

# USE OF ENZYMES AND LEAVENING AGENTS AS A STRATEGY TO REDUCE THE PRESENCE OF CADMIUM IN THE FERMENTATION PROCESS OF *Theobroma cacao* L. ALMONDS

Jaime Fabián Vera Chang<sup>1</sup>, Rocio Natividad Morejon Lucio<sup>2</sup>, Iván Patricio Salgado Tello<sup>3</sup>, Cesar Iván Flores Mancheno<sup>4</sup>, Mauricio Renato Morejón Centeno<sup>5</sup>

<sup>1</sup>Faculty of Industry and Production. Food Engineering, Universidad Técnica Estatal de Quevedo, Campus Experimental La María Km, 7 vía Empalme, Los Ríos Ecuador; Doctoral candidate Universidad Americana de Europa. Cancún - México

<sup>2,3,5</sup>Faculty of Livestock Sciences School and Institute of Postgraduate and Continuing Education Master's Degree in Agroindustry, mention in quality management and food safety, Escuela Superior Polytechnical de Chimborazo, Panamericana Sur km 1 1/2, Riobamba, Ecuador.

<sup>4</sup>Institute of Pharmacy and Food of the University of Havana Cuba

Email: jverac@uteq.edu.ec

## Abstract

Ecuador is one of the leading producers of fine aroma cocoa worldwide. In 2019, Ecuador became the leading exporter of cocoa beans in the Americas and ranked fourth worldwide. The objective of this research was to use enzymes and yeasts as a strategy to decrease cadmium content during the fermentation of cocoa (*Theobroma cacao* L.). A completely randomized design with a bifactorial arrangement was used, where factor A corresponds to fermentation time (48, 96, and 120 hours); and factor B measured the type of starter culture (fermentation; fermentation + mother yeast; fermentation + mother yeast + PPO). A total of nine treatments were evaluated and treatments T1, T2, and T3 were the controls. Tukey's test was used with a  $p < 0.05$  to find differences between treatment means. Treatment T9 presented the highest fermentation percentage, with 89.33% in the cut-off test. T5 reached 5.48% moisture, and T7 presented 3.54% ash and 7.42 g.kg<sup>-1</sup> of glucose. At the same time, T2 registered 14.99% protein. Of the AxB interaction, T3 presented the lowest cadmium content 0.20 mg.kg<sup>-1</sup>, in the cocoa beans. In the sensory evaluation, there were no differences between treatments. In conclusion, the increase in fermentation time and the addition of yeast and enzymes decreased cadmium content in dry cocoa beans. Likewise, for the main effect of fermentation time, the lowest cadmium content was achieved when cocoa beans were fermented for 120 hours.

**Keywords:** Cadmium(Cd); Fermentation; Enzymes; Brewer's yeast (*Saccharomyces cerevisiae*); Cocoa almonds.

## INTRODUCTION

Ecuador is one of the leading producers of fine aroma cocoa worldwide, in 2018, it exported 315 metric tons; in 2019, Ecuador became the main exporter of cocoa beans in America and reached fourth place worldwide. In 2018, exports of dried almonds represented 93.67% and 6.33% of semi-finished products, of which the leading destinations for these products were the European Union and the United States (Anecacao, 2019; Borja et al., 2021).

The export of cocoa leaves is threatened due to the presence of high levels of cadmium in cocoa beans. Currently, the Codex Alimentarius establishes maximum cadmium levels for: Semi-sweet chocolate 0.3 mg.kg<sup>-1</sup>; bitter chocolate 0.8 mg.kg<sup>-1</sup> (Codex Alimentarius, 2018, pp. 46-68).

**Address for correspondence: Jaime Fabián Vera Chang**  
Doctoral candidate Universidad Americana de Europa. Cancún - México.  
Email: jverac@uteq.edu.ec

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While the European Union (EU) General Regulation established for both chocolate and cocoa sold to final consumers a level of 0.1 mg.kg<sup>-1</sup> and 0.8 mg.kg<sup>-1</sup> for concentrations of 30 and 70% cocoa mass, respectively (European Commission, 2014, p.78).

Research conducted in several areas of Ecuador has identified cocoa beans with high cadmium content and exceeding the permitted limit. For example, in the province of Los Ríos Mite et al. (2010, p.14), the province of Los Ríos, minimum and maximum values of 0.23 and 1.23 mg.kg<sup>-1</sup> of cadmium were found in samples of cocoa beans. Therefore, this situation generates excellent concern in the public and private sectors since it represents a problem for the entry of the product into the European market (Huamani and Huauya, 2018, p.21).

The presence of heavy metals in Ecuador, specific cadmium, raises the need to investigate techniques that allow its reduction through the addition of acid media, draining of almonds, types of fermenters, fermentation methods, types of drying, and application of chelating agents (Cedeño, 2020, p.13). In addition, the use of microorganisms has also been proposed due to their ability to absorb heavy metals such as cadmium in their cell walls thanks to peptidoglycans (Falcon, 2019, p.32).

The presence of cadmium in certain cocoa production areas in Ecuador raises the need to investigate techniques that allow the reduction of cadmium through the addition of acid media, draining of the beans, types of fermenters, fermentation methods, drying methods, and application of chelating agents. (Cedeño, 2020, p.13).

Many cocoa producers are vulnerable to the new regulations set by the European Union. Chocolate must contain a maximum of 0.3 mg.kg<sup>-1</sup> cadmium (Meter et al., 2019, p.1). For this reason, there is an urgent need to provide short, medium, and long-term solutions to reduce the problem.

Because cadmium can be transported and accumulated in cocoa beans, there is a significant risk to health due to ingesting food contaminated with cadmium. Once cadmium is absorbed it accumulates in the body for long periods. Depending on the amount, time, and type of exposure, cadmium can produce a series of disorders, including cancer. It can also damage organs such as the liver, kidney, lung, bones, placenta, and testicles (Alvarez, 2021; Martínez et al., 2013; Pérez and Azcona, 2012). This is why cadmium contamination in human food has generated significant concern worldwide.

Cadmium can produce a series of disorders, including cancer; it can also damage organs such as the liver, kidney, lung, bones, placenta, and testicles. (Alvarez, 2021; Martínez et al., 2013; Pérez and Azcona, 2012). Once cadmium is absorbed it accumulates in the body for a long time. This is why cadmium contamination in human food has generated significant concern worldwide. Cocoa plants can transport cadmium from the soil and accumulate it mainly in the almonds, which poses a significant health risk

by ingesting chocolate from almonds with high levels of cadmium.

Fermentation of cocoa beans is the biochemical process capable of transforming and eliminating macro and microelements from inside the cotyledon to the outside. Although yeasts play a fundamental role in many industrial processes, *Saccharomyces cerevisiae* can sequester cadmium in its cell walls and consequently decrease the concentration of cadmium in cocoa beans (Falcon 2019, p.32).

Polyphenol oxidases (PPOs) are essential in the agri-food industry and influence product quality (Morante y Agnieszka, 2017; Narro et al., 2003; Vázquez et al., 2016). Therefore, the objective was to evaluate the use of enzymes and yeasts as a strategy to reduce the presence of cadmium in the fermentation process of cocoa beans (*Theobroma cacao* L.).

Cadmium occurs naturally in the environment in small concentrations. (Llatance et al., 2018; Mite et al., 2010). This metal cannot be degraded or destroyed; physical and chemical agents can dissolve it. The cocoa plant absorbs the cadmium in the soil and concentrates it in the almonds, which are then incorporated into the food chain and can accumulate and alter human metabolism. (Piedraita et al., 2018, pp.13-14).

Other research has shown that during cocoa fermentation, biochemical processes occur that allow the transformation and elimination of organic substances from the cotyledon to the exterior (Lares et al., 2013, pp.41-43). In addition, it is known that microorganisms such as yeasts can sequester cadmium through their cell walls, thus decreasing the cadmium content of cocoa beans.

Currently, areas with high cadmium content in Ecuador have already been identified to organize, train, implement and manage cocoa production (Barraza, Schreck, Lévêque, et al., 2017; Sánchez, 2019; Zambrano, 2018). It has been shown that increasing the microbial population during cocoa fermentation is a different and innovative alternative to reducing cadmium content, which can be transmitted and replicated by producers, thus contributing to scientific and technological exchange (Intriago et al., 2019 pp.18-21 ).

Cadmium is a toxic heavy metal that is quickly mobilized and accumulates in different organs, causing severe damage to the consumer's health (CAOBISCO/AEC/FCC, 2015; Pavicich, 2019). The worldwide concern is increasing in the cocoa trade chain, specifically in the producing countries of Latin America and the Caribbean, due to the presence of cadmium in cocoa beans and its direct transfer to chocolate and cocoa derivatives. The finding of high levels of cadmium in cocoa beans of Ecuadorian origin will reduce the export of cocoa to the international market. (Intriago et al., 2019, p.18).

Foreign markets, specifically the European Union, have set maximum limits for cadmium in almonds and cocoa products. The lack of training and knowledge of new cocoa production technologies keeps producers adapting to conventional forms,

which affects their economy. Faced with this problem, Ecuador needs to implement several strategies to reduce the mobility and accumulation of cadmium from production to industrialization of cocoa.

Likewise, the addition of the enzyme polyphenol oxidase is used to act in the initial phase of fermentation and help break down glycoproteins, forming peptides and sugars, which allows more time for yeast action (Diaz et al., 2012, p.5). Thus, it is proposed that cocoa fermentation directly affects the cadmium reduction in cocoa beans. Therefore, this research is based on the need to improve the fermentation phase with the use of polyphenol oxidase enzymes and mother yeast that could reduce the presence of cadmium and improve the quality of the product and increase the income of producers. Therefore, the objective is to use enzymes and yeasts as a strategy to reduce the presence of cadmium in the fermentation process of *Theobroma cacao* L. almonds, which will generate a relevant scientific contribution to the problem and will allow establishing solutions to the cadmium content in cocoa and its derivatives for national consumption and export.

## MATERIALS AND METHODS

### Location

The cocoa samples were taken from the Experimental Farm "La Represa" of the State Technical University of Quevedo (UTEQ), province of Los Ríos. The geographical location is 1° 03' 18" South latitude and 79° 25' 24" West longitude and an altitude of 90 masl. The average annual temperature is 26°C and the average annual precipitation is 3229.3 mm/year. Laboratory analyses were carried out at the Bromatology Laboratory of the Faculty of Livestock Sciences of the Polytechnic School of Chimborazo. The cocoa beans of the Nacional variety were harvested fresh and with mucilage from physiologically mature pods.

### Experimental Design

The experiment was carried out as a Completely Randomized Design (CRD) with a bifactorial arrangement A×B, where factor A corresponds to the fermentation time of the cocoa beans (48, 96, and 120 hours); while factor B evaluated the type of starter culture (fermentation; fermentation + mother yeast (*Saccharomyces cerevisiae*); fermentation + mother yeast (*Saccharomyces cerevisiae*) + PPO). A total of nine treatments were evaluated; treatments T1, T2, and T3 corresponded to the control, in which only fermentation was carried out at different times. Each treatment had three replicates for a total of 27 experimental units. The Tukey test was used to determine differences between treatment means with a significance level of  $p < 0.05$ .

The experimental unit is represented by a wooden microfermenter box (16 × 29 × 16 cm), where 2 kg of cocoa beans were placed and the corresponding treatments were

randomly added for the fermentation process (Intriago et al., 2019). After 24 hours, the temperature was taken and 0.2 % *Saccharomyces cerevisiae* yeast and 10 % banana puree (polyphenol oxidases) were added to each experimental unit according to the characteristics of each treatment. The microfermenters were then covered with banana leaves to create an anaerobic environment and initiate the fermentation phase. The fermenting mass was stirred every 24 hours after 48 hours to ensure homogeneous fermentation. Subsequently, the almonds were air-dried until they reached approximately 7 % humidity. (Guevara 2017).

### Variables evaluated

The following variables were evaluated for this research:

Shear test: Reference Standard NTE INEN 176.

Temperature: It was recorded at 24, 48, 96, and 120 hours after the beginning of the fermentation process of the cocoa beans with a digital thermometer.

Fermentation time: 48, 96, and 120 hours, a stopwatch was used.

Humidity: Reference Standard NTE INEN 173.

Ash: According to No. 972.15.

Protein: It was determined according to the Kjeldahl method. Reference AOAC, 970.20.

pH: Reference No. 970.22.

Titrate acidity: Titration method with NaOH 01N.

Fermentable sugars: Acid hydrolysis was carried out in 125 mL Erlenmeyer flasks containing 10 g of crushed cocoa beans and 50 mL of H<sub>2</sub>SO<sub>4</sub> at concentrations of 0.5, 1, 2, and 3 N. The flasks were incubated at 30 °C at 150 rpm on an orbital shaker for 48 h. The concentration of total sugars was determined by the sulfuric phenol method (Dubois 1956). The concentration of total sugars was determined by the sulfuric phenol method (Dubois 1956).

Cadmium: According to AA (Flame) method PEE/B/09.

### Sensory analysis

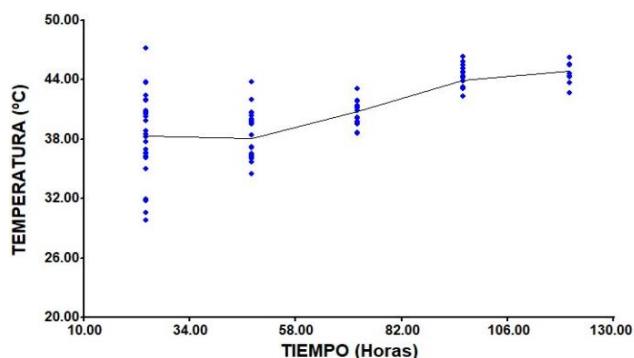
For the sensory analysis, the cocoa paste was prepared. Then, the sensory evaluation was carried out for each treatment, and the attributes associated with flavor and aroma were quantified: caramel, cocoa, bitter, nutty, floral, fruity, acid, and astringent. A hedonic scale of 0-10 was applied to quantify these variables, where 0 is absent, 1 and 2 are low, 3 and 5 are medium, 6 to 8 are high, and 9 to 10 are very high. (Solórzano et al., 2015, p.41).

## RESULTS AND DISCUSSION

The temperature of cocoa beans during fermentation

Figure 1 shows a positive third-degree polynomial model that explains 51 % of the temperature variability during the 24, 48, 72, 96, and 120 hours of fermentation ( $p < 0.05$ ). In addition, the proposed model does not present a lack of fit ( $p =$

0.2356). The results reflect a regular increase in temperature during fermentation.



**Figure 1.** Average values of regression analysis between fermentation time and temperature.

The pH of cocoa beans during fermentation

Table 2 shows significant differences between the treatments evaluated for the pH value in the fermentation of cocoa beans ( $p < 0.05$ ). T7 (48 h fermentation + mother yeast + PPO) presented the highest pH value of 5.26 at 48 hours, while T9 (120 h fermentation + mother yeast + PPO) obtained the lowest pH value of 4.15 at 120 h during the fermentation process. In all treatments, it was observed that

pH decreased as fermentation time elapsed (Table 2).

The acidity of cocoa beans during fermentation

In Table 2 there were significant differences in the cocoa beans' acidity percentage in the analyzed treatments ( $p < 0.05$ ). The highest percentage of acidity was identified in T8 (96 h fermentation + mother yeast + PPO) with 0.88 %. Acidity in cocoa beans increases due to the effect of fermentation. (Lares et al. 2013; Teneda 2016; Chávez 2020). In addition, a decrease in the percentage of acidity was observed in the treatments with 120 h of fermentation, enzymes, and PPO (T6 and T9), as shown in Table 2.

During the 48 and 96 h of fermentation, the samples evaluated showed an increase in acidity due to acetic acid resulting from the oxidation of ethanol produced by acetic acid and lactic acid bacteria at the beginning of aerobic fermentation between 48 and 72 h. However, at 120 h of fermentation, the percentage of acidity decreased because acetic acid decreased progressively from the cocoa beans because of its volatilization. However, at 120 h of fermentation, the percentage of acidity decreased because acetic acid decreased progressively from the cocoa beans because of its volatilization.

**Table 2.** pH and acidity of almonds during fermentation.

Treatment	pH	Acidity (%)
T1 (48 hours)	5.09 ± 0.61 ab	0.49 ± 0.02 d
T2 (96 hours)	4.38 ± 0.06 bc	0.87 ± 0.06 a
T3 (120 hours)	4.17 ± 0.01 c	0.60 ± 0.06 bcd
T4 (48 hours)	5.10 ± 0.16 ab	0.51 ± 0.03 cd
T5 (96 hours)	4.53 ± 0.39 abc	0.64 ± 0.08 bc
T6 (120 hours)	4.42 ± 0.23 bc	0.53 ± 0.08 cd
T7 (48 hours)	5.26 ± 0.14 a	0.52 ± 0.03 cd
T8 (96 hours)	4.20 ± 0.02 c	0.88 ± 0.02 a
T9 (120 hours)	4.15 ± 0.08 c	0.71 ± 0.05 b
Average	4.59	0.64
CV (%)	5.76	7.95
p-value	<0.0001	<0.0001
Lower limit 0.05	4.40	0.58
Upper limit 0.05	4.78	0.70

Different letters indicate statistical significance according to Tukey's test ( $p < 0.05$ ).

Physicochemical characteristics of dried cocoa beans

Cutting test

Table 3 shows the means of treatment of the cutting test of fermented and dried national cocoa beans using enzymes

and yeast. According to the analysis of variance, there is a significant difference of  $p < 0.05$  in the good, violet, and slaty fermentation. For moldy and defective fermentation, there were no significant differences of  $p > 0.05$  among the samples analyzed. The data obtained in this research show that in the

fermentation process, the highest percentage of good almonds and the lowest percentage of violet almonds were

found in T9 [fermentation 120 h + mother yeast + PPO] with 89.33 and 6.67 %, respectively (Table 3).

**Table 3.** Cocoa kernel shear test.

Treatment	Good		Violet		Slate		Mushrooms		Faulty	
T1 (48 hours)	71.33	d	16.00	a	12.00	a	0.00	a	0.67	a
T2 (96 hours)	76.00	cd	14.00	abc	9.33	abc	0.00	a	0.67	a
T3 (120 hours)	80.67	abc	11.33	abc	7.33	abc	0.00	a	0.67	a
T4 (48 hours)	76.67	cd	12.00	abc	11.33	abc	0.00	a	0.00	a
T5 (96 hours)	82.67	abc	10.00	abc	6.67	abc	0.00	a	0.67	a
T6 (120 hours)	86.00	ab	8.00	bc	5.33	bc	0.00	a	0.67	a
T7 (48 hours)	77.33	cd	12.67	abc	9.33	abc	0.00	a	0.67	a
T8 (96 hours)	82.67	abc	12.67	abc	4.00	c	0.00	a	0.67	a
T9 (120 hours)	89.33	a	6.00	c	3.33	c	0.67	a	0.67	a
Average	80.30		11.41		7.63		0.07		0.59	
CV (%)	3.36		24.10		28.09		519.6 2		183.7 1	
p-value	<0.000 1		0.010 3		0.000 6		0.469 0		0.997 3	
Lower limit 0.05	78.01		9.94		6.26		0.08		0.22	
Upper limit 0.05	82.58		12.88		9.00		0.23		0.96	

Different letters indicate statistical significance according to Tukey's test ( $p < 0.05$ ).

#### Humidity

Table 4 shows the moisture content of the almonds, which did not show significant differences with a  $p > 0.05$  among the treatments analyzed. However, T5 had the lowest moisture content,  $5.48 \% \pm 0.13$ . It is worth mentioning that all the samples were within a range of less than 7 %.

#### Ash

The ash content in the dry cocoa beans showed significant differences with a value of  $p < 0.05$ , the highest percentage of ash was found in T7 (48h fermentation + mother yeast + PPO) with  $3.54 \pm 0.15$  %, while T3 (120 h fermentation), obtained the lowest value of  $2.76 \pm 0.09$  %.

#### Total sugars (glucose g/kg)

Table 4 shows the average glucose values of the cocoa beans, and there are significant differences with a  $p < 0.05$ . T7 (48 h fermentation + mother yeast + PPO) presented the highest glucose content with  $7.42 \text{ g.kg}^{-1}$  and T5 (96 h fermentation and mother yeast) presented the lowest content with  $4.67 \text{ g.kg}^{-1}$  of glucose. This is because the yeasts can remain in the stationary phase after 24 h of fermentation. On the other hand, it is observed that the treatments with 48 h of

fermentation present the highest glucose content in the Nacional cocoa beans, and as the fermentation process progresses, glucose decreases until 120 h (Table 4). Finally, it is worth mentioning that T6 (120 h fermentation and yeast) presented  $5.55 \text{ g.kg}^{-1}$  of glucose.

#### pH

As shown in Table 4, there are significant differences with  $p < 0.05$ . T1 (control 1) presented the highest pH value with  $6.02 \pm 0.08$  compared to T3 (120 h fermentation control 3), which presented the lowest pH value with  $5.49 \pm 0.15$ . It is worth mentioning that the INEN Standard does not specify the pH measurement, although the presence of acidity favors the presence of acetic compounds during fermentation.

#### Acidity

Table 4 shows significant differences with a  $p < 0.05$  between the acidity values in the cocoa beans. T1 (48 h fermentation control 1) presented the lowest percentage of acidity with  $0.44 \% \pm 0.03$ , compared to T8 (96 h fermentation + yeast + PPO), which presented the highest percentage of acidity with  $0.50 \% \pm 0.03$ .

Protein

As shown in Table 4, there were significant differences with a  $p < 0.05$  between the treatments under study. T2 presented the highest percentage of crude protein with  $14.99 \% \pm 0.26$  and differs from T8 (96 h fermentation + mother yeast + PPO), which registered the lowest percentage of  $10.66 \% \pm 0.86$ .

Cadmium

According to the analysis of variance (ADEVA), the cadmium content showed that there were highly significant differences for the fermentation time (Factor A), there were also significant differences for the interaction A x B, and in the application of yeast and enzymes, there was no significant difference. As a result of this research, the lowest cadmium content was when the cocoa beans were fermented for 120 hours (Figure 2). In this research, the cadmium contents in the analyzed samples do not exceed the permissible limits of the European Commission Regulation (2014), establishing a maximum cadmium content of  $0.8 \text{ mg.k}^{-1}$ . Therefore, it is adduced that T3 (fermentation 120 h control 3) is the best treatment, followed by T9, T8, and T6 for presenting the lowest averages ( $0.21 \pm 0.01$ ) in cadmium content.

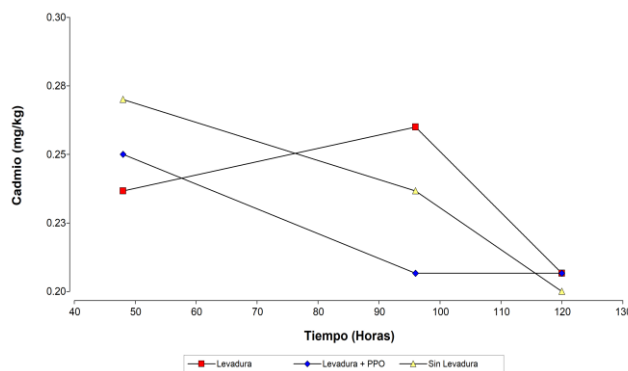


Figure 2 Interaction between yeast and enzyme fermentation times.

Source: Morejón Lucio Rocio (2021).

Sensory analysis of cocoa paste

No significant differences exist between the treatments analyzed in the results (Table 5) of the primary and specific flavors. According to the hedonic scale of the descriptive test, the cocoa flavor profile, the highest intensity was identified as 8.07 in T5. According to Table 5, the cocoa flavor presented greater intensity in all treatments for the specific flavors. T2 (fermentation 96 h control 2) stood out in the floral flavor with medium intensity. In the fruit profile, T7 (48 h fermentation + yeast + PPO) stood out with a medium scale. The nutty flavor stood out in T3 (fermentation 120 h control 3). The caramel flavor was more intense in T2 (fermentation 96 h control 2). The differences in fermentation quality explain the links between cocoa flavor, floral, fruity, nutty, and caramel flavor.

Table 4. Physicochemical analysis of cocoa beans dry.

Treatment	Humidity (%)	Ash (%)	Glucose (g.kg <sup>-1</sup> )	Cadmium (mg.kg <sup>-1</sup> )	pH	Acidity (%)	Protein (%)
T1 (48 hours)	5.80 ± 0.21 a	3.50 ± 0.18 ab	6.81 ± 0.28 abc	0.27 ± 0.02 a	6.02 ± 0.08 a	0.44 ± 0.03 b	14.66 ± 1.54 ab
T2 (96 hours)	5.73 ± 0.21 a	3.18 ± 0.15 abcd	6.47 ± 0.20 bc	0.24 ± 0.01 abc	5.65 ± 0.02 cd	0.45 ± 0.03 ab	14.99 ± 0.26 a
T3 (120 hours)	5.67 ± 0.07 a	2.76 ± 0.09 d	6.04 ± 0.28 cd	0.20 ± 0.00 c	5.49 ± 0.15 d	0.48 ± 0.03 ab	13.26 ± 0.12 abc
T4 (48 hours)	6.34 ± 0.99 a	3.11 ± 0.03 bcd	7.17 ± 0.07 ab	0.24 ± 0.03 abc	5.61 ± 0.06 cd	0.45 ± 0.01 ab	12.29 ± 0.40 bcd
T5 (96 hours)	5.48 ± 0.13 a	3.30 ± 0.15 ab	4.67 ± 0.38 e	0.26 ± 0.03 a	5.66 ± 0.07 cd	0.46 ± 0.01 ab	12.84 ± 1.64 abcd
T6 (120 hours)	5.64 ± 0.25 a	2.82 ± 0.09 cd	5.55 ± 0.22 d	0.21 ± 0.01 bc	5.76 ± 0.05 bc	0.48 ± 0.01 ab	12.11 ± 1.64 cd
T7 (48 hours)	5.82 ± 0.19 a	3.54 ± 0.15 a	7.42 ± 0.29 a	0.25 ± 0.01 ab	5.93 ± 0.01 ab	0.48 ± 0.01 ab	10.72 ± 0.22 d
T8 (96 hours)	5.98 ± 0.08 a	3.20 ± 0.23 abc	6.90 ± 0.01 abc	0.21 ± 0.01 bc	5.64 ± 0.01 cd	0.50 ± 0.03 a	10.66 ± 0.86 d
T9 (120 hours)	5.91 ± 0.17 a	2.77 ± 0.17 d	5.53 ± 0.58 d	0.21 ± 0.01 bc	5.63 ± 0.04 cd	0.48 ± 0.01 ab	12.73 ± 0.70 abcd

Average	5.82	3.13	6.28	0.23	5.71	0.47	12.70
CV (%)	6.35	4.68	4.81	6.74	1.20	3.92	6.73
p-value	0.2924	<0.0001	<0.0001	<0.0001	<0.0001	0.0069	<0.0001
Lower limit 0.05	5.67	3.01	5.93	0.22	5.64	0.46	12.06
Upper limit 0.05	5.97	3.25	6.64	0.24	5.78	0.48	13.33

Different letters indicate statistical significance according to Tukey’s test (p<0.05).

Sensory analysis of cocoa beans with the addition of polyphenol oxidase enzymes and mother yeast (*Saccharomyces cerevisiae*).

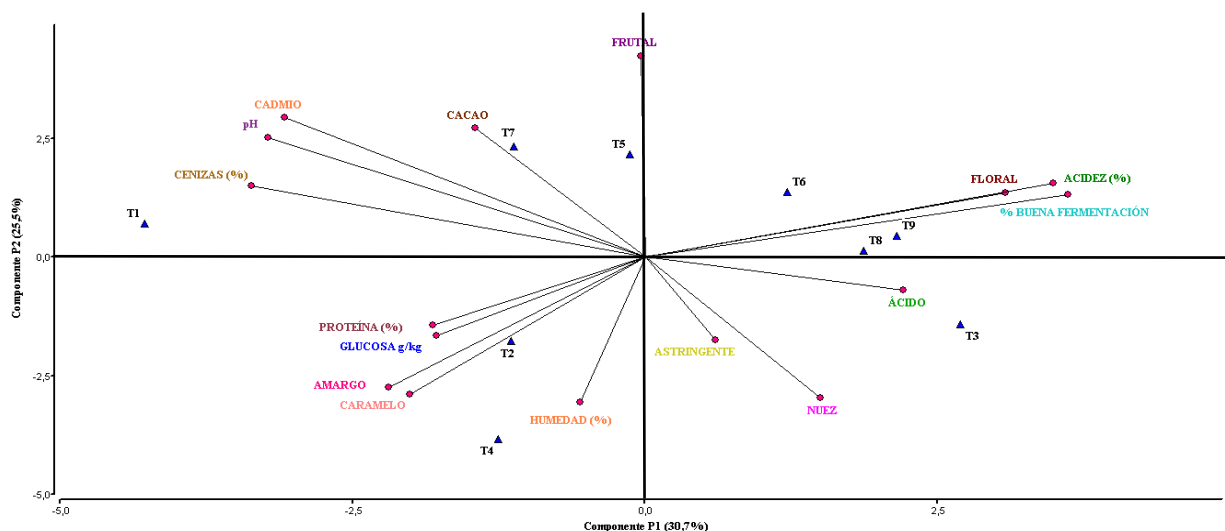
Treatment	Flavors (0-10)							
	Cocoa	Floral	Fruit tree	Walnut	Caramel	Bitter	Astringent	Acid
T1 (48 hours)	7.93 <sup>a</sup>	1.63 <sup>a</sup>	3.47 <sup>a</sup>	2.46 <sup>a</sup>	1.14 <sup>a</sup>	6.61 <sup>a</sup>	4.44 <sup>a</sup>	3.42 <sup>a</sup>
T2 (96 hours)	5.45 <sup>a</sup>	5.45 <sup>a</sup>	1.86 <sup>a</sup>	2.23 <sup>a</sup>	1.46 <sup>a</sup>	5.49 <sup>a</sup>	4.75 <sup>a</sup>	5.01 <sup>a</sup>
T3 (120 hours)	6.27 <sup>a</sup>	3.45 <sup>a</sup>	2.46 <sup>a</sup>	3.86 <sup>a</sup>	0.75 <sup>a</sup>	6.13 <sup>a</sup>	5.07 <sup>a</sup>	5.41 <sup>a</sup>
T4 (48 hours)	7.14 <sup>a</sup>	1.86 <sup>a</sup>	1.23 <sup>a</sup>	2.80 <sup>a</sup>	1.37 <sup>a</sup>	7.28 <sup>a</sup>	5.83 <sup>a</sup>	4.14 <sup>a</sup>
T5 (96 hours)	8.07 <sup>a</sup>	1.99 <sup>a</sup>	3.22 <sup>a</sup>	1.22 <sup>a</sup>	0.53 <sup>a</sup>	5.94 <sup>a</sup>	5.91 <sup>a</sup>	5.60 <sup>a</sup>
T6 (120 hours)	7.64 <sup>a</sup>	3.16 <sup>a</sup>	2.76 <sup>a</sup>	2.23 <sup>a</sup>	1.00 <sup>a</sup>	5.31 <sup>a</sup>	4.53 <sup>a</sup>	3.90 <sup>a</sup>
T7 (48 hours)	7.47 <sup>a</sup>	3.20 <sup>a</sup>	3.54 <sup>a</sup>	1.82 <sup>a</sup>	0.90 <sup>a</sup>	5.44 <sup>a</sup>	4.18 <sup>a</sup>	3.64 <sup>a</sup>
T8 (96 hours)	6.31 <sup>a</sup>	2.33 <sup>a</sup>	3.33 <sup>a</sup>	2.39 <sup>a</sup>	0.68 <sup>a</sup>	5.28 <sup>a</sup>	5.64 <sup>a</sup>	5.65 <sup>a</sup>
T9 (120 hours)	7.14 <sup>a</sup>	3.43 <sup>a</sup>	3.07 <sup>a</sup>	2.63 <sup>a</sup>	1.06 <sup>a</sup>	5.31 <sup>a</sup>	4.26 <sup>a</sup>	3.97 <sup>a</sup>
Average	7.05	2.54	2.77	2.40	0.99	5.86	4.95	4.53
p-value	0.3270	0.3977	0.4536	0.4795	0.7153	0.7148	0.8667	0.6713
Lower limit 0.05	6.51	2.05	2.24	1.91	0.74	5.31	4.32	3.84
Upper limit 0.05	7.58	3.03	3.30	2.89	1.23	6.42	5.59	5.21

Different letters indicate statistical significance according to Tukey’s test (p<0.05).

Source: Morejón, Rocio (2021).

Figure 4 shows a total variability of 56.2% in the 16 physicochemical and sensory variables evaluated. Principal component 1 includes the variables floral flavor, acidity, and good fermentation, while principal component 2 groups the variables cocoa flavor, cadmium, pH, and ash. Treatments T9, T8, and T6 are grouped by presenting good fermentation, a floral/acid tone, and acceptable acidity due to acetic acid, and also show low cadmium content. It can be seen that T1 has high ash, pH, and cadmium content. It should be noted that cadmium shows an interesting relationship with pH and the cocoa profile. Treatment T5 stands out for presenting a good combination of cocoa and

fruit flavors at high intensities, also showing a low percentage of moisture. T3 had the lowest cadmium content. The highest protein percentage was recorded in T2, in addition to a caramel profile. T7 presented high glucose content and a good level of fruity flavor.



**Figure 4:** Principal component analysis of physicochemical and sensory variables.

## DISCUSSION

### The temperature of cocoa beans during fermentation

The decrease in temperature after 24 hours agrees with Braudeau (1970) and Enriquez (1985), who indicate that in the first stage of fermentation, the microbial activity causes an increase in temperature until it reaches a maximum of 40 to 45 °C and then drops due to the inactivation of lactic acid bacteria and the death of the embryo (Vilchez, 2016, pp.63-69). Vera et al. (2014, p.23) reached 43°C as the average temperature during the micro fermentation of cocoa beans for five days. The temperature recorded during fermentation agrees with Mejía et al. (2018), who recorded values between 37 to 50°C, arguing that these temperatures are optimal for enzymes and yeasts to act.

### The pH of cocoa beans during fermentation

In the fermentation reactions, the yeasts consume the sugars and then the acetic acid bacteria consume the alcohol, which causes the pH to decrease over time and conditions a good fermentation (Lanza et al., 2016); Mejía et al., 2018; Rivera, 2018; and Santa, 2019). As cocoa fermentation progresses, the pH decreases, and this occurs because the testa of the cocoa beans is permeable and allows the entry of acetic acid, which causes acidification. The tendency for pH to decrease as fermentation time progresses was reported by Álvarez (2008). In the physicochemical characterization of fermented cocoa beans, who started with a pH of 6.17 in fresh beans and evaluated the pH at 24, 48, 72, 96, and 120 hours of fermentation and obtained pH values of 4.95, 4.73, 4.47, 4.29 and 4.22, respectively.

### The acidity of cocoa beans during fermentation

The acidity of almonds increases with fermentation time up to 96 h and then decreases because acetic acid decreases gradually due to acetic acid volatilization (Lares et al. 2013; Teneda 2016; Chávez 2020). Chavez (2020) found 0.64%

acidity at 0 days of fermentation, 1.77% at 48 hours, 2.63% at 96 hours and 2.59 % at 120 hours.

### Physicochemical characteristics of dried cocoa beans

#### Cutting test

Álvares (2008) indicates that the percentage of fermentation of fine aroma cocoa beans should be higher than 80%, which allows the acquisition of cocoa’s aroma and flavor characteristics. During fermentation, the first 48 hours are fundamental because, at this stage, the yeasts convert the starch and sugars of the mucilage into ethanol. Because of this, it can be deduced that treatments T7, T4, T2, and T1 with the shortest fermentation time did not reach the appropriate temperature before the first stirring, which is essential for the release of flavor and aroma precursors, and therefore these samples did not reach full quality.

According to Vera y Goya (2015, p.35), the degree of fermentation of cocoa almonds is proportional to the fermentation time; that is, the longer the fermentation time the higher the degree of fermentation of the almonds, as observed in treatments T9, T6, and T3. In other investigations, adding additives, fermentation time and soaking allowed for obtaining values higher than 83% of fermented almonds. This same effect was obtained by Guevara (2017, pp.42-44) when evaluating the addition of enzymes and yeasts to improve its fermentative process.

#### Humidity

During this investigation, the moisture content in the samples is within the range established by NTE-INEN 173.

#### Ash

Larez et al. (2013, pp.441-442) indicate that the decrease in ash in dry cocoa beans occurs due to the high microbiological activity during the fermentation process and the number of minerals required by the yeasts. The same author mentions that this variation is due to the draining effect that occurs

during the fermentation of cocoa beans. The ash content in the cocoa beans evaluated in this research is similar to the values obtained by Alvarez et al. (2007) and Perea et al. (2011), who reported values between 2.8 and 3.32 %.

#### Total sugars (glucose g/kg)

Rojas et al. (2021, pp.57-59) state that the highest sugar consumption is observed after the anaerobic phase at 72 h of fermentation. Camu et al. (2008) found glucose concentrations ranging from 1.13 to 3.09 mg.g-1 in fermented and dried cocoa beans, which are lower than the glucose content found in this research.

Thus, at very high concentrations, sugars prevent the multiplication of microorganisms, causing fermentation difficulties (Cárdenas, 2017, p.16). It is worth mentioning that T6 (120 h fermentation and yeast) presented 5.55 g.kg-1 of glucose. This could be associated with kinetic behavior, where microorganisms remain in the stationary phase. On the other hand, the initial glucose content can cause a well-marked effect on cell metabolism and become a potent inhibitor at high concentrations so that it may be related to the overproduction of ethanol and acetic acid, causing adverse effects at specific concentrations (Aguilar et al., 2015, p.1).

#### pH

Llerena and Uriña (2017) state that if the pH is less than 5.0, it indicates the presence of non-volatile acids that are undesirable for the development of cocoa aroma and flavor (Erazo and Mendoza, 2015, pp.72-73). Loureiro et al. (2016, p.5) ratify that pH is important in cocoa quality and should be between 5.0 and 5.4. On the other hand, for Perea et al. (2011, p.38), pH values between 5.3 and 5.6 correspond to average values and are accepted by the industry. Bertorelli et al. (2009), observed an increase in pH of 5.98 in fermented almonds and 6.07 in dried almonds. The same author argues that due to the volatilization of acetic acid by drying, the pH increases in cocoa almonds.

#### Acidity

According to Bermúdez y Mendoza (2016), lactic and acetic acids migrate towards the cotyledon's interior due to the pulp's microbial degradation, increasing the percentage of acidity during fermentation which is subsequently reduced during the drying of cocoa beans (Vázquez et al., 2016). Alvarez et al. (2010) and Perea et al. (2011) indicate that the percentage of acidity in cocoa beans depends on the fermentation and drying process, although it can also affect the variety and mucilage content.

#### Protein

The protein content in the National cocoa beans in this research was similar to the values found by Zambrano (2018), with a protein content between 9.90 and 15.07. On the other hand, Vilchez (2016) reported a decrease in the percentage of protein during fermentation from 12.59 to 12.40% due to the drying action of the fermented beans. Because, during fermentation, lactic and acetic acid enter the

cotyledon of the cocoa beans, these acids enzymatically degrade the proteins. In addition, at this stage, the yeasts require food to consume the proteins. Therefore, the decrease in protein is also attributed to drainage during fermentation.

#### Cadmium

Lara (2017) fermented Nacional cocoa beans for 96 hours, evaluated different drying methods, and finally reduced the cadmium content. The relationship between cadmium content and fermentation time agrees with Falcon (2019). Furthermore, the relationship between cadmium content and fermentation time agrees with the study of cocoa beans, which maintains that the cadmium content decreases as the fermentation time increases in cocoa beans. On the other hand, Intriago et al. (2019) state that during fermentation and drying of cocoa beans, the concentration of cadmium can decrease due to the loss of water with total soluble solids, the transformation of sugars into anaerobic and aerobic compounds that allow the loss of heavy metals. On the other hand, Lanza et al. (2016) found no significant differences in cadmium concentrations between cocoa beans.

#### Sensory analysis of cocoa paste

Solórzano et al. (2015) obtained an average of 3.50 cocoa flavors from different producing areas. On the other hand, Bermúdez and Mendoza (2016) argue that the intense cocoa flavor is characteristic of national cocoa and demonstrates that the post-harvest process was adequate. At the same time, Olarte and Rincon (2019) attribute that the perception of cocoa flavor was higher in the samples with natural drying.

In floral flavor, T2 stood out with medium intensity, surpassing the values obtained by Solórzano et al. (2015) in different producing areas except for the Miss Ecuador association, which presented a value of 4.20. The values obtained in this research are similar to those found by Vera et al. (2014), who highlighted clone DIRCYT-C114 with a medium intensity of floral flavor. Also, Guevara (2017) also presented similar values in the evaluation of cocoa paste in the study carried out with the addition of enzymes and mother yeast to improve the organoleptic characteristics of cocoa.

In the fruit profile, the values found are higher than those presented by Olarte and Rincon (2019; Solórzano et al., 2015), during the sensory evaluation of clone FTA-4, who at 144 h of fermentation obtained a more excellent perception of fruity flavor, so they deduced that the fermentation time influences the perception of fruity flavor, which is in agreement with this research. The nutty flavor in this research is superior to those found by Cedeño (2020) and Solórzano et al. (2015), with values of 1.17 and 3.00, respectively. On the other side, Olarte and Rincon (2019) state that the values found the highest value (4.00) at 192 h of fermentation with artificial drying. The caramel flavor surpassed the values found by (Solórzano et al., 2015; Vera et al., 2014). The differences in fermentation quality explain the links between cocoa flavor, floral, fruity, nutty, and caramel flavor.

## CONCLUSIONS

According to the results of this research, T9 presented the best fermentation percentage. While the highest total sugar content (glucose) was obtained in T7 (48 h fermentation + mother yeast + PPO).

In the interaction between fermentation time and yeast and enzymes, it is concluded that the increase in fermentation time and the addition of yeast and enzymes allowed a decrease in cadmium content in the dry cocoa beans.

For the main effect of fermentation times, the lowest cadmium content in cocoa beans is obtained by fermenting for 120 hours.

The pH values obtained in this investigation decrease with fermentation time.

The sensory evaluation showed that cocoa flavor was the attribute with the highest score in all the treatments evaluated. All the treatments analyzed were generally characterized by moderate acidity, astringency, and bitterness. On the other hand, the addition of enzymes and yeasts in the amounts used in this research did not affect sensory quality.

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