BRAZILIAN JOURNAL OF FOOD TECHNOLOGY

ISSN 1981-6723
Online version

ORIGINAL ARTICLE

Physicochemical changes during controlled laboratory fermentation of cocoa (CCN-51) with the inclusion of fruits and on-farm inoculation

María Alicia Peña González[1,2], Jennifer Pamela Ortiz Urgiles[1], Fernanda Alegría Santander Pérez[1], Marco Antonio Lazo Vélez[1], Rodrigo Sebastián Caroca Cáceres[1*]

[1]Universidad del Azuay, Laboratorio de Biotecnología, Cuenca/Azuay - Ecuador
[2]Universidad Nacional del Cuyo, Facultad de Ciencias aplicadas a la Industria, San Rafael/Mendoza - Argentina

***Corresponding Author:** Rodrigo Sebastián Caroca Cáceres, Universidad del Azuay, Laboratorio de Biotecnología, Av. 24 de mayo, 7-77, Apartado, Cuenca/Azuay - Ecuador, e-mail: rcaroca@uazuay.edu.ec

Cite as: Peña González, M. A., Ortiz Urgiles, J. P., Santander Pérez, F. A., Lazo Vélez, M. A., & Caroca Cáceres, R. S. (2023). Physicochemical changes during controlled laboratory fermentation of cocoa (CCN-51) with the inclusion of fruits and on-farm inoculation. *Brazilian Journal of Food Technology*, *26*, e2023013. https://doi.org/10.1590/1981-6723.01323

Abstract

Fermentation is key to developing the organoleptic characteristics of cocoa beans, as dynamic changes in metabolites have a significant impact on flavors and aromas, hence modifications of this process have been investigated. In this research, the mucilage of CCN-51 cocoa beans was replaced by a mixture of passion fruit (*Passiflora edulis*) and plantain (*Musa paradisiaca* L.) pulp, and a controlled fermentation of this mixture was carried out after its spontaneous on-farm inoculation. The physicochemical changes and correlations during the five days of fermentation were evaluated. At the end of the process, the temperature reached 47 ºC in the fermentation mass and pH 5.64 was recorded in the cotyledon. In the first 48 hours, citric acid and fructose were high but at the end of fermentation were 71% and 41.17% lower than at the start of fermentation, respectively. As glucose and fructose were consumed during fermentation, acetic acid and lactic acid levels increased from day two onward, reaching values at the end of the process of 22.48 mg/g and 16.01 mg/g, respectively. In contrast, the bromatological parameters did not show greater variability when comparing each day of fermentation. The data generated and results presented in this study will contribute to the knowledge of possible sensory improvements achieved with the inclusion of pulp fruits in the fermentation stage.

Keywords: Cocoa bean; Modified fermentation; Proximal composition; Dynamics; Cocoa quality; Organic acids.

Highlights

- Proximal composition of cocoa beans showed no significant changes during fermentation
- Sugar concentration decreased
- Acetic acid and lactic acid increased during five days of fermentation while citric and malic acids showed the opposite behavior

1 Introduction

Flavour is one of the main attributes analyzed when purchasing cocoa beans since the quality of the processed chocolate will depend on it. The properties of the final product are influenced by the processing of the raw material, with postharvest handling having the greatest impact (Afoakwa, 2016). In this regard, the fermentation stage is fundamental to obtain a product of good quality. The dynamic physicochemical interactions that occur between the resulting metabolites during the fermentation processes of cocoa beans have a significant impact on the organoleptic quality of fermented cocoa beans; in particular, the interactions of organic acids and sugars, mediated by temperature, and microorganisms, have a significant impact on the organoleptic quality of fermented cocoa beans. Therefore, having greater control over this process could improve the sensory profile of the resulting chocolate.

Several factors affect the quality and the final flavour of chocolate, among them are genetics, environmental conditions, cocoa variety, geographical origin, degree of maturity, quality of fermentation, drying, and roasting (Kongor et al., 2016). It is important to note that proper fermentation plays a fundamental role in the development of the sensory attributes of cocoa. Unfermented cocoa beans have a dark gray color and are astringent (Engeseth & Pangan, 2018), whereas partially fermented beans are purple colored, and their flavour profile is bitter and harsh (Aprotosoaie et al., 2016). The bean microbiota and its metabolic activities during fermentation contribute to the elimination of the mucilage and it influences the development of certain volatile compounds related to fruity and floral aromas, as well as the elimination of undesirable characteristics such as astringency (De Vuyst & Weckx, 2016; Tigrero-Vaca et al., 2022).

The microorganisms involved in cocoa fermentation are mainly yeasts, lactic acid bacteria (LABs) and acetic acid bacteria (AABs) (Ganeswari et al., 2015; Giacometti et al., 2015; Ho et al., 2015; Kouamé et al., 2015; Aprotosoaie et al., 2016; Caligiani et al., 2016; De Vuyst & Weckx, 2016; Pereira et al., 2016; Hernández et al., 2019). In anaerobic conditions, yeasts metabolize the sugars present in the cocoa mucilage to produce ethanol. Low oxygen availability, in conjunction with increasing temperature and pH, promotes the growth of LABs and AABs. Towards the end of the process, AABs dominate and yield acetic acid from the ethanol are produced by the yeasts (De Vuyst & Weckx, 2016). The acetic acid diffuses into the cocoa beans and, along with the high temperature and ethanol, is responsible for killing the embryo (Schwan & Wheals, 2004). Remarkably, a good drying of the almonds does not, in any way, improve the sensory quality of insufficiently fermented cocoa (Dzelagha et al., 2020).

In previous studies, a fermentation of CCN-51 cacao beans has been carried out with fruit replacement (passion fruit and banana pulp) and incorporating mixtures of probiotic microorganisms (Vizcaino-Almeida et al., 2022). So far, no results have been reported where the replacement pulps are inoculated with the natural microbiota from the environment and fermented under controlled conditions. In addition, no data regarding the dynamics of transformations of organic acids and sugars and the variations in the bromatological profile during the five days of fermentation are available. This information will shed light on the impact that fruit pulp inclusion can have on the development of metabolites and bromatological parameters during a controlled fermentation process of CCN-51 cocoa. Moreover, relevant knowledge could be obtained to understand the possible sensory improvements achieved with this modification in the fermentation stage. Therefore, this research aimed to evaluate the dynamic of organic acids and sugars during controlled laboratory fermentation of cocoa beans (CCN-51) with the inclusion of fruit pulp and spontaneous *on-farm* inoculation.

2 Material and methods

2.1 Controlled laboratory fermentation

CCN-51 cocoa pods, passion fruit (*Passiflora edulis*) and banana (*Musa paradisiaca* L.) were obtained from a farm located in Putucay, Ecuador (2°32’43.7” S, 79°23’03.8” W). Ripe cocoa pods were collected

(purple-reddish coloration), opened with a machete and the mucilage was manually extracted. The bananas and passion fruits selected did not present any irregularities that could affect the quality of these fruits. The pulp from 36 kg of cocoa beans was treated with 0.5 mL of pectinase/kg cocoa beans (AB Enzyme, Granotec, Guayaquil, Ecuador) to remove the mucilage. The mixture was thoroughly homogenized and incubated for one hour at ambient temperature (30 °C). Once the mucilage was removed, the cocoa beans were mixed with passion fruit and banana pulps, according to the protocol described by Vizcaino-Almeida et al. (2022). The cocoa beans fruit pulps mixture was placed in plastic containers and left for four hours (mixing every 30 minutes) outdoors, allowing the inoculation with the native microorganisms present in the environment. Afterward, the mixture was fermented under controlled conditions for five days in a climatic chamber (KBF 240, BINDER, Germany). The mixture of cocoa beans and fruit pulp was divided into three equal parts in plastic containers with perforations to perform the fermentation in triplicate. The relative humidity was fixed to 75% HR, and the temperature was controlled during fermentation, as follows: 25 °C from 0 to 48 hours, 35 °C from 48 to 96 hours, and 45 °C from 96 to 120 hours. Every 24 hours during the five days of fermentation, samples of 150 g were obtained from five different points of the fermented cocoa bean mass and were placed in sterile bags and then frozen at -80 °C (ARCTIKO, Denmark) for later tests. The temperature was measured three times a day with a thermometer probe placed in the thermal center of the fermented cocoa batches.

2.2 Proximate analysis

For proximate analysis, fifteen cocoa beans were taken from each of the five fermentation days and immediately processed. The beans were manually peeled to remove completely the mucilage and ground with an electric grinder (Krups, Mexico) avoiding the formation of a paste.

Moisture, fat, protein, and ash were measured following the reference methods AOAC 931.04, AOAC 963.15-1973, AOAC 970.22, and AOAC 972.15, respectively. Total carbohydrates were determined by the difference method. Dry samples were used for protein and ash determinations.

2.3 Physicochemical characterization

For physicochemical characterization, 10 g of the whole mixture (cocoa beans plus fruit pulps) taken from the fermenting mass samples, were grounded and 10 g were mixed with 60 mL of distilled water. Subsequently, Carrez I, Carrez II and NaOH solution were added, in the quantities described in the corresponding Megazyme protocol (Megazyme International, Granotec, Guayaquil, Ecuador). The flask was filled to the mark, mixed, and filtered. For clarification, 1.5% - 2% of activated charcoal was added to the filtered sample and allowed to stand refrigerated (4 ºC ± 1) for 24 hours. Afterward, the samples were centrifuged for 10 min and then transferred to 1.5 mL tubes, labeled and frozen at -20 ºC for later use.

The determination of D-glucose, D-fructose and sucrose concentration in the cocoa beans with the fruit's pulp attached was determined with the Megazyme K - SUFRG kit (Megazyme, Ireland), following the manufacturer's protocol in a spectrophotometer (Epoch, Biotek).

The concentrations of acetic, malic, citric, and lactic acids in the cocoa beans with the fruits pulp attached were determined using Megazyme K-ACETRM 04/20, Megazyme K-LMAL-58A/K-LMAL-116A 09/19, Megazyme K-CITR 05/21 and Megazyme K-DLATE 08/21, Megazyme K-DLATE 08/18, respectively. The manufacturer's protocols were followed, and measurements were performed in a spectrophotometer (Epoch, Biotek).

Finally, the pH measurements of the cocoa beans with the fruit pulp attached were determined according to the methods described by Senanayake et al. (1997) with a potentiometer (Seven Easy, Mettler Toledo, Switzerland).

2.4 Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was performed by applying a single-factor Analysis of Variance (ANOVA) and a Tukey's test to identify significant differences between

the different fermentation days. Differences between means were compared at a significance level of $\rho > 0.05$. Each determination was performed with a minimum of three replicates. Data were analyzed with Minitab 18 Software (State College, Pennsylvania, USA).

3 Results and discussion

3.1 Proximal analysis

The variation of the proximal composition of the cocoa bean cotyledon during the five days of fermentation is shown in Table 1. The five parameters analyzed are expressed on dry weight (DW). As the fermentation progresses, the content of protein, fat, and ash did not show marked differences. The protein content in the cotyledons ranged from 8 to 10.6% as well as reported by Rawel et al. (2019). During the fermentation process, a slight decrease in protein concentration was found, presumably due to degradation in free amino acids and oligopeptides which are central to the development of aromatic compounds (Barišić et al., 2019). On the other hand, the fat content in fermented cocoa beans (54%) was like the values described by Teneda Llerena (2016) and Graziani de Fariñas et al. (2003). It is known that cocoa beans have high-fat content, especially the CCN-51 clone, which has about 52% fat (Samaniego et al., 2021). Unexpectedly, the moisture contents tend to increase. Other studies show that the pulp attached to the beans is eliminated by the action of microorganisms causing a gradual loss of moisture (Ortiz de Bertorelli et al., 2009). In the fermentation reported in this study, it could be noted that deficient pulp drainage was observed by the type of container used. Besides, the constant relative humidity inside the fermentation chamber prevented the loss of exudate, providing a plausible explanation for the moisture increase on the fifth day.

Table 1. Proximate composition (% DW) of the cocoa bean during five days of fermentation with the inclusion of fruit pulp and spontaneous on-farm inoculation.

Parameters	Fermentation Days					
	D0	D1	D2	D3	D4	D5
Moisture	33.86 ± 1.12^{d}	33.36 ± 0.47^{d}	38.31 ± 1.12^{c}	39.24 ± 0.61^{bc}	38.83 ± 0.65^{c}	41.12 ± 1.38^{ab}
Fat	50.13 ± 0.63^{ab}	49.45 ± 0.30^{b}	52.36 ± 1.53^{ab}	51.46 ± 2.35^{ab}	53.21 ± 2.39^{ab}	54.08 ± 1.46^{a}
Protein	9.37 ± 0.36^{b}	10.64 ± 0.63^{a}	8.25 ± 0.34^{c}	8.03 ± 0.07^{c}	8.25 ± 0.33^{c}	8.52 ± 0.28^{c}
Ash	3.42 ± 0.02^{b}	3.25 ± 0.02^{bc}	4.18 ± 0.18^{a}	4.14 ± 0.13^{a}	3.39 ± 0.06^{b}	3.02 ± 0.09^{c}
Total Carbohydrates	37.07 ± 0.34^{a}	36.65 ± 0.53^{a}	35.21 ± 1.52^{a}	36.36 ± 2.29^{a}	35.14 ± 2.52^{a}	34.37 ± 1.49^{a}

Each value represents the mean ± standard deviation for triplicate determinations. Mean values in each raw bearing different superscript letters are significantly different ($p < 0.05$). Abbreviations: D0 – D5: cocoa beans fermented for zero, one, two, three, four, and five days.

3.2 Dynamic of temperature during controlled laboratory fermentation of cocoa beans.

The temperature of the cocoa bean mass increased during the fermentation (Figure 1). The fermenting mash temperatures from D1 to D3 behaved similarly to those of the climatic chamber (25 °C to 35 °C). However, in D3 - D4 a markedly higher temperature was registered in the fermented beans compared to the climatic chamber. As the fermentation process progressed, the temperature increased steadily, from 26.5 °C to 46.5 °C, in agreement with other cocoa fermentations where a temperature between 47 °C to 49 °C has been recorded at the end of the process (da Veiga Moreira et al., 2013; Ramos et al., 2014; Ganeswari et al., 2015; Peláez et al., 2016; Tunjung-Sari et al., 2021). This constant increase might result from the exothermic reactions taking place in the cocoa mass during the process, due to the fermentative activities developed by yeasts, BALs, and BAAs. As ethanol oxidation and acetic acid over-oxidation occur, temperatures rise near 50 °C, leading to a decrease in microbiological activity and death of cotyledons (Schwan & Wheals, 2004; Afoakwa et al., 2014; Batista et al., 2016; Castro-Alayo et al., 2019; Hernández et al., 2019; Schwan & Wheals, 2004).

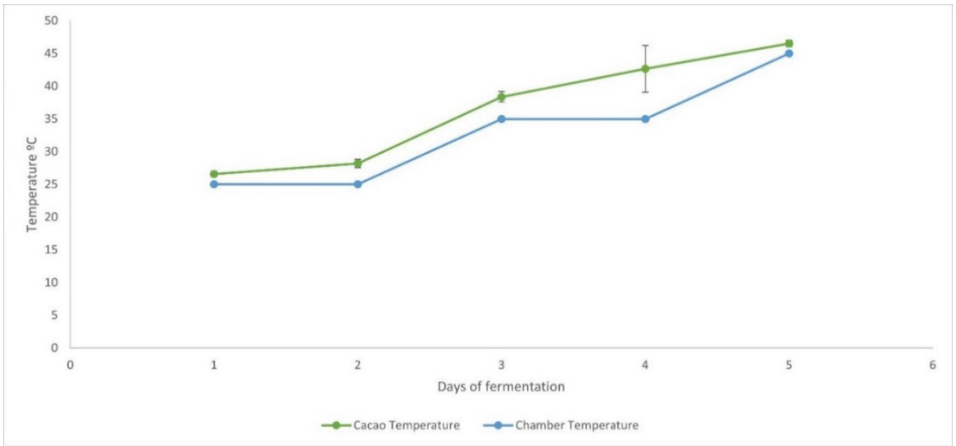

Figure 1. Temperature changes during cocoa fermentation.

3.3 Dynamic of organic acids and sugars during controlled laboratory fermentation of cocoa beans

The changes in the concentrations of sugars present in the cocoa beans with the fruit pulp attached throughout the five days of fermentation are shown in Table 2. High concentrations of glucose and fructose were observed during the first days, while sucrose presented low concentrations, being undetectable on the fifth day. The concentrations of these sugars reported here are like those reported by Mota-Gutierrez et al. (2018), who obtained at the beginning of fermentation concentrations of glucose, fructose, and sucrose of 20 – 24, 23 – 25 and 8 – 10 mg/g, respectively. Barbosa Santos et al. (2021) reported that glucose and fructose are the main sugars present in passion fruit and banana, which explains their high levels in this modified fermentation. Other authors observed a different behavior, where the main sugar was sucrose, while glucose and fructose were at low concentrations (Ho et al., 2015; Vizcaino-Almeida et al., 2022). These values may vary depending on the origin of the cocoa, method of determination, degree of maturity of the pulps, sample preparation, type and time of fermentation, and microbial activity (Verce et al., 2021). During the first 48 hours of fermentation, no significant changes were observed in glucose and fructose, but on the contrary, sucrose showed a significant decrease (Table 2). Very likely, during this early fermentation period microorganisms use glucose and fructose as substrates, but they could be provided by invertase-mediated sucrose hydrolysis in the cocoa beans (Kongor et al., 2016). Therefore, although these sugars are being consumed, apparently their concentration does not vary, due to a compensation effect. From the second day, the concentration of glucose and fructose began to decrease (Table 2). Most likely, as the fermentation progresses, yeasts, BALs, and BAAs consume these sugars to produce ethanol and some organic acids (Kouamé et al., 2015; Aprotosoaie et al., 2016; Pereira et al., 2016; Castro-Alayo et al., 2019; Hernández et al., 2019; Sarbu & Csutak, 2019).

At the start of the fermentation, the average pH value was 4.30±0.06, presumably due to the acidity of the incorporated fruits, especially the passion fruit which has a high content of citric acid (Oliveira et al., 2020). The pH value rose to 5.96 ± 0.06 and displayed a significant ($p < 0.05$) decrease to 5.64 ± 0.09 after 96 and 120 hours of fermentation, respectively. This increase in pH may be related to the loss of citric acid by the "sweatings" of the pulp, and the consumption of this metabolite because of microbial metabolism (Schwan & Wheals, 2004).

After the fourth day of fermentation, a slight decrease in pH was observed. It is expected that BAA predominates in this phase, and therefore the acetic acid produced by them penetrates the beans decreasing their pH and causing the death of the embryo (Caligiani et al., 2016; Giacometti et al., 2015; Kouamé et al., 2015; Aprotosoaie et al., 2016; De Vuyst & Weckx, 2016; Pereira et al., 2016; Castro-Alayo et al., 2019; Hernández et al., 2019; Sarbu & Csutak, 2019). Several authors suggest that the ideal pH of fermented cocoa beans at the end of the process should be between 4.8 and 5.2 (reviewed by Horta-Téllez et al., 2019). When pH is lower than 4.5, flavour precursors will be reduced and slow diffusion of

organic acids through the cotyledon is promoted. In contrast, if the pH is above 5.5 at the end of the process, the beans are not properly fermented, showing a slow diffusion of organic acids through the cotyledon, presenting problems of astringency and bitterness. The results obtained in this research were close to 5.5, consequently, it can be expected to obtain a good quality of cocoa.

Table 2. Organic acids and sugars of cocoa beans during five days of fermentation with the inclusion of fruit pulp and spontaneous on-farm inoculation.

Parameters	Fermentation Days				
	D1	D2	D3	D4	D5
Organic Acids					
Acetic Acid (mg/g)	ND	ND	15.96 ± 0.29^{c}	20.97 ± 0.47^{b}	22.48± 0.53^{ab}
Citric Acid (mg/g)	46.9 ± 0.36^{a}	36.95 ± 0.36^{b}	18.99 ± 0.12^{c}	13.95 ± 0.15^{d}	13.59 ± 0.19^{d}
Malic Acid (mg/g)	12.01 ± 0.18^{a}	3.32 ± 0.06^{bc}	4.25 ± 0.03^{b}	3.07 ± 0.04^{bc}	2.72 ± 0.03^{c}
D – lactic Acid (mg/g)	1.01 ± 0.02^{c}	0.71 ± 0.01^{c}	1.54 ± 0.02^{c}	3.40 ± 0.02^{b}	5.06 ± 0.05^{a}
L – lactic Acid (mg/g)	1.39 ± 0.03^{d}	2.64 ± 0.04^{d}	6.56 ± 0.07^{c}	8.49 ± 0.07^{b}	10.95 ± 0.04^{a}
Total lactic Acid (mg/g)	2.40 ± 0.04^{d}	3.35 ± 0.04^{d}	8.10 ± 0.09^{c}	11.89 ± 0.06^{b}	16.01 ± 0.07^{a}
Sugars					
Glucose (mg/g)	21.91 ± 0.20^{a}	21.91 ± 0.21^{a}	17.06 ± 0.14^{b}	12.12 ± 0.11^{c}	10.44 ± 0.11^{cd}
Fructose (mg/g)	24.75 ± 0.24^{a}	23.75 ± 0.31^{a}	23.08 ± 0.19^{ab}	19.83 ± 0.21^{b}	14.56 ± 0.10^{c}
Sucrose (mg/g)	5.79 ± 0.07^{a}	2.55 ± 0.03^{b}	1.82 ± 0.04^{c}	1.28 ± 0.01^{c}	ND

ND: Not detected. Each value represents the mean ± standard deviation for triplicate determinations. Mean values in each raw bearing different superscript letters are significantly different ($p < 0.05$). Abbreviations: D0 – D5: cocoa beans fermented for zero, one, two, three, four, and five days.

The changes in the concentrations of organic acids in the cocoa beans throughout the five days of fermentation are shown in Table 2. At the beginning of the process, citric acid was the most abundant organic acid, followed by malic and lactic acid. It could be noted that acetic acid was not detected in the first two days of fermentation, in agreement with Vizcaino-Almeida et al. (2022). According to Ho et al. (2015), the acids of interest for the proper development of chocolate flavour precursors are citric, acetic, and lactic. Citric acid is naturally present in the pulp of cocoa beans and is also one of the main acids present in fresh passion fruit (Barbosa Santos et al., 2021). Malic acid was also found in high concentration on the first day of fermentation since passion fruit and banana pulp provided this type of acid. As the fermentation process progressed, a statistically significant decrease in citric and malic acid concentrations was observed, while acetic and lactic acid concentrations increased steadily (Table 2), in agreement with Camu et al. (2007). In the case of citric acid, its decrease could be observed because it is partially or fully utilized during fermentation by the growth of yeasts and LABs. For instance, some species of lactic acid bacteria, especially *Lactobacillus fermentum*, utilize large amounts of this acid, causing its concentration to decrease during fermentation. Malic acid levels on the other hand, might be decreasing due to yeast metabolic activities (e.g., *Pichia kudriavzevii*.), as shown by Ho et al. (2015).

As previously mentioned, the production of organic acids during the cocoa fermentation process is of utmost importance for the final quality of the chocolate, and among them, lactic and acetic acids play a crucial role. Lactic acid is a product of microbial fermentation of simple carbohydrates and is produced as D (-) or L (+) acid or as its racemic mixture. The ratios of these two isomers were obtained from the five days of fermentation (D:L; 42:58, 21:79, 19:81, 29:71, and 31:68), observing a higher ratio of the L (+) lactic acid. Elevated levels of the D (-) isomer are detrimental for humans, while L (+) lactic acid is the preferred isomer in food industries (Cizeikiene et al., 2018). The L (+) isomer can be produced by LAB of the genera *Streptococcus, Pediococcus, Lactococcus* and *Lactobacillus,* while the D (-) isomer can be produced by certain strains of the genera *Lactobacillus, Leuconostoc* and *Oenococcus* (Trontel et al., 2011; Juturu & Wu, 2016).

Until 48 hours of fermentation, no acetic acid was detected possibly because in the first days of fermentation low or absent activity of BAAs is expected. However, during aerobic conditions at the end of fermentation, BAAs species dominate, which can oxidize the ethanol produced by yeasts to acetic acid (Peláez et al., 2016; De Vuyst & Weckx, 2016). The biochemical reactions inside the cocoa bean during fermentation are due to the penetration of acetic and lactic acid produced by microbial metabolism. BAAs play a key role in the fermentation of cocoa beans, as their main product, acetate, is one of the most important factors for the development of desired flavours in cocoa (Adler et al., 2014). At the same time as this acid penetrates the cotyledon, compounds such as alkaloids and polyphenols leach out of the fermentation, reducing the bitterness and astringency of the fermented beans (Pereira et al., 2016; Sarbu & Csutak, 2019).

Noteworthy, the concentrations of acids obtained in this study vary compared to those reported by Vizcaino-Almeida et al. (2022), despite the fact that the same cocoa variety and pulp replacement were evaluated. This difference may be due to the microbiological diversity and the conditions under which the fermentation was carried out, since in that study probiotic microorganisms were inoculated. Also, the differences in the concentrations of organic acids may be due to the state of maturity of the fruits used.

4 Conclusions

The inclusion of passion fruit and banana pulp had little influence on the kinetics of sugars and organic acids during a cocoa controlled laboratory fermentation, as the tendencies observed were similar to that reported in other investigations with traditional fermentation. In the proximate analyses, it was found that they did not present important changes in the different parameters analyzed. However, it could be expected that the passion fruit and banana used in this fermentation would contribute positively to the organoleptic characteristics of flavour and aroma, with fruity notes, which are very desirable in the chocolate industry.

Acknowledgements

The authors acknowledge the financial support of the Universidad del Azuay (Cuenca, Ecuador), project 2021-0067, and Diego Montero and Nicole Sarmiento for their help during the sample analysis.

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Funding: None.

Received: Feb. 16, 2023; Accepted: June 27, 2023

Section Editor: Marta Hiromi Taniwaki.