrolysis (Aprotosoaie et al., 2016) epimerization (Kothe et al., 2013), and polymerization (Racine et al., 2019b), depending on the cocoa processing conditions. Under the conditions of this study, the addition of CS during fermentation was found to maintain the contents of (-)-epicatechin and procyanidin B2 over fermentation time in unroasted cocoa powder, while samples without the addition of CS showed a decrease in these two flavan-3-ols with increasing fermentation time (**Table 4**). This is a similar trend to the one seen for trans-clovamide. Therefore, one could speculate that the composition of CS may have played a protective role for these flavan-3-ols, by limiting their degradation at advanced stages of fermentation compared to the unroasted samples fermented without the addition of CS where degradation was found (**Table 4**). Further studies are necessary to determine which mechanisms of transformation of polyphenols are repressed over time in unroasted cocoa powder when CS are added to the fermenting mass. The content of (-)-epicatechin and procyanidin B2 are comparable to what Żyżelewicz et al. (2016) reported for dried cocoa beans (1962 and 505.4 μg/g of (-)Epicatechin and procyanidin B2, respectively, vs. 1114 and 649 μg/g of (-)-Epicatechin and procyanidin B2 found here). For the last measured flavan-3-ol, (+/-)-catechin, the concentration found in this study is lower to what has been previously quantified (here up to 87.4 vs. 188 μg catechin/g (Żyżelewicz et al. 2016)); catechin levels were not significantly affected by either fermentation time or addition of CS (**Table 4**).

Polyphenol glucosides, similar to the flavan-3-ols, are altered by the interaction with oxidative enzymes and other degradation mechanisms that reduce their content during processing (Albertini et al., 2015; Aprotosoaie et al., 2016; Racine et al., 2019b; Santander et al., 2020). In this study, Isoquercetin and Hyperoside were quantified (**Table 4**) and their contents are within the ranges reported by Kauz et al. (2021) (9-90 and 3-18 μg/g of isoquercetin and hyperoside, respectively). The trends related to the addition of CS and time of fermentation on the polyphenol glucosides evaluated diverge. On one hand, unroasted cocoa powder fermented with the addition of CS showed a significantly higher concentration of Isoquercetin (with increases between 1.62-13.41 ug/g, **table 4**) and no degradation over fermentation time was observed in these samples. On the other hand, regardless of the addition of CS, hyperoside content decreased from 5 days of fermentation to 7 days of fermentation (25 and 32% in cocoa liquor with and without addition of CS, respectively). These results indicated that the potential protective effect of CS on polyphenols could preserve and encourage the production of isoquercetin, while the addition of CS is not able to prevent degradation of hyperoside. However, the mechanisms behind the stimulation and preservation of isoquercitin in fermentations with the addition of CS are still unknown and require additional studies.

The addition of CS was shown to have the potential to provide unroasted cocoa powders with higher and more stable (over fermentation time) concentrations of non-volatile compounds that are contributing to astringency and bitterness in cocoa. Since cocoa processing aims to reduce bitterness and astringency, the addition of CS may be counteracting these aims. Steps such as cocoa pod storage, pre-drying or de-pulping of cocoa seed prior to fermentation have shown to lower the acidification of the beans, leading to less acidic, bitter, and astringent cocoa products (Santander et al., 2020). These preconditioning steps may be better strategies to decrease astringency and bitterness in cocoa compared to adding CS during fermentation. However, rising concern regarding health and the consumption of highly polyphenol food such as dark chocolate (Pacyniak, 2018), could encourage the production of cocoa products with higher content of non-volatile compounds such as polyphenols, and therefore support the addition of CS in cocoa fermentation. Moreover, roasting also plays a key role in cocoa flavor development, where the addition of CS and fermentation time may affect flavor precursors that are transformed during such operation. Therefore, it is needed to assess how CS and fermentation time affects the non-volatile composition of roasted cocoa liquor and compare if the trends found in unroasted cocoa powder continue after roasting and grinding.

The content of non-fat-nonvolatile compounds in unroasted cocoa powder was less variable in the samples with the addition of CS. Except for caffeine, all the non-fat-nonvolatile presented a smaller %CV when CS were added compared to cocoa powder without the addition of CS. This shows a similar pattern to what was found for volatile compounds and supports the idea that the addition of CS has the potential of generate less variable batches of cocoa.

Table 4. Effect of fermentation time (i.e., 5, 6, or 7 days) and fermentation type (i.e., with or without the addition of CS) in the quantification of non-volatile compounds in unroasted cocoa powder. The quantification is expressed in terms of μg/g. Arrows indicate the effect of the addition of CS in the compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs; $p \leq 0.05$).

Compound	CS effect	With addition of CS (EMM ± standard error)			Without addition of CS (EMM ± standard error)		
		5 (n=3)	6 (n=2)	7 (n=3)	5 (n=3)	6 (n=3)	7 (n=3)
Alkaloids							
Theobromine		15949 ± 627.88A	15974 ± 812.96A	17447 ± 627.88A	16622 ± 627.88a	15857 ± 627.88a	14796 ± 627.88a
Caffeine	↑	4264 ± 105.2A	4109 ± 136.2A	4165 ± 105.2A	4031 ± 105.2a	3904 ± 105.2a	3942 ± 105.2a
Acid amide							
Trans-clovamide		28.3 ± 2.2A	28.3 ± 2.9A	31.3 ± 2.2A	33.3 ± 2.2a	31.1 ± 2.2ab	23.4 ± 2.2b
2,5-Diketopiperazine							
Cyclo(L-Leu-L-Pro)		23.7 ± 10.3A	19.4 ± 12.2A	41.0 ± 10.3A	35.0 ± 10.3a	32.7 ± 10.3a	37.3 ± 10.3a
Cyclo(-Pro-Val)		23.0 ± 11.7A	23.4 ± 14.2A	46.0 ± 11.7A	38.3 ± 11.7a	35.8 ± 11.7a	40.4 ± 11.7a
Flavan-3-ol							
(-)-Epicatechin		1114 ± 96.42A	977.6 ± 118.2A	1104 ± 96.42A	1105 ± 96.42a	1101 ± 96.42a	773.6 ± 96.42b
(+/-) Catechin		49.8 ± 27.0A	47.0 ± 34.8A	82.1 ± 27.0A	87.4 ± 27.0a	80.2 ± 27.0a	72.3 ± 27.0a
Procyanidin B2		648.7 ± 46.92A	524.6 ± 57.45A	598.3 ± 46.92A	614.5 ± 46.92a	614.3 ± 46.92a	442.9 ± 46.92b
Polyphenol glucoside							
Quercetin-3-O-glucoside (Isoquercetin)	↑	78.4 ± 3.28A	69.3 ± 4.25A	73.2 ± 3.28A	71.6 ± 3.28a	67.7 ± 3.28ab	59.8 ± 3.28b
Quercetin 3-galactoside (Hyperoside)		5.9 ± 0.3A	4.9 ± 0.4AB	4.3 ± 0.3B	5.4 ± 0.3a	4.7 ± 0.3a	3.7 ± 0.3b

1.1.1.4 Roasted cocoa liquor

Roasting affects the content of non-volatile compounds that are responsible for bitterness and astringency in cocoa (Kothe et al., 2013; McClure et al., 2021; Ziegleder, 2017). To evaluate how non-fat non-volatiles are affected by fermentation type and fermentation time post roasting, and how these changes compare to the results found for unroasted cocoa powder, the same 10 non-volatile compounds were quantified in roasted cocoa liquor (**Table 5**). In terms of alkaloids, neither theobromine nor caffeine showed any significant changes due to time of fermentation, similar to the unroasted cocoa powder (**Table 4**). The addition of CS did not have any significant effect on theobromine nor caffeine content in roasted cocoa liquor (**Table 5**), which was found only for theobromine in unroasted cocoa powder (**Table 4**). Despite caffeine increased in content with the addition of CS in unroasted cocoa powder (**Table 4**), this increase was not found in roasted cocoa liquor (**Table 5**). The results for alkaloids in roasted cocoa liquor of this study agree with reports by McClure et al. (2021) and Ziegleder (2017) who also reported no significant changes in theobromine and caffeine due to fermentation and roasting.

The content of trans-clovamide was not affected by the addition of CS but decreased in cocoa liquor from 5 days to 7 days of fermentation with (34.2 ± 1.43 to 27.3 ± 1.43 µg /g) and without (32.5 ± 1.43 to 26.7 ± 1.43 µg /g) the addition of CS (**Table 5**). These results differ from what was found in unroasted cocoa powder (**Table 4**), where the addition of CS was able to maintain trans-clovamide levels over fermentation time while samples fermented without the addition of CS decreased significantly from day 5 to day 7 (**Table 4**). This potential preventative effect of the addition of CS on trans-clovamide levels throughout the monitored fermentation time however, was not seen in the roasted samples that were fermented with the addition of CS: trans-clovamide levels were significantly lower in roasted samples fermented for 7 days regardless of the addition of CS (decrease of about 17% and 20% in liquors fermented with and without addition of CS, correspondingly, **Table 5**). Roasting has been previously identified as detrimental for acid amides such as N-caffeoyl-L-DOPA (clovamide) (Arlorio et al., 2008). Therefore, despite the protective effect of CS in trans-clovamide before roasting, roasting appears to counter that effect and degrade trans-clovamide based on the time of fermentation regardless of the addition of CS; the longer the fermentation, the lower the content of trans-clovamide in roasted cocoa liquors.

2,5-Diketopiperazines are heat-induced compounds produced from peptides (Ziegleder, 2017) and are mainly generated during roasting. In fact, roasted cocoa liquor contained on average 67.1% and 64.2% more Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) than unroasted cocoa powder samples (**Table 4 and 5**). The increase of 2,5-diketopyperazines in roasted cocoa liquor has been reported by McClure et al. (2021) who modeled the formation of cyclo(Pro-Val) as a function of roasting time and temperature: prolonged times and higher roasting temperature cause higher concentrations of cyclo(Pro-Val). In terms of the factors evaluated in this study, the addition of CS during fermentation stabilized the content of Cyclo(L-Leu-L-Pro) and Cyclo(Pro-Val) in roasted cocoa liquor over fermentation time. In contrast, the content of these compounds increased over time in the samples fermented without the addition of CS. From these results, it appears that the addition of CS promoted the production of precursors of Cyclo(L-Leu-L-Pro) and Cyclo(Pro-Val) in early stages of fermentation (prior to 5 days of fermentation). In samples without the addition of CS, the production of these precursors appears to progressively increase over time. Enzymes such aspartic endoprotease and carboxypeptidazse play a key role in breaking vicilin-class (7S) globulin and other storage proteins in the cocoa bean (Santander et al., 2020). Therefore, the acidic conditions of the CS treatment may have encouraged the activity of these enzymes in early stages of

fermentation and accelerated the production of peptides. Otherwise, the peptides would have been produced gradually with longer times of fermentation. The stimulation of peptidases in samples fermented with the addition of CS would have consequently increased the content of 2,5-diketopyperazines and stabilized their levels in later days of fermentation. Further studies regarding the CS characterization and its effect on proteases are required to explain the changes of 2,5-diketopyperazines content in roasted cocoa liquor samples.

Roasting affects the composition of flavan-3-ols in cocoa under mechanisms such as epimerization (Kothe et al., 2013) and polymerization (Racine et al., 2019b), and these may change the trends observed in unroasted cocoa powder (**Table 4**). According to the results (**Table 5**), the addition of CS increased both (-)-epicatechin and Procyanidin B2 concentrations in roasted cocoa samples, and further, maintained levels of all quantified flavan-3-ols over fermentation time. In contrast, roasted cocoa liquors fermented without the addition of CS showed a significant decrease in (-)-epicatechin and procyanidin B2 with increasing fermentation time; in these samples these two compounds decreased by 11 and 15% from day 5 to day 7. These results, together with the results for the unroasted cocoa powder (**Table 4**) suggest that the addition of CS is favorable in increasing and stabilizing (over time of fermentation) flavan-3-ols in cocoa before and after roasting. This could potentially increase bitterness and astringency perception of these cocoa products. Although (+/-)-catechin did not change significantly either due to time of fermentation nor addition of CS, compared to the unroasted samples, catechin concentration was approximately 70% higher in roasted cocoa liquor (**Table 4 and 5**). Kothe et al. (2013) reported that the increase of (+/-)-catechin during roasting was related the epimerization from (-)-epicatechin to (-)-catechin. However, since not evident decrease of (-)-Epicatechin was found in roasted cocoa liquor compared to unroasted cocoa powder (**Table 4 and 5**), other reactions that encourage the production of (+/-)-catechin during roasting may have occurred.

Conversely to what was shown for flavan-3-ols, levels in the two quantified polyphenol glucosides, isoquercetin and hyperoside, in roasted cocoa liquors were not significantly affected by the addition of CS (**Table 5**). Further, the longer the fermentation, the lower the concentration of these polyphenol glucosides regardless of the addition of CS. These results were different from what was found for unroasted cocoa powder (**Table 4**), where unroasted cocoa samples fermented with the addition of CS led to increased levels of isoquercetin that were maintained throughout the fermentation time. This trend is similar to what was found for trans-clovamide and reveals that cocoa polyphenol kinetics during roasting differ between compounds. Whereas the addition of CS during fermentation keeps the content of (-)-Epicatechin and procyanidin B2 constant over time, other polyphenols such as trans-clovamide, isoquercetin and hyperoside decrease over time regardless of fermentation type. Despite literature reporting the degradation of polyphenols due to roasting (Żyżelewicz et al., 2016a), there is not work regarding the effect of cocoa processing specifically on polyphenol glucosides. Thus, further studies are required to elucidate which factors in the CS affect the degradation/formation kinetics of the different cocoa polyphenols.

The non-volatile results for roasted cocoa liquor (**Table 5**) demonstrate that the addition of CS during fermentation conveys a protective role for some polyphenols, namely, the flavan-3-ols (-)-epicatechin, (+/-)-catechin and procyanidin B2, and even increased the content of (-)-epicatechin and procyanidin B2. Since similar tendencies were shown in unroasted cocoa powder, one can conclude that the addition of CS increases and stabilizes the content of flavan-3-ols. The mechanisms behind the protective role of CS during fermentation remains unknown but may be related to the composition of the CS and its effect on

enzymatic reactions during fermentation which are evident even after roasting. More studies are necessary to clarify which polyphenol reaction mechanisms are triggered and/or repressed by the addition of CS during cocoa fermentation.

In addition to the protective role that CS seems to provide to non-fat-nonvolaties in roasted liquor. The addition of CS generated more consistent nonvolatile content within biological replicates. In fact, by the exception of hyperoside, all the nonvolatile compounds quantified had a smaller %CV compared to cocoa liquors without the addition of CS. This performance was similar before and after roasting and suggest that the use of CS in fermentation may lead to more consistent batches of unroasted cocoa powder and roasted cocoa liquor. Further studies are required to understand which mechanisms generate this consistency, which is highly desirable for mainstream cocoa products.

Table 5. Effect of fermentation time (i.e., 5, 6, or 7 days) and fermentation type (i.e., with or without the addition of CS) in the quantification of non-volatile compounds in roasted cocoa liquor. The quantification is expressed in terms of μg/g. Arrows indicate the effect of the addition of CS in the compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs; $p \leq 0.05$).

Compound	CS effect	With addition of CS (EMM ± standard error)			Without addition of CS (EMM ± standard error)		
		5 (n=3)	6 (n=3)	7 (n=3)	5 (n=3)	6 (n=3)	7 (n=3)
Alkaloids							
Theobromine		19352 ± 502.31A	18781 ± 502.31A	18642 ± 502.31A	19371 ± 502.31a	18823 ± 502.31a	18946 ± 502.31a
Caffeine		4288 ± 77.28A	4220 ± 77.28A	4360 ± 77.28A	4243 ± 77.28a	4232 ± 77.28a	4303 ± 77.28a
Acid amide							
Trans-clovamide		34.2 ± 1.43A	34.0 ± 1.43A	27.3 ± 1.43B	32.5 ± 1.43a	31.6 ± 1.43a	26.7 ± 1.43b
2,5-Diketopiperazine							
Cyclo(L-Leu-L-Pro)		85.1 ± 6.17A	85.3 ± 6.17A	92.7 ± 6.17A	76.0 ± 6.17b	96.2 ± 6.17a	94.2 ± 6.17ab
Cyclo(-Pro-Val)		100 ± 6.67A	103 ± 6.67A	114 ± 6.67A	89.0 ± 6.67b	110 ± 6.67ab	113 ± 6.67a
Flavan-3-ol							
(-)-Epicatechin	↑	1267 ± 45.71A	1275 ± 45.71A	1222 ± 45.71A	1172 ± 45.71ab	1245 ± 45.71a	1107 ± 45.71b
(+/-) Catechin		260.9 ± 18.36A	259.0 ± 18.36A	247.1 ± 18.36A	211.8 ± 18.36a	250.4 ± 18.36a	223.2 ± 18.36a
Procyanidin B2	↑	676.2 ± 33.85A	693.5 ± 33.85A	631.0 ± 33.85A	630.4 ± 33.85a	646.7 ± 33.85a	547.8 ± 33.85b
Polyphenol glucoside							
Quercetin-3-O-glucoside (Isoquercetin)		68.8 ± 2.14AB	69.9 ± 2.14A	63.6 ± 2.14B	67.1 ± 2.14a	66.4 ± 2.14ab	60.6 ± 2.14b
Quercetin 3-galactoside (Hyperoside)		4.95 ± 0.32A	4.66 ± 0.32AB	3.83 ± 0.32B	4.75 ± 0.32a	4.25 ± 0.32ab	3.55 ± 0.32b

Sensory Analysis

Table 6 contains the results of the sensory analysis that tested the effects of the addition of CS and time of fermentation on perception of roasted cocoa liquor, using several triangle tests. I assumed that the effects of fermentation type and fermentation time on sensory perception may be small, therefore, a more sensitive discrimination task was chosen, to determine whether regular chocolate consumers would be able to differentiate between the samples. A subset of the samples was evaluated, as shown in **Table 6** to test (1) the effect of fermentation type (with vs. without the addition of CS for both 5 and 7 days of fermentation), (2) the effect of fermentation time (5 days vs. 7 days for both with and without the addition of CS), and (3) the interaction (5 days with vs. 7 days without the addition of CS), for perceivable sensory differences.

Table 6. Results of the sensory evaluation on the effects of the addition of CS and fermentation time in cocoa liquor using triangle tests with 99 to 103 regular dark chocolate consumers (at least 1/month).

	Effect of fermentation type (with vs. without CS)		**Effect of fermentation time (5 days vs. 7 days)**		**Interaction effect**
	5 days without CS vs. 5 days with CS	7 days without CS vs. 7 days with CS	5 days with CS vs. 7 days with CS	7 days without CS vs. 5 days without CS	5 days with CS vs. 7 days without CS
Difference test (α=0.05)					
N	99	100	103	101	99
Correct answers	43	39	37	43	33
d'	1.1	0.81	0.54	1.05	0
p-Value	***0.02***	*0.14*	*0.34*	***0.03***	*0.54*
Similarity test (β=0.10; α=0.05)					
Correct answers		39	37		33
Correct answers required		40	41		40
Estimated Pd ± standard error		0.09 ± 0.08	0.04 ± 0.07		0

Cocoa liquor after 5 days of fermentation and made with the addition of CS was found to differ significantly in perception from the cocoa liquor after 5 days of fermentation made without addition of CS (43 correct out of 99; p = 0.02; **Table 6**). Open comments collected from participants that correctly identified the odd sample indicate that the most quoted sensory quality was bitterness (**Supplemental table 4**), with 37% of commenters stating that the cocoa liquor fermented for 5 days with the addition of CS was more bitter. Despite the sensory difference between cocoa liquors fermented for 5 days with vs. without the addition of CS, for the cocoa liquor samples fermented for 7 days with vs. without the addition of CS, 100 regular chocolate consumer could not detect a significant sensory difference (39 correct out of 100; p = 0.14; **Table 6**); in fact, using a type II error limit of 10%, sensory similarity between these two samples can be concluded, as only 9% of the participants correctly identified the odd sample. These results imply that the sensory perception of cocoa liquors fermented with and without

addition of CS is apparent (albeit a small sensory difference with a d' of 1.1) after 5 days of fermentation but becomes indistinguishable after 7 days of fermentation.

Regarding the effect of fermentation time on sensory perception within fermentation type, cocoa liquors made with the addition of CS could not be discriminated by regular chocolate consumers when comparing samples fermented for 5 vs. 7 days (37 correct out of 103; $p = 0.34$; **Table 6**). Indeed, fermenting cocoa with the addition of CS leads to sensorily similar samples when fermenting either for 5 or 7 days ($\beta = 0.10$; 4% discriminators; **Table 6**). Conversely, when comparing cocoa liquors fermented without the addition of CS for 5 vs. 7 days, 101 regular chocolate consumers detected a significant difference between these samples (43 correct out of 101; $p = 0.03$; **Table 6**). Of those that correctly picked the odd sample, 39% of the comments (**Supplemental table 5**) indicate that the cocoa liquor fermented for 5 days without the addition of CS was less bitter than the cocoa liquor after 7 days.

For the last triangle test, cocoa liquor fermented with the addition of CS for 5 days was tested against cocoa liquor fermented without the addition of CS for 7 days (**Table 6**). Ninety-nine regular chocolate consumers were not able to discriminate between the samples (33 correct out of 99; $p = 0.54$). In fact, sensory similarity could be concluded at the 10% beta-level (pd = 0%). These results may infer two things: First, the addition of CS appears to lead to a more bitter-tasting roasted cocoa liquor, and this suggested bitterness remains stable over fermentation time. Second, cocoa liquors fermented without the addition of CS for 5 days are presumably less bitter than those fermented for 7 days. Taken together, the addition of CS during fermentation appears to generate roasted cocoa liquors that are similar in sensory perception to samples fermented without the addition of CS, however, in a shorter time of fermentation.

Based on the results from this study, it appears that the addition of CS during fermentation stabilizes sensory perception and potentially cocoa quality. This could be related to the protective effect of CS in terms of concentration of certain volatile and non-volatile compounds. The addition of CS stabilized volatile compounds such as alcohols (2-methyl-1-butanol), aldehydes (α-ethylidene-benzeneacetaldehyde), esters (ethyl 2-methylbutanoate; ethyl 3-methylbutanoate; and 3-methylbutyl acetate), furans (furfural, 4-butyrolactone and pantolactone), and 4-methyl-2-hexanone throughout 5 to 7 days of fermentation (**Table 3**). Similarly, the addition of CS stabilized the content of Cyclo(L-Leu-L-Pro), Cyclo(Pro-Val), (-)-Epicatechin and procyanidin B2 (**Table 5**) over fermentation time, all of these being attributed to elicit bitterness in cocoa (Kauz et al., 2021; McClure et al., 2021, 2022). Furthermore, the content of these compounds in cocoa liquor without the addition of CS after 7 days of fermentation did not show statistical difference compared to the cocoa liquor fermented with the addition of CS at 5 days of fermentation, except for α-ethylidene-benzeneacetaldehyde, 4-butyrolactone, (-)-epicatechin and procyanidin B2, based on Tukey post-hoc test $p < 0.05$. This could be one of the reasons for the statistically similar perception of these two samples in the triangle test. Consequently, the addition of CS preserved some volatile and non-volatiles compounds over fermentation time and led to similar sensory perception compared to cocoa liquors made without the addition of CS at advanced stages of fermentation. The mechanisms for the inhibition or delay in degradation of these compounds are unknown, but the composition of the CS may play a key role in it.

The triangle test is a basic discrimination analysis that does not give any indications of the direction of the difference (Sinkinson, 2017). However, the open comments from the participants suggest a potential trend of at least one of the sensory attributes that changed in this study (bitterness) (**Supplemental**

table 4 and 5). Although the sample differences were small with d' values just slightly above 1, regular consumers of dark chocolate (more than 1 time/month) with minimal training perceived differences between the roasted cocoa liquors. Further training and the use of a different sensory methodology could reveal qualitative and quantitative differences, but this study demonstrated that even without that training, there are recognizable sensory changes in roasted cocoa liquor due to the addition of CS and time of fermentation. McClure et al. (2022) reported that the reduction of bitterness is correlated with increased acceptability of unsweetened chocolate samples. Based on this, we could speculate that the cocoa liquors made with the addition of CS may not be highly acceptable to consumers. However, bitterness may not be sensory descriptor that drivers the differentiation in this samples. Since the participants were not trained, other disliking attributes could have been covered by the term bitterness. For instance, over-fermented beans can arise a hammy off-flavor that could have been associated to bitterness by the untrained panel (Ziegleder, 2017) Therefore, further studies are necessary to quantitate changes in bitterness and maybe other sensory attributes in cocoa liquors due to the addition of CS and time of fermentation.

Other sensory techniques such as descriptive analysis (DA) have been used to determine quantitative and qualitative differences due to cocoa processing (Crafack et al., 2014; Escobar et al., 2021; Santander et al., 2021). For instance, longer times of fermentation either increased or kept constant the bitterness perception in chocolates made from cocoa from different regions of Colombia (Escobar et al., 2021), which agrees with what was found here. However, the authors also found changes in flavor attributes such as fruitiness, floral, spice, and nuttiness during fermentation. Similarly, Santander et al. (2021) reported significant differences in fruitiness, floral, and nuttiness when using lactic acid and acidic acid to control fermentation, whereas bitterness remained similar. Therefore, despite this research is not able to clearly determine what the sensory differences are, bitterness appears to be one of the likely discriminants between cocoa liquors fermented with and without the addition of CS, however, other sensory attributes may also differ between the samples. Future studies that involve descriptive analysis are necessary to identify the main sensory drivers of differentiation between cocoa liquor made with and without the addition of CS over fermentation time.

CONCLUSIONS

Overall conclusions

This thesis evaluated the effect of the addition of CS during fermentation and the effect of fermentation time (5, 6, and 7 days) on volatile and non-volatile compounds in unroasted cocoa powder and roasted cocoa liquor, and sensory perception of differences in roasted cocoa liquor. Returning to my initial hypotheses for this research, I am concluding the following:

(1) The addition of CS produced in the first 18 h of fermentation change the flavor composition of roasted cocoa powder and roasted liquor, but did not always increase the content of such compounds, and their change can also depend on the time of fermentation. The addition of CS increased fat content by 0.3% and 0.4% in unroasted cocoa powder and roasted cocoa liquor, respectively. However, **the changes in fat reported in this study are very small and likely not cause a significant difference in flavor perception**.

In terms of volatile composition, **48 compounds were tentatively identified in unroasted cocoa powder but only 27 were classified as drivers of differentiation between samples fermented with and without the addition of CS and different fermentation time (5, 6, 7 days)** based on the PLS-DA models. The change within these 27 compounds followed different patterns. The addition of CS increased the content of some esters (Ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, 2-ethoxyethyl butanoate and ethyl-2-phenylacetate) benzaldehyde, 2-methylpropanoic acid, acetophenone and 2,3-dimethylpyrazine, but it reduced the content of furans (up to 0.9 µg naphthalene D8/g). Despite the reduction of furans in all cocoa powder fermented with the addition of CS, their content did not show statistical differences over fermentation time. This was in contrast to the cocoa powders fermented without the addition of CS where furan levels significantly decreased over fermentation time. According to the results, **it is presumed that CS may have some type of protective role in furan content over fermentation time for unroasted cocoa powder.**
In roasted cocoa liquor, 57 compounds were tentatively identified but only 37 were classified as drivers of differentiation between samples fermented with and without the addition of CS for 5, 6 or 7 days of fermentation. Similar to unroasted cocoa powder, the 37 compounds showed different patterns in concentration changes with the addition of CS over time. The addition of CS increased the content of five esters (ethyl acetate, ethyl hexanoate, ethyl, 2-phenylacetate, 2-phenylethyl acetate and the undefined ester), three alcohols (2-heptanol, benzy alcohol, and the undefined alcohol) and acetophenone. On the opposite, 3-methylbutanoic acid, 2-methyl-1-butanol, phenylethyl alcohol, α-ethylidene-benzeneacetaldehyde, 3-methylbutyl acetate, 4-methyl-2-hexanone and all the furans (except for 2-pentylfuran) decreased with the addition of CS. Cocoa liquor fermented with the addition of CS did not show any significant differences over fermentation time in 2-methyl-1-butanol, α-ethylidene-benzeneacetaldehyde, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 4-methyl-2-hexanone, acetophenone, furfural, 4-butyrolactone and pantolactone. In contrast, these compounds generally decrease over fermentation time in cocoa liquors fermented without the addition of CS. Therefore, **one could assume that CS play a protective role for ethylidene-benzeneacetaldehyde, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 4-methyl-2-hexanone, acetophenone, furfural, 4-butyrolactone and pantolactone in roasted cocoa liquor, due to the stabilization of the content of these compounds over fermentation time compared to samples fermented without the addition of CS.**

The addition of CS provided unroasted cocoa powders with higher content of isoquercetin and caffeine. Additionally, cocoa powders fermented with the addition of CS showed constant (over fermentation time) concentration levels of transclovamide, (-)-epicatechin, procyanidin B2, and isoquercetin, which implies that **CS may have a protective effect on the polyphenols evaluated in this study in unroasted cocoa powder**.

In roasted cocoa liquors, the addition of CS during fermentation generated samples with higher concentrations of (-)-epicatechin and procyanidin B2. Furthermore, the addition of CS during fermentation stabilized the levels of Cyclo(L-Leu-L-Pro), Cyclo(Pro-Val), (-)-Epicatechin, and Procyanidin B2 over time compared to cocoa liquors fermented without the addition of CS: For these samples, Cyclo(L-Leu-L-Pro), and Cyclo(Pro-Val) increased over time, whereas (-)-epicatechin and procyanidin B2) decreased. Therefore, **one can presume that CS stabilizes and protects the content of Cyclo(L-Leu-L-Pro), Cyclo(Pro-Val), (-)-epicatechin, and procyanidin B2 over fermentation time compared to cocoa liquors fermented without the addition of CS.**

(2) The fermentation time (5, 6, and 7 days) significantly affects volatile and non-fat non-volatile flavor composition of unroasted cocoa, and the effect can be detected after roasting. However, the changes in compound concentrations over fermentation time do not necessarily align between unroasted and roasted cocoa samples, and are affected by the addition of CS. From the volatiles that differentiate between fermentation type and time, only organic acids, furans, and pyrazines followed a similar trend in unroasted cocoa powder and roasted cocoa liquor over the time of fermentation. Organic acids decreased over time of fermentation, furans were stable over time of fermentation only in samples with the addition of CS, and pyrazines content did not change due to fermentation time regardless of the addition of CS. Ketones and esters showed content stabilization over fermentation time exclusively in roasted cocoa liquor fermented with the addition of CS. In unroasted cocoa powder conversely, ketones did not significantly change over fermentation time, and most of the esters decreased regardless of fermentation type. Ethylidene-benzeneacetaldehyde, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, 4-methyl-2-hexanone, and acetophenone, showed stabilization over fermentation time only in rosted cocoa liquors fermented with the addition of CS while samples fermented without the addition of CS showed significant changes. The above was only seen for furans for unroasted cocoa powder. Therefore, **the changes over fermentation time in volatile composition in roasted cocoa liquor may be driven by the changes in the precursors generated due to the addition of CS. During roasting, these precursors are transformed into flavor compounds, and therefore the fermentation time trends in volatile composition evidenced in cocoa powder prior to roasting are different from the changes found in roasted cocoa liquor samples**.

For non-volatiles Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) different trends before and after roasting were found, modulated by the addition of CS during fermentation: Whereas no changes over time were identified regardless of the addition of CS in unroasted cocoa powder, after roasting, cocoa liquor samples fermented without the addition of CS showed a significant increase in Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) over time, until reaching similar levels to the ones quantified in roasted liquors fermented with the addition of CS. Thus, **one can assume that the addition of CS produced Cyclo(Pro-Val) and Cyclo(L-Leu-L-Pro) in cocoa liquor in earlier stages of fermentation, which causes the stabilization over later stages of fermentation, whereas these levels were reached only after 7 days of fermentation for the cocoa liquors fermented without the addition of CS.**

(3) <u>Cocoa liquors produced with the addition of CS are perceptually similar after 5 days of fermentation compared to cocoa liquors fermented without the addition of CS after 7 days of fermentation.</u> Based on the sensory analysis, the addition of CS potentially leads to a more bitter-tasting roasted cocoa liquor, which remains stable over fermentation time: only 4% of participants who successfully picked the odd sample when presented with cocoa liquors fermented with the addition of CS after 5 vs. 7 days of fermentation, allowing me to conclude sensory similarity between these two samples. The addition of CS during fermentation appears to generate roasted cocoa liquor that is similar in sensory perception to samples fermented without the addition of CS, but in a shorter time of fermentation (concluding similarity at the 10% beta-level).

The sensory similarity between samples fermented with the addition of CS appears over different fermentation times can be related to the protective effect of CS found for several volatile and non-volatile compounds. The addition of CS stabilized the volatile compounds: 2-methyl-1-butanol; α-ethylidene-benzeneacetaldehyde; ethyl 2-methylbutanoate; ethyl 3-methylbutanoate; 3-methylbutyl acetate; furfural; 4-butyrolactone; pantolactone; and 4-methyl-2-hexanone throughout 5 to 7 days of fermentation. Additionally, the addition of CS stabilized the content of Cyclo(L-Leu-L-Pro), Cyclo(Pro-Val), (-)-Epicatechin and procyanidin B2 over fermentation time, all of these being attributed to elicit bitterness in cocoa.

As an ultimate conclusion, this study showed that the addition of CS during fermentation stabilized the content of 7 compounds in unroasted cocoa powder (Benzyl alcohol, 2-acetylfuran, pantolactone, transclovamide, (-)-epicatechin, procyanidin B2, and isoquercetin) and 17 compounds in roasted cocoa liquor (2-methyl-1-butanol, octanal, benzaldehyce, α-ethylidene-benzeneacetaldehyde, ethyl 2-methyl butanoate, ethyl 3-methylbutanoate, 3-methylbutyl acetate, furfural, 4-butyrolactone, 3-Furanmethanol, pantolactone, 4-methyl-2-hexanone, acetophenone, (-)-epicatechin, Procyanidin B2, Cyclo(Pro-Val), Cyclo(L-Leu-L-Pro)) over time of fermentation. Additionally, not differences in sensory perception over fermentation time were identified only in cocoa liquor fermented with the addition of CS. Therefore, this study suggests that CS have a protective effect in stabilizing the content of some flavor compounds and consequently generate cocoa with similar sensory perception in shorter times of fermentation. On the opposite, cocoa samples without addition of CS may develop similar sensory perception but in longer times of fermentation. Lastly, most of the compounds quantified shower a smaller coefficient of variation in the samples with the addition of CS. Therefore, this study also suggest that the addition of CS may have the potential for providing more consistent batches of cocoa with similar flavor composition. The above may be highly appreciated for the market of mainstream cocoa products, but further studies are required to evaluate which mechanisms are affected to provide such consistency.

Future directions

This exploratory study showed that the addition of CS and the time of fermentation affect flavor composition and sensory perception of cocoa. It is presumed that CS have a protective role in stabilizing the content of certain volatile and non-volatile compounds, and consequently generate more bitter cocoa in shorter fermentation times compared to cocoa fermented without the addition of CS. The addition of CS seems to provide more consistent flavor composition. However, further studies are necessary to understand the flavor formation mechanisms that CS trigger/inhibit during cocoa processing. This study focused only on the late stages of fermentation (5, 6 and 7 days of fermentation).

Therefore, evaluating how the addition of CS affects flavor composition during the early stages of fermentation would provide more insights about flavor kinetics from the pod opening till the final fermented bean. For example, with the monitoring of flavor formation in the early stages of fermentation, the role of compounds such as acetic acid and ethanol, which were not significant different between the experimental factors in this study, may be revealed. Since the presumed compounds that may affect sensory perception were stable over fermentation time with the addition of CS, this study was unable to determine if the use of CS speeded up or slowed down fermentation. Therefore, future studies that involve sampling at earlier times of fermentation may reveal if the stability of these compounds is through all fermentation time, or if there is any increase/decrease in early stages. Similarly, evaluating early stages of fermentation would also be valuable to determine if the cocoa produced with the addition of CS would always be perceived as more bitter, or if bitterness may be lower at shorter times of fermentation.

Flavor compounds are important factors in final product quality and are affected by cocoa processing. Monitoring substrates of flavor compounds, so called flavor precursors, during processing could provide more insights about flavor kinetics. Techniques such as untargeted non-volatile analysis may be valuable tools to quantify polyphenols, carbohydrates, and peptides, which are the bean main constituents that are experiencing changes during processing. By quantifying the changes of flavor precursors along with resulting flavor compounds, it would be possible to determine the effect of CS over fermentation time from the perspective of substrates and products, and ultimately, propose mechanisms for this behavior. With knowledge about substrates and products in fermentation plus monitoring physicochemical properties of the fermentation mass, it would be possible the design of heterogeneous fermentation systems. Research from other fermented products such as wine (Miller et al., 2020), showed that it is possible to generate models that estimate the fermentation kinetics, mass/heat transfer, fluid flow and phenolic extraction kinetics by physicochemical properties such as temperature and ethanol concentration. Further studies regarding the modeling of flavor precursor/compounds formation based on physicochemical properties during fermentation will be highly valuable in the cocoa industry.

It has been documented that changes in acidification may alter enzymatic reactions in the beans during fermentation, affecting flavor precursor levels and subsequently flavor compounds. Since CS physicochemical properties (such as pH, titratable acidity, and sugar content) vary across time of fermentation, future studies characterizing CS are fundamental to understand how CS interacts with the fermentation mass and participates in the changes in flavor composition. This study did not characterize the CS employed during the experiments, and this as a pitfall of this study. However, it opens the perspective over the use of CS as potential tool to add and preserve some volatile and non-volatile compounds that likely affect bitterness in cocoa. The type of compounds that CS stabilizes may depend on the physicochemical characteristics of the CS. A not very acidic CS could open the possibility to generate cocoa with high levels of hydrophobic amino acids and hydrophilic peptides, which have been related to high flavor potential in cocoa, and therefore, quality.

Bitterness was the attribute that the participants used the most to differentiate between the cocoa liquors in this study. However, other sensory methodologies such as DA need to be used in the future to identify and quantitate all sensory attributes in cocoa that are affected by the addition of CS during fermentation. Additionally, since the participants were not trained to identify specific sensory attributes, other sensory perceptions could have been masked by the bitterness and/or not been properly described by the untrained participants. It is known that the term bitter is often used to describe

unpleasant sensations that are not bitter *per se* but rather a lack of more descriptive language. Therefore, DA in future studies will be necessary to quantitatively and qualitative determine the effect of the addition of CS during fermentation on cocoa liquor sensory profiles.

REFERENCES

Aculey, P. C., Snitkjaer, P., Owusu, M., Bassompiere, M., Takrama, J., Nørgaard, L., Petersen, M. A., & Nielsen, D. S. (2010). Ghanaian cocoa bean fermentation characterized by spectroscopic and chromatographic methods and chemometrics. *Journal of Food Science*, *75*(6). https://doi.org/10.1111/j.1750-3841.2010.01710.x

Adams, M. R., Dougan, J., Glossop, E. J., & Twiddy, D. R. (1982). Cocoa sweatings—an effluent of potential value. *Agricultural Wastes*, *4*(3), 225–229. https://doi.org/10.1016/0141-4607(82)90014-2

Afoakwa, E. O., Kongor, J. E., Takrama, J. F., & Budu, A. S. (2013). Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp pre-conditioned cocoa (Theobroma cacao) beans. *International Food Research Journal*, *20*(3), 1215–1222. http://www.ifrj.upm.edu.my/20 (03) 2013/26 IFRJ 20 (03) 2013 Afoakwa (397).pdf

Afoakwa, E. O., Paterson, A., Fowler, M., & Ryan, A. (2008). Flavor formation and character in cocoa and chocolate: A critical review. *Critical Reviews in Food Science and Nutrition*, *48*(9), 840–857. https://doi.org/10.1080/10408390701719272

Albertini, B., Schoubben, A., Guarnaccia, D., Pinelli, F., della Vecchia, M., Ricci, M., di Renzo, G. C., & Blasi, P. (2015). Effect of Fermentation and Drying on Cocoa Polyphenols. *Journal of Agricultural and Food Chemistry*, *63*(45), 9948–9953. https://doi.org/10.1021/acs.jafc.5b01062

Andersen, C. M., & Bro, R. (2010). Variable selection in regression-a tutorial. *Journal of Chemometrics*, *24*(11–12), 728–737. https://doi.org/10.1002/cem.1360

Aprotosoaie, A. C., Luca, S. V., & Miron, A. (2016). Flavor Chemistry of Cocoa and Cocoa Products-An Overview. *Comprehensive Reviews in Food Science and Food Safety*, *15*(1), 73–91. https://doi.org/10.1111/1541-4337.12180

Ardhana, M. M., & Fleet, G. H. (2003). The microbial ecology of cocoa bean fermentations in Indonesia. *International Journal of Food Microbiology*, *86*(1–2), 87–99. https://doi.org/10.1016/S0168-1605(03)00081-3

Arlorio, M., Locatelli, M., Travaglia, F., Coïsson, J. D., Grosso, E. del, Minassi, A., Appendino, G., & Martelli, A. (2008). Roasting impact on the contents of clovamide (N-caffeoyl-L-DOPA) and the antioxidant activity of cocoa beans (Theobroma cacao L.). *Food Chemistry*, *106*(3), 967–975. https://doi.org/10.1016/j.foodchem.2007.07.009

Assa, A., Rosniati, & Yunus, M. R. (2019). Effects of cocoa clones and fermentation times on physical and chemical characteristics of cocoa beans (Theobroma cacao L.). *IOP Conference Series: Materials Science and Engineering*, *528*(1), 012079. https://doi.org/10.1088/1757-899X/528/1/012079

Audouin, V., Bonnet, F., Vickers, Z. M., & Reineccius, G. A. (2001). Limitations in the Use of Odor Activity Values to Determine Important Odorants in Foods. *Gas Chromatography-Olfactometry*, 156–174. https://doi.org/10.1021/bk-2001-0782.ch014

Bartoshuk, L. M., Sims, C. A., Colquhoun, T. A., & Snyder, D. J. (2019). What Aristotle didn't know about flavor. *American Psychologist*, *74*(9), 1003–1011. https://doi.org/10.1037/amp0000577

Breen, S. P., Etter, N. M., Ziegler, G. R., & Hayes, J. E. (2019). Oral somatosensatory acuity is related to particle size perception in chocolate. *Scientific Reports*, *9*(1), 7437. https://doi.org/10.1038/s41598-019-43944-7

Brown, A. L. (2021). *Understanding flavor in fine or flavor Theobroma cacao: A multidisciplinary human subjects research approach*. The Pennsylvania State University.

CAOBISCO/ECA/FCC. (2015). *Cocoa Beans: Chocolate and Cocoa Industry Quality Requirements* (M. J. End & R. Dand, Eds.).

Crafack, M., Keul, H., Eskildsen, C. E., Petersen, M. A., Saerens, S., Blennow, A., Skovmand-Larsen, M., Swiegers, J. H., Petersen, G. B., Heimdal, H., & Nielsen, D. S. (2014). Impact of starter cultures and fermentation techniques on the volatile aroma and sensory profile of chocolate. *Food Research International*, *63*, 306–316. https://doi.org/10.1016/j.foodres.2014.04.032

Dand, R. (2011). The physical market in the international cocoa trade. In *The International Cocoa Trade* (pp. 94–118). Elsevier. https://doi.org/10.1016/B978-0-85709-125-3.50004-5

Dang, Y. K. T., & Nguyen, H. V. H. (2019). Effects of Maturity at Harvest and Fermentation Conditions on Bioactive Compounds of Cocoa Beans. *Plant Foods for Human Nutrition*, *74*(1), 54–60. https://doi.org/10.1007/s11130-018-0700-3

de Vuyst, L., & Weckx, S. (2016). The cocoa bean fermentation process: from ecosystem analysis to starter culture development. *Journal of Applied Microbiology*, *121*(1), 5–17. https://doi.org/10.1111/jam.13045

Dias, D. R., Schwan, R. F., Freire, E. S., & Serôdio, R. dos S. (2007). Elaboration of a fruit wine from cocoa (Theobroma cacao L.) pulp. *International Journal of Food Science & Technology*, *42*(3), 319–329. https://doi.org/10.1111/j.1365-2621.2006.01226.x

Escobar, S., Santander, M., Zuluaga, M., Chacón, I., Rodríguez, J., & Vaillant, F. (2021). Fine cocoa beans production: Tracking aroma precursors through a comprehensive analysis of flavor attributes formation. *Food Chemistry*, *365*. https://doi.org/10.1016/j.foodchem.2021.130627

FAO. (2021). *Food and agriculture data*. http://www.fao.org/faostat/en/#home

Ferreira, V. (2010). Volatile aroma compounds and wine sensory attributes. In *Managing Wine Quality* (pp. 3–28). Elsevier. https://doi.org/10.1533/9781845699284.1.3

Fowler, M. S., & Coutel, F. (2017). Cocoa beans: from tree to factory. In S. T. Beckett, M. S. Fowler, & G. R. Ziegler (Eds.), *Beckett's Industrial Chocolate Manufacture and Use* (5th ed., pp. 9–47). Wiley-Blackwell.

Frauendorfer, F., & Schieberle, P. (2006). Identification of the Key Aroma Compounds in Cocoa Powder Based on Molecular Sensory Correlations. *Journal of Agricultural and Food Chemistry*, *54*(15), 5521–5529. https://doi.org/10.1021/jf060728k

Frauendorfer, F., & Schieberle, P. (2019). Key aroma compounds in fermented Forastero cocoa beans and changes induced by roasting. *European Food Research and Technology*, *245*(9), 1907–1915. https://doi.org/10.1007/s00217-019-03292-2

Grosch, W. (2001). Evaluation of the Key Odorants of Foods by Dilution Experiments, Aroma Models and Omission. *Chemical Senses*, *26*(5), 533–545. https://doi.org/10.1093/chemse/26.5.533

Guirlanda, C. P., da Silva, G. G., & Takahashi, J. A. (2021). Cocoa honey: Agro-industrial waste or underutilized cocoa by-product? *Future Foods*, *4*, 100061. https://doi.org/10.1016/j.fufo.2021.100061

Gutiérrez, T. J. (2017). State-of-the-Art Chocolate Manufacture: A Review. *Comprehensive Reviews in Food Science and Food Safety*, *16*(6), 1313–1344. https://doi.org/10.1111/1541-4337.12301

Hamada, T. Y., Brown, A., Hopfer, H., & Ziegler, G. R. (2020). Flavor and mouthfeel of pseudo-cocoa liquor: Effects of polyphenols, fat content, and training method. *Journal of Sensory Studies*, *35*(1). https://doi.org/10.1111/joss.12541

Hamdouche, Y., Meile, J. C., Lebrun, M., Guehi, T., Boulanger, R., Teyssier, C., & Montet, D. (2019). Impact of turning, pod storage and fermentation time on microbial ecology and volatile composition of cocoa beans. *Food Research International*, *119*, 477–491. https://doi.org/10.1016/j.foodres.2019.01.001

Hinneh, M., Semanhyia, E., van de Walle, D., de Wine, A., Tzompa-Sosa, D. A., Scalone, G. L. L., de Meulenaer, B., Messens, K., van Durme, J., Afoakwa, E. O., de Cooman, L., & Dewettinck, K. (2018). Assessing the influence of pod storage on sugar and free amino acid profiles and the implications on some Maillard reaction related flavor volatiles in Forastero cocoa beans. *Food Research International*, *111*, 607–620. https://doi.org/10.1016/j.foodres.2018.05.064

Ho, V. T. T., Zhao, J., & Fleet, G. (2014). Yeasts are essential for cocoa bean fermentation. *International Journal of Food Microbiology*, *174*, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014

Ho, V. T. T., Zhao, J., & Fleet, G. (2015). The effect of lactic acid bacteria on cocoa bean fermentation. *International Journal of Food Microbiology*, *205*, 54–67. https://doi.org/10.1016/j.ijfoodmicro.2015.03.031

ICCO. (2021). *Fine or Flavor Cocoa*. https://www.icco.org/fine-or-flavor-cocoa/

Johnsen, L. G., Skou, P. B., Khakimov, B., & Bro, R. (2017). Gas chromatography – mass spectrometry data processing made easy. *Journal of Chromatography A*, *1503*, 57–64. https://doi.org/10.1016/j.chroma.2017.04.052

Kauz, T., Dunkel, A., & Hofmann, T. (2021). High-Throughput Quantitation of Key Cocoa Tastants by Means of Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry and Application to a Global Sample Set. *Journal of Agricultural and Food Chemistry*, *69*, 8200–8212. https://doi.org/10.1021/acs.jafc.1c01987

Koné, K. M., Assi-Clair, B. J., Kouassi, A. D. D., Yao, A. K., Ban-Koffi, L., Durand, N., Lebrun, M., Maraval, I., Bonlanger, R., & Guehi, T. S. (2021). Pod storage time and spontaneous fermentation treatments and their impact on the generation of cocoa flavour precursor compounds. *International Journal of Food Science & Technology*, *56*(5), 2516–2529. https://doi.org/10.1111/ijfs.14890

Kothe, L., Zimmermann, B. F., & Galensa, R. (2013). Temperature influences epimerization and composition of flavanol monomers, dimers and trimers during cocoa bean roasting. *Food Chemistry*, *141*(4), 3656–3663. https://doi.org/10.1016/j.foodchem.2013.06.049

Kucheryavskiy, S. (2020). mdatools – R package for chemometrics. *Chemometrics and Intelligent Laboratory Systems*, *198*, 103937. https://doi.org/10.1016/j.chemolab.2020.103937

Kucheryavskiy, S. (2022). *Getting started with mdatools for R*. https://mdatools.com/docs/index.html

Kuznetsova, A., Brockhoff, P. B., & Christensen, R. H. B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software*, *82*(13). https://doi.org/10.18637/jss.v082.i13

Lenth, R. V., Buerkner, P., Herve, M., Love, J., Miguez, F., Riebl, H., & Singmann, H. (2022). *emmeans: Estimated Marginal Means, aka Least-Squares Means*. https://cran.r-project.org/package=emmeans

Liland, K., Mevik, B., Wehrens, R., & Hiemstra, P. (2021). *pls: Partial Least Squares and Principal Component Regression*. https://cran.r-project.org/package=pls

Marseglia, A., Musci, M., Rinaldi, M., Palla, G., & Caligiani, A. (2020). Volatile fingerprint of unroasted and roasted cocoa beans (Theobroma cacao L.) from different geographical origins. *Food Research International*, *132*, 109101. https://doi.org/10.1016/j.foodres.2020.109101

McClure, A. P., Hopfer, H., & Grün, I. U. (2022). Optimizing consumer acceptability of 100% chocolate through roasting treatments and effects on bitterness and other important sensory characteristics. *Current Research in Food Science*, *5*, 167–174. https://doi.org/10.1016/j.crfs.2022.01.005

McClure, A. P., Spinka, C. M., & Grün, I. U. (2021). Quantitative analysis and response surface modeling of important bitter compounds in chocolate made from cocoa beans with eight roast profiles across three origins. *Journal of Food Science*, *86*(11), 4901–4913. https://doi.org/10.1111/1750-3841.15924

Miller, K. v., Noguera, R., Beaver, J., Oberholster, A., & Block, D. E. (2020). A combined phenolic extraction and fermentation reactor engineering model for multiphase red wine fermentation. *Biotechnology and Bioengineering*, *117*(1), 109–116. https://doi.org/10.1002/bit.27178

Miyazawa, T., Gallagher, M., Preti, G., & Wise, P. M. (2008). Synergistic Mixture Interactions in Detection of Perithreshold Odors by Humans. *Chemical Senses*, *33*(4), 363–369. https://doi.org/10.1093/chemse/bjn004

Mohamadi Alasti, F., Asefi, N., Maleki, R., & SeiiedlouHeris, S. S. (2019). Investigating the flavor compounds in the cocoa powder production process. *Food Science & Nutrition*, *7*(12), 3892–3901. https://doi.org/10.1002/fsn3.1244

Moser, A. (2015). Alkalizing cocoa and chocolate. *The Manufacturing Confectioner*, *95*(5), 31–38.

NIST. (2014a). *NIST/EPA/NIH Mass Spectral Library*.

NIST. (2014b). *NIST/EPA/NIH Mass Spectral Library*.

Oracz, J., Nebesny, E., & Zyzelewicz, D. (2014). Effect of roasting conditions on the fat, tocopherol, and phytosterol content and antioxidant capacity of the lipid fraction from cocoa beans of different Theobroma cacao L. cultivars. *European Journal of Lipid Science and Technology*, *116*(8), 1002–1014. https://doi.org/10.1002/ejlt.201300474

Ortega, N., Romero, M.-P., Macià, A., Reguant, J., Anglès, N., Morelló, J.-R., & Motilva, M.-J. (2010). Comparative study of UPLC–MS/MS and HPLC–MS/MS to determine procyanidins and alkaloids in cocoa samples. *Journal of Food Composition and Analysis2*, *23*(3), 298–305. https://doi.org/10.1016/j.jfca.2009.10.005

Owusu, M., Petersen, M. A., & Heimdal, H. (2012). Effect of fermentation method, roasting and conching conditions on the aroma volatiles of dark chocolate. *Journal of Food Processing and Preservation*, *36*(5), 446–456. https://doi.org/10.1111/j.1745-4549.2011.00602.x

Pacyniak, B. (2018). $9.9-billion cocoa market to grow 3.76 percent annually. *Candy Industry*, *183*(2), 11–12. https://www.candyindustry.com/articles/87991-99-billion-cocoa-market-to-grow-376-percent-annually

Perry, D., & Hayes, J. (2016). Effects of Matrix Composition on Detection Threshold Estimates for Methyl Anthranilate and 2-Aminoacetophenone. *Foods*, *5*(4), 35. https://doi.org/10.3390/foods5020035

Racine, K. C., Wiersema, B. D., Griffin, L. E., Essenmacher, L. A., Lee, A. H., Hopfer, H., Lambert, J. D., Stewart, A. C., & Neilson, A. P. (2019a). Flavanol polymerization is a superior predictor of α-glucosidase inhibitory activity compared to flavanol or total polyphenol concentrations in cocoas prepared by variations in controlled fermentation and roasting of the same raw cocoa beans. *Antioxidants*, *8*(12). https://doi.org/10.3390/antiox8120635

Racine, K. C., Wiersema, B. D., Griffin, L. E., Essenmacher, L. A., Lee, A. H., Hopfer, H., Lambert, J. D., Stewart, A. C., & Neilson, A. P. (2019b). Flavanol polymerization is a superior predictor of α-glucosidase inhibitory activity compared to flavanol or total polyphenol concentrations in cocoas prepared by variations in controlled fermentation and roasting of the same raw cocoa beans. *Antioxidants*, *8*(12). https://doi.org/10.3390/antiox8120635

Reineccius, G. A., Keeney, P. G., & Weissberger, W. (1972). Factors affecting the concentration of pyrazines in cocoa beans. *Journal of Agricultural and Food Chemistry*, *20*(2), 202–206. https://doi.org/10.1021/jf60180a032

Rodriguez-Campos, J., Escalona-Buendía, H. B., Contreras-Ramos, S. M., Orozco-Avila, I., Jaramillo-Flores, E., & Lugo-Cervantes, E. (2012). Effect of fermentation time and drying temperature

on volatile compounds in cocoa. *Food Chemistry*, *132*, 277–288. https://doi.org/10.1016/j.foodchem.2011.10.078

Rojas, M., Osorio, J., Heeres, H. J., & Chejne, F. (2021). Kinetic Studies on Cocoa Roasting Including Volatile Characterization. *ACS Food Science and Technology*. https://doi.org/10.1021/acsfoodscitech.1c00481

Santander, M., Rodríguez, J., Eric, F., & Escobar, S. (2020). An overview of the physical and biochemical transformation of cocoa seeds to beans and to chocolate: Flavor formation. *Critical Reviews in Food Science and Nutrition*, *60*(10). https://doi.org/10.1080/10408398.2019.1581726

Santander, M., Vaillant, F., Sinuco, D., Rodríguez, J., & Escobar, S. (2021). Enhancement of fine flavour cocoa attributes under a controlled postharvest process. *Food Research International*, *143*. https://doi.org/10.1016/j.foodres.2021.110236

Schwan, R., & Wheals, A. E. (2004). The Microbiology of Cocoa Fermentation and its Role in Chocolate Quality. *Critical Reviews in Food Science and Nutrition*, *44*(4), 205–221. https://doi.org/10.1080/10408690490464104

Sinkinson, C. (2017). Triangle test. In L. Rogers (Ed.), *Discrimination Testing in Sensory Science* (pp. 153–195).

Sirbu, D., Grimbs, A., Corno, M., Ullrich, M. S., & Kuhnert, N. (2018). Variation of triacylglycerol profiles in unfermented and dried fermented cocoa beans of different origins. *Food Research International*, *111*, 361–370. https://doi.org/10.1016/j.foodres.2018.05.025

Stanley, T. H., van Buiten, C. B., Baker, S. A., Elias, R. J., Anantheswaran, R. C., & Lambert, J. D. (2018). Impact of roasting on the flavan-3-ol composition, sensory-related chemistry, and in vitro pancreatic lipase inhibitory activity of cocoa beans. *Food Chemistry*, *255*, 414–420. https://doi.org/10.1016/j.foodchem.2018.02.036

Stark, T., Bareuther, S., & Hofmann, T. (2006). Molecular Definition of the Taste of Roasted Cocoa Nibs (Theobroma cacao) by Means of Quantitative Studies and Sensory Experiments. *Journal of Agricultural and Food Chemistry*, *54*(15), 5530–5539. https://doi.org/10.1021/jf0608726

Stats and R. (2020). *Outliers detection in R*. https://statsandr.com/blog/outliers-detection-in-r/#boxplot

Suh, N. N., Njimanted, G. F., Thalut, N., & Yildiz, F. (2020). Effect of farmers' management practices on safety and quality standards of cocoa production: A structural equation modeling approach. *Cogent Food & Agriculture*, *6*(1). https://doi.org/10.1080/23311932.2020.1844848

Told, H., Guthausen, G., Burk, W., Schmalbein, D., & Kamlowski, A. (2006). Water/moisture and fat analysis by time-domain NMR. *Food Chemistry*, *96*, 436–440. https://doi.org/10.1016/j.foodchem.2005.04.032

Tuenter, E., Delbaere, C., de Winne, A., Bijttebier, S., Custers, D., Foubert, K., van Durme, J., Messens, K., Dewettinck, K., & Pieters, L. (2020). Non-volatile and volatile composition of

West African bulk and Ecuadorian fine-flavor cocoa liquor and chocolate. *Food Research International*, *130*, 108943. https://doi.org/10.1016/j.foodres.2019.108943

Tuenter, E., Delbaere, C., De Winne, A., Bijttebier, S., Custers, D., Foubert, K., Van Durme, J., Messens, K., Dewettinck, K., & Pieters, L. (2020). Non-volatile and volatile composition of West African bulk and Ecuadorian fine-flavor cocoa liquor and chocolate. *Food Research Internationa2l*, *130*, 108943. https://doi.org/10.1016/j.foodres.2019.108943

Vallejo T., C., Díaz, R., Morales, W., Soria, R., Baren, C., & Vera, J. (2010). Use of cocoa mucilage, national and trinitarian type, in obtaining jelly. *Revista ESPAMCIENCIA*, *7*(1), 51–58. http://revistasespam.espam.edu.ec/index.php/Revista_ESPAMCIENCIA/article/view/116

van Den Dool, H., & Dec. Kratz, P. (1963). A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *Journal of Chromatography A*, *11*, 463–471. https://doi.org/10.1016/S0021-9673(01)80947-X

Vásquez, Z. S., de Carvalho Neto, D. P., Pereira, G. V. M., Vandenberghe, L. P. S., de Oliveira, P. Z., Tiburcio, P. B., Rogez, H. L. G., Góes Neto, A., & Soccol, C. R. (2019). Biotechnological approaches for cocoa waste management: A review. *Waste Management*, *90*, 72–83. https://doi.org/10.1016/j.wasman.2019.04.030

Ziegleder, G. (2017). Flavour Development in Cocoa and Chocolate. In S. T. Beckett, M. S. Fowler, & G. R. Ziegler (Eds.), *Industrial Chocolate Manufacture and Use: Fifth Edition* (5th ed., pp. 169–191). https://doi.org/10.1002/9781444301588.ch8

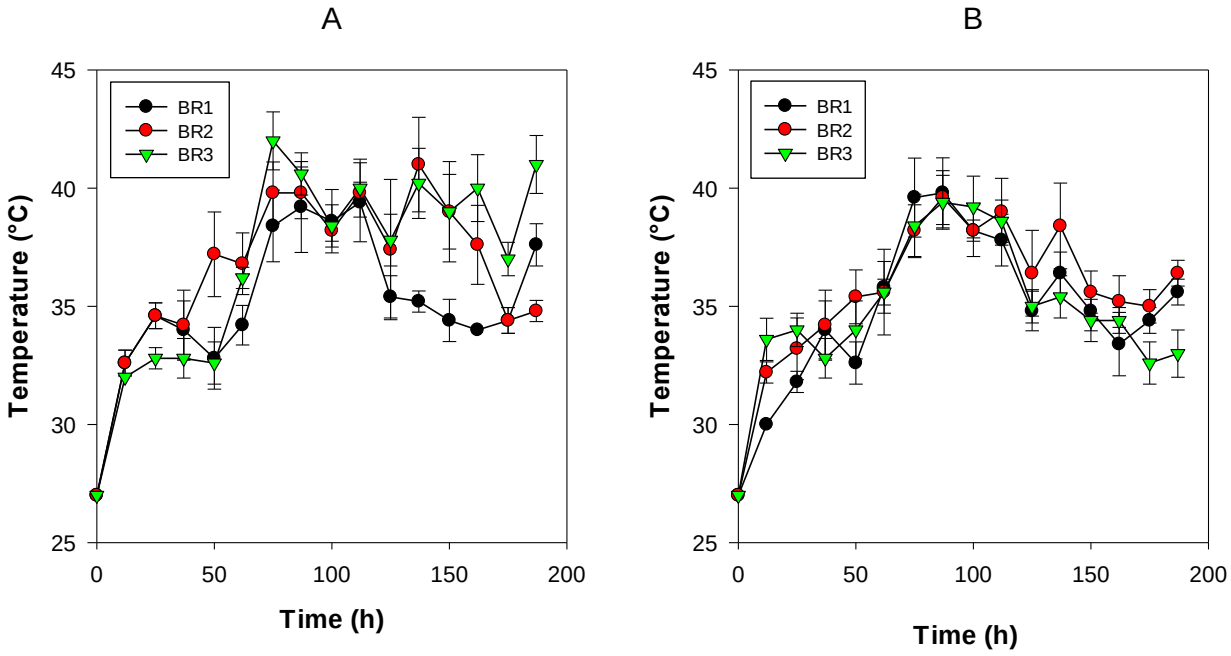
Ziegler, G., Mongia, G., & Hollender, R. (2001). The role of particle size distribution of suspended solids in defining the sensory properties of milk chocolate. *International Journal of Food Properties*, *4*(2), 353–370.

Żyżelewicz, D., Krysiak, W., Oracz, J., Sosnowska, D., Budryn, G., & Nebesny, E. (2016a). The influence of the roasting process conditions on the polyphenol content in cocoa beans, nibs and chocolates. *Food Research International*, *89*, 918–929. https://doi.org/10.1016/j.foodres.2016.03.026

Żyżelewicz, D., Krysiak, W., Oracz, J., Sosnowska, D., Budryn, G., & Nebesny, E. (2016b). The influence of the roasting process conditions on the polyphenol content in cocoa beans, nibs and chocolates. *Food Research International*, *89*, 918–929. https://doi.org/10.1016/j.foodres.2016.03.026

APPENDIX

Supplemental Figures



Supplemental figure 1. Temperature profile during fermentation of cocoa beans. **A**. Without the addition of CS, **B.** With addition of CS. Shown are all 3 biological replicates (BR 1 in black, BR 2 in red, and BR3 in green) over the 7 days of fermentation.

Supplemental Tables

Supplemental table 1. Effect of fermentation type (i.e., with or without the addition of CS) and fermentation time (i.e., 5, 6, or 7 days) on fat content of unroasted and unroasted cocoa liquor, and physicochemical properties of cocoa liquor. Arrows indicate the effect of the addition of CS in the compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs; $p \leq 0.05$).

Compound	**CS effect**	**With addition of CS (estimated marginal mean ± standard error)**			**Without addition of CS (estimated marginal mean ± standard error)**		
		5 (n=2)	**6 (n=2)**	**7 (n=2)**	**5 (n=2)**	**6 (n=2)**	**7 (n=2)**
Fat content (%)							
unroasted cocoa powder (n=3)		55.9 ± 0.18A	55.5 ± 0.23A	55.7 ± 0.18A	55.4 ± 0.18ab	54.8 ± 0.18b	55.6 ± 0.18a
roasted cocoa liquor (n=3)	↑	57.0 ± 0.26A	57.6 ± 0.23B	56.8 ± 0.23A	56.4 ± 0.23a	56.9 ± 0.23a	56.8 ± 0.26a
Physicochemical properties Cocoa liquor							
pH (n=10)		4.86 ± 0.04A	4.82 ± 0.04A	4.72 ± 0.04B	4.85 ± 0.04a	4.81 ± 0.04a	4.71 ± 0.04b
Titratable acidity (TA) (g citric acid/L) (n=10)		1.39 ± 0.04A	1.46 ± 0.04A	1.44 ± 0.04A	1.47 ± 0.03a	1.47 ± 0.04a	1.43 ± 0.04a

Supplemental table 2. List of volatile compounds detected in unroasted cocoa powder samples expressed as internal standard equivalent (ISE) per weigh of sample (µg naphthalene D8/g). All listed compounds showed the highest VIP values obtained in either the PLS-DA model for fermentation type (i.e., with or without the addition of CS) and/or fermentation time (i.e., 5, 6, or 7 days). Arrows indicate the effect of the addition of CS on compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs ; $p < 0.05$).

Compound	Retention index		Highest VIP	CS effect	With addition of CS (EMM ± standard error)			Without addition of CS (EMM ± standard error)		
	Sample	Standard			5 days (n=3)	6 days (n=2)	7 days (n=3)	5 days (n=3)	6 days (n=3)	7 days (n=3)
Acids										
Acetic acid	1392		0.37		464.99 ± 52.69A	370.35 ± 68.12A	474.82 ± 52.69A	451.7 ± 52.69a	357.46 ± 52.69a	424.69 ± 52.69a
2-methylpropanoic acid	1537		**1.06**	↑	14.03 ± 2.3B	16.79 ± 2.77AB	23.74 ± 2.3A	11.84 ± 2.3a	11.54 ± 2.3a	17.3 ± 2.3a
2-methyl-butanoic acid	1637	1640	**2.13**		10.36 ± 0.84A	1.77 ± 1.05B	2.23 ± 0.84B	7.77 ± 0.84a	1.94 ± 0.84b	2.08 ± 0.84b
3-methyl-butanoic acid	1636	1639	**1.44**		15.96 ± 1.52A	8.26 ± 1.97B	10.62 ± 1.52B	15.33 ± 1.52a	8.59 ± 1.52b	9.9 ± 1.52b
Total acids					505.34 ± 56.37A	396.44 ± 72.88A	511.42 ± 56.37A	486.63 ± 56.37a	379.53 ± 56.37a	453.98 ± 56.37a
Alcohols										
Ethanol	921	925	0.39		1.25 ± 0.28A	1.39 ± 0.31A	1.6 ± 0.28A	1.17 ± 0.28a	1.27 ± 0.28a	1.37 ± 0.28a
2-methyl-1-propanol	1078		0.73		2.55 ± 0.38A	1.86 ± 0.43A	1.67 ± 0.38A	2.19 ± 0.38a	1.95 ± 0.38a	2.02 ± 0.38a
3-methyl-1-butanol	1192		0.88		12.85 ± 2.18A	9.35 ± 2.4AB	7.4 ± 2.18B	11.46 ± 2.18a	9.89 ± 2.18a	9.42 ± 2.18a
Unknown	1510		0.93		68.86 ± 10.59A	63.21 ± 11.94A	71.9 ± 10.59A	54.46 ± 10.59a	48.73 ± 10.59a	59.73 ± 10.59a
2,3-Butanediol, [R-(R*,R*)]-	1547	1549	0.47		38.5 ± 6.95A	38.69 ± 7.96A	46.59 ± 6.95A	38.31 ± 6.95a	36.61 ± 6.95a	40.97 ± 6.95a
Benzyl alcohol	1824	1840	**1.16**		0.46 ± 0.04A	0.38 ± 0.05A	0.4 ± 0.04A	0.45 ± 0.04a	0.35 ± 0.04b	0.36 ± 0.04b
Phenylethyl Alcohol	1857	1873	0.63		23.94 ± 3.82A	21.93 ± 4.11A	19.4 ± 3.82A	23.87 ± 3.82a	19.78 ± 3.82a	21.02 ± 3.82a
Total alcohols					148.27 ± 23.81A	136.82 ± 26.6A	148.82 ± 23.81A	131.75 ± 23.81a	118.45 ± 23.81a	134.77 ± 23.81a
Aldehydes										
Butanal	803		0.87		1.07 ± 0.11A	0.92 ± 0.15A	0.93 ± 0.11A	1.14 ± 0.11a	1.05 ± 0.11a	0.8 ± 0.11a
2-methylbutanal	901	906	**1.04**		1.43 ± 0.11A	1.34 ± 0.14A	1.15 ± 0.11A	1.2 ± 0.11a	1.05 ± 0.11a	1.19 ± 0.11a
3-methylbutanal	905	909	**1.18**		1.59 ± 0.14A	1.34 ± 0.18AB	1.09 ± 0.14B	1.57 ± 0.14a	1.15 ± 0.14a	1.17 ± 0.14a
Benzaldehyde	1476	1483	**1.00**	↑	18.9 ± 1.81A	17.51 ± 2.15A	19.49 ± 1.81A	16.86 ± 1.81a	12.89 ± 1.81a	16.64 ± 1.81a

α-ethylidene-benzeneacetaldehyde	1871		**1.25**		0.22 ± 0.03B	0.44 ± 0.03A	0.35 ± 0.03A	0.26 ± 0.03b	0.2 ± 0.03b	0.48 ± 0.03a
Total aldehydes				↑	23.21 ± 2.04A	21.55 ± 2.45A	23.01 ± 2.04A	21.02 ± 2.04a	16.34 ± 2.04a	20.28 ± 2.04a
Esters										
Acetic acid, methyl ester	816		0.64		50.9 ± 6.76A	36.18 ± 8.42A	42.03 ± 6.76A	44.67 ± 6.76a	32.34 ± 6.76a	40.61 ± 6.76a
Ethyl Acetate	873	877	**1.09**		3.84 ± 1.1B	5.64 ± 1.29AB	8.53 ± 1.1A	4.21 ± 1.1a	5.39 ± 1.1a	6.85 ± 1.1a
Methyl 2-methylpropanoate	912	916	**2.07**		0.39 ± 0.04A	0.09 ± 0.05B	0.09 ± 0.04B	0.31 ± 0.04a	0.08 ± 0.04b	0.09 ± 0.04b
Ethyl 2-methylpropanoate	952	955	**1.88**	↑	1.03 ± 0.1A	0.29 ± 0.12B	0.26 ± 0.1B	0.63 ± 0.1a	0.27 ± 0.1b	0.28 ± 0.1b
2-methylpropyl acetate	1003	1005	**1.06**		10.8 ± 1.44A	8.34 ± 1.86A	9.7 ± 1.44A	9.75 ± 1.44a	9.36 ± 1.44a	11.84 ± 1.44a
Methyl 3-methylbutanoate	1007	1009	**2.05**		1.02 ± 0.09A	0.31 ± 0.11B	0.37 ± 0.09B	0.95 ± 0.09a	0.34 ± 0.09b	0.34 ± 0.09b
Ethyl 2-methylbutanoate	1037	1040	**1.99**		2.31 ± 0.23A	0.2 ± 0.26B	0.18 ± 0.23B	1.28 ± 0.23a	0.43 ± 0.23b	0.33 ± 0.23b
Ethyl 3-methylbutanoate	1053	1055	**1.80**	↑	1.36 ± 0.11A	0.52 ± 0.13B	0.54 ± 0.11B	0.87 ± 0.11a	0.49 ± 0.11b	0.49 ± 0.11b
2-Pentanol, acetate	1059		0.99	↑	8.14 ± 0.97A	6.12 ± 1.21A	7.52 ± 0.97A	5.46 ± 0.97a	5.18 ± 0.97a	5.84 ± 0.97a
1-Butanol, 3-methyl-, acetate	1110	1110	0.66		88.79 ± 12.03A	63.74 ± 14.39A	66.84 ± 12.03A	71.77 ± 12.03a	62.31 ± 12.03a	75.96 ± 12.03a
Methyl 2-hydroxypropanoate (Methyl lactate)	1291		**1.20**		2.32 ± 0.63A	2.25 ± 0.76A	2.47 ± 0.63A	3.14 ± 0.63a	2.55 ± 0.63a	2.97 ± 0.63a
2,3-Butanediol diacetate	1483		**1.04**		1.67 ± 0.44B	2.09 ± 0.57B	4.72 ± 0.44A	1.77 ± 0.44a	1.79 ± 0.44a	3.19 ± 0.44a
2-Ethoxyethyl butanoate	1583		**1.53**	↑	1.09 ± 0.09A	0.3 ± 0.11C	0.66 ± 0.09B	0.63 ± 0.09a	0.2 ± 0.09b	0.39 ± 0.09ab
Pentanoic acid, 2-methyl-, methyl ester	1672		0.99	↑	0.89 ± 0.13A	0.68 ± 0.14AB	0.62 ± 0.13B	0.62 ± 0.13a	0.51 ± 0.13a	0.54 ± 0.13a
Methyl 2-phenylacetate	1715	1726	**1.82**	↓	0.73 ± 0.07A	0.37 ± 0.09B	0.52 ± 0.07AB	1.16 ± 0.07a	0.45 ± 0.07b	0.45 ± 0.07b
Ethyl 2-phenylacetate	1744	1754	**1.04**	↑	1.17 ± 0.08A	1.03 ± 0.1A	1.1 ± 0.08A	0.96 ± 0.08a	0.89 ± 0.08a	1.02 ± 0.08a
Acetic acid, 2-phenylethyl ester	1771	1782	0.80		8.72 ± 1.01A	8.95 ± 1.23A	10.64 ± 1.01A	9.27 ± 1.01a	8.23 ± 1.01a	10.7 ± 1.01a
Total Esters					185.17 ± 23.62A	137.6 ± 28.61A	156.78 ± 23.62A	157.46 ± 23.62a	130.82 ± 23.62a	161.9 ± 23.62a

Furans										
2-Acetylfuran	1465	1471	**1.43**	↓	0.2 ± 0.04A	0.14 ± 0.04A	0.14 ± 0.04A	0.31 ± 0.04a	0.19 ± 0.04b	0.17 ± 0.04b
4-Butyrolactone	1569	1576	**2.34**	↓	2.6 ± 0.44A	2.3 ± 0.52A	2.28 ± 0.44A	3.26 ± 0.44a	3.04 ± 0.44a	3.2 ± 0.44a
2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-(Pantolactone)	1973	1991	**1.25**		0.26 ± 0.02A	0.22 ± 0.03A	0.24 ± 0.02A	0.32 ± 0.02a	0.23 ± 0.02b	0.25 ± 0.02ab
5-Acetyldihydrofuran-2(3H)-one	1988		**2.46**	↓	0.1 ± 0.02B	0.13 ± 0.02AB	0.15 ± 0.02A	0.16 ± 0.02b	0.17 ± 0.02b	0.23 ± 0.02a
Total Furans				↓	3.16 ± 0.47A	2.78 ± 0.57A	2.82 ± 0.47A	4.06 ± 0.47a	3.63 ± 0.47a	3.84 ± 0.47a
Hydrocarbons										
Styrene	1227	1231	0.88		3.75 ± 0.76AB	2.23 ± 0.85B	4.46 ± 0.76A	3.27 ± 0.76a	2.26 ± 0.76a	3.11 ± 0.76a
Total hydrocarbons					3.75 ± 0.76AB	2.23 ± 0.85B	4.46 ± 0.76A	3.27 ± 0.76a	2.26 ± 0.76a	3.11 ± 0.76a
Ketones										
2-Heptanone	1163		0.94		1.29 ± 0.13A	0.83 ± 0.16AB	0.79 ± 0.13B	0.94 ± 0.13a	0.78 ± 0.13a	0.89 ± 0.13a
Acetoin	1251	1254	**1.10**		10.37 ± 1.19A	8.4 ± 1.54A	6.34 ± 1.19A	11.62 ± 1.19a	9.27 ± 1.19a	7.81 ± 1.19a
2-Nonanone	1365		0.99		1.19 ± 0.21A	0.72 ± 0.26A	1.25 ± 0.21A	0.88 ± 0.21a	0.79 ± 0.21a	1.39 ± 0.21a
Acetophenone	1598	1607	**1.35**	↑	2.07 ± 0.14A	1.73 ± 0.18A	2.17 ± 0.14A	1.57 ± 0.14a	1.47 ± 0.14a	1.87 ± 0.14a
Total ketones					14.93 ± 1.49A	11.65 ± 1.93A	10.55 ± 1.49A	15.01 ± 1.49a	12.32 ± 1.49a	11.96 ± 1.49a
Pyrazines										
2,3-Dimethylpyrazine	1313	1315	**1.74**	↑	2.16 ± 0.19A	1.93 ± 0.22A	2.02 ± 0.19A	1.72 ± 0.19a	1.31 ± 0.19a	1.51 ± 0.19a
2,3,5-trimethylpyrazine	1376		0.92		3.47 ± 0.47A	2.94 ± 0.49A	2.85 ± 0.47A	3.48 ± 0.47a	2.92 ± 0.47ab	2.6 ± 0.47b
tetramethyl pyrazine,	1442		0.44		36.28 ± 8.01A	32.23 ± 8.37A	33.53 ± 8.01A	38.65 ± 8.01a	31.81 ± 8.01a	32.76 ± 8.01a
Total pyrazines					41.91 ± 8.46A	37.13 ± 8.86A	38.4 ± 8.46A	43.85 ± 8.46a	36.04 ± 8.46a	36.88 ± 8.46a
Pyrroles										
Ethanone, 1-(1H-pyrrol-2-yl)-	1910		0.62		0.58 ± 0.06A	0.6 ± 0.07A	0.67 ± 0.06A	0.65 ± 0.06a	0.63 ± 0.06a	0.61 ± 0.06a
Total pyrroles					0.58 ± 0.06A	0.6 ± 0.07A	0.67 ± 0.06A	0.65 ± 0.06a	0.63 ± 0.06a	0.61 ± 0.06a
Terpenes										
β-Myrcene	1149		0.72		1.21 ± 0.26A	0.22 ± 0.3B	0.09 ± 0.26B	0.07 ± 0.26b	0.24 ± 0.26ab	0.73 ± 0.26a
trans-β-Ocimene	1220		0.70		0.52 ± 0.11A	0.11 ± 0.13B	0.05 ± 0.11B	0.04 ± 0.11b	0.11 ± 0.11ab	0.31 ± 0.11a
Total Terpenes					1.73 ± 0.37A	0.33 ± 0.42B	0.14 ± 0.37B	0.11 ± 0.37b	0.35 ± 0.37ab	1.05 ± 0.37a

Others									
Unknown	956	0.82	↑	12.03 ± 1.01A	8.53 ± 1.27AB	8.06 ± 1.01B	6.68 ± 1.01a	5.38 ± 1.01a	6.57 ± 1.01a
Total others	1357		↑	12.03 ± 1.01A	8.53 ± 1.27AB	8.06 ± 1.01B	6.68 ± 1.01a	5.38 ± 1.01a	6.57 ± 1.01a

Supplemental table 3. List of volatile compounds detected in roasted cocoa liquor samples expressed as internal standard equivalent (ISE) per weigh of sample (µg naphthalene D8/g). All listed compounds showed the highest VIP values obtained in either the PLS-DA model for fermentation type (i.e., with or without the addition of CS) and/or fermentation time (i.e., 5, 6, or 7 days). Arrows indicate the effect of the addition of CS on compound concentration: ↑ significant increase, ↓ significant decrease. Same letters in rows indicate no significant differences between samples across fermentation days within one fermentation type (upper case letters with addition of CS; lowercase letters without addition of CS) based on Tukey post-hoc comparison of estimated marginal means (EMMs ; $p < 0.05$).

Compound	Retention Index		Highest VIP	Sweating effect	With addition of sweating (mean ± standard error)			Without addition of sweating (mean ± standard error)		
	Sample	Standard			5 days (n=3)	6 days (n=3)	7 days (n=3)	5 days (n=3)	6 days (n=3)	7 days (n=3)
Acids										
Acetic acid	1392		0.79		125.91 ± 15.92B	177 ± 15.92AB	199.51 ± 15.92A	191.47 ± 15.92a	147.55 ± 15.92a	155.41 ± 15.92a
2-methylbutanoic acid	1637	1640	**2.27**		3.33 ± 0.28A	1.04 ± 0.28B	0.78 ± 0.28B	4.16 ± 0.28a	0.72 ± 0.28b	0.95 ± 0.28b
3-methylbutanoic acid	1636	1639	**2.39**	↓	4.86 ± 0.55A	2.96 ± 0.55B	4.24 ± 0.55A	7.25 ± 0.55a	3.2 ± 0.55C	4.8 ± 0.55b
Total acids					134.09 ± 16.39B	180.74 ± 16.39AB	204.79 ± 16.39A	202.88 ± 16.39a	151.47 ± 16.39a	161.16 ± 16.39a
Alcohols										
Ethanol	921	925	**2.04**		0.21 ± 0.04B	0.45 ± 0.04A	0.25 ± 0.04B	0.28 ± 0.04ab	0.31 ± 0.04a	0.18 ± 0.04b
2-methyl-1-Butanol	1191	1192	**1.01**	↓	1248.83 ± 236.1A	1776.05 ± 236.1A	1218.4 ± 236.1A	2512.8 ± 236.1a	1816.43 ± 236.1ab	1142.62 ± 236.1b
4-methyl-3-heptanol	1306		0.81	↓	0.3 ± 0.05A	0.4 ± 0.05A	0.33 ± 0.05A	0.56 ± 0.05a	0.39 ± 0.05b	0.3 ± 0.05b
2-Heptanol	1306	1306	**1.42**	↑	0.31 ± 0.05A	0.39 ± 0.05A	0.37 ± 0.05A	0.28 ± 0.05a	0.3 ± 0.05a	0.28 ± 0.05a
Unknown	1510	1549	**1.22**	↑	16.26 ± 2.24B	24.46 ± 2.24A	26.54 ± 2.24A	20.55 ± 2.24a	16.82 ± 2.24a	18.26 ± 2.24a
2,3-Butanediol	1547	1549	0.83		8.1 ± 1.39B	14.32 ± 1.39A	15.45 ± 1.39A	13.19 ± 1.39a	11.88 ± 1.39a	11.53 ± 1.39a
Benzyl alcohol	1824	1840	**1.53**	↑	0.13 ± 0.01A	0.09 ± 0.01B	0.1 ± 0.01B	0.09 ± 0.01a	0.09 ± 0.01a	0.1 ± 0.01a
Phenylethyl Alcohol	1857	1873	**1.36**	↓	7.36 ± 0.69A	7.07 ± 0.69A	6.52 ± 0.69A	8.61 ± 0.69a	8.32 ± 0.69a	7.47 ± 0.69a
Total alcohols				↓	1281.5 ± 238.7A	1823.2 ± 238.8A	1268.0 ± 238.7A	2556.3 ± 238.8a	1854.5 ± 238.7ab	1180.8 ± 238.7b
Aldehydes										
2-methyl-butanal	901	906	0.58		0.3 ± 0.04A	0.39 ± 0.04A	0.36 ± 0.04A	0.45 ± 0.04a	0.33 ± 0.04a	0.33 ± 0.04a
3-methyl-butanal	905	909	0.56		1.83 ± 0.18A	2.01 ± 0.18A	2.01 ± 0.18A	2.07 ± 0.18a	1.74 ± 0.18a	1.89 ± 0.18a
Pentanal	959	963	**1.17**		0.59 ± 0.08A	0.29 ± 0.08B	0.2 ± 0.08B	0.35 ± 0.08a	0.28 ± 0.08a	0.55 ± 0.08a
Hexanal	1061	1065	**1.23**		1.99 ± 0.43A	0.6 ± 0.43B	0.84 ± 0.43AB	0.62 ± 0.43b	0.58 ± 0.43b	2.21 ± 0.43a
Heptanal	1164	1166	**1.26**		1 ± 0.17A	0.42 ± 0.17B	0.61 ± 0.17AB	0.48 ± 0.17b	0.42 ± 0.17b	1.04 ± 0.17a

Octanal	1267	1271	**1.70**		0.43 ± 0.08A	0.12 ± 0.08B	0.41 ± 0.08A	0.24 ± 0.08ab	0.14 ± 0.08b	0.45 ± 0.08a
Benzaldehyde	1476	1483	**2.01**		2.83 ± 0.21A	2 ± 0.21B	2.89 ± 0.21A	2.35 ± 0.21b	2.13 ± 0.21b	3.63 ± 0.21a
α-ethylidene-benzeneacetaldehyde	1871		**1.04**	↓	0.47 ± 0.08A	0.45 ± 0.08A	0.56 ± 0.08A	0.51 ± 0.08b	0.59 ± 0.08ab	0.7 ± 0.08a
Total aldehydes					9.44 ± 0.93A	6.3 ± 0.93A	7.87 ± 0.93A	7.07 ± 0.93b	6.21 ± 0.93b	10.79 ± 0.93a
Esters										
1-Propen-2-ol, acetate	803		0.61		0.6 ± 0.06A	0.72 ± 0.06A	0.72 ± 0.06A	0.73 ± 0.06a	0.58 ± 0.06a	0.65 ± 0.06a
Acetic acid, methyl ester	816		0.85		0.81 ± 0.12B	1.26 ± 0.12A	1.33 ± 0.12A	1.05 ± 0.12a	0.93 ± 0.12a	0.95 ± 0.12a
Ethyl Acetate	873	877	**1.54**	↑	0.82 ± 0.15B	1.96 ± 0.15A	1.67 ± 0.15A	0.97 ± 0.15a	1.34 ± 0.15a	0.98 ± 0.15a
Isobutyl acetate	1003	1005	0.81	↓	1.58 ± 0.71A	2.86 ± 0.71A	1.96 ± 0.71A	4.13 ± 0.71a	3.42 ± 0.71a	1.89 ± 0.71b
Ethyl 2-methylbutanoate	1037	1040	**1.49**		0.36 ± 0.05A	0.29 ± 0.05A	0.23 ± 0.05A	0.63 ± 0.05a	0.28 ± 0.05b	0.22 ± 0.05b
Ethyl 3-methylbutanoate	1053	1055	**1.68**		0.34 ± 0.04A	0.25 ± 0.04A	0.2 ± 0.04A	0.53 ± 0.04a	0.24 ± 0.04b	0.18 ± 0.04b
2-Pentanol, acetate	1059		0.75		2.69 ± 0.55A	4.08 ± 0.55A	3.3 ± 0.55A	4.18 ± 0.55a	3.65 ± 0.55a	2.77 ± 0.55a
3-methylbutyl acetate	1110	1110	**1.15**	↓	18.24 ± 3.06A	26.05 ± 3.06A	22.82 ± 3.06A	34.58 ± 3.06a	26.66 ± 3.06ab	22.94 ± 3.06b
Ethyl hexanoate	1218	1220	**1.49**	↑	1.07 ± 0.06B	1.46 ± 0.06A	1.35 ± 0.06A	1.2 ± 0.06a	1.29 ± 0.06a	1.1 ± 0.06a
2-Buten-1-ol, 3-methyl-, acetate	1234	1238	**1.22**		0.73 ± 0.13B	1.18 ± 0.13A	1.17 ± 0.13A	1.31 ± 0.13a	1.11 ± 0.13ab	0.98 ± 0.13b
Methyl heptanoate	1269		**2.44**		0.1 ± 0A	0.04 ± 0B	0.04 ± 0B	0.09 ± 0a	0.04 ± 0b	0.04 ± 0b
2,3-Butanediol, diacetate	1483		**2.09**		1.01 ± 0.22C	1.99 ± 0.22B	3.19 ± 0.22A	1.4 ± 0.22b	1.92 ± 0.22b	2.74 ± 0.22a
Unknown	1542		**1.86**	↑	15.15 ± 1.09C	21.51 ± 1.09B	28.28 ± 1.09A	18.8 ± 1.09ab	16.41 ± 1.09b	21.41 ± 1.09a
Ethyl 2-phenylacetate	1744	1754	**1.44**	↑	0.74 ± 0.04A	0.76 ± 0.04A	0.76 ± 0.04A	0.6 ± 0.04b	0.75 ± 0.04a	0.67 ± 0.04ab
2-phenylethyl acetate	1771	1782	**2.08**	↑	8.2 ± 0.34B	9.26 ± 0.34B	10.44 ± 0.34A	9.81 ± 0.34b	10.46 ± 0.34ab	11.59 ± 0.34a
Total Esters					52.42 ± 5.39B	73.67 ± 5.39A	77.46 ± 5.39A	80 ± 5.39a	69.06 ± 5.39a	69.12 ± 5.39a
Furans										
2-pentylfuran	1214	1216	**1.21**		0.83 ± 0.2A	0.17 ± 0.2B	0.31 ± 0.2AB	0.16 ± 0.2b	0.19 ± 0.2b	1.02 ± 0.2a
2-Furanmethanol, 5-ethenyltetrahydro-α,α,5-trimethyl-, cis-	1414		0.92	↓	1.07 ± 0.08A	1.07 ± 0.08A	1.1 ± 0.08A	1.13 ± 0.08a	1.15 ± 0.08a	1.2 ± 0.08a
Furfural	1426	1432	**1.18**	↓	0.32 ± 0.06A	0.37 ± 0.06A	0.39 ± 0.06A	0.61 ± 0.06a	0.43 ± 0.06b	0.42 ± 0.06b
4-Butyrolactone	1569	1576	**2.13**	↓	0.56 ± 0.09A	0.77 ± 0.09A	0.76 ± 0.09A	1.23 ± 0.09a	1.05 ± 0.09ab	0.93 ± 0.09b
3-Furanmethanol	1623		**1.39**	↓	0.82 ± 0.15A	0.83 ± 0.15A	0.58 ± 0.15A	1.26 ± 0.15a	0.96 ± 0.15ab	0.8 ± 0.15b

2(3H)-Furanone, dihydro-3-hydroxy-4,4-dimethyl-, (.+/-.)-(Pantolactone)	1973	1991	**1.87**	↓	0.18 ± 0.01A	0.17 ± 0.01A	0.17 ± 0.01A	0.22 ± 0.01a	0.19 ± 0.01b	0.19 ± 0.01b
2(3H)-Furanone-5-acetyldihydro-	1988		**2.45**	↓	0.06 ± 0.01B	0.07 ± 0.01AB	0.08 ± 0.01A	0.09 ± 0.01a	0.1 ± 0.01a	0.11 ± 0.01a
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-			0.95	↓	0.09 ± 0.02A	0.06 ± 0.02A	0.06 ± 0.02A	0.11 ± 0.02a	0.15 ± 0.02a	0.09 ± 0.02a
Total Furans				↓	3.92 ± 0.33A	3.51 ± 0.33A	3.45 ± 0.33A	4.82 ± 0.33a	4.22 ± 0.33a	4.74 ± 0.33a
Hydrocarbons										
Octane	801	800	**1.53**	↑	1.22 ± 0.23B	1.22 ± 0.23B	2.71 ± 0.23A	1.56 ± 0.23a	0.99 ± 0.23a	1.34 ± 0.23a
Styrene	1227	1231	**1.27**		2.18 ± 0.33A	2.62 ± 0.33A	2.53 ± 0.33A	2.55 ± 0.33a	2.25 ± 0.33a	2 ± 0.33a
Total hydrocarbons				↑	3.4 ± 0.4B	3.84 ± 0.4B	5.23 ± 0.4A	4.11 ± 0.4a	3.24 ± 0.4a	3.34 ± 0.4a
Ketones										
2,3-Butanedione	961		**1.08**	↓	0.54 ± 0.09AB	0.61 ± 0.09A	0.37 ± 0.09B	0.78 ± 0.09a	0.65 ± 0.09ab	0.49 ± 0.09b
1-Penten-3-one	1003	1005	**1.19**		0.13 ± 0.02A	0.05 ± 0.02B	0.05 ± 0.02B	0.05 ± 0.02a	0.05 ± 0.02a	0.12 ± 0.02a
4-methyl-2-hexanone	1161		**1.23**	↓	0.72 ± 0.06A	0.77 ± 0.06A	0.6 ± 0.06A	1 ± 0.06a	0.8 ± 0.06ab	0.62 ± 0.06b
Acetoin	1251	1254	0.88	↓	1.84 ± 0.41A	2.57 ± 0.41A	1.88 ± 0.41A	3.54 ± 0.41a	2.78 ± 0.41ab	1.71 ± 0.41b
1-hydroxy-2-Propanone	1261		0.85		0.41 ± 0.06A	0.51 ± 0.06A	0.44 ± 0.06A	0.71 ± 0.06a	0.49 ± 0.06b	0.39 ± 0.06b
2-Nonanone	1365		0.77		0.45 ± 0.02A	0.4 ± 0.02A	0.41 ± 0.02A	0.37 ± 0.02a	0.41 ± 0.02a	0.41 ± 0.02a
Acetophenone	1598	1607	**1.17**	↑	0.48 ± 0.1A	0.52 ± 0.1A	0.47 ± 0.1A	0.18 ± 0.1b	0.44 ± 0.1a	0.48 ± 0.1a
Total ketones				↓	4.57 ± 0.6A	5.44 ± 0.6A	4.22 ± 0.6A	6.63 ± 0.6a	5.61 ± 0.6ab	4.22 ± 0.6b
Pyrazines										
2,5-dimethylpyrazine	1290		0.72		0.84 ± 0.07B	1.04 ± 0.07A	0.94 ± 0.07AB	1.18 ± 0.07a	0.94 ± 0.07b	0.8 ± 0.07b
2,6-dimethylpyrazine	1296		0.76		0.88 ± 0.08A	1 ± 0.08A	1 ± 0.08A	1.2 ± 0.08a	0.98 ± 0.08ab	0.91 ± 0.08b
2,3-dimethylpyrazine	1313	1315	0.73		1.12 ± 0.17A	1.35 ± 0.17A	1.42 ± 0.17A	1.55 ± 0.17a	1.25 ± 0.17a	1.31 ± 0.17a
2,3,5-trimethylpyrazine	1376		0.84		5.62 ± 0.72A	6.29 ± 0.72A	5.41 ± 0.72A	6.93 ± 0.72a	6.17 ± 0.72a	5.57 ± 0.72a
2-ethyl-3,5-dimethylpyrazine	1413		0.69		1.45 ± 0.05A	1.34 ± 0.05A	1.28 ± 0.05A	1.3 ± 0.05a	1.36 ± 0.05a	1.43 ± 0.05a
2,3-Dimethyl-5-ethylpyrazine	1428		0.61		0.96 ± 0.16A	1.06 ± 0.16A	1.07 ± 0.16A	1.08 ± 0.16a	1.11 ± 0.16a	1.25 ± 0.16a
Tetramethylpyrazine	1442		0.65		22.94 ± 5.41A	26.05 ± 5.41A	23.28 ± 5.41A	28.42 ± 5.41a	26.51 ± 5.41a	27.54 ± 5.41a

2,3,5-Trimethyl-6-ethylpyrazine	1482	0.64		2.53 ± 0.34A	2.6 ± 0.34A	2.52 ± 0.34A	2.56 ± 0.34a	2.76 ± 0.34a	2.97 ± 0.34a
Total pyrazines				36.34 ± 6.83A	40.74 ± 6.83A	36.93 ± 6.83A	44.23 ± 6.83a	41.09 ± 6.83a	41.78 ± 6.83a
Pyrroles									
Ethanone, 1-(1H-pyrrol-2-yl)-	1910	0.91	↓	0.73 ± 0.05A	0.76 ± 0.05A	0.78 ± 0.05A	0.82 ± 0.05a	0.86 ± 0.05a	0.82 ± 0.05a
Total pyrroles			↓	0.73 ± 0.05A	0.76 ± 0.05A	0.78 ± 0.05A	0.82 ± 0.05a	0.86 ± 0.05a	0.82 ± 0.05a
Others									
Unknown	956	0.62		0.35 ± 0.19B	0.81 ± 0.19A	0.57 ± 0.19AB	0.73 ± 0.19a	0.58 ± 0.19ab	0.28 ± 0.19b
Unknown	1357	0.71	↓	6.26 ± 1.48A	9.58 ± 1.48A	8.15 ± 1.48A	10.96 ± 1.48a	9.81 ± 1.48a	8.22 ± 1.48a
Total others				6.61 ± 1.65B	10.39 ± 1.65A	8.72 ± 1.65AB	11.69 ± 1.65a	10.39 ± 1.65a	8.5 ± 1.65a

Supplemental table 4. Open comments from those participants who picked the correct odd sample in the triangle test comparing cocoa liquors after 5 days of fermentation without the addition of CS vs. 5 days of fermentation with the addition of CS.

Sample identified	Comment	Comment categorization
5 days without addition of CS	it was the most different - sour	sourer
	not as strong or bitter	**less bitter**
	it had a more bitter aftertaste	more bitter
	less bitter	**less bitter**
	strongest flavor	strong flavor
	Less bitter after taste than the others	**less bitter**
	While all very bitter, 541 was even harsher in flavor. All tasted sour, but it was slightly more so.Tester next to me has very strong perfume, so smell is an issue.	hard flavor
	much more bitter tahn the others	more bitter
	very mild and not as acidic as the other two samples	sourer
	less bitter than the other two samples	**less bitter**
	772 was less bitter than the other two.	**less bitter**
	it was less sour than the others	less sour
	slighlty less bitter	**less bitter**
	This sample was very bitter.	more bitter
	this sample was not as bitter as the other two - much more smooth	**less bitter**
	more sour	sourer
	I think it is more bitter than the other two.	more bitter
	The texture and flavor was different from 644 and 769	na
	taste	na
	it did not taste as bitter	**less bitter**
	AGAIN,,Not as bitter	**less bitter**
	more bitter	more bitter
	This one had a stronger taste. The other two didn't have an intense flavor until the aftertaste. The intenseness of this one happened sooner.	more bitter
5 days with addition of CS	it was more bitter	**more bitter**
	The bitter, yucky taste was different on this sample. More chauky tasting.	**more bitter**
	seemed more mild, the consistency some thicker, not as much after taste.	less bitter
	it was a tad sweeter	na
	The sample had nutty undertones that the others didn't possess. The others had a faint taste of liquor that sample 769 lacked.	na
	slightly more powdery texture, more bitter	**more bitter**

	seemed most "musty" earthen type flavor	na
	I don't have the right vocabulary, so I'll explain it by the analogy of musical notes.Sample 769 has the dark acidic bitterness of all dark chocolates, but the aftertaste felt like it resolved into a light, high musical plane. In contrast, Sample 772 felt like it fell into a much "lower" register of bitterness, which had a stronger acidic quality to it and the bitterness lasted longer. Sample 541, if it had matched 769, I was expecting to feel like the "note" had shifted up, but it didn't. So, I think it resembled 772. That leaves 769 with its "higher" note and more mild acidity as the outlier.I noticed that Sample 772 had a "lower"	**more bitter**
	less sour than the other two	less sour
	a little less bitter than the others	less bitter
	taste bitter and the previous two contains little bit wine taste	**more bitter**
	Similar but not as much flavor in this sample	no flavor
	769 had a different (less?) dark chocolate taste.	na
	The finishing taste was not as bitter and nasty/burny.	less bitter
	it seemed to have less choc tasting than the others	less chocolate
	Other two were very bitter, dry, and acrid tasting. #644 not as much	less bitter
	stronger sour taste	sourer
	This sample was less bitter.	less bitter
	as they dissolved in my mouth 772 and 541 had a sourer taste. 769 was more bitter.	**more bitter**
	more bitter/astringent than the other two. Melted quicker in the mouth and had a bit of an aftertaste	**more bitter** and astringent

Supplemental table 5. Open comments from those participants who picked the correct odd sample in the triangle test comparing cocoa liquors after 5 days of fermentation without the addition of CS vs. 7 days of fermentation without the addition of CS.

Sample identified	Comment	Comment categorization
5 days without addition of CS	more bitter	more bitter
	more bitter	more bitter
	not as sour/bitter	**less bitter**/less sour
	More bitter	more bitter
	Not as bitter as other 2	**less bitter**
	this sample was more bitter than the other two	more bitter
	This one had a bad aftertaste.	na
	this sample was less bitter.	**less bitter**
	more acidic and astringent than the other 2 samples	more sour more astringent
	more sour and bitter	more sour and more bitter
	this sample was less bitter and also harder than the other two samples. It feels less soft and melts slower in the mouth, but the bitterness was more acceptable.	**less bitter**
	aftertaste	
	it was less acidic and sour than the others	less sour
	It was a tab bit sweeter	more sweet
	883 had more of a creamier texture to it when it melted on my tongue	na
	The bitter taste did not hit until later than the other samples...it was not as strong of a bitter taste either	**less bitter**
	Less bitter. Dissolved more quickly. Less lingering taste.	**less bitter**
	im not sure of this answer. they were all very close in bitterness and taste. not good	na
	less bitter	**less bitter**
7 days without addition of CS	sample 339 was not as bitter and the other two samples	less bitter
	Nutty flavor	na
	all were dry and bitter but 339 was the most dry and bitter	**more bitter**
	The bitter aftertaste (or release) felt muted compared to the strong bitter aftertaste of the other samples. It didn't nearly carry as strong a punch. The astringency of 221 also seemed slightly different in tone/flavor.	less bitter
	339 was more bitter than the others, also less sweet	**more bitter**
	The other 2 were more salty.	na

	has the worst aftertaste	na
	Not a bitter aftertaste like the other two samples, a bit milder taste	less bitter
	Sample was less bitter than the other two.	less bitter
	It was the most bitter.	**more bitter**
	the flavor and aftertaste are stronger compared to the other samples	more flavor
	more bitter, bitter to back of throat	**more bitter**
	was more bitter than other two	**more bitter**
	It was foul. Extremely bitter. Aftertaste that lingered for a long time.	**more bitter**
	not as bitter and a bit of a richer taste	less bitter
	this one tasted a bit more bitter than the other two samples. although they were all very bitter	**more bitter**
	alot less bitter	less bitter
	It had a stronger aftertaste. I felt it was similar to 168, but the after taste was stronger. It could have been because it had them after one another.	stronger
	seemed saltiest - most "bite" to it	na
	So very bad, but this one was the worst.	na
	It had a weird (maybe just more bitter) flavor than the others.	**more bitter**
	more bitter than the other two	**more bitter**
	more astringent than the other two with a peanut after taste	more astringent
	Slightly more bitter than the others. The bitterness taste came faster on my tongue than the other two.	**more bitter**

Sensory test screener