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Induction of rhizobium japonicum in the fermentative mass of two varieties of cacao (Theobroma Cacao L.) as a strategy for the decrease of cadmium

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***Abstract*---**Introduction: Ecuadorian cocoa paste used for chocolate production must comply with regulation 488/2014 regarding permissible levels of cadmium for marketing in the European Union. Objective: The main objective of the research was to evaluate the effect of the application of Rhizobium japonicum in the fermentation on the reduction of cadmium content in cocoa beans. Likewise, the capacity to improve sensory aspects was determined with the induction of the microorganism. Materials and Methods: Once harvested, different concentrations of 3% and 5% were inoculated. During the fermentation time, physicochemical analysis was performed (pH, °Brix and temperature). At the end of the fermentation process, the almonds were dried in the sun for 6 days. In the dried almonds, the cut test was performed to know the fermentative state according to INEN 176/2018 standard. In cocoa paste, a sensory analysis was applied

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where aroma, flavor, and bitterness were evaluated with the help of a panel of experts. Results and Discussion: The application of the microorganism made it possible to reduce the cadmium content from 36 to 29 mg/kg (ppm), which is within the admitted parameters of the European Union. Based on a cost analysis, the profitability of this type of treatment was determined, resulting in a cost of 52.02 USD for the control samples, T1 and T4, and 53.94 USD for the inductions T2, T3, T5 and T6. Conclusions: The inclusion of the microorganisms contributed to the decrease of cadmium content in the cocoa samples.

Keywords---cocoa, analysis, cadmium, sensory, treatment.

Introduction

Theobroma cacao L. is a species native to tropical and subtropical regions of America (tropical America) originating in the twentieth century BC, there are studies that its origin dates back about 5500 years ago, but remains and chemical and physical evidence have been found in the Amazon basins of Ecuador found in Zamora Chinchipe, of the variety "fine aroma" highly appreciated and requested by international chocolate industries (ANECACAO, 2014). Ecuador produces approximately 530 thousand hectares that generate 275 thousand tons, which is why Ecuador is one of the largest producers of fine aroma cocoa in the world, cocoa is one of the main products of Ecuadorian exports since ancient times. According to the National Institute of Statistics and Census (INEC), the cocoa sector represents 5% of the national economically active population (EAP) and 15% of the rural EAP, which forms a fundamental basis of family subsistence in the coastal areas of the country, the foothills of the Andean mountains and the Ecuadorian Amazon (Espinoza and Arteaga, 2015).

At present, exports of fine aroma cocoa especially to European markets may be threatened by signs of heavy metal contamination, creating uncertainty for the population and the consumer because of its consequences, causing critical lung damage accompanied by bone softening that causes this terrible itai-itai disease that had a resurgence in Japan by this metal, it is estimated that its origin of contamination can prevent naturally or induced by man (Ramírez, 2002). The process to investigate and mitigate the cadmium content present in the cocoa bean is detrimental to the production of chocolate, so it is intended to induce a large negative bacterium *Rhizobium japonicum* that characteristic, belongs to the genus bradhyrhizobium of the family bradyrhizobiaceae, is fixing atmospheric nitrogen, in turn providing fixed "N" that is usable by plants, where different doses of *Rhizobium* concentrations are applied, there have been studies where its use can reduce the content of heavy metals (Deltha, 1982). The research department of the State Technical University of Quevedo intends to mitigate this problem of high cadmium content in cocoa production, despite the limitations due to the pandemic, sustainable alternatives have been proposed for producers and industrialists. The objective was to induce *Rhizobium japonicum* in the fermentative mass of two varieties of cocoa (*Theobroma cacao* L.) Nacional and Trinitarian as a strategy for cadmium reduction.

Materials and Methods

The research used a Completely Randomized Bifactorial Design (CRBD) with a bifactorial model with 3 replications, as the first factor of induction *Rhizobium japonicum* of liquid product (0, 3, 5% per two kilos of fermentative mass or 0. 60 and 100 mL of liquid product, respectively) as second-factor cocoa varieties (Nacional and Trinitarian), to determine the difference between means was applied Tukey's multiple range test at ($p \leq 0.05$), if there is difference or equality in treatments

Table 1
Arrangement of treatments

N°	Code	Description
1	v_{0j0}	National Cocoa without *Rhizobium japonicum* (control)
2	v_{0j1}	National Cocoa with induction of *Rhizobium japonicum* (3 %)
3	v_{0j2}	National Cocoa with induction of *Rhizobium japonicum* (5%)
4	v_{1j0}	Trinitarian cocoa without *Rhizobium japonicum* (control)
5	v_{1j1}	Trinitarian Cocoa with induction of *Rhizobium japonicum* (3%)
6	v_{1j2}	Trinitarian Cocoa with induction of *Rhizobium japonicum* (5%)

Post-harvest process

About 30 cobs were harvested to reach the required amount for each treatment, at the time of harvesting the cobs were taken into account that these cobs are in good condition avoiding collecting fruits with monilla considering that it was of the Nacional and Trinitarian varieties.

Fermentation

The placenta was separated from the cocoa bean to be able to extract the seed without mixing the varieties in each cell of the micro-fermenter 2kg, giving rise to the fermentation process covering with the help of green leaves to help improve the process, making the first removal at 48 hours and therefore every 24 hours for the remaining days that are in total 4 days of fermentation for both varieties, taking data from variables such as Temperature, pH, °Brix, taking into account that it should not exceed 50°C; the micro - fermenter boxes whose capacity is 24 spaces, of which 18 separations correspond to the present research. Each separation can contain 2kg of cocoa beans, having a total of 36kg of total fresh mass

Induction of *Rhizobium japonicum*

Rhizobium japonicum was induced to the fresh mass depending on the experimental sketch with the doses required for each treatment and repetitions were 3% (60ml) and 5% (100ml) its application allows to accelerate the drainage of mucilage and in turn determines its effect on the decrease of cadmium present in the fresh fermentative mass of cocoa and thus improve the quality, productivity prior to its industrialization and export, determining whether the concentrate of the microorganism can penetrate the wall of the kernel, in this case, the testa.

Drying

The fermented and dried cocoa beans (aerobic phase) were exposed to the sun to reduce the humidity from 8 to 6%, avoiding the generation of moldy almonds and not altering their conservation, which lasted 6 days.

Storage

The grains were taken separately in different paper bags, with their identification to avoid confusing the treatments, it must be emphasized that storage will improve the quality of the grains.

Physical-chemical variables
Seed rate

For this variable, 100 fermented and dried cocoa beans were randomly selected and weighed in grams on a precision analytical balance and averaged as follows. The following formula was used and applied:

$$\text{IS} = \frac{\text{Weight in grams of 100 fermented and dried cocoa almonds}}{100}$$

Testa and cotyledon test

The testa test was obtained by weighing 30 grams of fermented and dried cocoa beans and thus obtaining the percentage of cocoa beans by applying the following formula:

$$\%\ \text{Testa} = \frac{(\text{Weight of the testa})}{\text{Weight of 30 grams of cocoa}} * 100$$

Cutting test

100 cocoa beans were randomly selected and weighed, and with the help of a stylus, a transversal cut was made in the dry cocoa beans to evaluate through a visual test if there was an optimal fermentation or a defect, according to INEN 176:2008 standards.

Determination of pH

For the determination of pH, 10 grams of almonds were crushed and then diluted in 100 ml of warm distilled water (40°C) in a beaker. This process was repeated for all treatments and repetitions for 4 days, in this way the data was obtained with the help of a pH meter.

Brix Grades

For the measurement of the Brix degrees 10 grams of cocoa, and almonds were taken at random which were macerated in 100 ml of warm distilled water, later with the help of the Brixometer instrument, 3 drops of the obtained mixture were placed in the main prism. Later the prism cover was lowered and the prism was left for 60 seconds to read the reading and record it, it is advisable to look for a place that has exposure to the outside light for better observation, taking the reading through the ocular lens of the refractometer, this process was determined during the days of fermentation.

Sensory analysis

A test was carried out by ten semi-trained tasters, each judge was given a sample of 20 grams for each sample, and the following sensory attributes were evaluated:
Main flavors: acidity, bitterness, astringency, cocoa.
General: aroma, flavor, intensity.
Defects: burnt, moldy, chemical substances.
Specific: floral, fruity, sweetness, nutty.
Respectively the values were made from an ordinal scale of test that was 1=Absent, 2=Low, 3=Medium, 4=High, 5=Very high or strong.

Cadmium test

The values of cadmium present in the fresh fermentative mass after the induction of *Rhizobium japonicum* were analyzed to determine and evaluate if there is a decrease in this metal and if they are within the average values allowed by the European Union. The analysis of cadmium is read by the atomic adsorption equipment by graphite furnace, for which 1 gram of sample is weighed, the dry cocoa beans were crushed by wet digestion using nitric acid through microwaves, followed by filtering the outgoing sample and proceeding to read in the graphite furnace equipment.

Preliminary steps for obtaining cocoa paste with a purity of 100%

Each roasting sample corresponds to 1 kg of dried cocoa beans per treatment, which were roasted in an earthenware vessel for a prolonged period of 15 to 20 minutes at a temperature of 130°C with constant stirring to avoid burnt beans or smoky odors. After the roasting process, the cocoa beans were manually shelled to remove the head of the cotyledon and thus discard the moisture contained in the cocoa bean, with the help of a manual or traditional mill, the beans were ground to reduce the thickness of the cotyledon to a size that facilitates the refining process, After the refining process, the final quality will be improved so that there are no granules and this does not have any inconvenience when consuming or that affects or is found by the taste buds, it is recommended that the granules for the elaboration of the cocoa paste play a fundamental role in the production of the cocoa paste. The cocoa sample was introduced gradually so that the shelling machine traps all the raw material that remains on the wall of the shelling machine, and goes diluting, this process was maintained for 6 hours for each treatment. Next, the cocoa paste was waited to temper, that is to say, to reduce the temperature to be transferred to the molds where it rested until it had consistency The cocoa paste was wrapped in aluminum foil for its storage in refrigeration at a temperature of 4°C, the wrappings were kept in a cover with their respective codification to avoid any cross contamination.

Steps for organoleptic evaluation

After having the cocoa paste wrapped and stored, the sample is placed in containers heated in a bain-marie to melt at a temperature of approximately 45 degrees Celsius for tasting by the panelists. Subsequently, the melted paste was placed in plastic sample cups, placing the paste uniformly to carry out the tasting, each panelist was given 6 samples accompanied by paddles and a napkin, it is indicated to them that they should be placed for tasting what fits on the paddle the sample on the taste buds for about 15 to 20 seconds for the appearance of flavors and aromas. It should be indicated that what is perceived should be noted in the attributes found on the sheet intended for tasting. This process should be continued for each sample individually, it is advisable to repeat the tasting 2 to 3 times per sample or depending on the taster; it is advisable to rinse the mouth with water before tasting. There should be a resting time to continue to consider between 1 to 2 between samples. The remaining sample should be discarded and should not be stored again.

Results and Discussion

Effect of cocoa varieties on physical-chemical, sensory and cadmium content variables in cocoa beans
Temperature

Table 2 shows the averages that report the effect of cocoa varieties on fermentation temperature, showing that there were no statistical differences (P>0.05), which indicates that both national and Trinidadian cocoa showed the same behavior in the four events recorded for this variable.

Table 1
Effect of variety on the cocoa temperature in the fermentation process

Varieties	Temperature (day 1)	Temperature (day 2)	Temperature (day 3)	Temperature (day 4)
National	28.22a	36.89a	39.10a	39.77a
Trinitarian	28.00a	36.78a	39.12a	41.38a
EEM	0.3333	0.1665	0.0331	5.416
P<0.05	0.5893	0.4930	0.9136	0.3947

pH

In this sense, it was observed that the pH variable in the fermentation process was not affected (P>0.05) by the effect of the cocoa variable used in the last moments of its evaluation, except in the first moment of the fermentation process (day 1) where the highest value (P<0.05) was shown by the variety called national (Table 3)

Table 2
Effect of variety on the cocoa temperature in the fermentation process

Varieties	pH (día 1)	pH (día 2)	pH (day 3)	pH (day 4)
National	3.63a	4.79a	4.97a	4.92a
Trinitarian	3.44b	4.79a	4.79a	4.80a
EEM	0.28	<0.00000	0.266	0.1833
P<0.05	0.0035	1.000	0.1008	0.2039

Brix Grades

Regarding the quantification of the °Brix, which indicates the percentage of soluble solids based on the total solution, it can be mentioned that the cocoa variety had a characteristic effect, showing differences in the study and quantification process, with a higher value ($P<0.05$) for the national cocoa variety (Table 4).

Table 3
Effect of variety on the °Brix of cocoa during fermentation process

Varieties	°Brix (day 1)	°Brix (day 2)	°Brix (day 3)	°Brix (day 4)
National	13.14a	12.71a	11.34a	9.57a
Trinitarian	12.84b	11.91b	11.01b	8.02b
EEM	0.458	1.196	0.518	2.326
P<0.05	<0.001	<0.001	0.0019	<0.0001

Variables of the cut-off test

Regarding the cut test variables, among which were evaluated the percentage of testa, cotyledon, seed index, fermented elements, and the presentation of violet almonds and slates; it can be indicated that there were no statistical differences ($P>0.05$) due to the effect of cocoa variety. In all cases, these variables showed similar results, although the numerical performance of the variables was slightly higher in the national cocoa variety, except for the fermented variable, in which the Trinitarian cocoa variety showed better numerical performance, although not statistically (Table 5).

Table 4
Effect of variety on the variables of the cocoa cutting test in the fermentation process

Varieties	%Testa	% Cotyledon	Seed Index	Fermented products	Violets	Slate
National	14.06a	84.30a	1.54a	62.22a	30.88a	1.77a
Trinitarian	14.04a	83.33a	1.46a	74.78a	32.55a	1.66a
EEM	0.031	0.031	12.33	2.33	2.50	0.16
P<0.05	0.9882	0.9882	0.0888	0.5369	0.4882	0.7121

Sensory variables

Similarly, when evaluating the sensory variables, the results of this research showed that except for the cocoa and intensity variables, which showed differences (P<0.05), the other studies (Table 6) did not show changes in their behavior due to the effect of the cocoa varieties studied. In the case of the cocoa flavor variable, it was much deeper and determinant for the national variety, while this one presented a lower intensity.

Table 5
Effect of variety on cocoa sensory variables after fermentation process

Varieties	Smell	Acidity	Bitterness	Cocoa	Walnut	Dry Fruits	Floral	Spices	Others	Intensity
National	3.70	0.83	4.00	3.93a	2.53	2.50	2.63	0.23	0.83	3.20b
Trinitarian	3.70	0.53	3.87	3.50b	2.16	2.43	2.37	0.23	0.47	4.33a
EEM	<0.0001	0.8215	0.3651	1.1867	1.004	0.1826	0.7303	<0.0000	1.0041	3.1037
P<0.05	1.0000	0.103	0.5286	0.044	0.2081	0.8334	0.4874	1.00000	0.1219	0.0005

Effect of induction with Rhizobium Japonicum on physical-chemical, sensory and cadmium content variables in cocoa beans.
Temperature

able 7 shows the effect of the induction with *Rhizobium Japonicum* on the temperature of the cocoa in the fermentation process; it is evident that at the beginning and end of the study (day 1 and 4 of fermentation, respectively) there were no statistical differences (P>0. 05) in the temperature values, however, in the intermediate estimations (day 2 and 3 of fermentation, respectively), the addition of the higher value of the microbiological inducer of fermentation (*Rhizobium Japonicum*), caused the existence of significant (P<0.05) higher values of temperature.

Table 6
Effect of induction with Rhizobium Japonicum on the temperature of cocoa in its fermentation process

Rhizobium Japonicum	Temperature (día 1)	Temperature (día 2)	Temperature (día 3)	Temperature (día 4)
0%	28.17a	36.00b	38.78b	40.00a
3%	28.17a	36.50b	38.90b	41.67a
5%	28.00a	38.00a	39.65a	42.05a
EEM	0.1360	1.471	0.6651	5.606
P<0.05	0.9264	<0.0001	0.0083	0.3504

pH

Regarding the effect of induction with *Rhizobium japonicum* on the pH of cocoa in the fermentation process, there is no generalized linear consequence of the values (except on day 1, linear decreasing); however, there were statistical differences (P<0.05) in days 1 and 3, but not on days 2 and 4 (P<0.05), respectively. This indicates that for this research the induction with *Rhizobium japonicum* is not consistent and is even less correlated with the decrease or increase of pH.

Table 7
Effect of induction with Rhizobium Japonicum on the pH of cocoa in its fermentative process

Rhizobium Japonicum	pH (day 1)	pH (day 2)	pH (day 3)	pH (day 4)
0%	3.67a	5.00a	5.02a	4.85a
3%	3.61a	4.62a	4.58b	4.83a
5%	3.33b	4.75a	5.03a	4.90a
EEM	0.254	0.275	0.360	0.0489
P<0.05	0.0004	0.0747	0.0047	0.8263

Brix Grades

Regarding the percentage of soluble solids based on the total solution (quantification of °Brix), the induction with *Rhizobium japonicum* caused variable data in terms of the linearity of the values in response to the incremental addition (0, 3 and 5%) of inducer; however, there were changes in the statistical responses (P<0.05). These data also show that the older the age of evaluation (day 4), the °Brix values decrease as a mechanism of the fermentative processes, as evidenced by the effect of the induction with the microorganism (Table 9).

Table 8
Effect of induction with Rhizobium Japonicum on the °Brix of cocoa in its fermentation process

Rhizobium japonicum	°Brix1	°Brix2	°Brix3	°Brix4
0%	12.94c	12.43a	11.22ab	9.09a
3%	12.99b	12.26b	11.38a	9.03a
5%	13.03a	12.26b	10.96b	8.27b
EEM	0.055	0.1367	0.297	0.6506
P<0.05	<0.001	<0.001	0.0071	0.0006

Cut-off test variables

In the case of the variables testa percentage, cotyledon, and seed index, there were similar statistical results (P>0. 05) were similar, without showing a pattern of linear numerical behavior in response to induction with *Rhizobium japonicum*, except for the fermented variable (increases), presentation of violet almonds (decreases) and slates (decreases), which are shown in Table 10; it should be emphasized that a positive value (P<0.05) of the induction procedure was evident for these last variables.

Table 9
Effect of induction with Rhizobium japonicum on cocoa cutting test variables in the fermentation process

Rhizobium japonicum	% Testa	% Cotyledon	Seed Index	Fermented products	Violets	Slate
0%	13.33a	86.66a	1.51a	58.83b	38.83a	2.33a
3%	14.46a	85.52a	1.57a	67.50a	30.33 b	2.166a
5%	14.33a	85.66a	1.51a	73.33a	26.00 b	0.66b
EEM	0.87	0.87	6.27	10.31	9.23	1.29
P<0.05	0.7731	0.7731	0.3000	0.0014	0.0023	0.0010

Sensory variables

Regarding the sensory variables, the results of the present investigation did not show statistical differences (P<0.05), except for the other variable (indeterminate tastes and odors), which showed the presence of differences (P<0.05) (Table 11) without a linear decreasing growth criterion.

Table 10
Effect of induction with *Rhizobium Japonicum* on cocoa sensory variables after fermentation process

Rhizobium Japonicum	Smell	Acidity	Bitterness	Cocoa	Walnut	Dried fruit	Floral	Spices	Others	Intensity
0%	3.75a	0.80a	3.95a	3.65a	2.40a	2.40a	2.85a	0.15a	0.25b	4.00a
3%	3.55a	0.60a	3.65a	3.65a	2.35a	2.60a	2.25a	0.25a	1.00a	3.80a
5%	3.80a	0.65a	4.20a	3.85a	2.30a	2.40a	2.40a	0.30a	0.70ab	3.50a
EEM	0.3415	0.2687	0.711	0.2981	0.129	0.2981	0.8062	0.1971	0.9747	0.6496
P<0.05	0.7045	0.6454	0.1113	0.6716	0.9606	0.8369	0.4150	0.6045	0.0376	0.4088

Effect of cocoa variety interaction by Rhizobium japonicum induction on physical-chemical, sensory and cadmium content variables in cocoa beans
Temperature

Table 12 shows the values of the temperature variable as a response to the interaction of cocoa varieties by the induction of *Rhizobium japonicum*, showing that in the first days (1, 2 and 3) of the fermentation process, there were no statistical differences (P>0.05); however, on day 4, an interaction between the factors studied was observed, in addition to the clear increase in the value of this variable for the treatment constituted by the national cocoa variable with the induction of 5% of the product containing the microorganism (*Rhizobium japonicum*). Regardless of the method that has been applied in the fermentation of cocoa, according to Cardona Velásquez *et al.* (2016), covering the fermentation box helps to increase the temperature so that it has a favorable interaction with the appearance of the proliferation and biological activity of microorganisms and improving the chemical and biochemical properties and having better oxidative catabolism of organic substances, recommends that fresh almonds should be covered with banana leaves that have glucose and nutrient contents and help to improve fermentation and quality of cocoa beans ensuring the heating of temperature homogeneously helps the purification of air to the fermenter.

Arévalo *et al.,* (2017) affirm that the temperature is due to the activity of the fermentation stage where there are alterations in the properties mentioned that it is called the third phase in which beneficial microorganisms such as acetic acid bacteria participate by modifying the ethanol that are produced by the yeast (helping to have an optimal fermentation) in acetic acid by having an adequate temperature rise will exist the presence of Bacillus (bacteria) these contribute to the flavor so it produces organic acids and flavorings such as butanediol to have optimal temperatures is obtained an excellent fermentation and microbial activity.

Table 11
Effect of the interaction of cocoa varieties by the induction of *Rhizobium japonicum* on the cocoa temperature in the fermentation process

Varieties	*Rhizobium japonicum*	Temperature (day 1)	Temperature (day 2)	Temperature (day 3)	Temperature (day 4)
National	0%	28.00	36.00	38.90	41.16b
	3%	28.33	36.67	38.67	41.70b
	5%	27.67	38.00	39.73	42.76a
Trinitarian	0%	28.33	36.00	38.67	41.16b
	3%	28.00	36.33	39.13	41.63b
	5%	28.33	38.00	39.57	41.33b
EEM		0.2545	0.0961	0.1931	0.4044
P<0.05		0.5171	0.6186	0.3250	0.0009

pH

Table 13 shows the values of the pH variable as a response to the interaction of cocoa varieties by the induction of *Rhizobium japonicum*, showing that on the second day of the fermentation process, there were no statistical differences

(P>0.05); however, on days 1, 3 and 4, an interaction between the factors studied was observed (P<0.05), although without a clear pattern that would allow to deduce that the interaction responded according to the incremental level of the microorganism studied.

Álvarez *et al.* (2010) ratify that the pH are parameters to consider and expressed in the quality of cocoa and final product by having an excellent fermentation used by the chocolate production factories highlights that the excessive increase of acetic acid this has an unfavorable effect on the fermentation altering its properties and its adverse on the flavor. The adverse effects on the flavor by having a high pH content in the cotyledons is an indication that there is a bad fermentation cataloged as over fermentation of cocoa beans in the fermentation process this has an effect by the appearance of carboxylic acids and biogenic amine belonging to the amino acids. According to Vega (2018), a noble fermentation should have a pH range between 5.0-6.1 the optimal pH according to Armijos (2002) should be 5.1 to 5.7 so that there is an adequate fermentation but on the contrary, if the pH is less than 5. 0 the appearance of non-volatile acids is manifested, which are not desirable for the chocolate aromas, which are very unpalatable. This happens when there is incomplete fermentation, according to the research, and it has been shown that the use of this microorganism helps to improve the pH so that there is a favorable fermentation. When pH levels are lower than usual cocoa is below good quality. Castañeda *et al.* (2016) found that cocoa beans that have a pH below 4.5 generate low potential aromatic precursors. Zambrano *et al.* (2010) indicate that if the pH is in a range of 5.5-5.8, it can be estimated that it is likely to be fermented, but if the range is between 4.7 and 5.2, it can be assessed that there has been an adequate fermentation.

Table 12
Effect of the interaction of cocoa varieties by the induction of Rhizobium japonicum on the pH of cocoa in its fermentation process

Varieties	*Rhizobium Japonicum*	pH (day 1)	pH2 (day 2)	pH3 (day 3)	pH4 (day 4)
National	0%	3.83a	4.83	4.80ab	5.10ab
	3%	3.73ab	4.63	4.77ab	5.03ab
	5%	3.33c	4.90	4.80ab	5.63b
Trinitarian	0%	3.50bc	5.17	5.23b	4.60b
	3%	3.50bc	4.60	5.40a	4.63b
	5%	3.33c	4.60	5.27a	5.17a
EEM		0.085	0.1589	0.235	0.2849
P<0.05		0.059	0.1573	0.0080	0.0010

Brix Grades

On days 1, 2, and 4 of the fermentation process, the experimental values of the °Brix variable showed an interaction between the factors studied (P<0.05), although without a clear linear pattern that would allow to deduce that the interaction of the factors was directly proportional to the increase in the level of microorganism used (Table 14), however, on the third day of the fermentation process, there was no statistical difference (P>0.05).

Santana *et al.* (2019) mention that as time goes by the amount of mucilage decreases, this effect occurs due to the microbial activity that fulfills the function that exists when temperatures increase.

The Brix degrees to determine the soluble solids as mentioned in the fruit must have an acceptable sweetness for the final product, whether for a liqueur or nectar and not alter its organoleptic properties mentioned Quizphi (2016), in this case, there is evidence that by inducing the microorganism there is a decrease in the °Brix, therefore that there is bitter chocolate of good quality, the main factor is at the time of fermentation to meet the required properties such as cocoa paste data that are not represented or equal to such research there is the tendency of the decrease of °Brix. According to Garcia and Martinez (1967), the appearance and increase of yeasts is due to the content of total sugars in the cocoa bean cotyledon, the brix that are considerably higher favor the metabolic action of the yeasts to obtain an excellent fermentation of cocoa beans, having adequate values will have the effect of having available sugars for the proliferation of yeasts and these happen to make ethanol.

Table 13
Effect of the interaction of cocoa varieties by the induction of *Rhizobium japonicum* on the °Brix of cocoa in its fermentation process

Varieties	*Rhizobium Japonicum*	°Brix (day 1)	°Brix2 (day 2)	°Brix3 (day 3)	°Brix4 (day 4)
National	0%	13.12b	12.84a	11.40	10.03a
	3%	13.14ab	12.79a	11.62	10.10a
	5%	13.17a	12.50b	11.05	8.58b
Trinitarian	0%	12.78d	12.01c	11.04	8.16b
	3%	12.85c	11.72d	11.13	7.96b
	5%	12.88c	12.01c	10.87	7.95b
EEM		0.0135	0.1451	0.0803	0.4001
P<0.05		0.0154	<0.001	0.3573	0.0019

Cut-off test variables

Based on the results of the variables, testa percentage, cotyledon, seed index and slates, it can be indicated that there were no results that differed statistically (P>0.05) by the effect of the interaction of the factors cocoa varieties by the induction of *Rhizobium japonicum*, contrary to what was observed (P<0.05), with the variables fermented and presentation of violet almonds. Similarly, it can be indicated that there is no linear numerical behavior pattern in response to *Rhizobium japonicum* induction (Table 15). It is of relevance according to Aguilar (2016), that the cut test is of great importance since performing such cut analysis it is possible to evidence different evaluative factors such as fermented beans, violets, slates, fungi among others present in the cocoa beans, besides being an observatory analysis demonstrating the quality of fermentation that was generated in the time elapsed in that process, so the results obtained are acceptable and comparable with INEN 176 (2018), the appropriate requirements for the quality of cocoa and evidencing by visual examination according to standard 175 in the cut test.

Table 14
Effect of the interaction of cocoa varieties by the induction of Rhizobium japonicum on the variables of cocoa cut test in its fermentation process

Varieties	*Rhizobium Japonicum*	%Testa	% Cotyledon	Seed rate	Fermented products	Violets	Slates
National	0%	13.11	86.89	1.56	63.00bc	34.33ab	2.66
	3%	13.44	86.55	1.58	71.00ab	27.00ab	2.00
	5%	15.55	84.44	1.49	68.00abc	31.33abc	0.66
Trinitarian	0%	13.55	86.44	1.52	54.66c	43.33a	2.00
	3%	15.50	84.49	1.44	64.00bc	33.66abc	2.33
	5%	13.11	86.89	1.42	78.66a	20.66c	0.66
EEM		1.14	1.14	2.58	5.30	5.37	0.25
P<0.05		0.4375	0.4375	0.6470	0.0137	0.0093	0.3966

Sensory variables

For this group of variables, the results do not show statistical differences (P<0.05), except for the variables others and intensity, which showed changes in their statistical behavior (P<0.05), due to the effect of the interaction between the studied factors (Table 16), it should be reiterated that there was no evidence of a linear growth criterion of the response values due to the effect of the increasing addition of the studied microorganism. Portillo *et al.* (2009) and Álvarez *et al.* (2020) mentioned that the fundamental part for the generation of sensory properties is the fermentation process where the precursors of the aroma and final flavor of the finished product are developed, it has to do with the chemical properties are closely related to the sensory quality these are also due to thermal phenomena as a result of fermentation, the method of fermenting cocoa almonds has a link with the sensory characteristics emphasizes Quevedo *et al.* (2018), that it is important for the flavor and aroma the fermenting boxes covered with banana leaves help the fermentation of beneficial microorganisms that fulfill the function of eliminating the mucilage that are covered in the cocoa bean, therefore if there are optimal removals allow the elimination of the embryo that dies and there is no presence of bitter taste, reducing the loss of theobromine enhancing the flavor and aroma of chocolate that the research conducted show similar data between the variables evaluated for the sensory analysis.

Table 15
Effect of the interaction of cocoa varieties by *Rhizobium japonicum* induction on cocoa sensory variables in its fermentation process

Varieties	*Rhizobium Japonicum*	Smell	Acidity	Bitterness	Cocoa	Walnut	Dried fruit	Floral	Spices	Others	Intensity
National	0%	3.80	1.00	4.30	4	2.1	2.2	2.7	0.1	0.1	3.2
	3%	3.60	0.60	3.50	3.9	2.2	2.8	1.8	0.3	1.6	2.8
	5%	3.70	0.90	4.20	3.9	2.2	2.5	2.6	0.3	0.8	3.6
Trinitarian	0%	3.70	0.60	3.60	3.3	2.7	2.6	3	0.2	0.4	4.8
	3%	3.50	0.60	3.80	3.4	2.5	2.4	2.7	0.2	0.4	4.8
	5%	3.90	0.40	4.20	3.8	2.4	2.3	2.2	0.3	0.6	3.4
EEM		0.9448	0.242	0.468	0.2768	0.19	0.3800	0.5939	0.0912	0.6972	1.0697
P<0.05		0.8601	0.4946	0.1471	0.5001	0.8405	0.5630	0.3855	0.8050	0.0349	0.0108

Cadmium concentrations

Based on the experimental results on the concentration of Cadmium in cocoa beans, after the fermentation process, it can be said that after the descriptive analysis of these, both in the national and trinitarian cocoa varieties, the induction with *Rhizobium japonicum* had positive effects on the reduction of the concentration of this polluting chemical element (Table 17). Concerning the above Perez *et al.* (2012) indicate that cadmium as heavy metal is characteristic of being a bioaccumulated in the human body producing harmful and irreversible damage to the health of people by the consumption of food containing cd content would cause internal problems of the organism being a risk of serious care whether this is environmental exposure or consumable being this listed as toxic to know the percentages allowed to tolerate and permissible, it is essential to avoid any damage or harm to the consumer.

While Covarrubias *et al.* (2015) mention the use of bacteria that fulfill the role of being beneficial to behave as bioregulators when adding or inoculating in seed or in any plant that you want to reduce cadmium, in addition, these bioregulators help growth, strengthen, and improving productivity fulfills the function of a development enhancer allows the absorption of nutrients such as fertilizers by the use of microorganisms such as *Rhizobium japonicum*, which has the characteristic of fixing nitrogen.

Table 16
Interaction of cocoa varieties by *Rhizobium japonicum* induction on cadmium concentrations in cocoa

Varieties	*Rhizobium japonicum*	mg/ kg
National	0%	0.36
	3%	0.34

	5%	0.35
	0%	0.36
Trinitarian	3%	0.30
	5%	0.29

Conclusions

Based on the results obtained, it is concluded that the cadmium contents in the present investigation comply with the established standard N° 488/2014 of the European Union for the different treatments obtaining favorable results for the reduction of Cadmium in cocoa beans by induction with *Rhizobium Japonicum*. Inconclusive effects of the cocoa variety variable on the physicochemical, cutting test and sensory variables of the fermented cocoa beans were observed since there were no significant statistical differences for all the variables studied. It is important to mention that there is no specific regulation for the values of these variables according to the cocoa variety, and it is possible to indicate that, when compared with those found by other authors, the present results are closely related (temperature 28.00 to 41.38, pH 3.44 to 4.92, °Brix 13.14 to 8.02) with those of the literature. There are inconclusive effects of the induction variable with Rhizobium Japonicum on the physicochemical, cutting test and sensory variables of the fermented cocoa beans since there are no significant statistical differences for all the variables studied. Similarly, it is possible to indicate that there are no reference values in the literature for the physicochemical variables according to the use of a microbiological induction method, however, when compared with those found by other authors, the present results are closely related (temperature 28.00 to 42.05, pH 3.33 to 4.90, °Brix 12.94 to (9.09) with those in the literature.

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