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Effect of processing conditions on physical-chemical and structural characteristics of cacao (*Theobroma cacao L.*)

A thesis presented by

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Dedication

I want to dedicate this thesis to Ernesto and Lolita, whom I thank for the support and encouragement in my decisions; to my brothers Alejandra and Ernesto with whom I share the journey.

To Ter who has encouraged me to pursue the passions of life.

To those of us who believe that a different future is possible, in which the work of the entire production chain and the sacred work of the earth are valued.

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Effect of processing conditions on physical-chemical and structural characteristics of cocoa (*Theobroma cacao L.*)

by

Karla Maria Guillén Guerrero

Abstract

Three cocoa bean (*Theobroma cacao L.*) fermentations were carried out with a duration of 6 days simulating the box technique, with the aim to observe the biochemical changes and their effect on chemical and physicochemical characteristics, the temperature at the last fermentation stage was modified 50 °C for control, 40 °C (F40), and 60 °C (F60). Therefore, there were changes in pH into the cotyledons with 5.3, 5.7, and 5.0 for control, F40 and F60, respectively, which developed variations in the chemistry and structure of the cocoa bean. A proximal analysis was carried out (moisture, ash, proteins, lipids, and carbohydrates) as well as the determination of Free Amino Nitrogen (FAN), Phenolic Compounds (PC), Antioxidant Activity, Enzyme Activity, and Image structural Analysis. According to a Pearson Correlation Analysis and a Principal Component Analysis (PCA), the most significant response variables on day 6 of fermentation are FAN, with 11.41 ± 0.31, 10.38 ± 0.23, 12.50 ± 0.65 g / kg obtained for control, F40 and F60; and phenolic compounds with 42.00 ± 0.96, 66.43 ± 0.07 and 22.41 ± 0.96 mg Gallic Acid / g cocoa for control, F40 and F60 respectively. Changes in the behavior of enzymatic activity related to FAN and PC were also observed since proteases and amylases decreased their activity in F60, while they increased in lipases, inverse to the F40 treatments, compared with control. The degree of fermentation was determined with the cracking of the cotyledons as a parameter where 39% is observed for control, 32% for F40, and 49% for F60, showing that low and over fermentation temperatures cause variations in enzyme behavior, metabolite production and structure of cacao bean.

Keywords: Cacao, fermentation, enzyme activity, cracking area

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1. INTRODUCTION

Cacao (*Theobroma cacao. L*) is a crop that grows in tropical areas and is distributed in other areas of the world to produce chocolate. To obtain this product, it is necessary to go through several processes. As a first step, after being harvested, the cacao undergoes a fermentation process *in situ* to develop flavor precursors; the metabolites formed react later during roasting to develop desirable flavors characteristic of the chocolate (Sacchetti, Ioannone, Gregorio, Mattia, Serafini, & Mastrocola, 2015).

During fermentation, different microorganisms, including yeasts, bacteria, and even fungi, play a specific role in developing microenvironments in the batch of between 150 and 400 kg of beans. Fermentation is divided into two: anaerobic and aerobic. The first to occur is aerobic fermentation, where the pulp, rich in sugars, will serve to start the process with the native microorganisms of the area, specifically with yeasts. They consume the sugars and generate ethanol and CO_2 in a reaction that slightly increases the temperature (Schwan & Wheals, 2004). After most of the pulp is consumed, the aerobic fermentation begins. At this point, oxygen passes through the tissues and allows lactic acid bacteria to metabolize the ethanol, to generating lactic acid. Acetic acid bacteria use both, ethanol, and lactic acid, to generate acetic acid in an exothermic reaction that brings the bean cotyledons to a temperature of 50 ºC (Lefeber, Gobert, Vrancken, Camu, & De Vuyst, 2011).

For rise 50 ºC, the acetic acid will pass the permeable shell to get into the cotyledons and generate a particular pH and temperature conditions into, that will enhance the enzymatic activity of the embryo in a synergistic way. This stage during fermentation is commonly known as " kill the embryo"; and after all, the tissues will be modified due to a series of chemical reactions, that will impact the seed structure and organoleptic properties.

Different fermentation processes are carried out around the world as heap and boxes. Among them, the fermentation in boxes is outstanding (**Figure 1**). The greatest advantage observed of working with boxes as a process is ease of aeration, which is required every two days, since the drawers are staggered allows the continuous and efficient removal of the beans, as well as safety, keeping them away from the ground preventing contact with insects or pollutants.

The process on which this research is based is developed by the RAYEN cooperative, located in Tapachula, Chiapas, México, who kindly opened their doors and shared their knowledge as master growers and fermenters. Here, the fermentation is carried out in boxes (**Figure 1**),

made of a wood called “primavera” from the tree (*Tabebuia smithii*) (Galvez Del Cid, 2019), which takes place for more or less seven days, depending on the observations and parameters such as temperature, pH, and cracking of the bean, which is the parameter that will serve to stop the fermentation, as well as the bean tone that went from purple to pink.

This thesis covers the cacao bean and its physical, chemical, and structural behavior during fermentation and compares the metabolites obtained at the end of three different temperature treatments.

Figure 1. Cacao bean (*Theobroma cacao L.*) box fermentation developed in RAYEN cooperative.

2. LITERATURE REVIEW

2.1 Cacao (*Theobroma Cacao L.*)

Tree

Theobroma cacao is a tree native to South America and Mexico (**Figure 2**). It is a perennial vegetative cycle tree with foliage preserved throughout the year, its flowering and bear fruit begins after 2-3 years of age, but only harvest in their specific seasons that go from October to January as high harvests and from March to August as low harvest. The cacao tree can measure up to 3-5 m tall. It is composed of two types of branches: orthotropic and plagiotropic. Orthotropic branches are the main vertical trunk, and plagiotropic are those branches that grow outward. Be cauliflory is another of the outstanding characteristics of the cacao tree (Predan, Lazăr, & Lungu, 2019) since it is in the main trunk where the pacifiers give space for the flowers to sprout and the pods to grow. The cacao tree is also hermaphroditic, so it is possible that the pollen from a flower of the neighboring tree arrives and pollinates nearby flowers, so there is a genetic cross in the plantation, unless you have entirely separated trees to preserve a specific variety or hand pollination (Toledo, Tscharntke, Tjoa, Anshary, Cyio, & Wanger, 2020).

There are three recognized varieties: Criollo, Forastero, and Trinitarian; from which derive hundreds of varieties, even genetically modified ones. The Criollo variety has its origins in regions of Mexico and South America; it has the characteristic of having white seeds, while the Forastero is native to the Amazon areas, presenting seeds of purple - dark brown tones. The Trinitario variety is a cross between Forastero and Criollo and has intermediate characteristics of size and flavor (Bartley, 2005).

Figure 2. Cacao (*Theobroma cacao L.)* tree with pods.

Cacao Pod

After ovary maturation and pollination, the inflorescence (**Figure 3**) grows on an axillary bud's stem and main branches. As time passes, a bud, also called a pacifier, grows, and thickens to form a floral bottom; from which more than one flower can sprout, formed by thin pedicels that open and close at a certain time in the morning but only 0.5% - 5% of flowers develop into a mature indehiscent berry of fleshy pericarp fruit (Carr & Lockwood, 2011). It takes between 4 to 7 months from pollination to ripening, the formed pod (**Figure 4**) can measure between 12 to 20 cm in large; its surface can be smooth or rough and has a green, yellow, purple, or red color (depending on the variety of the fruit), supported by the peduncle from the ripening of the flower pedicel. Its thickness ranges from 1 to 2 cm, and it is hard, so a tool such as a rock or a machete is necessary to open it. Once mature, the pod is available for approximately 2 to 3 weeks to be cutter, since it is necessary to have many pods to open and be able to collect a large number of seeds since several kilograms are needed to develop the fermentation of seeds (Bariah, 2014).

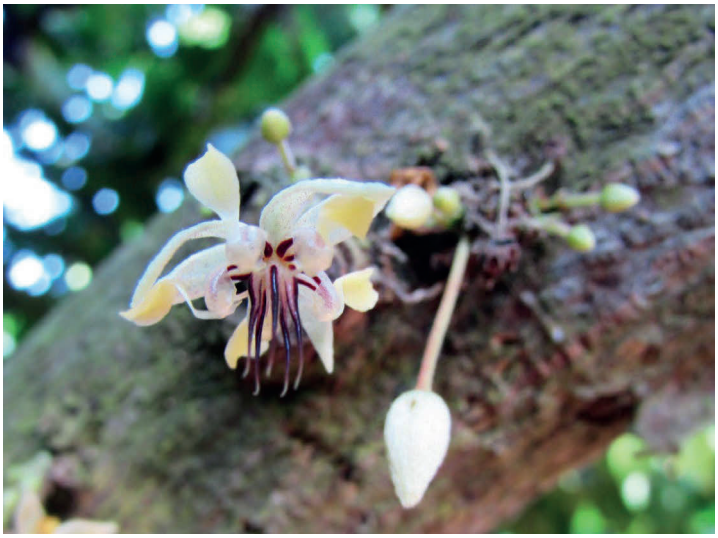


Figure 3. Open cacao *(T. cacao L.)* flower

Figure 4. Ripe cacao (*T. cacao L.*) pods

Cacao Seed - Pulp

Cacao is an angiosperm with dicot seeds; each pod is composed of 30 to 40 seeds measuring between 1 and 3 centimeters and weighing between 0.5 to 2.0 g, covered by a white pulp or mucilage (**Figure 5**), which comprise approximately 40% of the weight of fresh cacao bean. This pulp is composed of 82 - 87 % water and spongy parenchyma cells that are rich in sugars (10-15%) as glucose, fructose, and sucrose, pentosans (2-3%), citric acid (1-2%), pectin (1-1.5%) and other hemicellulose polysaccharides (1-2%) (López, 1986; Pettipher,1986); its pH varies from 3.0 and 4.0 depending on the content of citric acid. The pulp plays an essential role in the processing of the bean to begin the anaerobic fermentation due to the sugars and

their capacity to be fermented by yeast and produce ethanol and prevent the passage of air between the seeds at this stage of the process.

Figure 5. Cacao (*T. cacao L.*) pod open, the seeds are in a fleshy structure surrounded by pulp.

Cacao Seed - Seed coat

Between the pulp and the cotyledons, there is a layer of tissue known as the seed coat or testa. This cover is a multifunctional structure that functions as a protection against loss of water and solutes, prevents physical or biological damage, also coordinates the transport of water and nutrients between the vegetative plant and the seed by means of short-distance transport. Its structure is multilayer and bitegmic, having an internal and external tegument, as it consists of an external testa and an internal tegmen. It is also a hilum and an endosperm cuticle. It is known that the mechanism by which some substances such as nutrients and acetic acid, which plays an important role during fermentation, go through this structure through an apoplastic path (Andersson, Koch & Lieberei, 2006).

Cacao Seed - Cotiledons

Each seed consists of two cotyledons and a germ (Avendaño, Villareal, & Campos, 2011). These seeds are mainly composed of water (32-39%), total fats (30-32% with a majority of saturated fats), proteins (8-10%), carbohydrates (15%), of which cellulose (2-3%), starch (4-6 %) and sucrose (2-3%), polyphenols (5-6%), organic acids (1%), theobromine (1-3%) and caffeine (0.2-0.1%) (Bucheli, Rousseau, Alvarez, Laloi, & McCarthy, 2001). When it is fresh,

the color is purple due to the polyphenol composition; its pH is near 7 and decreases during fermentation.

Into the cotyledons (**Figure 6**), the leaf mesophyll is the dominating tissue, conformed of two types of cells which differ in composition; most of these cells, about 80%, are lipid/protein, which is made up of many lipid bodies of approximately 2 μm that surround one or more vacuoles of branched proteins and amyloplasts. The cells are a dispersed lipid phase, the lipid bodies, in a continuous aqueous phase, the cytoplasm. The other 20% of the cellular composition corresponds to storage cells of polyphenols and purines made up of a central vacuole (Macrae, Robinso., & Sadler, 1993). The subcellular structure controls the reactions after the fermentation peak which will be discussed later.

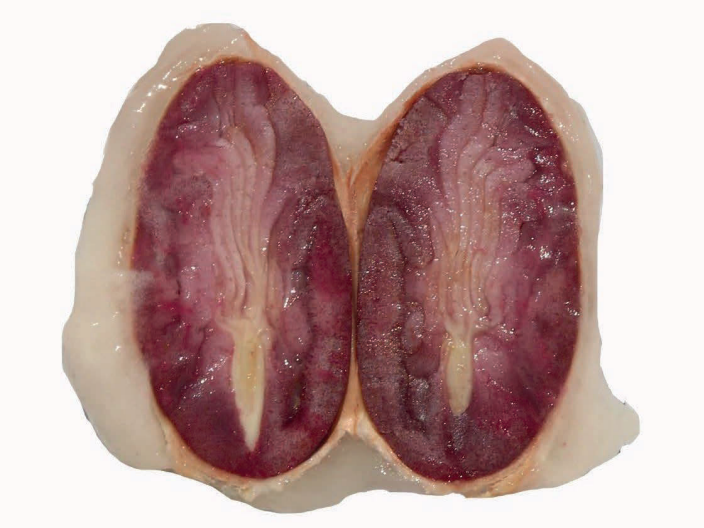


Figure 6. Open cotyledons of fresh cacao (*T. cacao L.*) bean

2.2 Importance and harvest of cacao in Mexico

2.2.1 Importance

Mexico ranks 13th worldwide in cacao production with 28 thousand tons (CEDRSSA,2021). The states with the most significant contribution to its production are Tabasco, Chiapas, Guerrero, and Oaxaca as the main producers due to their tropical climatic conditions, since these areas have 16-24 ºC and less than 1000 m at sea level where the plant can grow and bear fruit; of these, Tabasco contributes 66.4% of production, Chiapas 32.9%, and Guerrero 0.2%, while Oaxaca contributes the remaining.

In Chiapas, the cacao-producing areas are worked in family groups with other trees and cacao, in called agroforestry systems. These species are for the region's population because they represent an income when commercially exploiting other crops, regardless of the cacao harvest.

2.2.2 Agroforestal system

In this work, the production system was observed in Raymundo Enriquez community (-*92.315833, 14.871389*), who works under an agroforestry system in which, in addition to cacao, there are mamey, coconut, lemon, hawaiian flower, banana, and mango as main crops, in addition to timber trees. This association or intercalation of crops allows the cacao tree to grow in the shade, buffering the environmental, climatic conditions. At the same time, providing a space for biodiversity (Salgado, Núñez, Macías, & López, 2007), in addition, this system is jointly increasing nutrient uptake with, being potassium the most responsive to the shade (Isaac, Timmer, & Quashie-Sam, 2007); also has the least contribution to global warming, acidification and eutrophication of the soil (Utomo, Prawoto, Bonnet, Bangviwat, & Gheewala, 2016). In terms of yield, some authors support that the agroforestry system has no influence on the yield of pods or beans per pod (Somarriba, & Beer, 2011). Still, others affirm less pod production but higher yield per pod than in monocultures (Armengot, Ferrari, Milz, Velásquez, Hohmann, & Schneider, 2019).

2.2.3 Fine aroma cacao

In recent years, the commercialization and demand of fine aroma cacao, consisting of fermented beans, produced, and processed in Central America has increased versus conventional or bulk cacao produced mainly by Ivory Coast, Ghana, and Nigeria (Duguma, Gockowski, & Bakala, 2001). Fine aroma cacao has a monitored fermentation as a post-harvest process, that promotes the development of flavor and odor precursors by the enzymatic activity of invertases, glucosidases, proteases, and lipases, as well as the degradation of pigments by polyphenol oxidase. This is a non-industrialized process since, after cutting, the pod is open, and that is where the process begins.

2.2.4 Diseases

The main disease that prevents optimal development and limits cocoa production is monilia, whose annual incidence ranges from 54.49% to 88.73% in the Chiapas and Tabasco regions. Monilia is caused by the fungus *Moniliophthora roreri*, a pathogen that only attacks the fruits

in any of its stages of development, its form of propagation is the spores, after invading it they can penetrate it, germinating inside it causing internal and external necrosis (Álvarez, Martínez, & Coy, 2014), the seeds become difficult to detach, being not suitable for either fermentation or consumption.

It tends to be present around 10 months across the year due to its long period of growth, development, and reproduction, since it incubates in a time of 38 days, its lactation period is 46 days and the infectious one is longer than five months, while conidia in the air last all yea (Jaimes & Aranzazu, 2010). These fungi have optimal conditions for progress in temperatures between 20 – 26 ºC and relative humidity greater than 60%, where the shadows caused by the leaves of the trees in the area help to maintain them.

3. CACAO FERMENTATION

After being removed from the pod, the mucilage-covered cacao beans can be fermented through various techniques, the most common are box and heap fermentation, but other alternatives such as basket, tray, even in holes in the ground already exist. The objective is not only to remove the pulp from the bean and prevent its germination but also to develop flavor precursors from the intrinsic macromolecules of the bean as the primary goal. A field was made to Raymundo Enriquez, Tapachula, where the RAYEN Cacao Cooperative is working with 23 cacao producers, who harvest cacao and takes to the fermentation center where the good quality cacao beans are recollected and processed 6 - 7 days, between 152 to 164 h, for fermentation and 7 days for sun drying. Some of the technical information is given by producers.

3.1 Box Fermentation

In some areas in Mexico, like Chiapas, the traditional box fermentation process begins after harvest. Cacao pods are cut and placed in a pile. The cacao pods are split in half with the help of a rock, the seeds covered with mucilage are removed and assembled in plastic cans, all these activities are carried out in the tree zone. Then, they are taken to the fermentation cooperative, where the plastic cans are emptied into the first of the fermenting boxes to begin the process. The fermenting boxes (**Figure 7**) are made of primavera (*Tabebuia donnell)* wood, measuring 80x80x80; they are placed continuously and uneven to form a ladder that allows the beans to be lowered to the next box after 2 and 4 days: two times of aeration. The second and third box are covered with “hoja blanca” *Calathea lutea* leaves which works as a barrier and temperature container material.

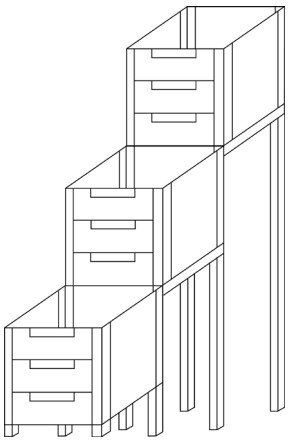


Figure 7. Structure of ladder boxes where cacao (*Theobroma cacao L.*) fermentation takes place.

The microorganisms that lead to fermentation are endogenous species that are added to the process in the form of natural contaminants. Although, these microorganisms are present on the surface of the pod, on the hands of workers as well as on their tools, even in the drawers used for processing. Other possible contaminants are insects, such as flies or bees that are found in the area.

3.2 Yeast

During the first two days, the cacao beans rest in the first drawer without any coating. The viscosity prevents oxygen from entering the bean mass due to the pulp pectin and polysaccharides, so that this part of the fermentation is known as anaerobic. Throughout these two days, the drained mucilage produces a liquid called mead. In this first phase, yeast growth occurs, primarily from the *Saccharomycetaceae* family, *Pichia membranifaciens,* and *Candida krusei* (Dujon & Louis, 2017). Some authors support the concentration of *S. cerevisiae* reaching a population of 10^7 CFU / g at 1 day of box fermentation, being the majority compared to other species (Pereira, Miguel, Ramos, & Schwan, 2012) which produce ethanol from carbohydrates, mainly glucose, which comes from the hydrolysis of the pulp sucrose by invertase activity, causing its drainage, also carbon dioxide and glycerol are produced. Other secondary by-products are aldehydes, ketones, organic acids, esters (Pereira, Alvarez, Neto & Soccol, 2017). The quantity and the quality of these compounds formed vary depending on the yeast species that predominate on the pulp.

After the conditions are not favorable for the yeast to stay, they die and provide micronutrients such as amino acids and vitamins that will be beneficial for bacterial growth (Fleet, 2008). Ethanol production from yeast is an exothermic process that causes the initial temperature to rise from 30 ºC to 40 ºC within these first two days (De Vuyst & Weckx, 2016). The environment is slightly acidic, around pH 3.5 - 4.0, due to the concentration of citric acid in the pulp. Another characteristic of yeasts is that they are pectinolytic and have a role during the degradation of the pulp and the hydrolysis of the pectin that makes it up. The enzyme polygalacturonases carry this out, pectin methyl esterases and pectin lyases (Silva, Borges, Medina, Piccoli, & Schwan, 2005), which cause the viscosity of the pulp to decrease, proving its leaching, as well as penetration of oxygen creating an optimal environment for the growth and reproduction of lactic acid bacteria (LAB) and acetic acid bacteria (AAB) that develop anaerobic fermentation.

3.3 Lactic Acid Bacteria

During fermentation, LAB performs three reactions in semi aerobic conditions, varying in concentration over time:

I. Fermentation of pulp sugars by homofermentative species (*L. plantarum, Lactococcus lactis and Pediococcus acidilactici*) produce mainly lactic acid; also, heterofermentative (*L. fermentum, Leuconostoc spp, and Wissella spp.*) produce lactic acid, ethanol, acetic acid, CO_2, and aromatic metabolites as diacetyl.
II. Citric acid from the pulp is converted into lactic and acetic acid, ethanol, and dimethyl by *L. fermentum* and *L. plantarum*.
III. Fructose is reduce to mannitol also by *L. fermentum* (De Melo Pereira, Magalhães, de Almeida, da Silva Coelho, & Schwan, 2013; Lefeber et. al 2011).

LAB is tolerant to the high concentration of ethanol and the temperature conditions of temperature. The activity of these bacteria in the consumption of citric acid is the main factor that produces an increment in the pH of the pulp, bringing the outside of the bean to a balance between 5.0 and 5.5 units, an ideal environment for enzymatic activities (Lefeber, Janssens, Camu, & De Vuyst, 2010). Their reproductive capacity has been investigated, and it is known that both *L. fermentum* and *L. plantarum* reach a maximum population of 10^7 – 10^8 CFU / g between 1 and 2 days, and then descend (Ardhana & Fleet, 2003). Some lactic acid diffuses into cotyledons because it is non-volatile, making an impact on acidity and flavor and some are used by AAB, especially *A. pasteurianus*, which oxidase 60% of it to produce carbon dioxide and water (Lefeber, et. al 2010).

3.4 Acetic Acid Bacteria (AAB)

When aerobic conditions arrive at 96 hours aeration, AAB reaches their potential growth, being *A. pasteurianus* the most frequently recovered. These bacterias oxidase ethanol produced by the yeast to acetic acid, then the oxidation of lactic acid to carbon dioxide and water.

Ethanol oxidation is an exothermic reaction, the rising temperature of the fermenting cacao bean mass to reach 45–50 °C and even higher between 5 to 6 days, also decreasing pH (Mozzi, Raya, & Vignolo, 2015). Although acetic acid is volatilized with vinegar odor, another amount (2% approximately) penetrates the seed coat to enter cotyledons and breaking the cell walls (Schwan et al. 2004). This low acidity causes color changes in the cotyledons, which turn from purple to pink because anthocyanin pigments, 3-β-D-galactosyl- and 3-α-L-arabinosyl-cyanidins, are hydrolyzed by glycosidases in the pH range 3.8-4.5 (Afoakwa, Quao,

Takrama, Budu, & Saalia, 2012; Biehl et al., 1989). At the end of fermentation, the cotyledons begin to crack due to the internal changes and enzymes in action; changes in proteins, carbohydrates, and phenols have been recorded and detailed. In RAYEN, fermentation stops when 7 of every 10 nibs looks visually cracked, and an acid juice is excreted when it matches in half.

3.5 Changes in chemical composition of T. cacao L during fermentation

3.5.1 Proteins

Proteins represent 8 – 10% of the dry weight in unfermented cacao beans composed of albumin 52% and 7S vicilin class 43% (Voigt, Biehl, & Wazir, 1993). During fermentation, they are hydrolyzed by aspartic endoprotease and serine carboxypeptidase enzymes simultaneously, depending on pH changes to develop. First, 7S vicilin is degraded by aspartic endoprotease (whit pH 3.5 and 48 ºC as optimal; inactive at >5.5 pH), generating a mix of hydrophobic oligopeptides, then by the action of carboxypeptidase (pH 5.5 and 45 ºC as optimal) (Amin, Jinap, & Jamilah, 1998), they are transformed into a mix of hydrophilic oligopeptides and hydrophobic free amino acids, precursors of flavor (Voigt, 2010).

Hydrophobic free amino acids are present in rages of leucine between 11% - 14%, phenylalanine 8% - 10%, valine 4% - 10%, alanine 4% - 13% and isoleucine 3% to 8%, and others 30 - 55% (Brunetto, Gallignani de Bernardi, Orozco, Clavijo, Delgado, Ayala, & Zambrano, 2020). The kinetics of degradation depends on the molecular weight of the protein, those with a molecular weight greater than 31 KDa are hydrolyzed in the first two days, while those with less than 21 KDa hydrolyze after four days (Hue, Gunata, Breysse, Davrieux, Boulanger, & Sauvage, 2016). In the first 48 hours, the peptides of > 21 amino acids (AA) decrease rapidly. At the third day 7 - 20 AA peptides are at their maximum, when the fermentation is nearing its end or 152 - 16, two-thirds thirds of the peptides have lengths between 2 - 5 AA (D'Souza, et al. 2018).

3.5.2 Carbohydrates

The carbohydrate content in fresh cacao beans is approximately 15%, composed mainly of 90% of sucrose and 5 - 6% of reducing sugars, and 1 - 2% of non-reducing sugars (Hashim, Selamat, Syed, & Ali,1998, Afoakwa, Quao, Takrama, Budu, & Saalia, 2011). Its composition during fermentation is affected by endogenous invertase, that hydrolyzes sucrose with an increase of fructose, glucose, mannitol, and inositol. At the fourth day, reducing sugars are at

maximum concentration, playing a role as flavor precursors intermediates for Maillard products in roasting, as alpha dicarbonyl compounds that interact with amino acids through the Strecker degradation reaction, resulting in aroma compounds such pyrazines and pyrroles (Yatlayan, 2003). Also, starch granules have been identified as < 16% of the total carbohydrate content (De Brito, García, Gallao Cortelazzo, Fevereiro & Braga, 2000).

3.5.3 Phenolic Compounds

Polyphenols in cacao seeds are reduced after the fermentation process from 16% to almost 5%. They are made up of proanthocyanins or condensed tannins (58%), flavan-3-ols (37%), and anthocyanins (4%) (Wollgast, 2000). Into the flavan-3-ols, (-) epicatechin is the most abundant compound, and the main polyphenol oxidase (PPO) substrate in parallel, (-) epicatechin is reduced until 90% of its initial value at the end of fermentation (Brito, 2000). PPO action is optimal activity at pH 6 and 35.5 ºC, the reason why it is inactivated at the fifth day (120 h) since 50 ºC has been reached (Brito, García & Amancio, 2000; López & Dimick, 1995), also scavenging capacity show a decrease resulting in loss of 39% after 7 days of fermentation, but its activity remains high, giving the antioxidant capacity to cacao products(Aikpokpodion & Dongo, 2010; Afoakwa, Paterson, Fowler, & Ryan, 2008).

3.5.4 Lipids

Lipids are the major component of unfermented cacao beans around 30 - 32%, composed by 97% of glycerolipids assemblages in lipid bodies (Bayés, Aguilar, Calvet, Koyano, & Sato, 2019). The glyceride composition is conformed by 35% oleic acid, 34% stearic acid and 26% palmitic acid, with 23 - 37 ºC melting capacity (Afoakwa, 2010). However some authors assumed that it is stable, and it is not affected because fermentation only acts on compounds with hydrophilic bridges (Guehi, Dadie, Koffi, Dabonne, Ban-Koffi, Kedjebo, & Nemlin, 2010), but the lipid bodies that conform the lipid/protein cells, suffer a reposition (Lopez, Dimick & Walsh, 1987). Also, some latest published studies (Servent, Boulange, Davrieux, Pinot, Tardan, Forestier-Chiron, & Hue, 2018) explain that there is an increase in free fat during fermentation, but it is largely due to the method used for extraction, reinforcing the idea that lipid molecular interactions are stronger than the polarity of the solvent. The present work aims to analyze the behavior of fats because of temperature and to speculate how their respond to internal microenvironment changes into the cacao bean.

4. JUSTIFICATION

During the cacao fermentation process, the changes in temperature and pH microenvironments activate enzymes that trigger shifts in cacao macromolecules, that are reflected in the behavior of their physicochemical composition and molecular characteristics. By modifying such temperatures at 40 ºC and 60 ºC, and taking 50 ºC as control, due traditional fermentation parameters carried out in Tapachula, Chiapas, Mexico; these may promote changes in such microenvironment and composition having a direct impact on micro structural characteristics.

5. OBJECTIVES

Analyze the impact of fermentation temperature on the physical-chemical and molecular characteristics of the cacao bean (*Theobroma cacao L.*)

5.1 Specific objectives

Evaluate the cacao bean with a bromatological study and compare the moisture, carbohydrates, lipids, proteins, and ashes content at different temperatures of fermentation.

Evaluate the changes in the enzymatic behavior of the cacao bean by analyzing proteases and lipases at different fermentation temperatures and comparing them with the change in total proteins, total free amino acids, and total lipids.

Evaluate the changes in the lipid profile of the cacao bean, specifically of saturated fatty acids such as oleic acid, stearic acid, linoleic acid.

Evaluate the changes in phenolic compounds and their antioxidant capacity, at different fermentation temperatures.

Evaluate the changes in the structure of cacao bean through fermentation with different temperatures.

6. METHODS

6.1 Sample

Cacao pods (*Theobroma cacao L.*) in a mature state were collected from the RAYEN Cooperative, from Tapachula, Chiapas for each fermentation. The parameters used for their selection were the absence of external and internal monilia (*Moniliophthora roreri*) once opened. The shell colors varied in green, yellow, and reddish and large between 12 to 19 cm.

6.2 Fermentations

The control treatment emulated the fermentation temperature that takes place in the RAYEN cooperative, reaching 50 ºC at the 5th day; treatment T40 consist of maintained 40 ºC from 3$^{rd.}$ day until the end of fermentation, 6th; while in treatment T60 consist of gradually increased the temperature from 3rd day until reaching 60 ºC at 5th day and kept there until the end of fermentation, 6th day (**Figure 8**). Two aerations were simulated at 2 and 4 days with a bag change.

The pods (**Figure 9**) were opened, and 1500 g of cacao with mucilage were placed in a 45 x 35 cm polyethylene bag as a retainer; they were inoculated with the pods where native microorganisms exist (**Figure 10**); the bag was closed with a wire. A total of 3 treatments were carried out, modifying the temperature in stage 3 that goes from 4 to 6 days.

To maintain the temperature, the incubator with a digital thermometer (**Figure 11, 12**), programmable to control the temperature (Hamilton Beach, 32100A) was used as a fermenter in a temperature range of +/- 3 ºC. Inside there was a bag of fermentation cacao which was monitored with an internal thermometer. The temperature was programmed to increase in each variable, and the steps shown in **Table 1** were carried out. The fermentations were stopped at 6th day, removing the bags from the fermentation chamber, and placing the beans on a mesh in the sun to be dried for 7 days (**Figure 13**).

Throughout the process, samples were taken on days 0, 3, and 6. In each of the samplings, ten beans were randomly taken, weighed, their length, width, and thickness were measured, and they were split in half.

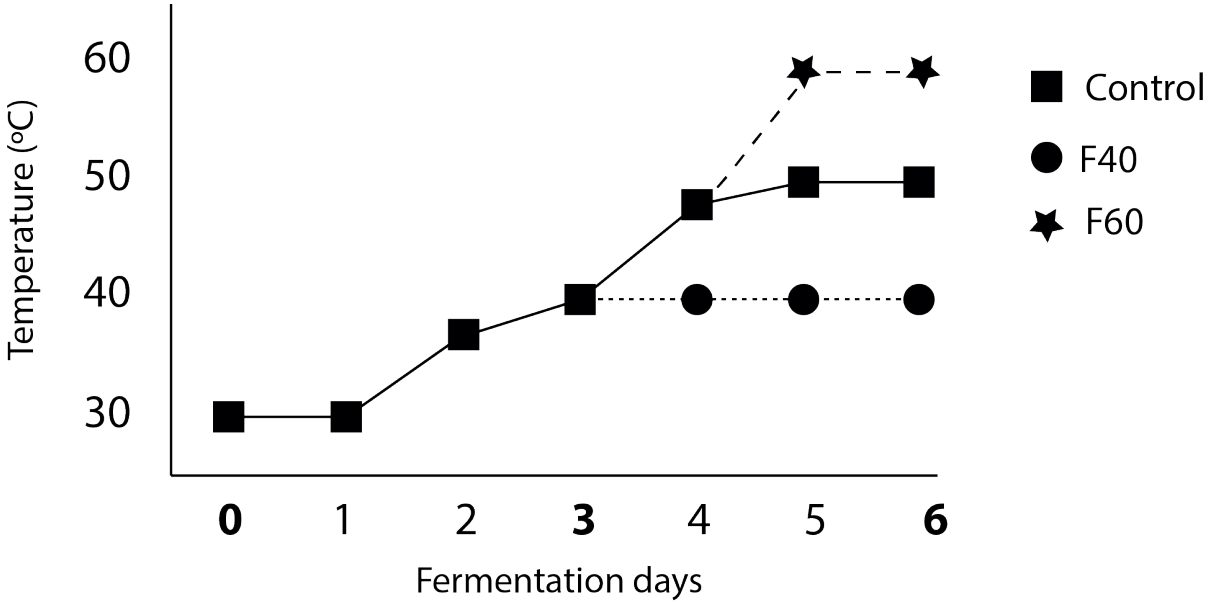


Figure 8. Emulated treatments temperature, F40: maintained 40 ºC from 3rd day until the end of fermentation, 6th day F60: on gradually increased the temperature from 3rd day until reaching 60 ºC at 5th day and kept there until the end of fermentation, 6th day.

Table 1. Steps in method treatments cacao (*Theobroma cacao L.*) bean fermentation.

Step	Detailed process information
1.	Cacao pods were received, healthy pods were selected
2.	The pods were opened five days after their harvest, the beans were placed in the polyethylene bags and the pods were rolled over them to inoculate (**Figure 9**) native microorganisms. The temperature was set at 30 ºC, here it starts at hour 0. A sample was taken and frozen.
3.	After 1 day, the bag that had already been inflated by yeast action was perforated.
4.	On day 2 the bags were opened, and the beans were changed to a new one. It was left slightly open for air exchange.
5.	On day 3 a sample was taken and frozen.
6.	On day 4 the bag was reopened, and the bag was changed again and left open for air exchange.
7.	On day 6 the bag was opened, and the beans were taken out to dry in the sun.

Figure 9. Pods of *T. cocoa* from the same plot

Figure 10. Inoculation of native microorganisms to the bag of cacao *(T. cacao L.)* beans taken from the pods.

Figure 11. Incubation of cacao (*T. cacao L.)* fermentation treatments carried out in Hamilton Beach, 32100A.

Figure 12. Temperature control in the fermentation treatments of *cacao* (*T. cacao L.*)

Figure 13. Drying of cacao (*T. cacao* L.) beans under the sun.

6.3 Experimental diagram

The general diagram of cacao experimentation is shown in **Figure 14**.

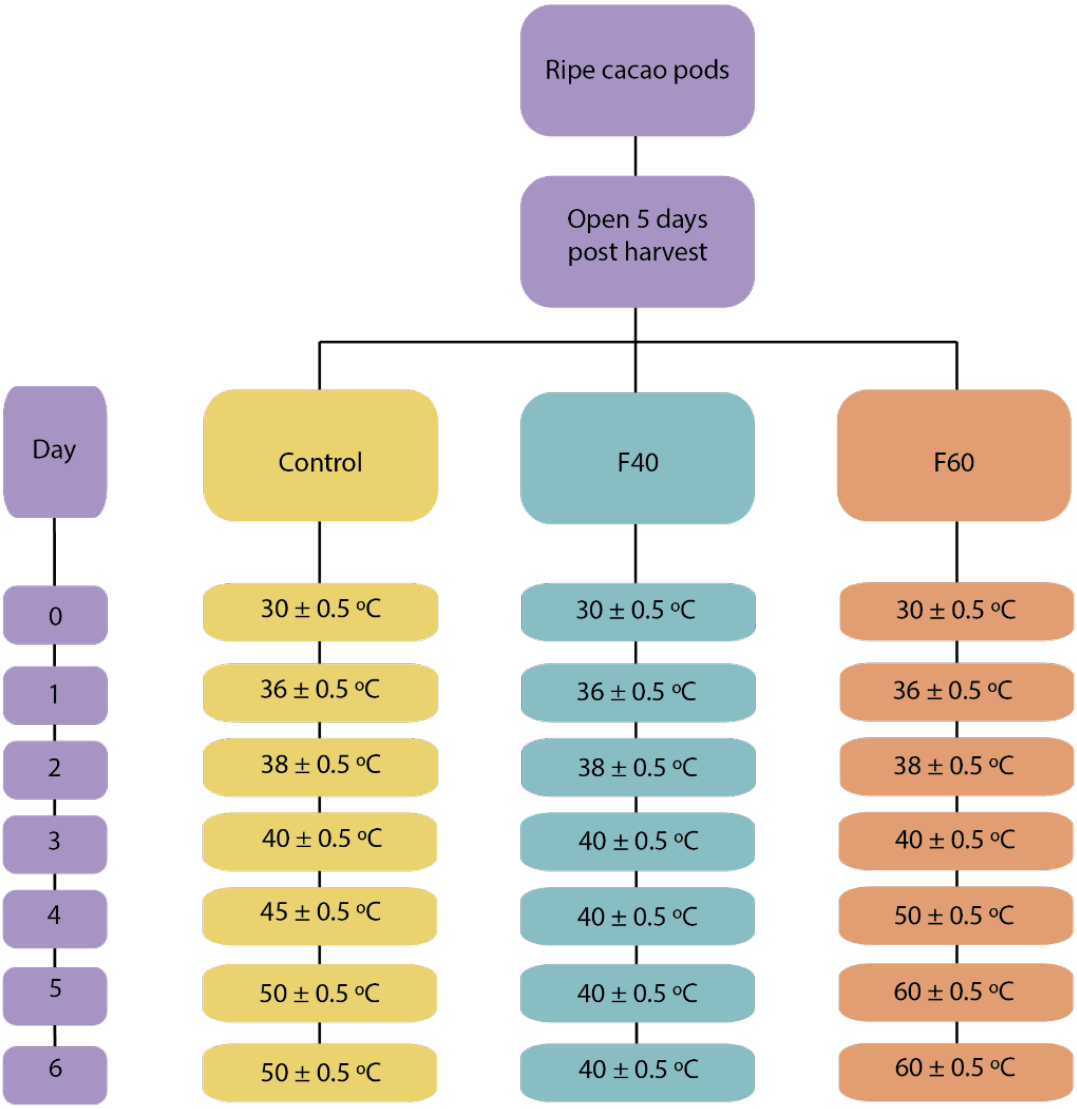


Figure 14. General diagram of the fermentation process and its treatments with a temperature variation on box 3 that include 5th and 6th day, where Control: follow the traditional fermentation, rising 50 ºC, F40: Fermentation 40, maintained at 40 ºC and F60: Fermentation 60, rising 60 ºC.

6.4 pH pulp and cotyledons

The pH of the pulp was measured in triplicate at 0, 3 and 6 days, for this a pH probe (HM Digital, PH-2000) was used, which was inserted into three different points of the polyethylene bag verifying that it had direct contact with the surface of at least one bean.

The pH of the cotyledons was measured in triplicate in each of the samples, for this the seed coat was detached along with the residual pulp, 10 g of pure cotyledon were ground in 90 ml of distilled water, and the measurement was taken.

6.5 Cacao proximal analysis

6.5.1 Moisture

Method 44-19 (AACC, 2000) was used, for this, 2 g of sample were weighed into aluminum trays, and the rum was placed in a convection oven (Scorpion scientific, Maryland, USA) set at a temperature of 110 ± 1 °C for 3 hours. Moisture content was calculated by weight difference.

6.5.2 Protein

The protein content was determined with the Kjeldahl method for total nitrogen (Bhüchi, Labortechnik, Germany). For this, 1 g of previously defatted sample, 1 g of copper sulfate and 10 g of anhydrous potassium sulfate were weighed and transferred to a Kjeldahl tube, in which 15 ml of concentrated sulfuric acid were added. The tube was placed in the digester where it was gradually heated up to 400 °C, the visual signal to reduce the temperature was the green coloration. When it was, 50 ml of 40% sodium hydroxide were added. Subsequently, it was placed in a digester until obtaining a sample volume of 100 ml in a flask with 50 ml of 4% boric acid as well as 10 drops of Tashiro indicator. The sample was titrated with 0.1 N hydrochloric acid. The protein content was determined with a conversion factor of N x 6.25.

6.5.3 Lipids

It is determined using AOAC method 931.15 of fat Soxhlet extraction using 3 grams of sample with 100 ml of petroleum ether for each beaker, the sample was recirculated for 4 hours, then the beakers were dried at a temperature of 60 °C. for solvent evaporation. The lipid content was calculated by temperature difference.

6.5.4 Carbohydrates

Carbohydrates were calculated by difference, where the accumulated of moisture, protein, lipids, and ashes represent 100%.

6.5.5 Ashes

The method used was AACC 08-01, 2000, for which 1 g of sample was weighed in a porcelain crucible. The sample was charred in a calcination oven for 6 hours at 550 °C, then the crucible was taken out to bring it to room temperature and weighed, to calculate the ash content by weight difference.

6.6 Fatty Acid determination

The fat extract obtained was converted into fatty acid methyl esters (FAME). For this, it was subjected to esterification according to the AOAC 963-22 (2000) method and subsequently injected into the chromatograph.

The equipment conditions were those used by Moreira and Mancini Filho (2004) with a TSQ DUO GC system, the result calculated using the Chromeleon V 3.1, (Fischer, Wisconsin, USA) software. The column used was SUPELCOWAX 10 fused silica (polyethylene glycol, 30 x 0.25 mm x 0.25 µ). The conditions follow the process:

1. Gradient temperature: the initial temperature was 170 °C and to 225°C at a rate of 1 °C / min
2. Vaporization temperature: 250 °C
3. Temperature detector: 270 °C
4. Carrier: Helium (He) with a flow rate of 1 ml / in and distribution ratio of sample to injector 1/150.

Finally, Sigma Aldrich standards were used for comparison.

6.7 Free Amino Nitrogen (FAN)

For the determination of Free Amino Nitrogen, the ninhydrin method was used according to (Boffill, Gallardo & de Almeida 2017). For this, 2 ml of cocoa previously defatted with distilled water were placed in test tubes, simultaneously other tubes with known amounts of glycine were placed, it was added 1 ml of ninhydrin reagent with pH 6.6. The mixtures are placed in a

water bath for 15 min, cooled to 20 °C. After 20 minutes, the absorbance at 570 nm is measured against reagent blank in a spectrophotometer (Genesys 10S, Maryland, USA), and the absorbance calculated from the sample tests is divided by the measurement in the assays with the glycine solution and multiplied by the dilution factor.

6.8 Polyphenols and antioxidant capacity

6.8.1 Extraction of phenolic compounds

For the extraction of phenolic compounds from the cocoa bean, the methodology proposed by Summa et al. (2006) was used. Cocoa beans were grounded in a coffee grinder for 30 seconds. To defat, 5 g of ground cocoa were placed with 25 milliliters of n-hexane and mixed for 3 minutes. This mixture was centrifuged for 15 min at 3,000 rpm using a Beckman Coulter Avanti J-15 centrifuge (Detroit, USA). The solvent was then allowed to evaporate. To proceed to extract the phenolic compounds, 2 g of the previously defatted sample were used, and it was mixed with 50 ml of a solution composed of 80% methanol and was magnetically stirred for 2 hours at 50 °C with a stirrer. Subsequently, the sample passed through a Whatman No.1 filter paper, and the extract was capped in a 50 ml volumetric flask.

6.8.2 Total phenolic compounds

For the determination of the phenolic compounds in cocoa bean, the Folin -Ciocalteu method was used where a 100 ml beaker was determined to mix in order 1 ml of diluted extract, 50 ml of deionized water, 5 ml of Folin reagent and 20 ml of sodium carbonate in solution 20% (w / v) and volumetric. After 30 minutes and with the help of a spectrophotometer (Genesys 10S, Maryland, USA), the absorbance was measured at a wavelenght of 750 nm. The standard curve was determined with gallic acid (GA) in concentrations from 0 to 600 mg / L and the result was expressed as GA equivalents.

6.8.3 Antioxidant activity

To determine the antioxidant activity, the methodology proposed by Rivero-Pérez et al. (2007) where a mixture of 60 µM a methanolic solution of DPPH (2,940 µL) with 60 µL extract and a polystyrene cuvette is used to measure the absorbance at 515 nm on two occasions: before adding the extract, and 60 min after adding it with a spectrophotometer (Genesys 10S, Maryland, USA). The standard curve was determined with Trolox and the results were expressed as Trolox equivalent mmol.

6.9 Enzyme activity

6.9.1 Protease

A Sigma Aldrich Universal Protease Assay was used with casein as substrate, when it is consumed by proteases the amino acid tyrosine is released along with other amino acids and peptide fragments. Folin's Reagent is added to react with free tyrosine resulting in a blue chromophore that is quantified in the spectrophotometer (Genesys 10S, Maryland, USA) as an absorbance value, compared to a standard curve of 1.1 mM tyrosine at concentrations of 0.05, 0.10, 0.20, 0.40, 0.50 ml with a capacity of 2 ml and Folin reagent. The absorbance generated is used to determine protease activity in terms of units, which is the amount in micromole equivalents of tyrosine from casein per minute.

To carry out the reaction 15 ml vials were used, one as a blank and three with protease dilutions to compare the results, to each vial 5 ml of 0.65% casein solution was added, prepared with 6.5 mg / ml of 50 mM phosphate buffer. of potassium, the buffer was prepared with 11.4 mg / ml of dibasic phosphate trihydrate in water and pH adjusted to 7.5 with 1M HCl. To each vial, except the blank, the enzyme solution was also added. They were mixed and incubated at 37 °C for 10 minutes. After, 5 ml of sodium carbonate was added to regulate the change in pH caused by the Folin reagent, which is added immediately and interacts with tyrosine, mixed, and incubated at 37 ºC for 30 minutes. It is then read on the spectrophotometer at 660 nm. And the following equations are used to determine the units per ml of enzyme and the units per milligrams of solid:

$$\frac{\text{Units}}{\text{ml enzyme}} = \frac{(\mu\text{mol tyrosine equivalents}) \text{ x } (11)}{(1) \text{ x } (10) \text{ x } (2)}$$

Where:
11: total volume in ml of the assay
10: time per unit in minutes of the test
1: volume of enzyme used
2: volume in milliliters used in colorimetric determination

6.9.2 Lipase

For the determination of the enzymatic activity of the lipase, a Lipase Activity Kit was used (Sigma Aldrich, USA), which follows the Bulletin technique, which consists of a couple of reactions that result in a colorimetric product proportional to the activity of the enzyme, one unit being the amount of enzyme that generates 1.0 µmol of glycerol from triglycerides per minute at 37 ºC.

For this assay, 10 µL of 100 mM glycerol were used as a standard, which was diluted in 990 µL of buffer and 0, 2, 4, 6, 8 and 10 µL of the standard were added to a 96-well plate. It was filled to 50 uL each with a buffer.

For sample preparation, 5 mg of grounded cacao was homogenized in the buffer and centrifuged at 13,000 x g for 10 minutes to remove the insoluble material; then it was gauged at 50 µL.

A compilation of the Reaction Mix was made to carry out the reactions, which consisted of 93 µL of Buffer, 2 µL of Peroxidase Substrate, 2 µL enzymatic Mix and 3 µL lipase substrate; the lipase substrate was excepted for the blank.

Of the Reaction Mix, 100 µL was added to each of the wells. The plate was incubated at 37 °C and the absorbance at 570 nm was measured after 2 minutes, initial Time (T). It was incubated again at 37 °C and the absorbance was measured after 60 minutes (final T). This rhythm was continued until the value of the most active sample exceeded the value of the highest standard, the final T value being the last within the standard.

The change in absorbance was calculated as from initial T to final T as:

$$\Delta A570 = A(570)_f - A(570)_i$$

Where:

$\Delta A570$ Delta absorbance
$A(570)_i$ Initial absorbance
$A(570)_f$ Final absorbance

The delta A570 value of each sample was compared with the standard curve to terminate the amount of glycerol (B) generated by the lipase between initial T and final T. The lipase activity was determined with the following equation:

$$\text{Lipase activity} = \frac{(\text{B x sample dilution factor})}{(\text{Reaction time x V})} \ \frac{\text{milliunit}}{\text{ml}}$$

Where:
B: amount in nmoles of glycerol generated between initial T and final T
Reaction time: Final T - Initial T (minutes)
V: ml of sample added to the well

6.9.3 Amylase

The used kit (Sigma Aldrich, USA) operates under the reaction that alpha amylase hydrolyzes starch to produce maltose. For this, it is necessary to prepare the following reagents using ultrapure water (>18 MΩ x cm)

- Buffer solution (20 mM Sodium Phosphate with 6.7 mM Sodium Chloride, pH 6.9 at 20 °C)
- Starch solution [1.0% (w/v) Soluble Starch Solution
- 2 M Sodium Hydroxide (NaOH) solution
- 5.3 M potassium sodium tartrate, tetrahydrate solution
- 96 mM 3,5-Dinitrosalicylic acid solution
- 0.2% (w/v) Maltose Standard
- Color Reagent solution – 12.0 mL of ultrapure water is added with 8.0 mL of 5.3 M potassium sodium tetrahydrate solution and 20 mL of 96 mM 3,5-Dinitrosalicylic acid solution, all in 70 °C temperature.

For the procedure, it was considered that the final concentration of Sample 1,2,3 and Blank, was 10 ml. The following reagents are pipetted in the order listed:

- Starch solution - 1 ml for sample 1, 2 and 3 and 1 and for blank
- Alpha Amylase - 0.50, 0.70 and 1.00 ml in sample 1,2 and 3, does not add to the blank.

Mix by swirling and incubate for *exactly* 3.0 minutes at 20 °C.

- Color reagent - 1 ml in sample1,2,3 and for blank

The containers are covered and placed in a hot water bath for 15 min.

- Alpha amylase - 0.50, 0,30, 0 for samples and 1.00 ml for blank,

Then cool to room temperature.

- 9 ml of ultrapure water for sample 1,2,3 and for blank

Mix and take a spectrophotometer reading at 540 nm.

The standard curve is prepared with 7 standard points with 0.2% maltose concentrations of 0.05, 0.20, 0.40, 0.60, 0.80, 1.00, 2.00, and 0 mL for blank; ultra-pure water with concentrations of 1.95, 1.80, 1.60, 1.40, 1.20, 1.00, 0, 2.00, respectively and reagent color with concentrations of 1.00, for all standards. They are placed in a hot water bath for 15 minutes, and 9 ml of ultrapure water are added to each one. It was mixed and read at 540 nm.

The determination of the delta readings for the standard curve is made with A540 (standard) - A540 Blank) and linear regression is calculated. The difference of the samples was then determined with A540 (sample) - A540 (sample blank), and the mg of maltose was determined using the standard curve.

6.10 Structural analysis

To quantify the size and the percentage of area that was cracked in the cotyledons of the bean through fermentation, 10 beans were selected, which were divided in half with the help of a knife and a photograph was taken in a leaflet. The Image J (Java, USA) program was used to process the images, convert them into an 8-bit image, and select the sink area to apply thresholds and quantify it.

6.11 Colorimetry

For the determination of changes in the color of the bean, 10 beans were taken as a sample at 0, 3 and 6 days in each fermentation to which an open cotyledon photograph was taken. Then the ColorColl tool (Taki Hirata, 2020) was used to determine the RGB coordinates of each cotyledon, after they were translated into Lab with the Colormine tool (USA, 2021), the average of the data was determined.

6.12 Statistical Analysis

A statistic of Correlations by fermentation was carried out to determine which variables had correlation with others in the process for each treatment, using Minitab (USA, 2021) with α = 0.05

Also, a principal component analysis was performed with all treatments on day 6 to explain the maximum variance with the least number of principal components.

7. RESULTS AND DISCUSSION

7.1 Observations on the bean micro structural behavior during fermentations

The first hours after place the beans in the bag, mead was observed reaching at least one sixth of the beans. At 12 hours, the bag was inflated and with a layer of foam, due to the production of CO_2 by the yeasts, while the passage of oxygen was limited with a wire in the bag, simulating how tight they are beans in the mass of 150 to 400 kg. At this time, the viscous structure of the pulp made up of pectin and polysaccharides, generate an anaerobic environment. Is after this moment where perforations were made in the bag to remove most of the leaching made by the pulp, to give way to oxygen into the bean mass.

On day 2 the bag was opened to simulate aeration and change it. An alcoholic odor was perceived. On day 3, the pulp began to show the seed coat marks as well as to reveal a tone no longer white but slightly pink on the outside, when opened a juice of reddish-purple tones came out as squeezed from the cotyledons, that is, the intracellular liquid. It is from this day where the fermentations treat temperature change.

On day 4, its perceiving acidity in its odor, when bean is split the amount of internal juice reached in control is notable (**Figure 15**), and some slightly cracked areas began to be perceived, giving the bean a disarmed appearance (**Figure 16**). Some beans remained purple and others pink. At the arrival of the 6th day 9 of the 10 beans showed a very perceptible crackle and all a pink color, except for the criollo beans, that came out fortuitously into the mass, with a white tone due to their nature (**Figure 17**).

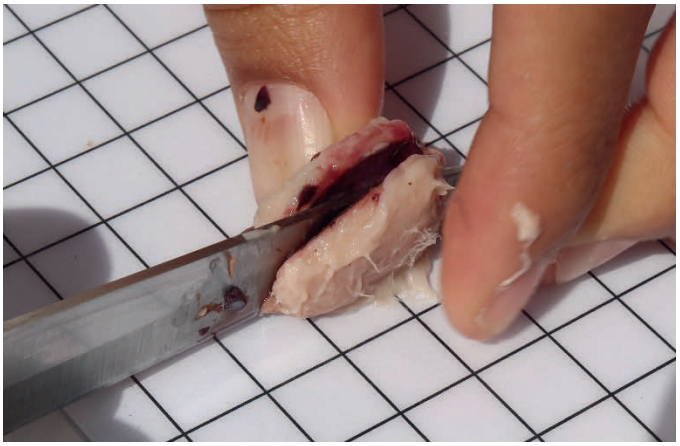


Figure 15. Cacao bean split (*T. cacao*) of the control treatment, on day 4.

Figure 16. Opened cacao bean (*T. cacao*) of control treatment, on day 4, where cracked is visible.

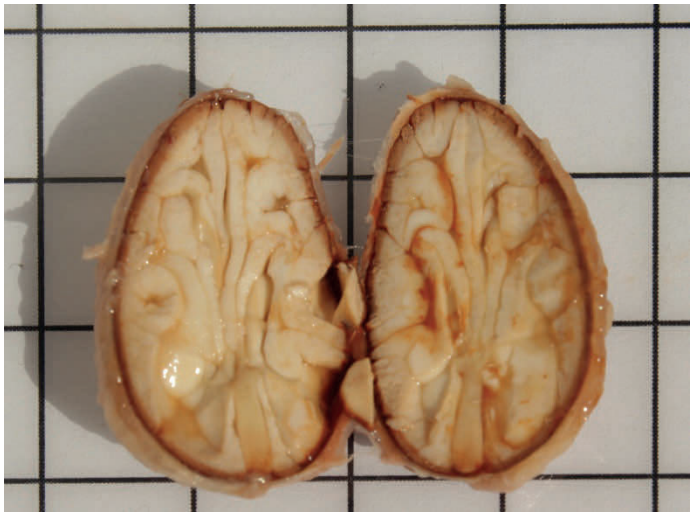


Figure 17. Criollo cacao bean located in the mass of beans fortuitously.

At the end of Fermentation 40, the beans developed an almost imperceptible crackle. They did not change their purple color because the anthocyanin compounds may not have been hydrolyzed (Afoakwa et. Al, 1989). While Fermentation 60, on day 5 the pulp has completely disappeared, and the seed coat of the bean turns a reddish-brown tone. When splitting the bean in half, a evident juice is released, and the crackle is very marked; visually it could be said to be ideal for fermentation (**Figure 18**)

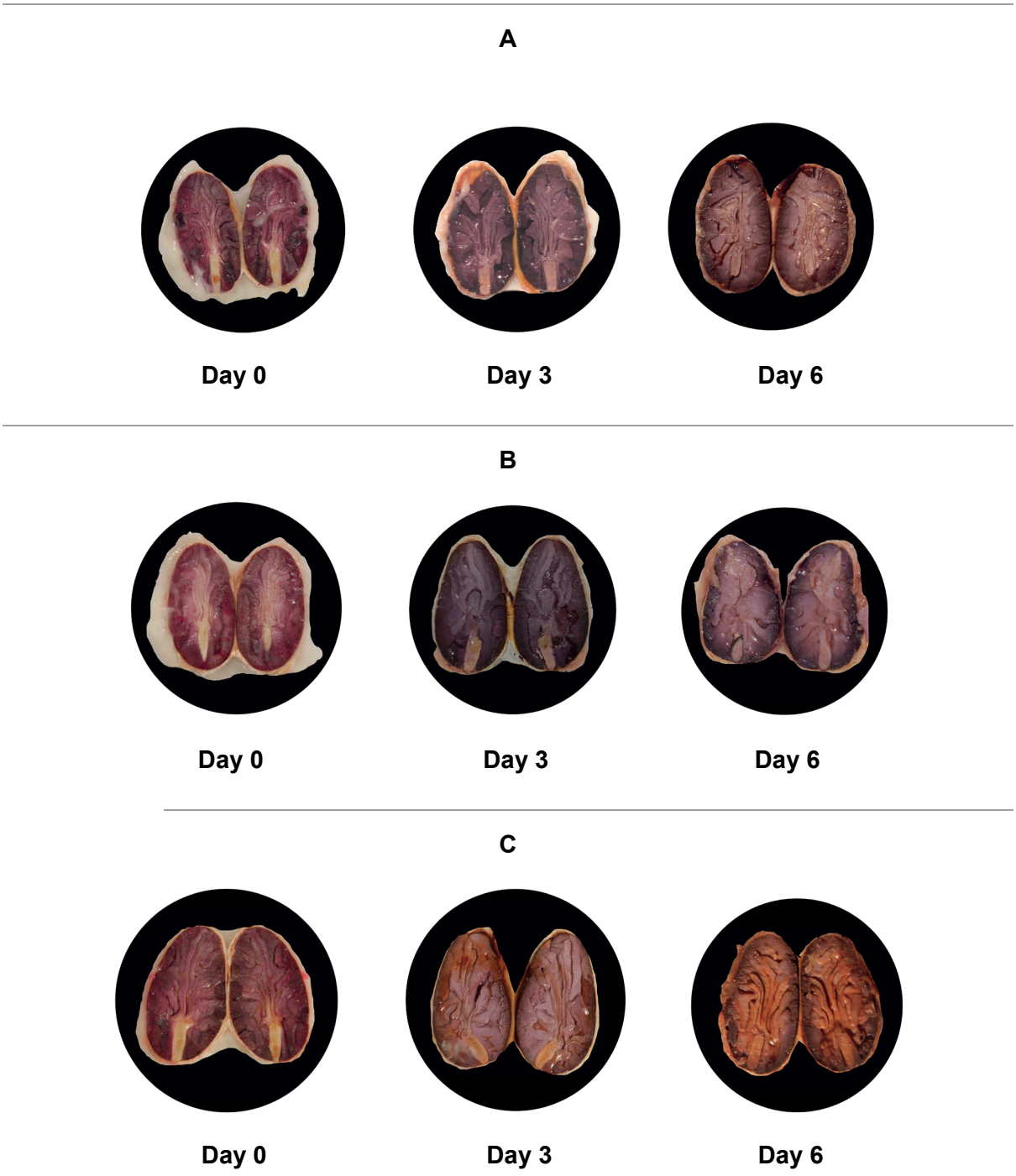


Figure 18. Cacao bean cotyledons of 0, 3 and 6 days, for each treatment. A) Change in the cocoa bean in the control treatment. B) Change in the cocoa bean in treatment F40. C) Change in the cocoa bean in treatment F60

7.2 Changes in pH in treatments

The results of the pH inside and outside the bean are shown in **Table 2**. On the outside of the bean, on the pulp side, the pH started between 3 and 3.5 units, this due to the content of citric acid that maintains an acidic pH (Koffi et. al, 2017), which together with the 23 – 27% of sugars (**Table 3**), is the ideal environment for the development of yeasts (Afoakwa et. al, 2011).

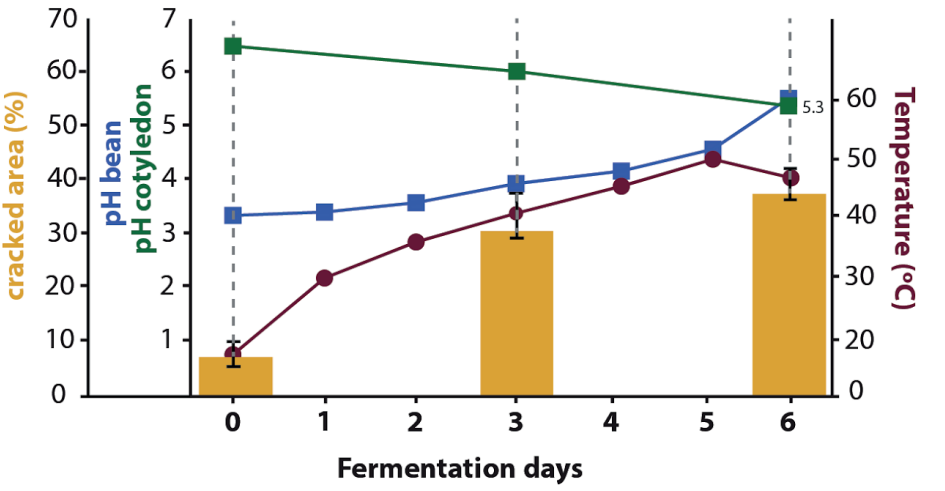
Yeast consumes not only the sugars, but also some strains, such as *Pichia kluyveri* and *Kluyveromyces marxianusm,* hydrolyze pectin by produce pectin lyase and pectinolytic enzymes (Koffi, Samagaci, Goualie, & Niamke, 2017) such as polygalacturonase, and pectin methylesterase (Dasilva, Borges & Medina, 2005). Other authors (Ouattara, Koffi, Karou, Sangaré, Niamke, & Diopoh, 2008) argue that it is an interaction between pectinolytic enzymes produced by yeasts and by strains of *Bacillus.spp.* Enzyme activity breaks the viscous structure of pectin, increasing oxygen difussion and a pH increase, since citric acid is consumed by the bacteria throughout the process (Afoakwa et. al, 2011).

Both the production of enzymes and the quantity and quality of the metabolites produced by the yeasts will be a consequence of the yeast strain that predominates (Pereira et al. 2012). For this reason, the pH conditions vary, despite coming from the same crop; since in addition to ethanol and carbon dioxide they can produce not only acetic acid succinic acid, but also other alcohols, aldehydes, ketones and fatty acid esters, which will form part of the development of flavor precursors, studies have been carried out using starter cultures to see the response of the strains on products as mentioned (De Vuyst, & Weckx, 2016).

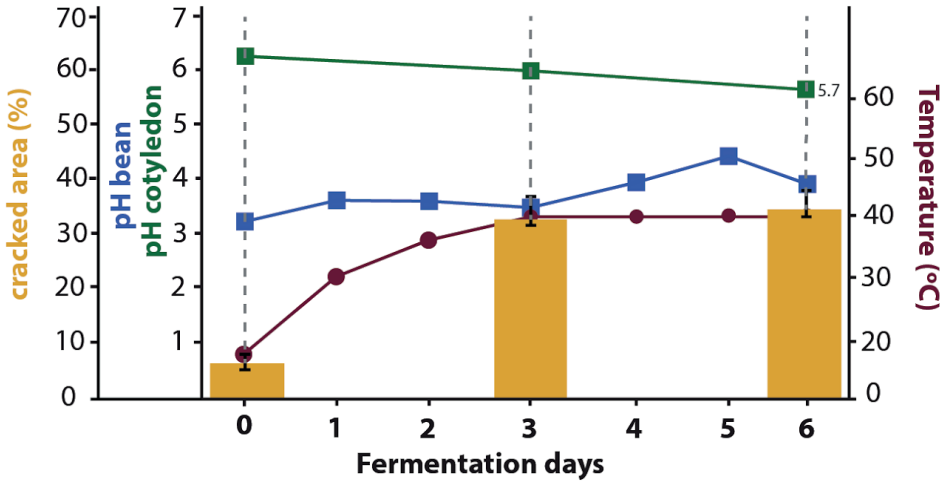
Table 2. Hydrogen potential during fermentation in pulp and cotyledon cacao (*T. cacao L*) beans.

	pH pulp			pH cotyledons		
Day	0	3	6	0	3	6
Control	3.30 ± 0.04^{a}	3.89 ± 0.07^{a}	5.58 ± 0.09^{a}	6.40 ± 0.02^{a}	5.96 ± 0.01^{a}	5.33 ± 0.02^{a}
F40	3.05 ± 0.09^{b}	3.28 ± 0.04^{b}	3.73 ± 0.10^{b}	6.41 ± 0.01^{a}	5.89 ± 0.01^{b}	5.73 ± 0.03^{b}
F60	3.04 ± 0.10^{b}	3.58 ± 0.50^{b}	4.53 ± 0.20^{b}	6.55 ± 0.01^{b}	5.98 ± 0.02^{a}	5.00 ± 0.02^{c}

Data are the average of three replicates ± standard deviation.
Different letters in the same column indicate significant differences ($P < 0.05$).



A.



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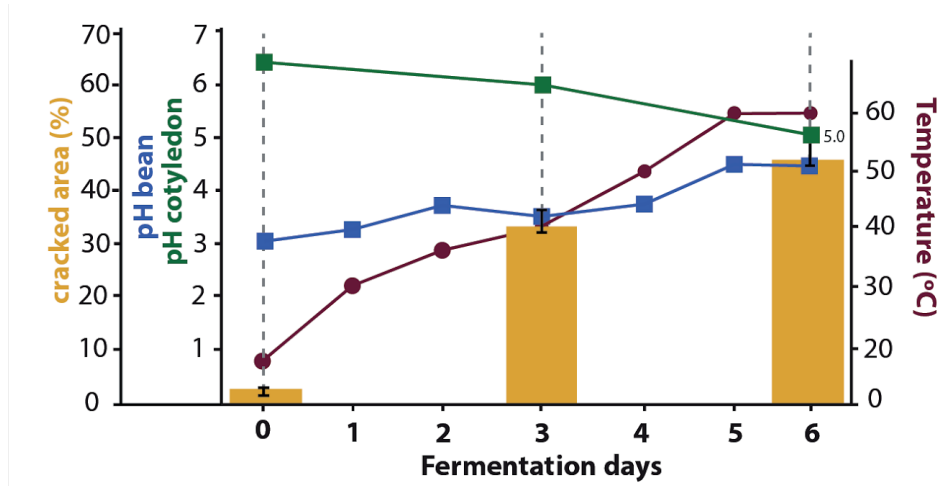

C.

Figure 19. Behavior of the treatments in temperature (°C, red Y axis), crackle area (%, yellow Y axis), pulp pH (blue Y axis) and cotyledon pH (green Y axis). A) Control treatment (50 °C). B) Treatment F40 (40 °C). C) Treatment F60 (60 °C).

On day 6, a significant difference was found between the pH of the cotyledons of the 3 fermentations, and as consequence of a higher temperature in the tree treatments during stage 3, the pH of the interior decreased in different proportions, but not the exterior. Although all three started from an internal pH of between 6.4 - 6.5, at the end Control shows a value of 5.33, while F40 and F60 had 5.7 and 5.0 respectively (**Figure 19**). With these values, it is confirmed that both control and Fermentation 60 reached an appropriate fermentation since according to Afoakwa et al. (2008), a poorly fermented cocoa has an acidity of between 5.5 to 5.8, while a properly fermented cocoa has a pH of between 4.7 to 5.3.

These changes in pH affect the entire fermentation process. At a lower pH, which is equivalent to a greater diffusion of acetic acid into the bean (as happened in the control fermentation and F60) the lipid bodies are fused and segregated in the cell center, allowing unrestricted diffusion of other water-soluble components, that would enable proteolysis and other reactions.

While at lower acetic acid entry, or lower pH as in F40 fermentation, a minimal breakdown of the cell structure and the enzymes present in the embryo could not be released and contribute to transformation because the low acetic acid concentration causes the formation of a hydrophilic dispersal phase, decreasing cell diffusion and enzyme activation (Caballero, Trugo, & Finglas, 2003).

7.3 *Cacao proximal analysis*

A compilation of the proximal analysis of the cocoa beans in the different fermentations at 0,3 and 6 days is shown in **Table 3.**

Table 3. Proximal Composition of cacao (*T. cacao L.*) bean across fermentation treatments.

Treatment	Day	Moisture (w.b.) (%)	Protein (w.b.) (%)	Lipids (w.b.) (%)	Carbohydrates (w.b.) (%)	Ashes (w.b.) (%)
Control	0	65.23 ± 0.69^{a}	17.23 ± 0.37^{a}	53.23 ± 0.60^{b}	24.21 ± 0.64^{a}	5.33 ± 0.56 ^{a}
	3	43.45 ± 0.05^{a}	16.50 ±0.97 ^{a}	54.21 ± 0.08^{a}	23.81 ± 0.85^{a}	5.46 ± 0.06 ^{a}
	6	24.55 ± 0.78^{a}	16.52 ± 0.48 ^{a}	53.98 ± 0.43^{a}	24.25 ± 0.51^{a}	5.21 ± 0.32^{a}
F40	0	57.58 ± 0.76^{c}	16.54 ± 0.01^{b}	50.57 ± 0.72^{c}	27.77 ± 0.13^{b}	5.12 ± 0.40 ^{a}
	3	39.62 ± 0.37^{c}	15.53 ± 0.3 ^{a}	50.96 ± 0.18^{b}	28.27 ± 0.52^{b}	5.24 ±0.43 ^{a}
	6	20.98 ± 0.47^{b}	15.57 ± 0.42 ^{b}	48.04 ± 0.41^{b}	31.39 ± 0.22^{b}	5.00 ± 0.81 ^{a}
F60	0	63.27 ± 0.69^{b}	17.23 ± 0.08^{a}	54.23 ± 0.37^{a}	23.11 ± 0.29^{c}	5.43 ± 0.09 ^{a}
	3	41.71 ± 0.05b^{a}	15.03 ± 0.35^{a}	54.31 ± 0.31^{a}	26.35 ± 0.14^{c}	4.31 ± 0.11^{b}
	6	23.81 ± 0.78 ^{a}	14.57 ± 0.1 ^{c}	54.21 ± 0.89^{a}	27.83 ± 0.63^{c}	3.39 ± 0.80 ^{a}

Data are the average of three replicates ± standard deviation.
Different letters in the same day of treatment indicate significant differences ($P < 0.05$).

7.3.1 Moisture

The percentage of humidity on day 0 of the fermentations resulted in a range between 58-65%, although they were significantly different, this variation may be due to the difference in varieties, such as the ripening of the fruit or the conditions, for example, temperature, during transport. At the end of the fermentations, the moisture content was significantly the same for Control and F60 with 23.81 ± 0.78 and 24.55 ± 0.78, respectively. The moisture retention throughout the process is because the cellular breakdown caused by high temperatures and low pH releases intracellular juices that spread through the cotyledons (Puerto, Guerra, & Contreras, 2016) and are visibly present when opening the bean in half, while for F40 it was 20.98 ± 0.47, where it is assumed that the water was trapped inside the cells that did not break their walls.

7.3.2 Protein

During all treatments, the total protein content decreased from day 0 to day 6, control treatment decreased by 4.02% and F40 by 5.8%, however in treatment F60 the greatest change was observed with a decrease in 15.4%, from 17.23 ± 0.08 to 14.57 ± 0.13%. Other authors have verified a reduction of similar percentages in different varieties of cacao (Assa, Alfian, Misnawi, Djide & Muliadi, 2017), assigning its reduction not only to the hydrolysis suffered by amino acids hydrophobic of the 7S vicilin-class by the amino endoprotease at the beginning of the process and by the carboxypeptidase, but also by the polymerization mechanism in which the proteins participate together with the phenolic compounds for the formation of condensed tannins. For the behavior in the protein of the F60 treatment, high enzymatic activity is assumed since its behavior is related to the content of Free Amino Nitrogen that is discussed later.

7.3.3 Lipids

The lipid content was significantly the same in Control and F60 at the end of the fermentation. Other authors state that there is no change in the proportion of fats during traditional fermentation. Still, changes occur in the conformation of the molecular structure (López & Quesnel, 1973). On the contrary, it is observed a significant decrease in the treatment F40 (**Table 3**), this could be due to the disposition in which the lipids are found to be extracted, depending on the behavior of the cell in response to pH and temperature conditions, since F40 did not reach a temperature that could not even destabilize the cell or break its walls, the lipid bodies could have formed a hydrophilic dispersal phase, decreasing cell diffusion inside the cell (Caballero, et al. 2003) as well as the agglomeration of lipid bodies that hinders its extraction (Servent et al. 2018).

Some authors (Guehi, Dingkuhn, Cros, Fourny, Ratomahenina, Moulin, & Clément, 2007) highlight that the impact of fermentation on the fatty acid is minimal or null because during the process, only hydrophilic compounds and bonds are affected, and cocoa butter is highly stable to oxidation. While others have reported the opposite, decrements during the process (Ndife, Bolaji, Atoyebi & Umezuruike, 2013)

7.3.4 Carbohydrates

Carbohydrates showed an increase in their concentration in all treatments, in the same way that they remained significantly different between them at the end of fermentation with values of 24.25 ± 0.51%, 31.39 ± 0.22% and 27.83 ± 0.63 for control, F40 and F60, respectively. The increase in carbohydrates is due to the increase in reducing sugars since the sugars present as sucrose that represents about 90% of the content are transformed by invertases as well as alpha-amylases to smaller molecules such as glucose.

7.3.5 Ashes

The ashes had variations in increase and decrease in the fermentations, even though all the treatments began above 5%, Control and F40 increased on day 3 and decreased on day 6 to 5.21 ± 0.32 and 5.00 ± 0.81 respectively, while that F60 gradually decreased from 5.43 ± 0.09 to 3.39 ± 0.80, as observed in **Table 3**. The primary concentration depends primarily on the soil of the crop, as well as the content and type of minerals present in the beans, determined by the variety, made up of macronutrients (Ca, Mg, Na and K) and micronutrients (Fe, Mn and Z). These macro and micronutrients are affected by fermentation (Assa, et al. 2017); for example, macronutrients such as Ca tend to increase with increasing fermentation time, as well as Mg.

7.4 Free Amino Nitrogen (FAN)

The content of FAN through the fermentation is summarized in **Table 4** and is related to the activity of protease, specifically with that of carboxypeptidase, which, being an exoprotease, cuts the amino acids from the ends of the peptide, leaving free hydrophobic amino acids (Silveira, Melo, Souza, Bispo & Soares, 2017). Although an increment in all treatments is observed, the lowest content of free amino acids was that of the F40 treatment with 10.38 ± 0.23 g/kg at the end of the fermentation, while the highest content was that produced in the F60 with 12.50 ± 0.65 g/kg and Control remained in the intermediate of both with 11.41 ± 0.31 g/kg, being significantly different.

Table 4. Free Amino Nitrogen concentration (g/kg) during cacao (*T. cacao L.*) fermentation treatments in 0,3 and 6 days.

Fermentation	Day	Free Amino Nitrogen (g/kg)
Control	0	3.36 ± 0.39^{a}
	3	8.33 ± 0.91^{b}
	6	11.41 ± 0.31^{b}
F40	0	3.19 ± 0.64^{a}
	3	7.58 ± 0.54^{b}
	6	10.38 ± 0.23^{c}
F60	0	3.36 ± 0.16^{a}
	3	10.75 ± 0.90^{a}
	6	12.50 ± 0.65^{a}

Data are the average of three replicates ± standard deviation.
Different letters in the same day of treatment indicate significant differences ($P < 0.05$).

However, even F40 managed to develop some free amino acids that could be flavor precursors due to its low temperature, since in non-fermented cocoa, the ranges shown in some varieties go from between 2.33 and 5.96 g / kg, while for the fermented ones they range from 9.05 to 13.73 g / kg (Brunetto, Gallignani, Orozco, Clavijo, Delgado, Ayala, & Zambrano, 2020) where all treatments come in, however, it is worth remembering that there may be variations between varieties. These results are related with the enzymatic activity of the proteases in 7.7.1.

7.5 Lipid Profile

The composition of cocoa butter depends on several factors such as variety, age of plantation, soil and climate conditions, and physiological maturity of the pod. Although several authors have studied the composition of pods of the tropical line, it is the same fatty acids that remain in higher percentages, agreeing that the main saturated fatty acids are oleic, stearic, and palmitic (Servent et al. 2018).

Our results shown that after the fermentations the fatty acid with the highest proportion in the three treatments was oleic acid with 40.33 ± 0.88, 38.31 ± 0.14, and 38.31 ± 0.78 for Control, F40, and F60, respectively (**Table 5**). Followed by stearic acid with 41.22 ± 0.60, 37.51 ± 0.18, and 39.57 ± 0.67, while the third in proportion is palmitic acid with 2.21 ± 0.64, 2.08 ± 0.64, and 2.14 ± 0.96.

The reason for maintaining, and not showing a change in proportion during fermentation, of these three fatty acids is what gives its properties to the final product such as chocolate, since saturated fatty acids, both palmitic and stearic, have a melting point of 62 ºC and 68 ºC respectively, while fatty acid unsaturated, oleic, melts at 16 ºC, in this way the product can be solid at room temperature and melt in the mouth (Mustiga, Morrissey, Stack, DuVal, Royaert, Jasen & Motamayor, 2019), in addition to providing its properties for crystallizing when it is being molded.

Not only that, but clinical studies have shown that cocoa consumption has neutral effects on LDL cholesterol due to high amounts of stearic acid (Kris & Mustad, 1994). At the same time, studies on HLD cholesterol have shown favorable effects as it is 4% higher if 22 g of cocoa is consumed per day, or a greater increase of between 11 - 14% if 75 g daily is consumed than compared to normal diets. Being a high concentration of HDL a variable that decrease risk factor for cardiovascular diseases (Mursu, Voutilainen, Nurmi, Rissanen, Virtanen, Kaikkonen, & Salonen, 2004)

Table 5. Changes in fatty acids percentage during cacao fermentation treatments in 0,3 and 6 days.

Fermentations	Day	Oleic Acid	Stearic Acid	Palmitic Acid
Control	0	42.00 ± 0.88[a]	38.00 ± 0.26[a]	11.00 ± 0.12[a]
	3	41. 22 ± 0.77[a]	39.40 ± 0.23[a]	9.26 ± 0.62[a]
	6	40.33 ± 0.59[a]	41.22 ± 0.60[a]	10.36 ± 0.87[a]
F40	0	39.06 ± 0.11[c]	36.10 ± 0.18[a]	10.45 ± 0.85[a]
	3	39.57 ± 0.15[b]	36.64 ±0.13[b]	8.33 ± 0.82[a]
	6	38.31 ± 0.14[b]	37.51 ±0.18[c]	9.95 ± 0.91[a]
F60	0	39.90 ± 0.39[b]	36.86 ± 0.96[a]	10.67 ± 0.41[a]
	3	39.16 ± 0.40[b]	37.43 ± 0.56[b]	8.98 ± 0.57[a]
	6	38.31 ± 0.78[b]	39.57 ± 0.67[b]	9.84 ± 0.17[a]

Data are the average of three replicates ± standard deviation.
Different letters in the same day of treatment indicate significant differences ($P < 0.05$).

7.6 Polyphenols and antioxidant capacity

7.6.1 Total Phenolic Compounds

Currently, phenolic compounds have a high focus in the diet due to their benefits as antioxidant, anti-inflammatory, hypocholesterolemic, and vasodilator activities (Indiarto, Pranoto, & Santoso, 2019) as well as the reduction of cardiovascular diseases.

Catechins are the main molecule that conform flava-3-ols, with (-) epicatechin as the major component of it with 35%. This molecule is degraded by the polyphenol oxidase activity, near to 90% of (-) epicatechin decrease versus its initial concentration during fermentation (Efraim, Pezoa, Jardim, Nishikawa, Haddad & Eberlin, 2010). It also can be explained by the

epimerization of (-) epicatechin into (-)-catechin, and degradation of compounds because of temperature.

The loss of catechin and epicatechin is time and temperature dependent, it has been shown that catechin degrades about 10% when exposed to a temperature of 50 °C for 60 minutes and even 5% if it is for 2 minutes, while epicatechin degradates 26.15% during 2 min heating at 100 ºC (Lončarić, Lamas, Guerra, Kopjar, & Lores, 2017) so a fermentation time 7 days can degrade significatively.

In the results obtained during the experimentation, it is observed that the three fermentations began with content of phenolic compounds of between 170 and 180 mg / g (**Table 6**). At the end of the fermentation, phenolic's content of the control decreased by 76%, close to what was observed in other studies where 70% of cacao polyphenols decrease after fermentation (Efraim et al, 2017). These results differ from those published by other authors (Romero, Salgado., García, García, del C Rodríguez, Hidalgo, & Robles, 2013), who observed a reduction of only 20% of phenolic compounds in a traditional fermentation using gallic acid as a reference. Other authors report decreases of 31% after 7 days (Brito et al, 2017).

However, in F40, a retention of the compounds was observed compared with the control, since it decreased only 61%, being visibly verifiable since the purple tone provided by the anthocyanins remained similar, as well as the astringent flavor that lasts in poorly fermented cocoa (Puerto et al. 2016). While in the F60 treatment, the decrease of the compounds was the greatest, with 87% of degraded phenolic compounds where, more than to the enzymes, this decrease is due to the low stability of the compounds before the high temperatures exposed for 6 days (Lončarić et al. 2017).

Table 6. Total phenolic compounds (mg gallic acid / g cacao) and Antioxidant activity (µM Trolox / g cacao) during cacao (*T. cacao L*) fermentation treatments in 0,3 and 6 days.

Fermentation	Day	Total Phenolic Compounds (mg gallic acid/g cocoa)	Antioxidant Activity (µM Trolox / g cacao)
Control	0	180.00± 0.21[a]	795.00± 0.12[a]
	3	86.00 ± 0.80[a]	243.84 ± 0.21[a]
	6	42.00 ± 0.96 [a]	196.00 ± 0.67[a]
F40	0	172.33 ± 0.84[b]	775.00 ± 0.53[b]
	3	101.12 ± 0.63[b]	291.00 ± 0.69[b]
	6	66.43 ± 0.07 [b]	204.00 ± 0.71[b]
F60	0	175.14 ± 0.03 [c]	777.00 ± 0.21[c]
	3	84.33 ± 0.09[c]	214.00 ± 0.71[c]
	6	22.41 ± 0.96[c]	91.00 ± 0.94[c]

Data are the average of three replicates ± standard deviation.
Different letters in the same day of treatment indicate significant differences ($P < 0.05$).

7.6.2 Antioxidant activity

Both epicatechin and catechin have a high potential to combat free radicals and inhibit lipid peroxidation, providing the antioxidant capacity of cocoa and its by-products such as chocolate or cocoa.

The results of the study showed, along with the decrease in phenolic compounds (**Table 6**), a reduction in antioxidant capacity with 75% less than at the beginning of the control fermentation, 73% for F40 and 88% for F60, being the most affected by thermosensitivity of the compounds that give it these properties.

For the control fermentation, the non-fermented cocoa had an antioxidant capacity of 709 +/- 17 µM Trolox, equivalent to day 0 in this experiment with a range of 777 +/- 0.21 µM Trolox for control, 775 +/- 0.53 µM Trolox for F40 and 777 +/- 0.21 µM Trolox for F60. While poorly fermented cocoa shows a concentration of 154 +/- 8 µM Trolox, which could be compared to

F40 with 204 +/- 0.71 µM Trolox. Meanwhile, F60 showed 91.00 +/- 0.94 µM Trolox, coinciding with those exposed by Suazo, Pardo, & Arozarena (2014).

It can be observed in the F40 treatment, its quantity of phenolic compounds decreased in greater quantity compared to Antioxidant Activity. This may be because phenolic compounds such as catechin and epicatechin tend to condense to form complex tannins, oligomers of catechin monomers as well as to form complexes. with amino acids, proteins and flavonoids, resulting in water-insoluble tannins that give brown tones (Afoakwa, 2008), and although they are not identified within the phenolic compounds, they can have antioxidant activity (Smeriglio, Barreca, Bellocco, & Trombetta 2016)

7.7 Enzymatic activity

Table 7 is shown below, with a summary of the enzymatic activity of proteases, lipases, and amylases activity in the cocoa samples from the Control, F40 and F60 fermentations.

Table 7. Enzymatic activity of protease (UAE/mg protein), lipase (µm glycerol/min-1) and α amylase (µm maltose/min-1) during cacao (*T. cacao L*) fermentation treatments in 0,3 and 6 days.

Fermentation	Day	Protease activity (UAE/mg protein)	Lipase activity (µm glycerol/min^{1})	Alpha amylase activity (µm maltose/min^{1})
Control	0	3.36 ± 1.12^{a}	0.55 ± 1.23^{a}	5.35 ± 0.89^{a}
	3	8.29 ± 2.16^{b}	6.26 ± 2.43^{a}	25.36 ± 2.36^{a}
	6	16.31 ± 2.15^{b}	16.36 ± 2.27^{a}	43.41 ± 3.31^{a}
F40	0	3.87 ± 1.00^{a}	1.00 ± 0.33^{a}	6.21 ± 1.1^{a}
	3	10.43 ± 0.80^{a}	7.21 ± 0.37^{a}	14.33 ± 3.23^{a}
	6	19.31 ± 0.72^{a}	12.40 ± 0.76^{b}	53.31 ± 4.31^{b}
F60	0	5.36 ± 0.92^{a}	1.10 ± 0.69^{a}	5.78 ± 0.14^{a}
	3	8.38 ± 0.29^{a}	8.43 ± 0.20^{a}	17.19 ± 0.96^{c}
	6	8.26 ± 0.02^{c}	16.16 ± 0.49^{a}	21.16 ± 0.61^{c}

Data are the average of three replicates ± standard deviation.
Different letters in the same day of treatment indicate significant differences ($P < 0.05$).

7.7.1 Effect on Protease activity

It is known that the characteristic flavors and aromas of cocoa are generated by the effect of Maillard reactions between flavor precursors previously generated in fermentation. In proteins, these precursors are derived from 7S vicilins by the coordinated action between aspartic endoprotease and carboxypeptidase with optimal pH of 3.5 and 5.8, respectively. The results of the universal activity of proteases in the control fermentations, 40 and 60, where an increase in the enzymatic activity is observed both in the control fermentation and in the F40 fermentation, which began with an activity of 3.36 ± 1.12 and 3.87 ± 1.00 (UAE/mg protein) and ended with values of 16.31 ± 2.15 and 19.31 ± 0.72 respectively, while it was in the F60

treatment where the value, although it increased in the middle of the process, decreased towards the end with an activity of 8.26 ± 0.02 (UAE/mg protein).

In past years, studies have been carried out to determine the activity of proteases, specifically, aspartic endoprotease increases up to 116% of its initial value at 72 hours of fermentation, as well as inactive at 144 hours. For its part, it has been observed that carboxypeptidase increases up to 157% of its original value at 96 hours, this due to the change in pH in the cotyledon (Amin, Jinap, and Jamilah, 1998). The enzymatic activity is directly proportional to the protein decrease.

For control fermentation, the proteolytic activity increases by 485% in general, without specifying the activity of a specific enzyme. The F40 and F60 treatments show values that are far from the end of the fermentation, for its part, the F40 treatment has higher enzymatic activity, even higher than the control, where it is assumed that the microenvironmental conditions for the development and activity of the proteolytic enzymes were adequate, also some studies have demonstrated that temperatures between 30 ºC to 40 ºC is where proteases shown higher activity in comparison with 50 ºC to 60 ºC (Brito, Pezoa & Amancio, 2004) while the F60 treatment maintains the units close to the value it had at 72 hours, plus a much higher value in FAN, assuming that the activity present at the end of the F60 treatment corresponds to the highly active carboxypeptidase.

7.7.2 Lipase

The activity of lipases in cocoa fermentation has been slightly studied since its presence is attributed to aerobic spore-forming bacteria, which have been found in concentrations close to 10^2 CFU / g and identified as *Bacillus spp. among which B. subtilis, B. cereus, B. pumilus*, *B. coagulans*, among others, stand out and their presence is less predictable as well as their importance is less understood in the fermentation process, however it is known that within the metabolic roles that it performs is capable of producing lipolytic enzymes, finding that in some batches is associated with off-odors produced from the generation of some free fatty acids (Running, Hayes & Ziegler, 2016).

Although little is known about the activity of lipases in the fermentation protocol, some data can be assumed with the findings of this experiment. An increase in its activity is observed during the three treatments (**Table 7**), being the Control treatment and the F60, those that present a greater activity with 16.36 ± 2.27 and 16.16 ± 0.49, respectively at the end of the fermentation. At the same time, F40 also increases until reach 12.40 ± 0.7.

There are some studies that support the inhibition of both lipases and alpha-amylases due to the effects of polyphenols (Gu, Hurst, Stuart, & Lambert, 2011), especially of procyanidins, which are part of the proanthocyanidins or condensed tannins present as 58% of the phenolic compounds in fresh cocoa, they are oligomeric compounds made up of catechin and epicatechin. The results of this study support that proanthocyanidins showed a stronger inhibitory activity against lipase by proanthocyanidins of different degrees of polymerization (2-10 especially those with major degree and concentration, from 1 to 10 μM proved, the near to 10 μM showed more inhibitions, also epicatechin (EC) was proved but its inhibition action result with less intensity.

This from the fact that it has been shown that the consumption of diets with cocoa extract of 1 3% (w / w) causes a decrease not only in body weight but also in glucose and total triglyceride levels in diabetic rats compared to fed animals by control (Ruzaidi, Amin, Nawalyah, Hamid, Faizul, 2005); others indicate that it re-reduces plasma glucose in diabetic rats after 60 to 90 min compared to untreated animals (Jalil, Ismail, Pei, Hamid, Kamaruddin, 2008). These reactions in living organisms are due to the inhibition of the behavior that enzymes must catalyze the hydrolysis of triglycerides to free fatty acids and of starch to maltose, being key to their absorption. In the same way, it may explain the low activity on lipids during fermentation, despite the presence of lipase enzymes found in the treatments.

7.7.3 α - Amylase

Amylase has been the subject of little study since, according to some authors (Redgwell et al., 2000) the enzymes cannot access the substrate, and the content of starch does not change significantly during fermentation and drying. These enzymes appear to be produced throughout the process as extracellular enzymes by fungi from *Aspergullus spp* strains (Gbolagade, Ajiboye, Asemoloye, Olawuyi & Babalola, 2016).

During the experimentation, a trend could be observed in the activity of alpha-amylase extracted from cocoa and tested on starch, where it was observed that it is in the F40 treatment where the highest presence and activity occurred with 53.31 +/- 4.31 μm maltose / min^1 at the end of fermentation since it has been reported that the effect of temperature on its activity is with a maximum of 37 ºC (Divakaran, Chandran & Pratap, 2011). The same reason why the lowest activity was reported for F60 21.16 +/- 0.61, followed by control with 43.41 +/- 3.31 at the end of the fermentations, as observed in **Table 7.**

Not only temperature is an important factor with respect to the activity of this enzyme, but also the presence of polyphenolic compounds such as proanthocyanidins, also known as condensed tannins, inhibit its activity within the cocoa bean. It has been observed that tannins of the degree of polymerization less than 5 shows <15% inhibition against α amylase at a concentration of 100 µM, while those with degree of polymerization 10 can inhibit it up to 50% (Gu et al. 2011).

7.8 Image analysis

During the structural evaluation by images carried out by Image J, an increase in the crackled area at day six could be observed in all treatments, visually evident in **Figure 20** where it can be observed differences between the 3 treatments at day 6. The first image corresponds to the original color photo of the bean, the second is the image in 8-bit format with a threshold application where the cracks are identified, while the third image from left to right corresponds to the area quantification. The control has deep cracks, as well as a slightly brown tone to the periphery of the bean while the center remains pink. The bean of the F40 treatment does not have pronounced cracks, but some slight holes can be noticed at the periphery, however, its color is obviously purple, very similar to a bean on day 0. While the F60 treatment shows several cracks that lengthen in the cotyledons, its color tends to be orange, and both here and in the control treatment, the embryo is the same color as the cotyledons, while in F40 it is slightly lighter.

The changes are the most significant at a higher fermentation temperature, as seen in **Table 8**. where it can be observed that at the beginning all fermentations have significantly the same cracking. By the third day both control and F40 have been behaving in a similar way, while F60 has already gone a bit higher. This may be due to some variation between the degrees Celsius of the fermentation since until now the fermentations are the same. It is on day 6 that the significant difference is evident because control had a cracking percentage close to 40%, while F40 lagged with 32.24 ± 1.14, and F60 had the largest cracked cotyledon area with 48.96 ± 0.3.

Table 8. Cracking area percentage across fermentation treatments of *T. cacao*

	Cracking area (%)		
Treatment	Day 0	Day 3	Day 6
Control	2.83 ± 0.96 [a]	16.76 ± 0.40 [a]	39.70 ± 1.50[a]
F40	3.14 ± 0.16 [a]	17.05 ± 0.03 [a]	32.24 ± 1.14[b]
F60	3.07 ± 0.33 [a]	19.67 ± 0.12 [b]	48.96 ± 0.31[c]

Data are the average of three replicates ± standard deviation.
Different letters in the same column indicate significant differences ($P < 0.05$).

Cracking occurs mainly in response to temperature changes according to the ANOVA shown in point 7.10, supported by a study where a notable increase continues to be observed during the drying and roasting processes (de Brito, García, Gallão, Cortelazzo, Fevereiro, & Braga, 2001) in which temperature is the changing variable. This study with microscopic analysis has observed the presence of cells rich in polyphenols on day 0, as well as non-fermented cacao, where these compounds spread throughout the cotyledon and after 72 which is equivalent to reaching 50 °C.

All these microstructural changes are reflected in the macrostructure of the collapsed cotyledon, which have been slightly addressed or studied. Growers use this parameter to determine if a batch is fermented by choosing 10 beans at random, fermentation stops when 7 or more of these shows both a clearly marked crackle and a pinkish hue. However, there is no numerical parameter that determines how much crackle is evidence of good fermentation.

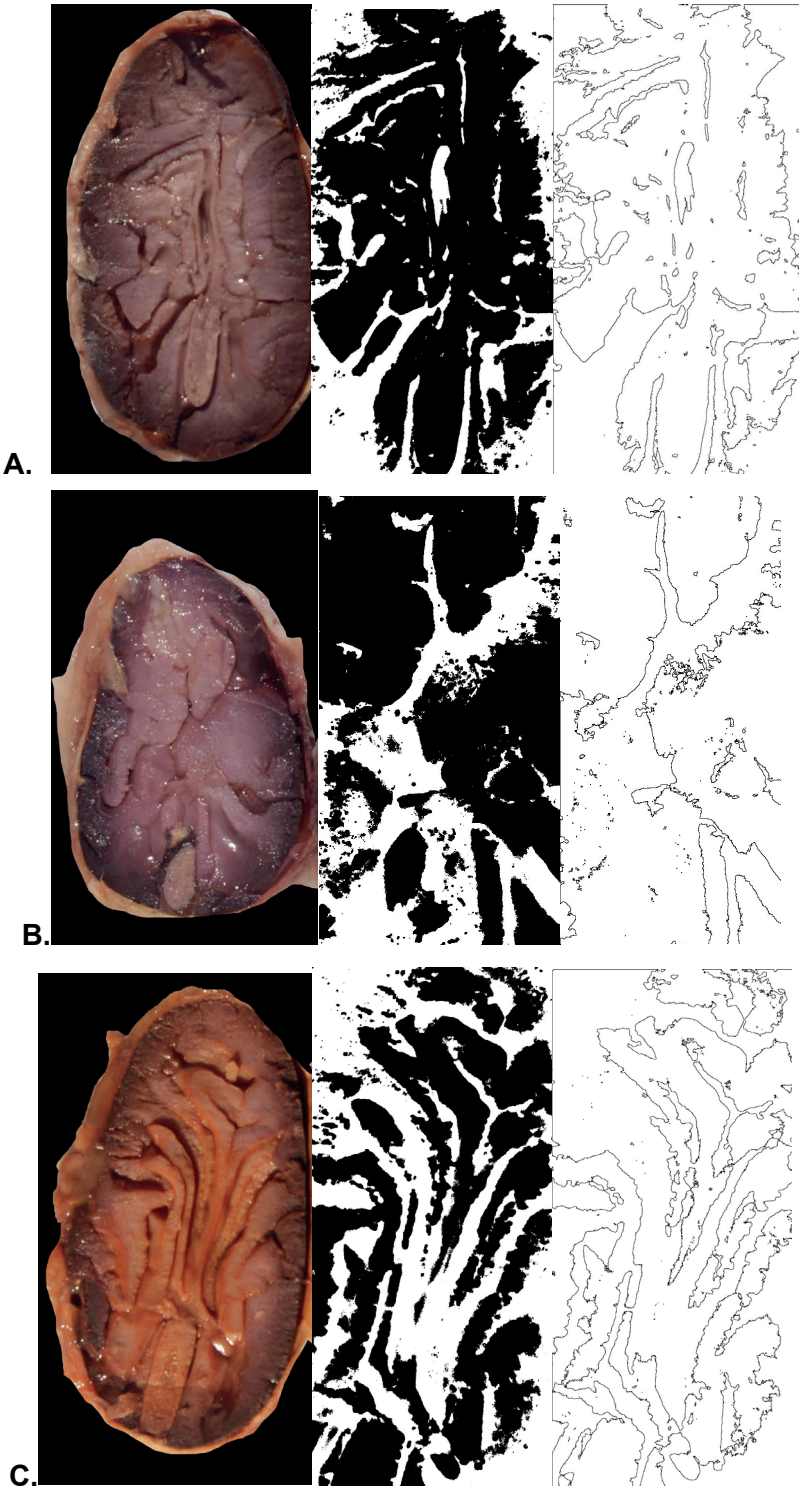

Figure 20. Images used for the analysis in Image J. From left to right, the image of the cotyledon, followed by the image under the effect of threshold and followed by the quantification of the area. Where a) Control fermentation, B) Fermentation at 40 ºC (F40) and c) Fermentation at 60 ºC (F60).

From the relationship between the percentage of cracking and the pH of the cotyledon, which is firmly determined, it can be assumed that a correctly fermented cocoa has a percentage of cracking of approximately 40%.

7.9 Colorimetry

As mentioned in the last paragraph, the color change is decisive to declare a bean as fermented. For this parameter, no Lab coordinates or any other magnitude reflect a specific data to be considered a control. Considering that the L * parameter goes from (+) white to (-) black, the a * from (+) red to (-) green and the b * from (+) yellow to (-) blue, it can be seen (**Table 9**) that in the control fermentation the parameter L* was closer to (+) white tones, while in the F40 treatment it was (-) darker. In the b* parameter that represents the yellow to blue tones, the control has (+)12.85, F40 (+) 03.65 while F60 (+) 24.38. While a* that gives the (+) red and (-) green, is in (+) 22.87 for control and (+)24.38 for F60 which have pink and orange tones respectively, while (+) 18.60 for F40.

The change in cotyledons in Control and F60, and the remaining purple tone in the F40 treatment is related to the reduction of 95% in the anthocyanin concentration. The pigments responsible for providing the color are known as 3-β-D-galactosyl- and 3-α-L-arabinosyl-cyanidins. In the fermentation process, these pigments undergo hydrolysis by glucosidases that is reflected in the whitening of the cotyledons (Afoakwa et al, 2012).

Table 9. Lab color parameters of cacao (*T. cacao L.*) in the fermentation treatments on day 0, 3 and 6

	Day 0	**Day 3**	**Day 6**
Control	**L* =** 27.28 **a*=** 27.31 **b*=** 08.31	**L* =** 27.92 **a*=** 17.22 **b*=** 03.52	**L* =** 45.86 **a*=** 22.87 **b*=** 12.85
F40	**L* =** 30.21 **a*=** 27.62 **b*=** 08.78	**L* =** 21.49 **a*=** 16.44 **b*=** 03.34	**L* =** 36.39 **a*=** 18.60 **b*=** 03.65
F60	**L* =** 34.22 **a*=** 25.99 **b*=** 11.39	**L* =** 50.92 **a*=** 19.00 **b*=** 09.32	**L* =** 50.71 **a*=** 25.94 **b*=** 24.38

7.10 Statistical Analysis

7.10.1 Pearson correlation

The Pearson correlation coefficient was used to determine the direction and strength of the linear relationship between the variables, to determine whether, at a given fermentation temperature, the increase of some variable measured such as humidity, lipids, proteins, carbohydrates, ash, FAN, polyphenols, antioxidant compounds, protease, lipase, or amylase activity are associated with decreases in some other.

The correlation value tends to vary between -1 and +1, while the greater the absolute value, the stronger the relationship between both variables. On the other hand, the sign indicates the direction of the relationship. If it is a positive sign, both variables request to increase or decrease at the same time. While if the sign is negative, one variable tends to increase while the other decreases.

Table 10. Set of variables with the highest correlation and significance (alpha = 0.05)

Treatment	Relation	r correlation
Control	Proteins - FAN	-0.905
Control	Proteins – Protease	-0.758
Control	FAN - Protease	0.963
Control	Proteins - Phenolic compounds	0.933
Control	Phenolic compounds - Antioxidant Activity	0.970
F40	Phenolic compounds - Antioxidant Activity	0.983
F40	Proteins - Phenolic compounds	0.936
F40	Proteins – Protease	-0.800
F40	FAN - Protease	0.977
F60	Proteins - FAN	-0.909
F60	Proteins - Phenolic compounds	0.968
F60	Proteins – Protease	-0.980
F60	Protease – FAN	0.967
F60	Phenolic compounds - Antioxidant activity	0.970

From the data obtained, the set of variables with the highest correlation and significance of the three treatments were taken in **Table 10** The relationship between the variables Protein - FAN had a strong correlation in the Control treatments -0.905 and F60 -0.909 but not in the F40, this highlights that when one of the variables as protein decreases the content of FAN tends to increase being the Control treatments and F60 (**Appendix B 1, 2 & 3**), the two with the highest content of FAN, also those with the lowest protein content on day 6 because, as mentioned, during the fermentation process the division of proteins into hydrophilic peptides and hydrophobic amino acid chains are from storage proteins (Rohsius, Matissek and Lieberei, 2005). However, in the F40 treatment this is not such a strong or significant relationship since the proportion of ANF is much lower with an increase of 325% versus 372% of F60.

This relationship continues in the variables Protein - Protease and FAN protease found in the 3 fermentations since the work carried out by protease is significant in the treatments. Other studies (Silveira, Melo, Souza, Bispo, & Soares, 2017) have supported that the protease activity in cotyledons varies with temperature, coinciding with the results shown in this experiment where the highest activity is shown in the F40 treatment as well as greater strength in its correlation.

Proteins also show a strong relationship with phenolic compounds during the three fermentations. This may be due to the molecular structure since they are the two main cell groups that contain the compounds to which the main changes occur (Lopez, Dimick, & Walsh, 1987) and no space is differentiated between them, so the lipid/protein cells contain protein and lipid bodies, and there are also larger cells that occur as a large vacuole where the polyphenolic material is stored. Both are released in response to high temperatures fermentation while the lipid bodies manage to maintain their structure more uniformly. Therefore, their correlations during the three fermentations are already positive at the three temperatures tested; in a greater or lesser proportion, the lipid / protein and polyphenolic cells are affected in parallel if the quantity of proteins decreases, also that of phenolic compounds.

The last of the relationships with greater significance have been that of Phenolic Compounds with Antioxidant Activity, marked with a positive sign in the three fermentations since the decrease in one of the variables also decreased the other. This is because they are the potential phenolic compounds for the elimination of free radicals, demonstrated in several studies (Mazor, Radojčić, Marković, Ivanec, & Delonga, 2011).

7.10.2 Principal Component Analysis

The experimental data were examined by PCA to visualize if the samples were grouped according to their differences between the results of their composition according to the variable temperature. As well as to observe which of the responses to be analyzed contributes to 64% of variation between the analyzed samples.

As shown in **Figure 21**. the three treatments are in different coordinates, in which phenolic compounds are found as the first component (**Figure 22**), followed by FAN. In contrast, Proteins is the second component, with the most variable results among the three treatments.

Subsequently, Phenolic Compounds and FAN were analyzed in a graph of main effects in **Figures 23** and **24**. It is clearly observed that the means for the three different treatments are significantly differentiated.

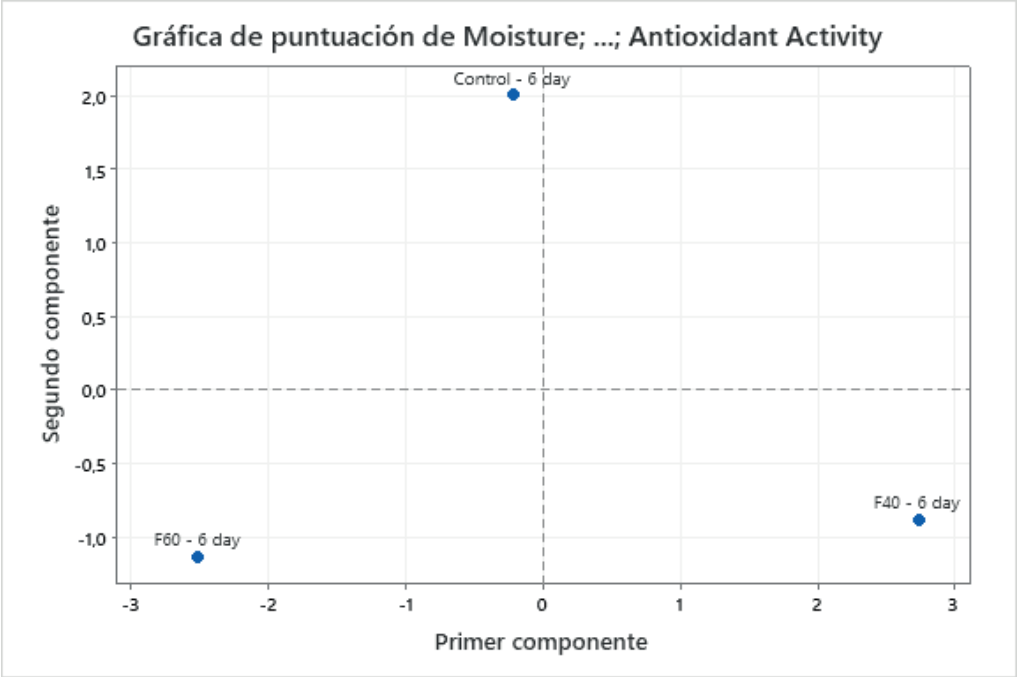

Figure 21. Score graph between the three control treatments, F40 and F60 at day 6 of fermentation

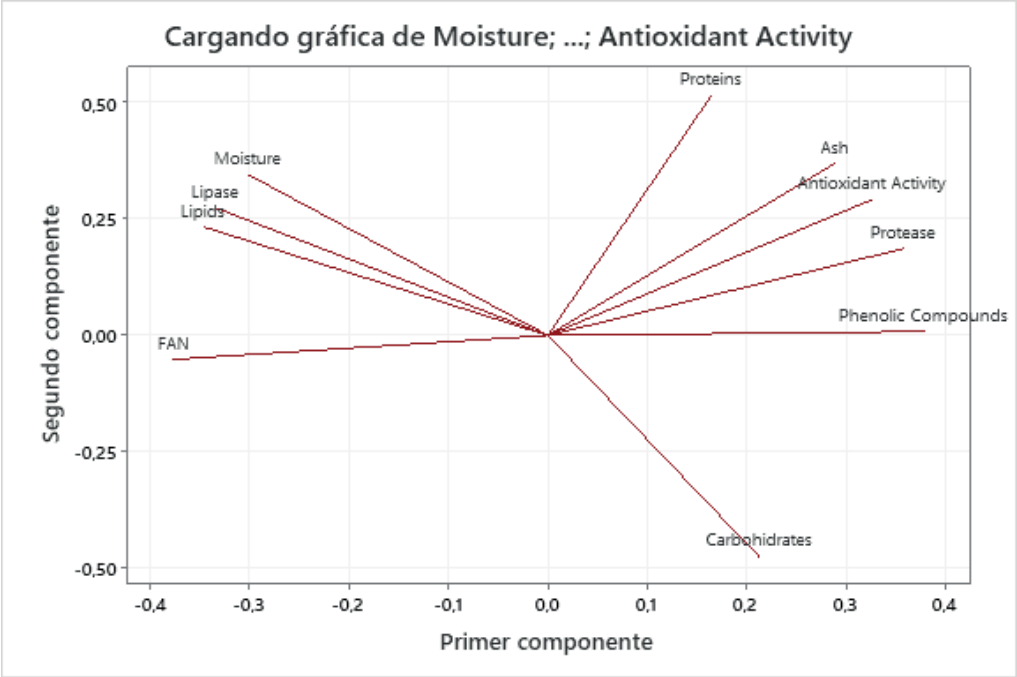

Figure 22. Main Components Chart of the control treatments, F40 and F60 at day 6of fermentation

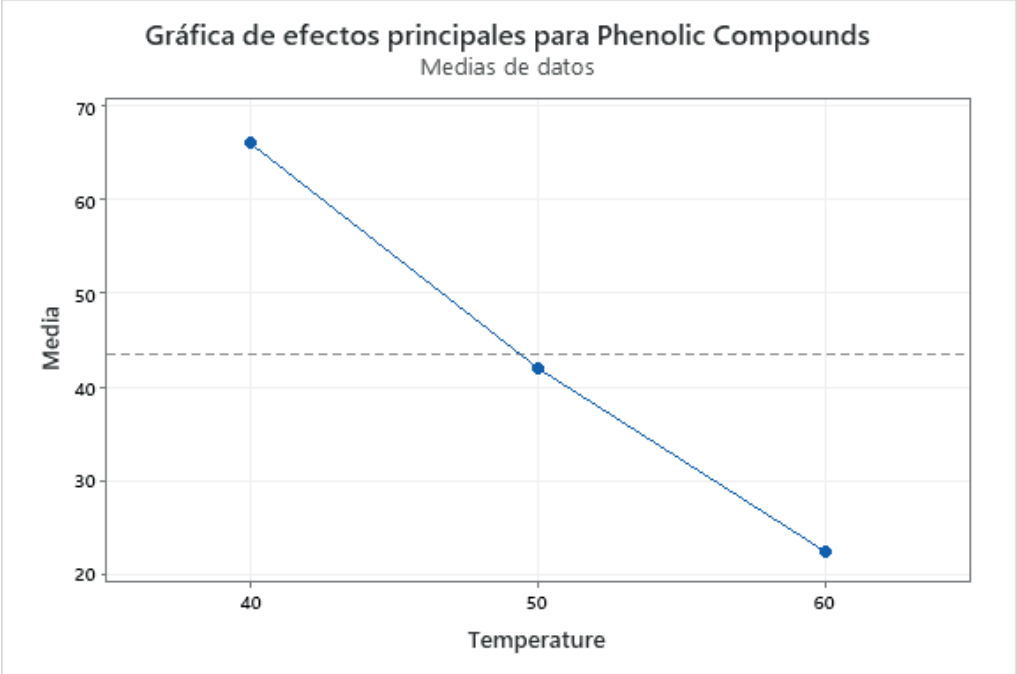

Figure 23. Graph of main effects for Phenolic Compounds of the three control treatments, F40 and F60 of fermentation

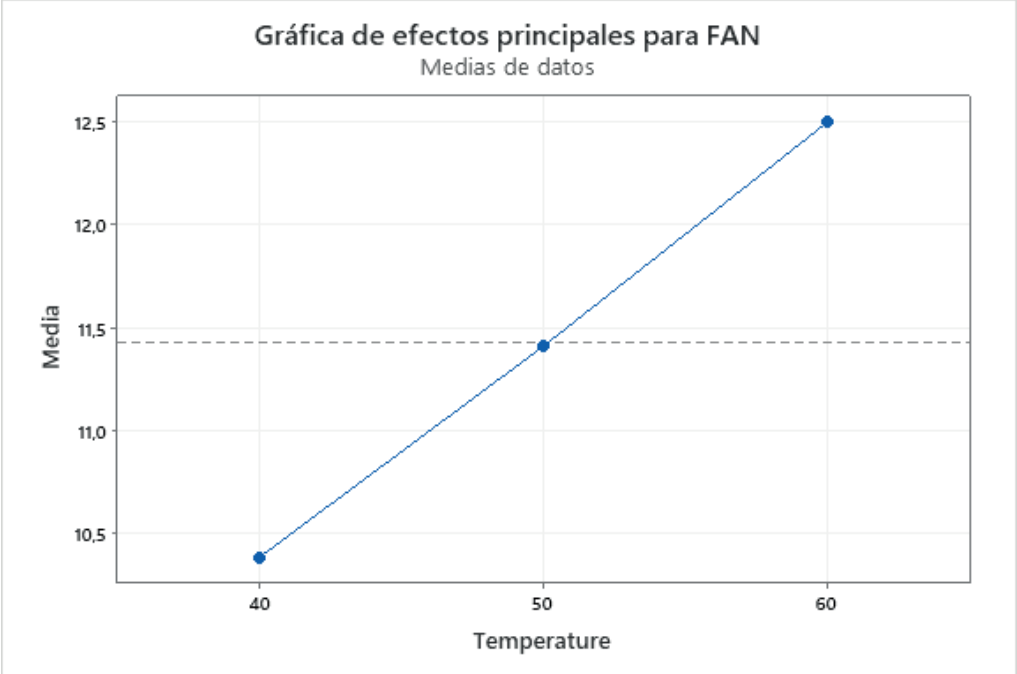

Figure 24. Graph of main effects for Free Amino Nitrogen of the three control treatments, F40 and F60

8 PROPOSALS FOR FUTURE RESEARCH

Based on the process carried out and the observations obtained, it is proposed to carry out a protein molecular weight profile that could allow to observe in more detail which and how many proteins are hydrolyzed throughout fermentation as well as those that remain unaltered. It is also recommended to do a more specific study on the activity of the proteases separately (amino endopeptidase and carboxypeptidase). Lastly, it would be of interest to evaluate the aromatic profile at different temperatures, with an emphasis on 60ºC since during the fermentations it showed intense odors during process.

9. CONCLUSIONS

Both the F40 and F60 treatments showed differences in the chemical, physical and structural behavior of the cacao bean compared to the control treatment. The most significant changes were observed on day 6, which means at the end of the fermentation. The most important change and from which variations in the internal reactions occurred has been the pH of the cotyledon where values of 5.7 and 5.0 were obtained for F40 and F60, in comparison with control with 5.3. The variation in the hydrogen potential is due to the amount of acetic acid that has been able to enter the cotyledons.

From the internal microenvironment of the grain, the proteins decreased 15% for the F60 treatment, due to the high participation of the proteolytic enzyme in this treatment, where the highest amount of free amino nitrogen compounds produced was also observed, assuming that the activity of the carboxypeptidase was the most present. On the contrary, in the F40 treatment, the protein content decreased only 7% in the same way that the lower FAN content was observed, assuming a more optimal activity for the amino endoprotease.

The changes in the phenolic compounds were significant for the treatments since the control treatment decreased by 76%, while F40 by 61% and F60 by 87% being the most affected by the thermosensitivity of the compounds, being parallel to the decrease in the antioxidant activity provided by them where control lost 75%, F40 lost 73% and F60 lost 88%, assuming not only the loss due to temperature but also due to the combination of phenolic compounds with amino acids to form compound tannins or proanthocyanidins.

It is these proanthocyanidins that have an inhibitory effect against lipases and amylases. The lipases showed the highest activity for the control treatment, followed by F60 and with less activity for F40, while the alpha amylase enzyme showed the highest activity for the F40 conditions, probably due to its optimal growth conditions. However, despite the activity of the lipases that turned out to vary, the lipids remained minimally altered without a significant deference in the fatty acid profile with a concentration of oleic acid close to 40%, followed by stearic acid close to 38% and palmitic acid about 10% for the three treatments.

These chemical changes visually led to a structural change in the cotyledon and the color of the cocoa bean with a percentage of cracking area referring to the degree of fermentation where 39% was obtained for control, 32% for F40 and 49% for F60, from which it can be assumed that in conjunction with the analyzed colorimetry, a fermented cocoa has around 40% cracked area.

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Appendix A

ABBREVIATIONS

(w/v)	
µL	Microliter
µmol	micromole
(µm glycerol/min^{1})	Glycerol micromole per minute
(µm maltose/min^{1})	Maltose micromole per minute
°C/min	Celsius per minute
AA	Amino acids
AAB	Acid Acetic Bacteria
AACC	American Association for Clinical Chemistry
AOAC	Association of Official Agricultural Chemists
C°	Celsius
Ca	Calcium
CFU/g	Colony-forming unit per gram
Cm	Centimeter
co2	Carbon Dioxide
DG	Degree of Polymerization
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAME	Fatty Acid Methyl Ester
FAN	Free Amino Nitrogen
Fe	Iron
G	Gram
g/kg	Gram per kilogram
GA	Gallic Acid
GC	Gas Comatography
H	Hours
HCl	Chloric Acid
HDL	High Density Lipoproteins

He	Helium
kDa	Kilodanton
Kg	Kilogram
LAB	Lactic Acid Bacteria
Lab	?
LDL	Low Density Lipoprotein
M	Meter
Mg	Magnesium
mg/L	Milligram per liter
mg/ml	Milligram per milliliter
Ml	Milliliter
ml/min	Milliliter per minute
mmol	Millimole
Mn	Magnesium
MΩ	Mega Ohmio
N	Nitrogen
Na	Sodium
Nm	Nanometer
nM	Nanomole
PCA	Principal Component Analysis
pH	Hydrogen Potential
PPO	Polyphenoloxidase
Rpm	Revolutions per minute
T	Temperature
(UAE/mg protein)	Urinary Protein Excretion per milligram of protein

Appendix B

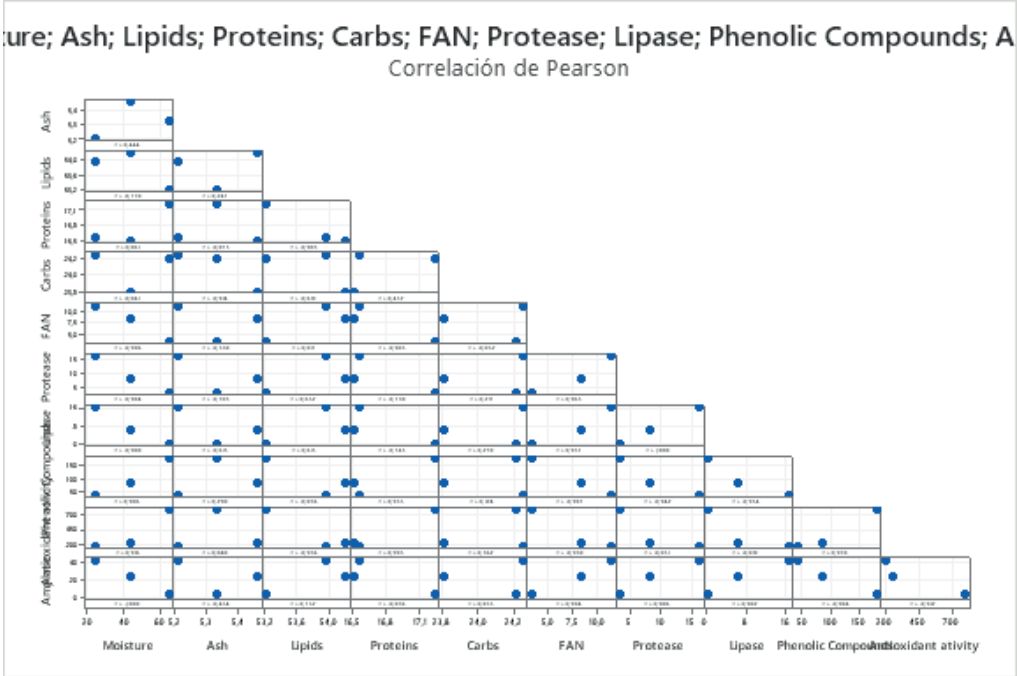

Figure 1. Pearson's correlations between control fermentation variables.

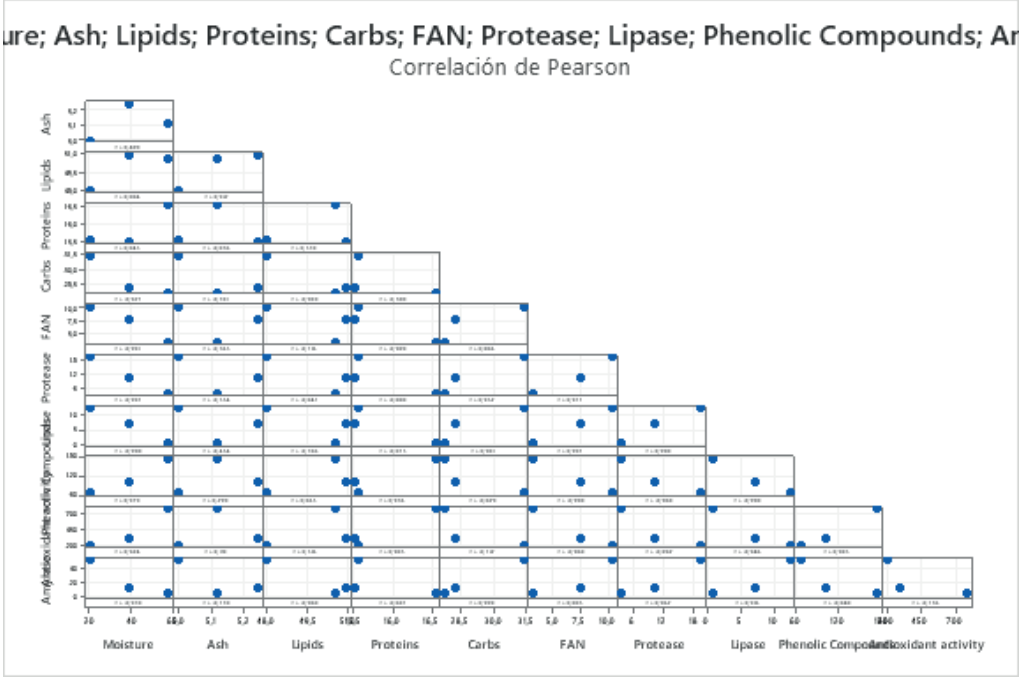

Figure 2. Pearson's correlations between F40 fermentation variables.

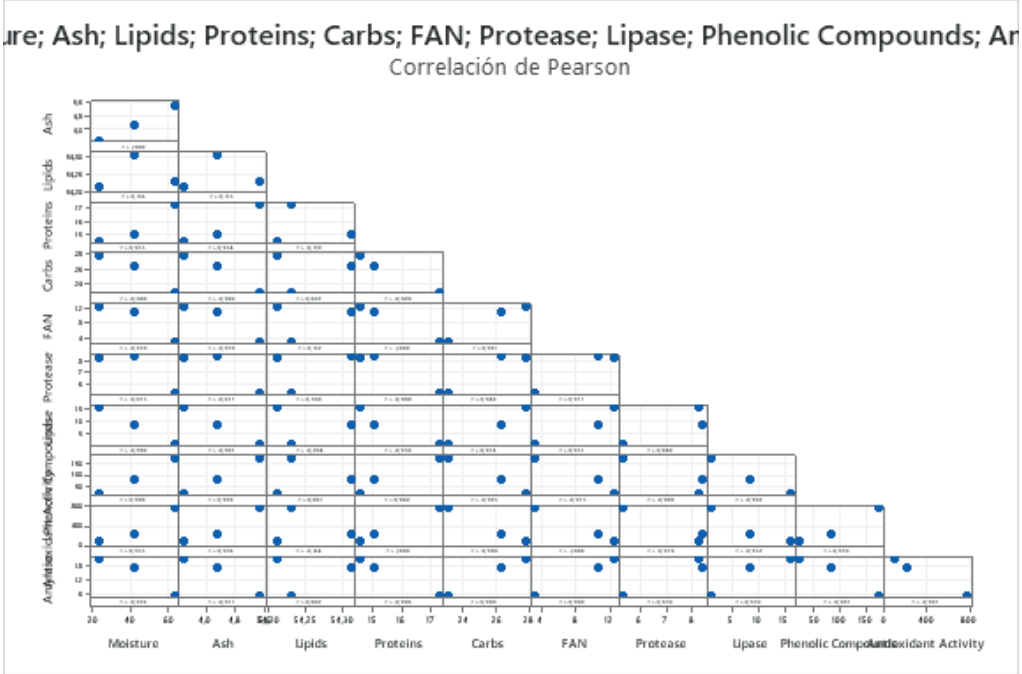

Figure 3. Pearson's correlations between F60 fermentation variables.

Curriculum Vitae

Karla María Guillén Guerrero, was born in Querétaro, Querétato Mexico, on December 29, 1994. She learned about food chemistry studying the degree of Engineering in Food Industries at the *Instituto Tecnológico y de Estudios Superiores de Monterrey*, Campus Querétaro where she graduated in May 2019. She did internships in some food production industries throughout this time. On August 2019, she returns to ITESM Qro to study the master's degree in Biotechnology with a specialty in Food - Cacao. In April 2021 he participated in the Functional Food Center congress with the work "Effect of processing conditions on physical-chemical and structural characteristics of cocoa (*Theobroma cacao L.*)" She is also passionate about chocolate, electronic music concerts, swimming, and pottery.