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Effect of Fermentation on Bacteria Isolates and Phytochemical Properties of Cocoa (*Theobroma cacao*) Beans

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Authors' contributions

This work was carried out in collaboration among all authors. Author FIO designed the study, wrote the protocol and wrote the first draft of the manuscript. Author ORA managed the laboratory analyses of the study and performed the statistical analysis while author AVT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

This investigation was carried out to assess the bacteria associated with cocoa beans at different stages of fermentation and determine the changes in the phytochemical constituents of the fermenting beans. The pour plate technique was used for bacterial isolation while phytochemicals were assessed based on standard qualitative chemical reactions. The total bacterial count on the cocoa beans reduced during fermentation from the initial 86.2±0.02 x 10^5 CFU/g (day 0) to 1.00±0.00 x 10^5 CFU/g on day 5. However, there was an increase in the lactic acid bacteria count from 48.7±0.03 x 10^5 CFU/g (day 0) to 111.7±0.03 x 10^5 CFU/g on day 3, then reduced to 51.4±0.01 x 10^5 CFU/g on day 5. *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus acidophilus* were isolated from the cocoa beans at different stages of the fermentation. There was a gradual increase in the temperature of the fermenting cocoa mass from the initial 25.6°C recorded at the beginning of the fermentation to 42.8°C recorded on day 5. Also, the pH of the fermenting cocoa beans reduced significantly from 6.1 at the commencement of the fermentation to 3.2 on day 5. In the total titratable acidity assay, the acidity of the cocoa beans increased from 3.12% at the beginning of the

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fermentation to 7.12% on day 5. Further, in the phytochemical screening, only alkaloids, phenols, steroids and flavonoids were detected in the beans throughout fermentation period whereas tannin and saponin were not found in the beans at any stage of fermentation. The preset phytochemicals got reduced in intensity as fermentation advanced. From these results, it can therefore be concluded that fermentation helps to improve the taste quality and phytochemical properties of Nigerian cocoa beans.

Keywords: Cocoa; microorganisms; fermentation; metabolites.

1. INTRODUCTION

Cocoa (*Theobroma cacao*, L.) is one of the most important agricultural produce in Nigeria, it is a source of foreign exchange, regional development, driving the development of agribusiness and agro-industry since pre-independence time [1]. Since the introduction of the crop to Africa from the native South America, the region has developed to become the leading producing region throughout the world. For instance, Ivory Coast is the leading cocoa producer in the region, followed by Nigeria with about one-fifth of the world's production [2].

Serra and Ventura [3] observed that cocoa beans from different parts of West Africa differs in several quality attributes such as amino acids and other secondary metabolites like phytochemicals. The differences in these parameters may be attributed to the difference in geographical indices of the various countries. Another possible reason may be due to the types and length of fermentation employed by the farmers. Here, microorganisms play a key role in the fermentation process ad therefore, contributes to the quality of the end product.

Moreover, the farming methods as well as post-harvest processes adopted by farmers are neither standardized nor uniform thereby making the cocoa bean quality to differ significantly between them [4]. Earlier. Rohsius et al. [5] have confirmed that the cocoa beans produced in West African countries are more quality than those produced in other parts of the continent, especially in the amino acid content while the level of substances like sugar, polyphenols and fat content in cocoa beans have been reported to be dependent on the origin of such cocoa beans [6].

Because of these differences in chemical composition of cocoa beans there are differences in the resulting flavour of the cocoa derived products produced from these cocoa beans. In an earlier study, Sukhat et al. [7] submitted that there exist significant differences in the flavor profiles of cocoa derived products produced from cocoa beans from different origins.

It has been established that flavors and their precursors are developed during the fermentation and drying processes of post-harvest handling of cocoa beans [8]. The flavor are developed by the activities of different microorganisms involved in the fermentation of the cocoa beans alongside the action of endogenous enzymes on macromolecules present in cocoa beans such as carbohydrates, proteins and polyphenols [9].

Phytochemicals have been described as naturally occurring bioactive substances that are produced by plants in response to infections by pathogens. These chemicals a time do work with nutrients to offer protection against the pathogens. It is reported that phytochemicals are the most surplus and widely distributed materials in plant system. Plant metabolites may be classified into primary and secondary metabolites where the primary metabolites consists of materials like proteins, triglycerides, saccharides and amino acids while the secondary metabolites include alkaloid, flavonoids, steroids, saponin, terpenoids, phenolics, etc., these are often referred as phytochemicals [10].

Some of these phytochemicals are known to confer special attributes to food substances. For instance, saponins may be an anti-nutrient as they are often bitter to taste, and so can reduce palatability, or even imbue them with life-threatening animal toxicity. Some saponins are toxic to coldblooded animals and insects at particular concentrations according to Edeoga and Eriata [11]. Similarly, alkaloids are reported to impute bitter taste food and feed. It is reported to act on different systems in man and other lower animals. Although, some animals have evolved the ability to detoxify alkaloids, some alkaloids can still produce developmental defects animals that consume but cannot detoxify the alkaloids [12]. Moreover, tannins are known to

confer astringent taste and smell to many plant based food ad it is reported to be responsible for dry and puckery feeling in the mouth following the consumption of unripened fruit or red wine.

During fermentation processes, there is bound to be changes in the chemical constituent of the substrate. These changes may be beneficial or otherwise. Some investigators have reported the reduction of some phytochemicals known as anti-nutrients during fermentation of plant materials. Although, the role of microorganisms in cocoa beans fermentation is reported to be limited to the removal of the pulp that surrounds the fresh beans [13], some important metabolites are also produced in the process. Yeasts are the main starter culture in the fermentation of cocoa where it covert sugars to alcohol while lactic acid, acetic acid and mannitol are produced by lactic acid bacteria (LAB), and conversion of ethanol into acetic acid is done by acetic acid bacteria (AAB). The heat released from this process and the diffusion of metabolic products into the beans put the bean germination into halt [14] and may alter the phytoconstituent of the cocoa beans. Also, other microbial metabolites such as esters and pyrazines, may enter the bean cotyledons and act as flavour precursors or directly as flavour compounds.

Therefore, this study was designed to assess the effect of fermentation on bacteria population associated with the cocoa bean fermentation and changes in its phytochemical contents at different stages of fermentation.

2. MATERIALS AND METHODS

2.1 Reagents

The analytical high grade purity reagents used were obtained from Sigma- Aldrich Inc (Missouri, USA) being used as procured without further purification.

2.2 Collection of Cocoa Samples

The cocoa beans used for this study were obtained from a cocoa plantation in Iyere, Owo, Ondo State Southwestern Nigeria. They were plucked directly from parent tree into sterile polythene bags and taken to the laboratory immediately for further processing. The plant material was authenticated at the Environmental Biology Unit of Science Laboratory Technology Department, Rufus Giwa Polytechnic, Owo and voucher specimens (TCC101B) was deposited at the Herbarium Section of the Unit.

2.3 Sterilization Protocol

The glass wares, such as conical flask, beaker, test-tubes etc. were duly soaked in detergent for 12 hours and then washed thoroughly using brush. They were subsequently rinsed in large quantity of clean water and finally with distilled water. The glass wares were air dried and then sterilized in hot air oven for 2 hours at holding temperature of 160°C. Inoculating wire loop used were sterilized by flaming with a Bunsen burner until red hot and then allowed to cool before using. The surfaces of the workbench were sterilized by cleaning with 75% alcohol before and after each working period.

2.4 Fermentation of Cocoa Beans

The fresh cocoa pods were broken and the beans evacuated aseptically into sterile wooden box previously inlaid with plantain leaves. It was then covered with another layer of plantain leaves and the box was sealed and allowed to ferment for 5 days according to the method of Afoakwa [1]. The set up was done in triplicates.

2.5 Physicochemical Analysis

2.5.1 Determination of ph and temperature

Ten gram of the sample was dissolved in 100 ml of distilled water. The pH and temperature of the samples were determined using a Geirincharz Thermos pH meter. This was done at 24, 48 and 72 hrs in triplicates.

2.5.2 Determination of titratable acidity

Total titratable acidity (TTA) was determined according to the method described by AOAC [15]. 50 g of the sample was blended, added to 100 ml of distilled water, agitated and allowed to settle. Then 20 ml of the supernatant was titrated against 0.1 M NaOH until a faint pink sharp end point was reached. Acidity was calculated as percentage/100 g of sample. This was done at 0, 24, 48 and 72 hrs in triplicates.

2.5.3 Isolation of bacterial species

At intervals of 24 hrs, the cocoa beans were analyzed for changes in microbial population. The pour plate method was adopted for the culturing of the organisms. 0.2 ml of the aliquot of 10^{-3} were dropped in pre-labeled separate sterile petri dishes in duplicates, 20 ml of molten agar at 45°C was poured on it and the petri dishes were swirled to homogenize. The plates were allowed to solidify on working bench and

then they were incubated inverted in incubator at 35°C for 18 to 24 hrs for nutrient agar plates while MRSA plates were incubated anaerobically for 48hrs. The colonies were counted after incubation and discrete colonies were sub-cultured on nutrient agar slant for further processing.

2.6 Characterization of Isolates

This was done based on the cultural and morphological characteristics of the colonies as well as appropriate biochemical properties as prescribed in the criteria of Berger's Manual of Determinative Bacteriology.

2.6.1 Qualitative phytochemical screening

All the tests described below were carried out on the extracts using the methods described by Sofowora [16].

2.6.2 Test for tannins

A 1ml of sample extract was boiled in 5 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for a green or a blue – black coloration which confirmed the presence of tannin.

2.6.3 Test for saponin

A part of the sample extract (1 ml) was boiled in 4 ml of distilled water in a water bath and filtered. 2 ml of the filtrate was mixed with 2 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with drops of olive oil and shaken vigorously, then observed for the formation of emulsion which confirmed the presence of Saponins.

2.6.4 Test for flavonoids

A measure of 3 ml of 1% Aluminum chloride solution was added to 3 ml of the sample extract. A yellow coloration was observed indicating the presence of flavonoids.

2.6.5 Test for steroids

An equal amount of acetic anhydride and sample extract (2 ml) was mixed thoroughly, this was followed by addition of 2 ml H_2SO_4. A color changed from violet to blue or green indicated the presence of steroids.

2.6.6 Test for alkaloids

A portion (1 ml) of the extract was mixed with 5 ml 1% $HCl_{(aq)}$ on a steam bath and filtered while hot. A part (1 ml) of the filtrate was treated with a few drops of Potassium mercuric iodide solution and a red-brown coloration was taken as a positive test for alkaloids.

2.6.7 Test for phenol

A portion (2 ml) of the extract was measured into a test tube, and then 4ml of distilled water was added to it. Later, 1 ml of NH_4OH solution and 2.5 ml of concentrated amyl alcohol were also added and left to react for 30min. Appearance of bluish-green colour was taken as a positive presence of phenol.

3. RESULTS AND DISCUSSION

The result of the bacterial count on the cocoa beans during fermentation is presented in Fig. 1. The table revealed that the total bacterial count on the cocoa beans reduced during fermentation from the initial 86.2±0.02 x 10^5 CFU/g (day 0) to 1.00±0.00 x 10^5 CFU/g on day 5. However, there was an increase in the lactic acid bacteria count from 48.7±0.03 x 10^5 CFU/g (day 0) to 111.7±0.03 x 10^5 CFU/g on day 3, then reduced to 51.4±0.01 x 10^5 CFU/g on day 5. The reduction in the total bacterial count from day 0 to day 5 may be due to the creation of acidic environment which do not support the growth of many bacteria species. Also the oxygen tension in the fermenting cocoa mass may be a limiting factor as all the aerobic bacteria are eliminated leaving the anaerobes. This agrees with the report of Schwan and Wheals [17].

A total of six bacteria species were isolated from the cocoa beans during fermentation, their characteristics are presented in Table 1 and Table 2 while their occurrence at different stages of fermentation is presented in Table 3. The bacteria include *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus acidophilus.* They were isolated at different point of the fermentation. Among these, *B. subtilis* was isolated throughout the fermentation stages while *S. aureus* and *M. luteus* were isolated only before the commencement of the fermentation whereas *L. fermentum* was isolated up to day 3 but *L. plantarum* and *L. acidophilus* appeared on day 1 and were isolated till the end of the fermentation period (Table 3). The presence of

S. aureus and *M. luteus* may be due to contamination from handling and may not be part of fermenting microbes [18]. Only *B. subtilis* was found all through the fermentation period, this may be due to its ability to produce spores which are resistant to harsh conditions [19]. Although *L. fermentum* was isolated from the beginning of the fermentation to day 3, it disappeared thereafter. Both *L. plantarum* and *L. acidophilus* appeared after 24 hours of fermentation and persisted to the end of the experimental period. These observations are in line with the earlier reports that Lactic acid bacteria are the major fermentation organisms at the second stage of cocoa beans fermentation which falls between day 1 and day 3 [18,19].

The results of the physicochemical changes in the cocoa beans at different stages of fermentation is shown in Fig. 1. The figure shows that there was a gradual increase in the temperature of the fermenting cocoa mass from the initial 25.6^0C recorded at the beginning of the fermentation to 42.8^0C recorded on day 5. The increase in the temperature of the fermenting mass of the cocoa beans may be due to the heat generated by the organisms causing the fermentation. Earlier reports suggested that during fermentation, sugar is converted to alcohol and carbon dioxide while heat is released as by product [1]. This heat is usually trapped by the carbon dioxide inside the fermentation container thereby increasing the temperature of the fermentation mass. This observation is in agreement with earlier report of Ardhana and dan Fleet [8].

Also, the pH of the fermenting cocoa beans reduced significantly from 6.1 at the commencement of the fermentation to 3.2 on day 2 but increased again to 5.3 on day 5. In the total titratable acidity assay, the acidity of the cocoa beans increased from 3.12 at the beginning of the fermentation to 6.87 on day 2 then started decreasing to 4.0 on day 5. The reduction in pH may be due to the production of organic acids by the fermenting bacteria. This further creates encouraging environment for the growth of these acidophiles thereby allowing *Lactobacillus* species to dominate fermentation process and converting the intermediate metabolites into lactic acids [20].

The pH and the titratable acidity were more appropriate indicator to measure the total acid level in any fermentation process and usually both parameters are negatively correlated. According to Afoakwa et al. [21], the synthesis of lactic acid was very common among lactic acid fermentation, especially cocoa beans fermentation that is carried out under anaerobic condition. The conversion of fermentable substrate into desired metabolite by-products was performed exothermically, and hence it assisted to the increase of temperature [17].

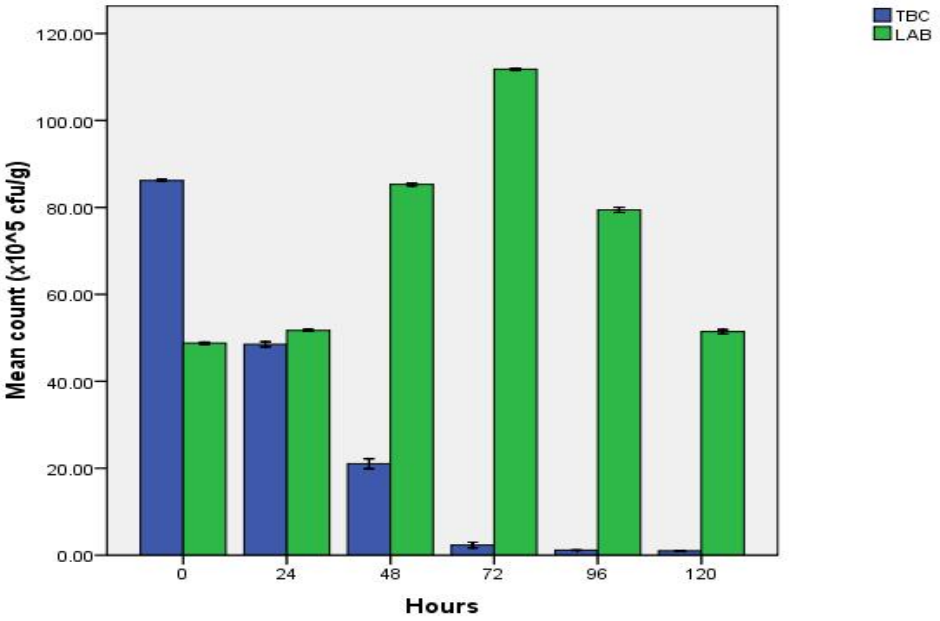

Fig. 1. Microbial count on cocoa beans at different stages of fermentation (x 10^5 CFU/g)

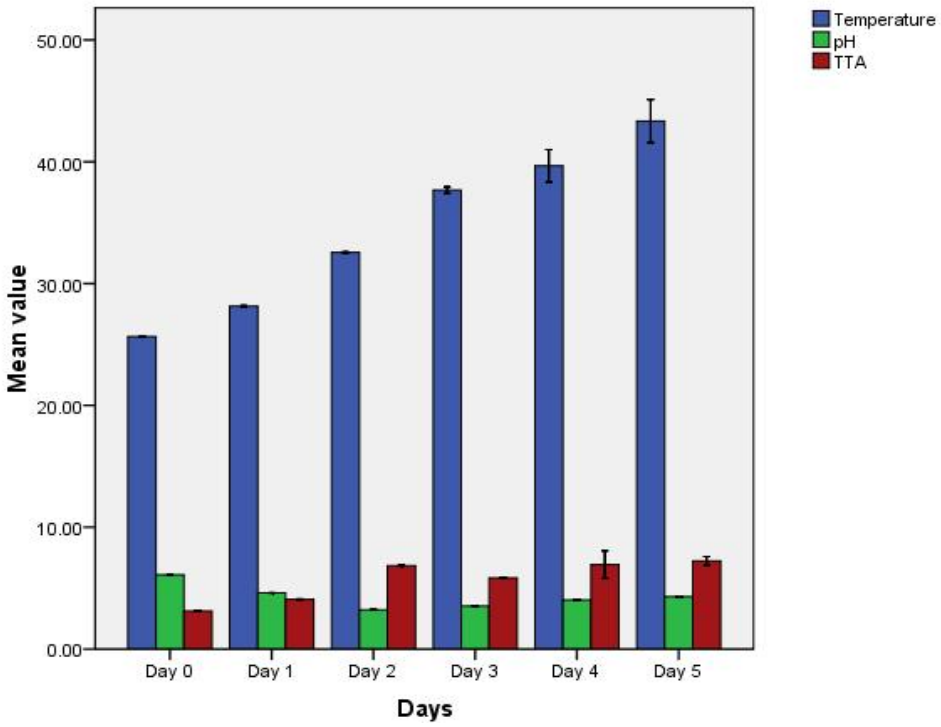

Fig. 2. Physicochemical changes of cocoa beans at different stages of fermentation

Table 1. Morphological characteristics of bacteria isolated from cocoa beans

Isolate codes	I	II	III	IV	V	VI
Colour	Pale yellow	Yellow	White	White	White	Creamy
Shape	Irregular	Circular	Irregular	Circular	Round	Circular
Edge	Entire	Entire	Entire	Entire	Entire	Entire
Elevation	Flat	Convex	Flat	Flat	Raised	Raised
Surface	Smooth	Smooth	Rough	Smooth	Smooth	Smooth

Table 2. Biochemical characteristics of bacteria isolated from cocoa beans

Isolate codes	I	II	III	IV	V	VI
Gram's reaction	+vecocci	+vecocci	+ve rod	+ve rod	+ve rod	+ve rod
Catalase	+	+	+	-	-	-
Coagulase	+	-	NA	NA	NA	NA
Spore	-	-	+	-	-	-
Glucose	AG	A	A	AG	+	AG
Lactose	A	L	A	AG	+	AG
Sucrose	A	-	A	AG	+	AG
Maltose	AG	-	-	-	+	-
Mannitol	A	-	+	AG	+	AG
Organism	*Stahylococcus aureus*	*Micrococcus luteus*	*Bacillus subtilis*	*Lactobacillus fermentum*	*Lactobacillus plantarum*	*Lactobacillus acidophilus*

Keys: + = Positive reaction, - = Negative reaction, A= Acid only, L= Late fermenter, AG= Acid and gas, NA= Not applicable

Table 3. Occurrence of bacteria isolates on cocoa beans at different stages of fermentation

Organisms	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Stahylococcus aureus	+	-	-	-	-	-
Micrococcus luteus	+	-	-	-	-	-
Bacillus subtilis	+	+	+	+	+	+
Lactobacillus fermentum	+	+	+	+	-	-
Lactobacillus plantarum	-	+	+	+	+	+
Lactobacillus acidophilus	-	+	+	+	+	+

+= present, -= absent

Table 4. Phytochemicals present in cocoa beans at different stages of fermentation

Phytochemical	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Alkaloids	+++	++	++	+	+	+
Tannins	-	-	-	-	-	-
Saponins	-	-	-	-	-	-
Flavonoids	++	++	+	+	+	+
Steroids	++	+	+	+	+	+
Phenols	+++	+++	++	++	++	++

+= present in trace amount, ++= present in moderate amount, +++= present in abundance, -= absent

In the phytochemical screening, only alkaloids, phenols, steroids and flavonoids were detected in the beans throughout fermentation period whereas tannin and saponin were not found in the beans at any stage of fermentation. These observations are in agreement with the reports of Afoakwa et al*.* [21]. Alkaloids like caffeine and theobromine alongside polyphenolic compounds like have been shown to impart bitterness and astringency in cocoa [13]. There was a reduction in the vigor of the reaction of the phytochemicals with advance in the time of fermentation. Only the phenols were still detected in moderate amount after the fermentation period while the others were detected in trace amount. The possible explanation for this may be due to the diffusion of the phytochemicals out of the beans during fermentation [22] or possible degradation by the action of the microorganisms. It is important to note that the phytochemicals were not removed totally from the cocoa beans as a result of fermentation, therefore it may be inferred that fermentation do modify the phytochemical content of cocoa beans and not outright removal.

4. CONCLUSION

Based on the results obtained from this study, it may be concluded that spontaneous fermentation modifies the phytochemicals present in cocoa beans. This action may be responsible for the lowering of bitter taste of the fermented cocoa beans which by extension helps to improve its taste quality.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Afoakwa E, Paterson A, Fowler M. Factors influencing rheological and textural qualities in chocolate e a review. Trends in Food Science and Technology. 2014; 18(6):290-298.
2. Afoakwa EO, Paterson A, Fowler M, Ryan A. Flavor formation and character in cocoa and chocolate: A critical review. Critical Reviews in Food Science and Nutrition. 2008;48:840–857.
3. Serra Bonvehí J, Ventura Coll F. Factors affecting the formation of alkylpyrazines during roasting treatment in natural and alkalinized cocoa powder. Journal of Agricultural and Food Chemistry. 2002; 50(13):3743-3750.
4. Lima LJR, Almeida MH, Nout MJR, Zwietering MH. *Theobroma cacao* L., the food of the gods: quality determinants of commercial cocoa beans, with particular reference to the impact of the fermentation. Critical Reviews in Food Science and Nutrition. 2011;51(8):731-761.
5. Rohsius C, Matissek R, Lieberei R. Free amino acid amounts in raw cocoas from different origins. European Food Research and Technology. 2006;222:432-438.

6. Arlorio M, Locatelli M, Travaglia F, Coïsson JD, Del Grosso E, Minassi A. Roasting impact on the contents of clovamide (N-caffeoyl-L-DOPA) and the antioxidant activity of cocoa beans (*Theobroma cacao L*.). Food Chemistry. 2008;106(3):967-975.
7. Sukha DA, Butler DR, Umaharan PE, Boult E. The use of an optimized organoleptic assessment protocol to describe and quantify different flavour attributes of cocoa liquors made from Ghana and Trinitario beans. European Journal of Food and Technology. 2008; 226:405–413.
8. Ardhana M, Fleet G. The microbial ecology of cocoa bean fermentations in Indonesia. International Journal of Food Microbiology. 2003;86:87–99.
9. de Brito ES, García NHP, Gallão MI, Cortelazzo AL, Fevereiro PS, Braga MR. Structural and chemical changes in cocoa (*Theobroma cacao L*) during fermentation, drying and roasting. Journal of the Science of Food and Agriculture. 2001;81(2):281-288.
10. Camu N, De Winter T, Addo SK, Takarama JS, Bernaert H, De Vuyst L. Fermentation of cocoa beans: Influence of microbial activities and polyphenol concentrations on the flavour of chocolate. Journal of the Science of Food and Agriculture. 2008;88:2288–2297.
11. Edeoga HO, Eriata DO, Alkaloid tannin. Saponin contents of some Nigeria medicinal plants. Journal of Medicinal Aromatic Plant Science. 2001;23:344-349.
12. Ogundipe OO, Moody JO, Houghton PJ, Odelola HA. Bioactive chemical constituents from *Alchornea laxiflora* (Benth.) Pax and Hoffman. J. Ethno-pharmacol. 2001;74:275-280.
13. Misnawi S. Physic-chemical changes during cocaoa fermentation and key enzymes involved. Review penelitian kopi dan kakao 2008;24(1):54-71.
14. Papalexandratou Z, Vranckena G, De Bruyneb K, Vandammeb P, De Vuys L. Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria. Food Microbiology. 2011;28:1326–1338.
15. Association of Official Analytical Chemist. Official methods of analysis, 18th Ed. Washington DC; 2005.
16. Sofowora A. Phytochemical screening of medicinal plants and traditional medicine in Africa. 2nd edition Spectrum Books Limited, Nigeria. 1993;150–156.
17. Schwan RF, Wheals AE. The microbiology of cocoa fermentation and its role in chocolate quality. Critical Reviews in Food Science and Nutrition. 2014;44:205–221.
18. Carr J, Davies P, Dougan J. Cocoa fermentation in Ghana and Malaysia. In 7th International Cocoa Research Conference. 2009:573-576.
19. LagunesGálvez S, Loiseau G, Paredes JL, Barel M, Guiraud JP. Study on the microflora and biochemistry of cocoa fermentation in the Dominican Republic. International Journal of Food Microbiology. 2007;114(1):124-130.
20. Nazaruddin R, Seng L, Hassan O, Said M. Effect of pulp preconditioning on the content of polyphenols in cocoa beans (*Theobroma cacao*) during fermentation. Industrial Crops and Products. 2006; 24(1):87-94.
21. Afoakwa EO, Quao J, Takrama J, Budu AS, Saalia FK. Effect of pulp preconditioning on acidification, proteolysis of sugars and free fatty acids concentration during fermentation of cocoa beans. Intr J. of Food science and nutrition. 2011;62(7):755-764.
22. Wollgast J, Anklam E. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. Food Research International. 2000;33:423-447.

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