Effect of Controlled Fermentation on Proximate Composition of Cocoa Bean

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ABSTRACT

The source of microorganisms used during fermentation is very important to the output of the fermentation process. The use of naturally predominant species from a fermentation process gives rise to improved fermentation than the use of microorganisms from culture collections. Natural and controlled fermentation of the cocoa bean inoculated with defined starter consortia of Acetobacter pasteurianus, Saccharomyces cerevisiae and Lactobacillus plantarum was done. Cocoa fermentation was carried out under two experimental conditions; beans naturally fermented with micro flora naturally present on the substrate and beans inoculated with a defined cocktail containing defined starters from a batch of previously fermented cocoa beans at different time course. The dynamics in microbial population and time course were determined by microbial count method. The chemical content of naturally fermented and controlled fermented cocoa beans were determined through proximate analysis respectively. The dynamics in microbial composition showed that the yeast, acetic acid bacteria and acetic acid bacteria populations increased slowly and reached maximum of 6.1 x 10^7, 4.4x 107 and 5.4x 10^7cfu\ml respectively, at days 3-4 for the natural fermentation, while they increased abruptly and reached maximum of 7.2x 10^7, 6.0x 10^7 and 6.1x 10^7cfu\ml respectively at day 2-3 for the controlled fermentation. Starter culture addition resulted in significant ($P<0.05$) decreases in ash (7.10%-4.50%), crude fiber (7.90-6.20%), crude protein (7.70-6.52%) content of the cocoa beans during natural and controlled fermentations, while carbohydrate and fat content increased from (62.00 - 69.70%) and (6.90 -7.70%) with starter addition. Thus, the fermentation of cocoa beans with starter culture addition produced fermented beans with higher natural quality and it potentially reduced the fermentation time to 3 days, as against 6-7 days recorded in natural fermentation.

INTRODUCTION

Cocoa (*Theobroma cacao L.*) belongs to the family *Sterculiaceae* and is economically important due to its valuable seed. In Nigeria, dry cocoa beans are exported as a foreign exchange earner while a small percentage of the cocoa beans serve as raw material for cocoa powder, cocoa butter and chocolate products (Adeyeye *et al*., 2010).

The fermented cocoa beans are the principal raw material for the chocolate preparation that imparts desirable flavor to the final chocolate (Afoakwa, 2010).The harvested cocoa pods are subjected to spontaneous fermentation through diverse natural micro flora from surroundings such as handling staff, transport containers, knives, pod surfaces, etc. (Daniel *et al*., 2009).

Cocoa fermentation is a crucial step in post-harvest processing of cocoa. It's a spontaneous process in which the different flavor and aroma precursors of cocoa beans desired in chocolate industry are produced, it occurs in two steps: the first stage involves microbial reactions that take place in the pulp and the outer part of the beans, the second phase involves several hydrolytic reactions that occur within the cotyledons (Schawn and Wheals, 2004). The microbial activities in the cocoa pulp is a well-defined temporal succession that is dominated by yeast at first, followed by lactic acid bacteria (LAB), which decline after 48hours of fermentation as they are overcome by acetic acid bacteria (AAB). Species of Bacilli, including other bacterial species and filamentous fungi may also grow during the fermentation and can affect bean quality and cocoa flavour (Schawn and Wheals, 2004).

The spontaneous nature of cocoa bean fermentations may be the source of variable cocoa beans broad quality (Maura *et al*., 2016). That is why many investigations suggest the use of microbial starter culture to improve the fermentation process. The use of selected starter culture improved cocoa fermentation process and the chemical quality of the cocoa beans, (for instance, the use of selected starter culture reduced the fermentation time from 6 days to 3 days this helped to eliminate the reduction in lipid content caused by prolonged fermentation) (Ndife *et al.,* 2013), but results obtained are still insufficient to understand the variations in the mineral composition and cocoa fermentation process standardization.

Therefore, the aim of this research is to assess the diversity of microbial strains involved in the fermentation of cocoa in Nigeria and select appropriate microbial starters that can reduce cocoa fermentation time and yield cocoa beans with higher export quality.

MATERIAL AND METHODS

Cocoa Beans Collection

One hundred (100) ripened cocoa pods were purchased from the local market (Eke Awka) in Awka South Local Government Area of Anambra State, Nigeria. These were transported in sacs to the Microbiology Laboratory of Nnamdi Azikiwe University, Awka for fermentation. The cocoa pods have characteristic size, peel, pulp, 16.32cm in length and 9.75cm in diameter with yellowish bark and 24-27 seeds per pod as identified at the Botany Department, Nnamdi Azikiwe University, Awka.

Proximate analysis of unfermented and fermented cocoa bean

Moisture Analysis

Moisture content was determined using the oven drying method described by AOAC (2012). Approximately 2g of cocoa sample was weighed into Petri dish prior to placing the tins into a forced air drying oven for 1 h at 150° C. Samples were again placed in an oven for another 1 hour until a steady result was obtained. Percent moisture content (%MC) was determined from the formula:

%MC = [(initial weight – dry weight) / initial weight] x 100.

Ash Analysis

Ash content was determined using the ashing method described by AOAC (2012). Approximately 2g of cocoa sample was placed into a pre-weighed, dry crucible prior to placing the crucible into a muffle furnace at 500° C for 3hours. Percent ash was calculated by dividing the ash weight by the initial sample weight and multiplying by 100.

$$\% \text{ Ash content} = \frac{W3 - W1}{W2 - W1} \times \frac{100}{1}$$

Where: W1 = weight of empty crucible, W2 = weight of crucible and sample, W3 = weight of crucible and ash

Determination of Crude Fiber

This was determined by the method described by AOAC (2012). Two gram of each cocoa sample was defatted with petroleum ether. The defatted sample was boiled in 200 ml of 1.25% H_2SO_4 solution under reflux for 30 minutes. After that, the samples were washed with several portion of hot boiling water using a two-fold muslin cloth to trap the particle. The washed samples were carefully transferred quantitatively back to the flask and 20 ml of 1.25% NaOH solution was added to it. Again, the samples were boiled for 30

minutes and washed as before with hot water. Then they were very carefully transferred to a weighed porcelain crucible and dried in the oven at 105°C for 3 hours. After cooling in a dessicator, they were reweighed (W2) and then put into a muffle furnace and burnt at 550°C for 2hours.

Again, they were cooled in a dessicator and weighed.

% Crude Fiber = $\frac{Weightoffibre}{weightofsample} \times 100$

Protein Analysis

Crude Protein (CP) was determined following the method of AOAC (2012)

using a nitrogen determinator (Leco TruSpec CN or Leco FP-2000; Leco Corporation, St. Joseph, MI and Rapid N cube, Elementar, Hanau, Germany).

Total percentage nitrogen was multiplied by a factor of 6.25 to calculate percent protein.

Determination of fat content

Fat content of the samples was determined by the continuous solvent extraction method using a Soxhlet apparatus model. The method used was that described by AOAC (2012). Five grams (5.0g) of each sample was put in a Soxhlet reflux flask containing 300ml of petroleum ether. The upper end of the reflux flask was connected to a condenser. By heating the solvent in the flask through electro-thermal heater, it vaporized and condensed into the reflux flask. The wrapped sample was completely immersed in the solvent and remained in contact with it, until the flask filled up and was siphoned over, thus carrying oil extract from the sample down to the boiling flask. This process was done repeatedly for about 6hours before the defatted sample was removed and reserved for Crude Fibre Analysis (CFA). The solvent was recovered and the oil content in the flask was dried in the oven at 60°C for 30 minutes to remove any residual solvent. After cooling in a dessicator, the flask was re-weighed.

By difference, the weight of fat (oil) extraction was determined and expressed as a percentage of the sample weight.

Determination of Carbohydrate

The carbohydrate content was calculated by method described by Pearson (1976). Using the formula below:

100 – (% protein + % Moisture + % Ash + % Fat + % Fibre)

Cocoa Bean fermentation

The cocoa fruits were manually cut open and the beans separated from the placenta. Five kilo gram cocoa beans was placed in a box fermenter underneath the banana leaves so as to trap the heat generated (act as insulator) during fermentation for six days as described by Ouattara *et al*., (2008).

The fermented cocoa beans were dried in a temperature controlled forced air oven for 24 hours at a temperature of 45 -50ºC by uniformly spreading cocoa beans on the clean tray. They were intermittently stirred (24 hours) with a turner to facilitate uniform drying, as described by Hamdouche *et al*., (2015)

Isolation and Identification of isolates

The method of Lefeber *et al.,* (2012) was used for culture dependent approach which was performed after sampling. Thereafter, 135 ml of 0.1% (w/v) buffered peptone water (Oxoid, Basingstoke, United Kingdom) were added to 15 g of pulp and beans in a sterile conical flask and placed in a shaker for 5 min to obtain a uniform homogenate. Samples (1.0 ml) of the homogenate were serially diluted 10-fold in 0.1 % normal saline, from which aliquots (0.1 ml) were platted on SDA medium (Sabouraud Dextrose Agar), supplemented with 2.5ml of chloramphenicol, to 250g/ml of SDA to inhibit bacterial growth.

Lactic acid bacteria (LAB) medium as constituted by Mann Rogosa Sharpe (MRS) agar (Oxoid) supplemented with 0.5ml/250g/ml (MRS Agar) of nystatin to inhibit yeast growth.

Glucose yeast extract peptone agar (GYPA) consisting of 50g/l of D-glucose, 10g/l of yeasts extract, 1g/ of peptone, 20g/l of glycerol, 15g/l of potato and 40g/l of ethanol (v/v), supplemented with 0.0016 % of

bromocresol green as color indicator and 0.5 ml of nystatin to 250/ml of (AAB medium) to inhibit yeast growth.

Nutrient agar also was supplemented with 0.5ml of nystatin for each 250/ml (NA) to inhibit yeast growth.

Samples were then incubated for 1 to 6 days in a standard incubator (Jouan, St. Herblain, France) at 30°C for monitoring and isolation of specific groups of microorganisms responsible for fermentation. Different colonies were picked up from a suitable dilution of each sample on SDA, MRS, GYPA and NA agar media, for morphological analysis and were purified through sub culturing and were stored at 4°C in the refrigerator available in the laboratory, in the same medium. All samples of microbial analysis were done in triplicates.

The isolates were identified by biochemical tests using the method described by Cheesbrough (2006)

Controlled fermentation

The pods were washed with distilled water and cleaned with 90% ethanol and broken open with sterilized knife, Five (5) kg of cocoa beans was inoculated aseptically with cultures from the spontaneous fermentation and fermented on sterilized foil (foils were sterilized by placing them in an oven for 24hours at 50°C) using the method described by (Schwan and Fleet, 2014). The isolate consortia were cultivated in MRS, SDA, and AAB broth respectively at 28°C in a 100ml conical flask and placed in a shaker for 48hours at 200 rpm. The cells were harvested by repeated centrifugation at 3000 rpm for 20 min, washed with sterile saline (0.85% NaCl w/v) and used as inoculum for the controlled fermentation. For comparison, fermentations without starter addition (natural fermentation) were also carried out, characterized by a succession of microbial activities by inherent microflora and also those from the environment and incubated for 6 days after which they were analyzed. The fermented cocoa beans were collected at a fixed time (24, 48, 72, 96,120 and 144 hours).

Microbial count of isolates during natural and controlled fermentation of cocoa beans

The cocoa beans were aseptically collected at every 12hour interval during the fermentation. Standard microbiological pour plate method was used for plating on SDA, MRS and GYPM (consisting of 50g/l of D-glucose, 10g/l of yeasts extract, 1g/l of peptone, 20g/l of glycerol, 15g/l of potato and 40g/l of ethanol). The agar plates were incubated at 30°C for 3days. After the incubation period, the number of colony- forming units (CFU\ml) was recorded. Colonies were picked at random in a number equal to the square root the total colonies present on the counted plate, seeking to ensure that all different colony morphologies were represented in each case (Senguna *et al*., 2009). All samples of microbial analysis were done in triplicates.

Statistical Analysis of Data

The results of the proximate analysis of natural and controlled fermentation from the study were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) and Duncan Multiple range test in Statistical Package for Social Sciences (SPSS) software (version 20).

RESULTS AND DISCUSSIONS

The values for the proximate analysis of dried unfermented cocoa beans are shown in Table 1.

Table 1: Proximate composition of dried unfermented (*Forastero*) cocoa

Parameters	**Composition**
Moisture	14.3 %
Ash	6.29 %
Crude fibre	7.25 %
Crude protein	7.66 %
Fat	7 .90 %
Carbohydrate	56.6 %

The diversity of microorganisms was studied in order to explain the variability of cocoa beans from Nigeria and identify key species for selecting appropriate starters for Nigerian cocoa fermentation. The yeast species isolated during the spontaneous fermentation process were *Saccharomyces cerevisiae*, which often dominates the main course of the fermentation process, because of its rapid growth at a slightly higher pH, pectinolytic

activity, ethanol and heat tolerance (Daniel *et al*. 2009; Papalexandratou and De Vuyst 2011; Hamdouche *et al*. 2015).Odilon *et al*. (2017) and Oauttara *et al*. (2008) reported diversity of yeast species involved in the spontaneous fermentation of cocoa beans, the limited species recorded in this study could be because the fermentation was performed in the laboratory.

The isolates obtained from MRS agar indicated that the fermentation process was dominated by *L. plantarum*. The predominance of homo fermentative LAB strains was also reported by other studies (Kostinek *et al*., 2008; Oauttara *et al*., 2008; Liliane *et al*., 2015). It is well known that homo fermentative LAB strains convert sugars almost exclusively into lactic acid while hetero fermentative strains produce lactic acid and ethanol and thus, compete with yeast for nutrients there by inhibiting their growth, slow down fermentation and impair the production of ethanol (Thomas *et al*., 2002). Therefore, homo fermentative strains producing solely lactic acid may be more interesting and desirable.

The isolates obtained from GYPA agar indicated the fermentation process was dominated by *Acetobacter spp* which has a well known acidification capacity desirable for production of cocoa beans and chocolate quality, although (Schawn and Wheal, 2004; Romero-Cortes *et al*., 2013; Liliane *et al*., 2015). Ouattara *et al*. (2008) isolated both *Acetobacter spp* and *Gluconobacter* species,the fermentation profiles obtained in this study were similar with respect to speed, microbial succession, and yield of metabolites to that recorded by (Daniel *et al*., 2009; De vuyst, 2010; Pereira *et al*., 2012; Samagaci *et al*., 2016; Odilon *et al*., 2017).

The dynamics of microbial population during natural and controlled fermentation showed that yeasts, lactic acid bacteria and acetic acid bacteria were the dominant micro organisms isolated during cocoa beans fermentation. Only yeast and acetic acid bacteria were observed significantly at the beginning of the fermentation, in succession at concentration 1.8 x 10^7(cfu\ml) and 2.1 x 10^7 (cfu\ml) for natural fermentation and 1.8 x10^7(cfu\ml) and 2.6 x10^7(CFU\ml) for controlled fermentation. Also the addition of yeast, LAB and AAB starters accelerated the concentration of LAB, it was observed that the concentration of LAB reached 1.7x10^7(cfu\ml) at 12hour of the controlled fermentation Table 3, whereas at the same time no LAB was observed in the natural fermentation as shown in Table 2.

The results in Table 2 showed that the yeast, lactic acid bacteria and acetic acid bacteria populations increased slowly and reached maximum of 6.1x10^7(cfu\ml), 4.4x10^7(cfu\ml) and 5.4x10^7 respectively at day 3-4 for the natural fermentation while the Yeast, lactic acid bacteria and acetic acid bacteria populations increased and reached maximum of 7.2x10^7(cfu\ml), 6.0x10^7(cfu\ml) and 6.1x10^7(cfu\ml) respectively at day 2-3 for controlled fermentations as shown in Table 3.

Table 2: Dynamics of microbial population during natural fermentation of cocoa beans

Time (hours)	**Natural (cfu/ml)**		
	(SDA)	(MRS)	(AAB)
0	2x10^6	NG	1.1 x10^7
12	1.8x10^7	NG	2.1 x10^7
24	2.4 x10^7	1.8 x10^7	3.3 x10^7
36	4.1 x10^7	2.4 x10^7	3.9 x10^7
48	6.9 x10^7	3.2 x10^7	4.3 x10^7
60	6.5 x10^7	4.5 x10^7	5.1 x10^7
72	6.1 x10^7	4.4 x10^7	5.4 x10^7
84	6.0 x10^7	2.3 x10^7	4.8 x10^7
96	4.5 x10^7	1.5 x10^7	2.2 x10^7
108	3.9 x10^7	5 x10^6	1.7 x10^7
120	3.1 x10^7	3 x10^6	8 x10^6
132	2.8 x10^7	2 x10^6	9 x10^6
144	1.8 x10^7	2 x10^6	7 x10^6

Key: NG= No growth, SDA=Sabouraud Dextrose Agar, MRS= Mann Rogosa Sharpe, AAB= Acetic Acid Bacteria

Table 3: Dynamics of microbial population during controlled fermentation of cocoa beans

Time (hours)	Controlled (cfu/ml)		
	(SDA)	(MRS)	(AAB)
0	$2x10^6$	NG	$1.2 x10^7$
12	$1.8x10^7$	$1.7 x10^7$	$2.6 x10^7$
24	$2.9 x10^7$	$2.5 x10^7$	$3.9 x10^7$
36	$4.1 x10^7$	$3.1 x10^7$	$4.5 x10^7$
48	$6.9 x10^7$	$5.9 x10^7$	$6.1 x10^7$
60	$6.5 x10^7$	$6.1 x10^7$	$5.9 x10^7$
72	$7.2 x10^7$	$6.0 x10^7$	$6.1 x10^7$
84	$7.0 x10^7$	$5.9 x10^7$	$5.7 x10^7$
96	$6.6 x10^7$	$5.4 x10^7$	$5.2 x10^7$
108	$5.9 x10^7$	$4.9 x10^7$	$4.9 x10^7$
120	$5.5 x10^7$	$4.1 x10^7$	$4.2 x10^7$
132	$5.1 x10^7$	$3.9 x10^7$	$3.9 x10^7$
144	$4.9 x10^7$	$3.6 x10^7$	$3.3 x10^7$

Key: NG= No growth, SDA= Sabouraud Dextrose Agar, **MRS=** Mann Rogosa Sharpe, **AAB=** Acetic Acid Bacteria

Mean results for proximate analysis of cocoa bean seed during natural and controlled fermentation

The mean results for proximate analysis of cocoa bean seed during natural and controlled fermentation are shown in Figures (1-6). Proximate analysis shows the values of the macronutrients in food samples. The Moisture, Ash, Crude fiber, Crude protein, total Lipid and total Carbohydrate were analyzed for both natural and controlled fermentation.

The cocoa bean of the natural fermentation had final moisture content of 11.40% at day 6 while that of the controlled fermentation had final moisture content of 11.20%, as shown in Fig. 1; unfermented cocoa bean had higher moisture content of 14.30% as shown in Table 1.

Fermentation introduced significant variation in the moisture levels (Fig. 1). The moisture content of a food gives an indication of the extent to which the nutritive value of the material can be maintained i.e. its shelf life, low moisture content is therefore required for a longer shelf life. Moisture levels were significantly lower ($p<0.05$) in both natural and controlled fermented cocoa beans than in the unfermented beans (Table 1), and this may be ascribed to the initial higher moisture levels of unfermented bean samples. The results obtained are similar to those reported by Afoakwa *et al*. (2011).

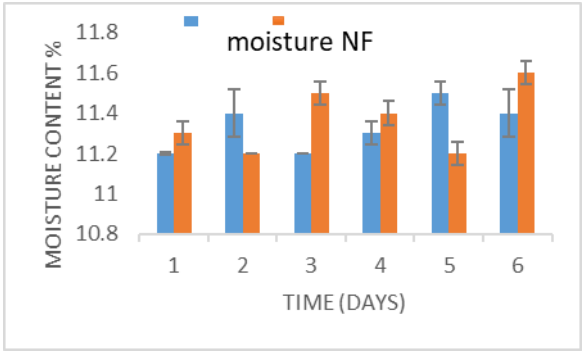

Fig 1: Moisture content of cocoa bean during natural and controlled fermentation

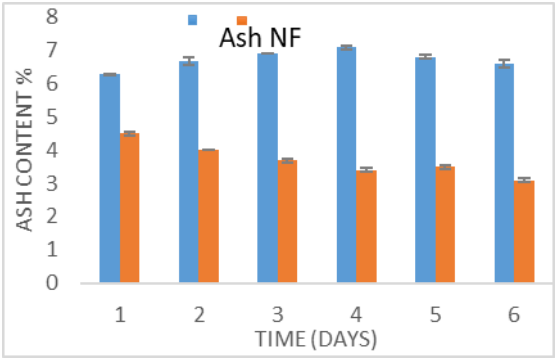

Fig. 2: Ash content of cocoa bean during natural and controlled fermentation

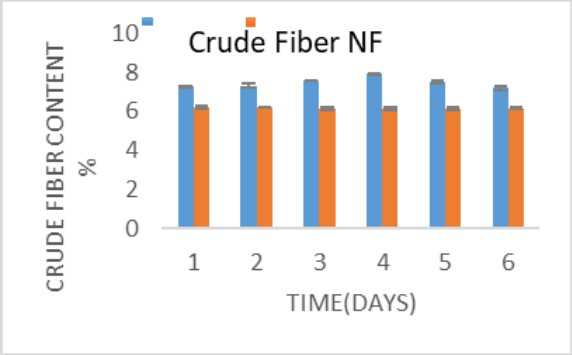

Fig. 3: Crude fiber content of cocoa bean during natural and controlled fermentation

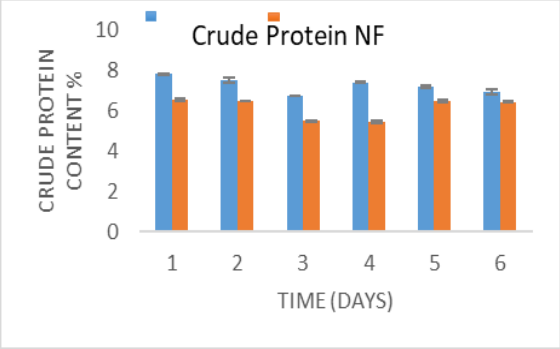

Fig. 4: Protein content of cocoa bean during natural and controlled fermentation

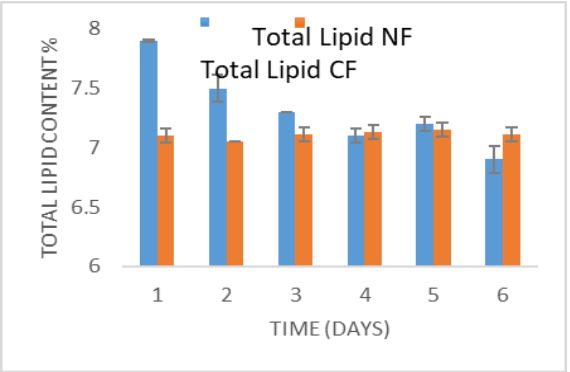

Fig. 5: Lipid content of cocoa bean during natural and controlled fermentation

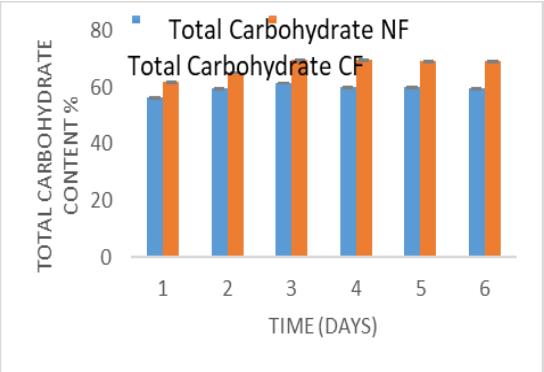

Fig. 6: Carbohydrate content of cocoa bean during natural and controlled fermentation

Key: Blue bar show results obtained during natural fermentation **(NF)**, Orange bar also showed results obtained during controlled fermentation (**CF)**

Also, the ash content of the controlled fermented cocoa beans decreased significantly ($p<0.05$) than that of the natural fermentation, with a value ranging from 3.10-4.50% and 6.28-7.10% respectively as shown in Fig. 2, but the unfermented cocoa bean had an ash content of 6.29% as shown in Table 1. The addition of microbial starter caused a decrease in the ash content (inorganic minerals) of the cocoa beans during the controlled fermentation (Fig. 2) with values ranging from (3.10-4.50%). Ash analysis is the burning away of organic minerals leaving the inorganic minerals, there was faster depletion of inorganic minerals in the controlled fermentation than in the natural fermentation leaving more organic minerals in the controlled fermented beans. Also, the body needs more organic minerals than inorganic minerals. Since organic minerals are gotten from plants and animals, cocoa beans are a good source of plant organic minerals. The decrease recorded in this study is similar to findings by Afoakwa *et al*. (2011) who recorded significant decrease in ash content with values ranging from 3.48-2.92%.

There was slight decrease in the crude fibre content with the controlled fermentation. Crude fibre content ranged from 7.20 – 7.90% for the natural fermentation and 6.12-6.20% for the controlled fermentation as shown in Fig. 3. The result recorded in this study is similar to that of Ndife *et al*. (2013) who also recorded reduction in crude fibre content with values ranging from 2.34-3.16%.

There was significant ($P<0.05$) decreases in Crude protein (Fig. 4) with values ranging from (5.45-6.52%) with the controlled fermentation. The result recorded in this study is similar to that reported by *Afoakwa et al*. (2011) who also recorded significant decrease in crude protein content with values ranging from 6.10-7.37% and in contrast to literature values of 15.2-19.8% reported by Afoakwa *et al.* (2008). The observed decreases in protein content in the cocoa bean with controlled fermentation might be due to protein breakdown during the curing process which occurred partly due to hydrolysis of amino acids and peptides and partly by conversion to insoluble forms by the action of polyphenols as well as losses by diffusion (Afoakwa *et al*. 2008).

The lipid content ranged from 6.90-7.90% for the natural fermentation and 7.05- 7.15% with the controlled as shown in Fig. 5. The fat content of the beans as observed in this study with values ranging from (7.8-9.1%) was similar to values of (10.05 to 12.65%) recorded by Ndife *et al*. (2013) and lower than values of (50.40% and 53.35%) reported by Afoakwa *et al.* (2011) and Aremu *et al.* (1995), who reported a decrease in lipid content of the cocoa beans from 62.9% to 55.7% by the sixth day of fermentation. In this study, there was slight increase in lipid content in the controlled fermentation from day 4 (reduced fermentation time) and it remained relatively stable throughout the fermentation period, unlike the lipid content in the naturally fermented cocoa beans that reduced by the end of the fermentation. This suggests that the reductions in fat content in cocoa beans could be avoided by reducing fermentation time. Variations in the bean sizes could account generally for the observed relatively lower fat content obtained in this study.

In addition, total carbohydrate content of the cocoa bean with controlled fermentation ranged from (62.00-69.70%) while that of the natural fermentation ranged from (56.00-61.50%) as shown in Fig. 6. Carbohydrate content was significantly ($p<0.05$) higher in the controlled fermentation with values ranging from (62.5-69.9%) than in the natural fermentation with values ranging from (56.6-59.90%) (Fig.6.). This increase could be as a result of an apparent inverse relationship which exists between the levels of fat and total carbohydrate in fermenting cocoa, thus, converting the lipid to carbohydrate via gluconeogenesis, employing the glyoxylate cycle. It has been indicated that this pathway normally operates in microorganisms and germinating oil seeds (Afoakwa *et al.* (2011). The unfermented cocoa bean had a carbohydrate content of (56.6%) (Table1). An increase in carbohydrate content was also recorded by Afoakwa *et al*. (2011) from 15.47% to 24.93% and by Ndife *et al.* (2013) from 43.92% to 61.74%.

CONCLUSION

The addition of microbial starters into cocoa bean fermentation as well as environmental factors (pH and temperature) reduced the fermentation process (food processing time) from 6 to 3 days, influenced to varied levels the mineral composition which resulted in consistent decreases in ash and protein, slight increase in fat content of the cocoa beans, and abrupt increase in carbohydrate content, resulting to a cocoa bean seed with potentially better and desirable export quality. The addition of microbial starter seemed to be the fundamental for the improvement of cocoa bean fermentation quality, demonstrating further application of these strains.

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