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Polyphenol and Microbial Profile of On-farm Cocoa Beans Fermented with Selected Microbial Consortia

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Abstract

Background and Objective: Quality and preference of cocoa as raw material for various cocoa products primarily depend on fermentation techniques that modulate the resultant flavour and the phytochemical properties. This study investigated the combined effect of selected microbial consortia and bioreactors on phytochemical profiles of fermented cocoa beans.

Material and Methods: Three microbial consortia labeled as Treatments (T-1, T-2, T-3) were used as starter culture ($\approx 10^5$cells ml^{-1}) for on-farm cocoa fermentation on three chambers (basket, woodbox, and plastic) for 7 days. These novel consortia were T-1, *Staphylococcus* spp + *Pseudomonas* spp+ *Bacillus* spp, T-2, *Staphylococcus* spp + *Pseudomonas* spp +*L. lactis*, and T-3, *Bacillus* spp+ *Lactobacillus* spp + *Saccharomyces* spp+ *Torulopsis* spp.

Results and Conclusion: The microbial profile were significantly ($P \leq 0.05$) altered by all treatments (T-1, T-2, T-3) and microbial frequency was enhanced by 5 -22.5%. T-3 and T-1 significantly altered phenolic content in basket chamber. Tannin was significantly ($p \leq 0.05$) varied by T-1(basket, plastic, wood box) and T-2(plastic). Tannin: polyphenol conversion ratio adopted as fermented cocoa bean quality benchmark was significantly enhanced by T-1 (basket, woodbox) and T-2 (plastic), but was significantly suppressed by T-3 (basket). This study evidently concluded that the appropriate synergy of microbial flora and fermenting chambers could achieve good cocoa quality with low polyphenol content (best for cocoa beverages) or high polyphenol content (best for pharmaceutical, confectionery and nutraceutical industries). These findings would avail an economic alternative to the expensive polyphenol reconstitution of cocoa butter used for various industrial products, thereby maximizing economic benefits for both cocoa farmers and industrialists.

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1. Introduction

The quality of fermented cocoa beans depend on cocoa variety, fermentation methods, starter culture, weather and physiological conditions of the beans prior to fermentation. Products of fermented Cocoa beans are mainly chocolates and wide range of beverages, pharmaceuticals, cosmetics and toiletries. The resultant flavour and phytochemical properties of the fermented cocoa beans determine its quality and preference as raw material for various cocoa products [1]. Generally, fermentation alters the phyto-chemical properties of cocoa bean and reconfigures the stereochemistry of the cocoa beans, such that polyphenols oxidize and complexed into high molecular mass of insoluble tannin, which is the major constituent of the flavour precursor [2]. Variations in phytochemical content between fermented and unfermented cocoa beans are due to the microbial metabolites (especially lactic acid and acetic acids) synthesized from cocoa pulp. These metabolites penetrate the bean cotyledon, coupled with a sharp drop in pH (6.5-4.8), increase in temperature (50-55^{o}C) and endogenous enzyme activities on carbohydrate, protein and polyphenols within the beans testa and cotyledon, collectively cause fatal disorientation of cotyledon and induce flavour precursors development [3,4]. Interestingly, unfermented cocoa beans lack the chocolate flavours that typify cocoa beverages [5-8]. These flavour precursors (especially reducing sugars, peptides, and amino acids) are further modified during drying and roasting through Maillard reactions [5-7].

Polyphenols and flavonoids constitute the major phytochemicals of interest in cocoa bean. Bitterness and astringency significantly reduces during fermentation as more than 30% alkaloids and 20% polyphenols diffuse away from the beans. However, alongside fermentation other factors that alter phenolic content include: cocoa bean type, pre and post fermentation activities, lengthy processing and high temperatures [5-8]. Thus, producer countries require an improved scientific methodology for processing (fer-mentation) techniques. Previous studies were designed toward developing different controlled fermentation methods (starter culture, fermenting chambers), drying and roasting techniques that would satisfy the need of the consumers [9].

Despite the numerous literatures on cocoa fermentation and its' phenol quality assessment, there seems to be no literature that attempt to modulate the phenolic quality of fermented cocoa using combined effects of selected microbial consortium (as starter cultures) and bioreactors. Thus, this study aimed to investigate this knowledge gap, to assess the impact of selected microbial consortia on the polyphenols content and microbial profile of fermented cocoa.

2. Materials and Methods

2.1. Sample collection

Freshly harvested and extracted cocoa beans (Amelonado) from 3 cocoa farms in Itunta-Ibere, Ikwuano LGA, Abia State, were bulked together to limit bias. A weigh of 15 kg of cocoa beans were placed on each of the three different fermenting chambers (plastic, wooden and baskets) insulated with plantain leaves. A total of 72 samples were collected from 12 fermenting chambers (3 treatments and 1 control for each of the 3 different chambers) at daily intervals for 6 days (2^{nd}-7^{th} day).

2.2. Microbial analysis

After each daily turning, about 10 g of cocoa beans were immersed in 1000 ml of physiological saline and agitated for 10 min. From this solution, 1 ml was serially diluted and 0.1 ml of 6^{th} index were plated in duplicates of appropriate culture media (for bacteria, yeast and mold) as described below.

2.2.1. Lactic Acid Bacterial (LAB) Isolation and Identification

Lactic acid bacteria were isolated on MRS medium (AEB, France) and incubated at 37ºC for 48-72h using spread plate method and pure isolates were stored in MRS agar at 4ºC for short term use. Isolates were characterized with culture morphology, growth at different temperature (10ºC, 45ºC, 60ºC) [10,11] and sugar fermentation test was determined with API 50CH kit (bio Merieux SA, France) under sterile paraffin oil and incubated at 30˚C for 24 - 48h.

2.2.2. Fungi (yeast and mold) Isolation and Identification

Yeast and molds were isolated by spread plating, 0.1 ml aliquot of cocoa beans homogenized in peptone water (0.1% w v^{-1}, Oxoid, Melbourne) on PDA (adjusted pH 3.5 and incubated for 3 days at 25°C) medium and Dichloran Glycerol Chloramphenicol Agar (incubated at 30- 35°C for 5-7) medium respectively. Pure isolates of yeast were characterized and identified based on their culture and microscopic morphologies, biochemical and sugar fermentation assays. Mold isolates were characterized using color atlas, culture and microscopic (lacto phenol stain) morphology [2,12].

2.2.3. Acetic Acid Bacteria (AAB) Isolation and Identification

Acetic acid bacteria were isolated on plates of Glucose yeast calcium (GYC) medium supplemented with 100 mg l^{-1} of Pimaricin (Sigma-Aldrich; Steinheim; Germany) to inhibit the growth of yeasts and molds. Acetobacter and Gluconobacter are distinguished based on pigment changes on Carr medium and acid production from calcium carbonate incubated at 30°C for 2 days [13].

2.2.4. Bacteria Isolation from fermenting cocoa samples

Spread plate method was used for isolation of bacteria on nutrient agar (Fluka), MacConkeyagar (Fluka), Mannitol salt agar (Brittania), Thiosulphate citrate Bile Salt agar (Oxoid) and Deoxycholate citrate agar (Oxoid) Culture plates were incubated (30°C) for 2 days. Pure isolates were identified using sugar assays, cultural, morphological and biochemical characterization [11,14]. These isolates were expressed as Total Mesophilic Bacteria (TMB).

2.3. Preparation of starter culture and fermentation protocol

All the microorganisms used as starter culture were isolated from spontaneous cocoa bean fermentation of the same farm area, and identified as described above (section 2.2). Predominant isolates (*Saccharomyces cerevisiae* and *Torulopsis* spp), Lactic acid bacteria (*Lactobacillus* (*L.*) *lactis*, and *L. plantarum*), Bacillus (*Bacillus* (*B.*). *subtilis* and *B. cereus*) and other bacteria (*Staphylococcus* spp and *Pseudomonas* spp) were designated as starter cultures. It is important to note that Staphylococcus and Pseudomonas are human normal flora and only opportunistic pathogens to immunosuppress patients. Predominance of these species in cocoa fermentation and the environment (soil, water and handlers), and ability to utilize sugar and non-sugar sources for lactate production were the reasons for their selection. They (Staphylococcus and Pseudomonas) were manually added as part of microbial consortium to ensure competitive dominancy over other normal floras that causes failed fermentation. Acetic acid bacteria were

excluded as starter culture because they are late successor of cocoa fermentation. Moreover, LABs among other bacteria (bacillus) can degrade lactic acid to acetic acid as well. An initial cell concentration ($\leq 10^1$ CFU g^{-1}) of freshly extracted cocoa beans placed in their respective chambers was determined prior manual addition of the consortium (starter culture). This was an insignificant value compared to 30ml consortium (average of 3×10^6 CFU g^{-1}), which is approximately 300,000 folds greater than normal flora. Thus, starter culture would competitively dominate and modulate the fermentation pathway.

Pure colonies of each isolate used as starter culture were first grown in 250 ml of Erlenmeyer flasks containing 100 ml of respective standard broth medium (yeast in PDA, LAB in MRS, Bacillus and other mesophilic bacteria in nutrient agar) and incubated at 30°C for 48 h. From each of these cultures, 1 ml was transferred to a fresh 100ml of the respective broths and incubated at 30ºC for 18-24 h. Cells were centrifuged at 2800 ×g for 20 min and re-suspended in 1000 ml of sterile peptone water containing 2% v v^{-1} of cocoa pulp incubated at 30ºC for 18-24h prior to its spray as inocula (starter culture) over the cocoa mass. The cultures were reconstituted to approximate cells concentration range of 10^4-10^5 cells ml^{-1} and verified with culture method. A total of 30 ml consortium (5 ml from each of six different starter cultures) were designed as treatments. This was collected using sterile 5 ml syringe. The consortia used as treatments (T-1, 2, 3) were:

T-1 = *S. aureus* + *S. epidermidis* + *P. aeruginosa* + *P. fluorescences*+ *B. subtilis* + *B. cereus*

T-2 = *S. aureus* + *S. epidermidis* + *P. aeruginosa* + *P. fluorescences* + *L. lactis* + *L. plantarum*

T-3 = *B. subtilis* + *B. cereus* + *L. lactis* + *L. plantarum* + *S. cerevisiae*+ *Torulopsis* spp.

Control = spontaneous fermentation without starter culture

Each treatment (T) was applied on 15 kg cocoa beans mass aseptically placed in each of the three different chambers (basket, plastic and woodbox). Each type of chamber has four replicates, designed as T-1, T-2, T-3 and CTRL. Fermentation conditions were standardized for all chambers and treatments throughout the 7 days period at an initial ambient temperature (28-30ºC) and pH (4.4-4.6).

2.4. Preparation and determination of total polyphenols chemicals and reagents

Total Phenolic content were determined using Folin-Ciocalteu's phenol reagent (Merck Chemicals Argentina, Buenos Aires), anhydrous sodium carbonate (99% purity, Anedra Argentina), and gallic acid (99% purity, Sigma, Argentina) as the standard.

2.5. Preparation of defatted cocoa samples for extraction analysis

Dried fermented cocoa beans were homogenized in an electric blender with addition of dry ice to check the mechanical generated heat from melting of cocoa lipids. Ground cocoa powder were sieved through a 710 µm screen and defatted for 16-18 h using petroleum ether (B.P. 35-60ºC). Resulting residue were dried in vacuum oven at 65ºC for 5 minutes and placed over silica gel desiccator in dark chamber prior to extraction procedure [15].

2.6. Qualitative screening for phytochemical constituents

Appropriate quantity of defatted cocoa powdered were screened for tannin, saponins, flavonoids and alkaloids [16]

2.7. Determination of Total polyphenol content.

Total Phenolic content was determined using Folin-Ciocalteu's phenol reagent (Merck Chemicals Argentina, Buenos Aires), anhydrous sodium carbonate (99% purity, Anedra Argentina), and gallic acid (99% purity, Sigma, Argentina) as the standard [17]. Total tannin content was evaluated using modified folin-denis method [18] as described by Ainsworth and Gillespie [19]. Spec-tro-photometric analysis at 735 nm (total tannins) and 760 nm (total polyphenol) absorbance readings were expressed as Gallic acid Equivalent (GAE g kg^{-1}).

2.8. Statistical analysis

Analysis of variance (ANOVA) was carried out using SPSS software version 20 (IBM SPSS Switzerland) to determine the variation in microbial and phytochemical profiles of fermented cocoa beans. Results were expressed as means of triplicate measures and subjected to T-test and linear regression. Only the values beyond 95% confidence intervals of the control values were designated as significant.

3. Results and Discussion

3.1. Microbial profile of cocoa beans during fermentation

Mesophilic bacteria predominated with more than 80% frequency were in descending order of B. *cereus*, *S. aureus*, *S. epidermidis*, *Escherichia coli*, while *Zymomonas* spp and *Chromobacterium* spp were less than 20% (Figure. 1). *L. lactis* and *L. plantarum* predominated the LAB group with 100% occurrence, while *Streptococcus cremolis* and *Enterococcus* spp were the least frequent (≤30%) LABs (Figure. 2). Among the AABs, only *Gluconoacetobacter hensenii* achieved 70%, while *Gluconoacetobacter diazotrophicus* was the least with 20% (Figure.3).

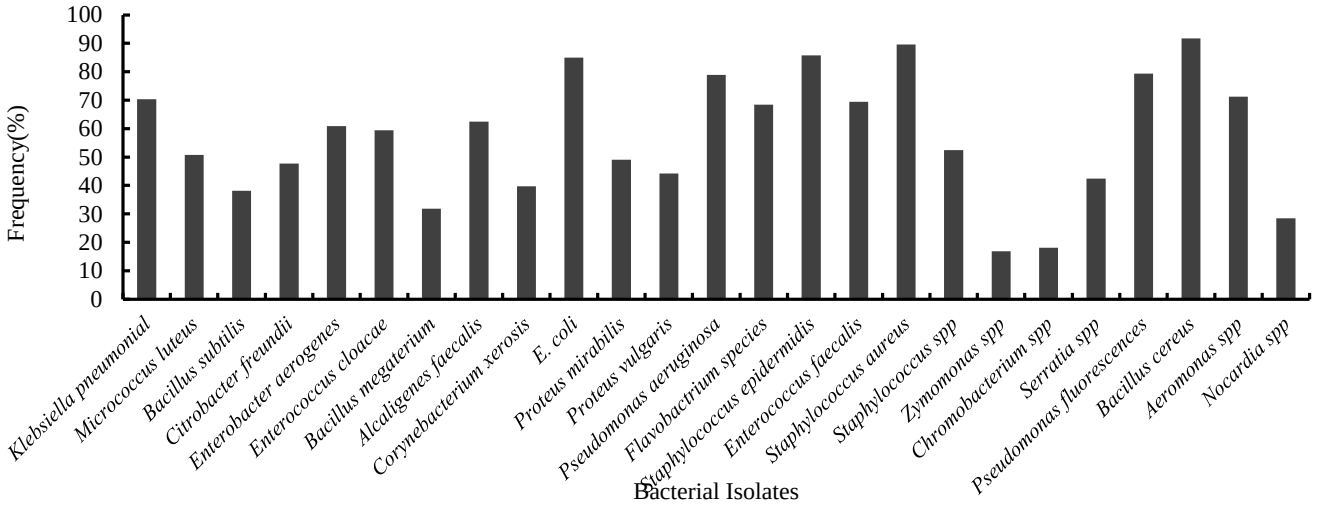

Figure 1. Frequency of total mesophilic bacterial isolates from fermented cocoa

NB: Frequency are expressed as (%) of affirmative cases among a total of 72 samples

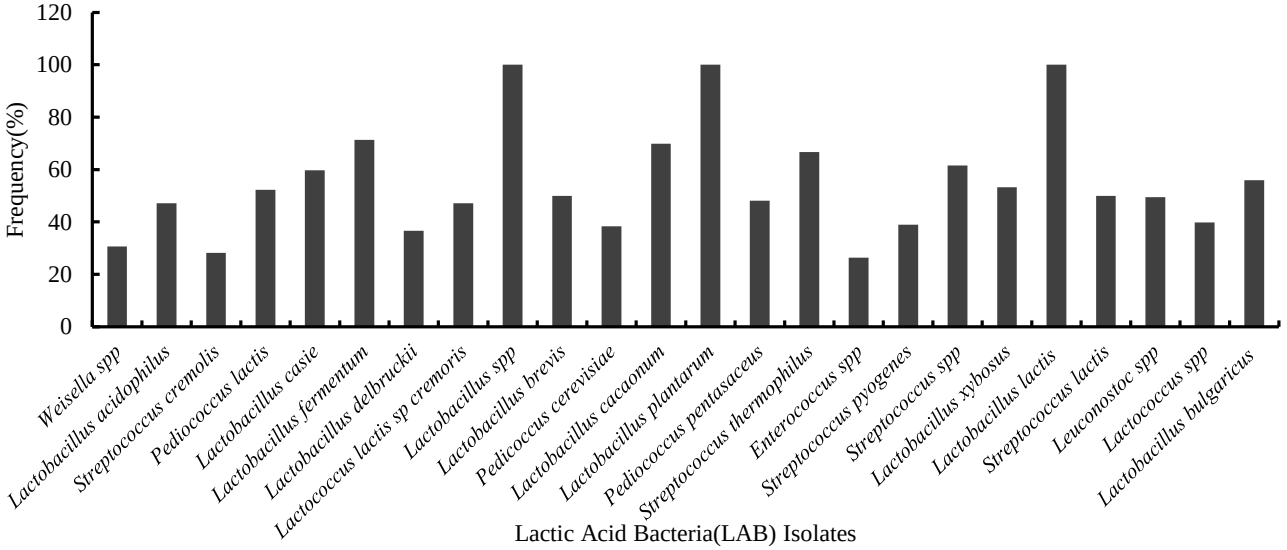

Figure 2. Frequency of LAB isolates from fermented cocoa

NB: Frequency are expressed as (%) of affirmative cases among a total of 72 samples

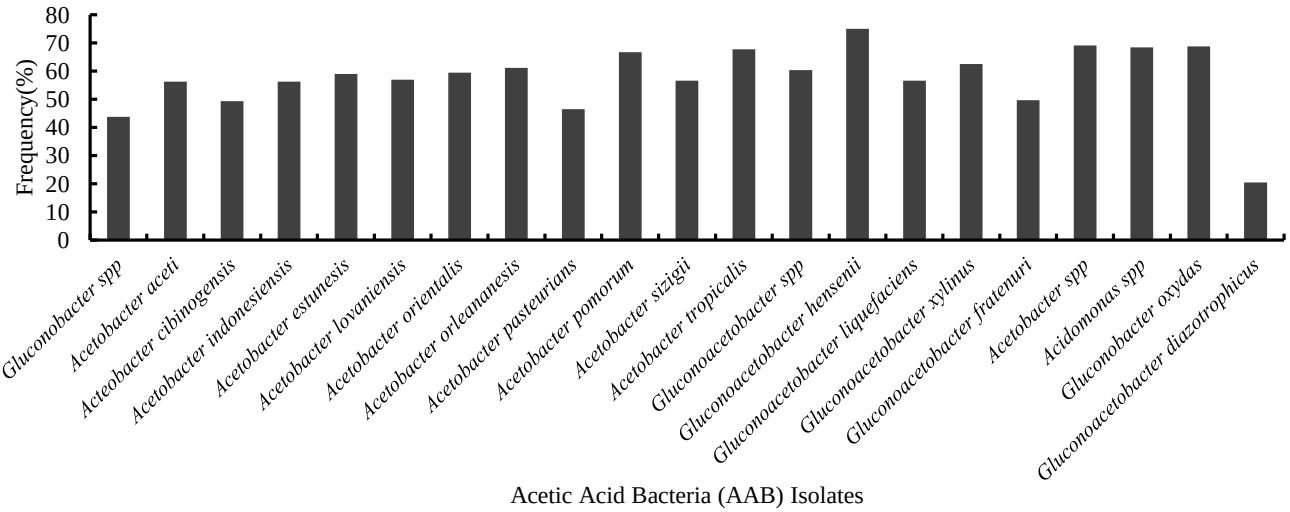

Figure 3. Frequency of AAB isolates from fermented cocoa

NB: Frequency are expressed as (%) of affirmative cases among a total of 72 samples

S. cerevisiae and *Torulopsis* spp were among the yeast that frequent more than 80% during fermentation, while the least (≤10%) include *Trichosporons cutaneum*, *Cryptococcus* spp and *Kloectra* spp (Figure. 4). Generally, molds were less than 40% with descending order of *Abisidia* spp, *Aspergillus niger*, *Mucor* spp, *Geotrichum candidum* (Figure. 5). Each of the applied treatments (T-1, 2 &3) was observed to induce a significant ($P \leq 0.05$) variation on the overall microbial profile of fermenting cocoa when compared to the controls samples (Figure. 6).

Microbial profile of cocoa beans assessed during fermentation reflected the associated sources of microbes as normal flora of farmers, farm environs, predominate water and air-borne microbes as reported in previous studies [4,20-22]. This justifies the high occurrence of microbes in the fermenting beans. Survivability of LABs, TMBs, AABs, yeast and mold were adversely affected by the different treatments throughout the fermenting period, as none of them were able to maintain 100% frequency irrespective of being members of the microbial consortium. Possibly, some limiting factors (temperature, pH, and rapid depletion of sugar, antimicrobial metabolites) within 2nd to 7th day of fermentation had adverse effects on the microbial population [5,23].

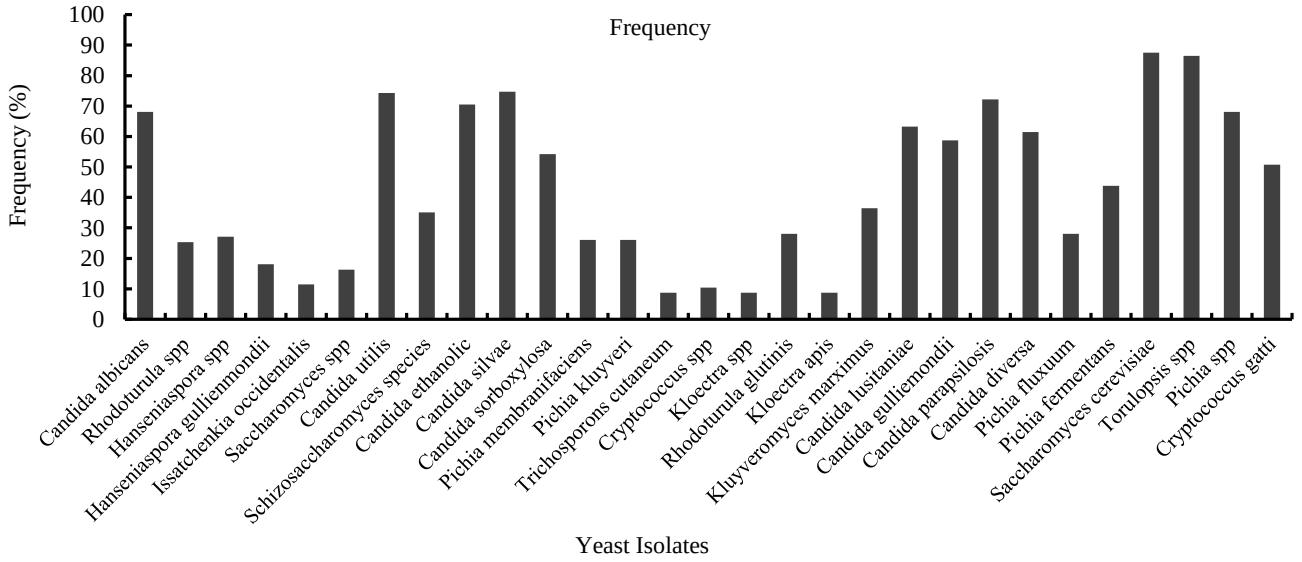

Figure 4. Frequency of yeast isolates from fermented cocoa
NB: Frequency are expressed as (%) of affirmative cases among a total of 72 samples

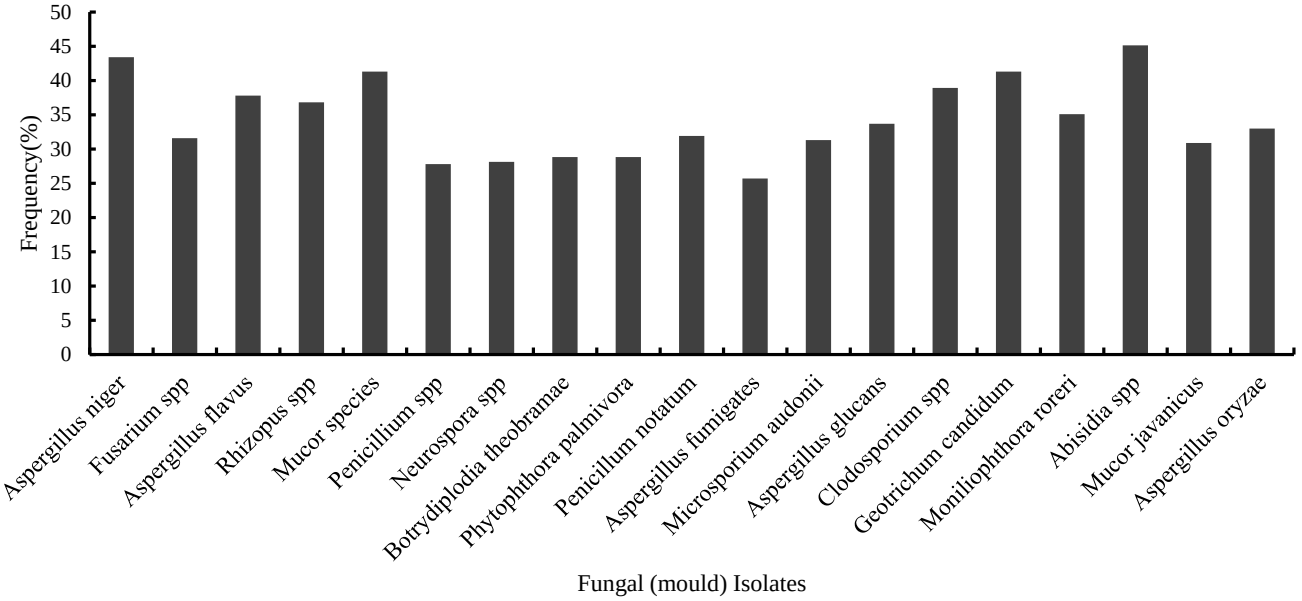

Figure 5. Frequency of fungi (mold) isolates from fermented cocoa
NB: Frequency are expressed as (%) of affirmative cases among a total of 72 samples

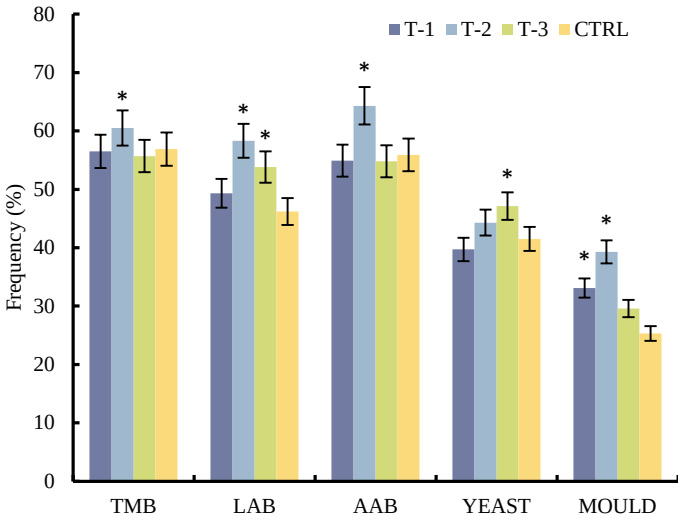

Figure 6. Microbial profile of cocoa beans fermented with different Treatments

*Treatment with significantly different (P≤0.05) microbial profile compared to the control. Frequency are expressed as (%) of affirmative cases among 18 samples

NB: Treatment-1=Staphylococcus + Pseudomonas + Bacillus
Treatment-2=Staphylococcus +Pseudomonas + LAB
Treatment-3=Bacillus + Yeast + LAB

3.2. Total phenolic content of fermented cocoa beans varied with different treatments.

On the overall Treatment across all the samples, only Basket method of T-1 (0.192 g kg^{-1}) and T-3 (0.376 g kg^{-1}) recorded significant difference (P≤0.05) in phenolic content when compared to the control value of 0.306 g kg^{-1} (Figure. 7).

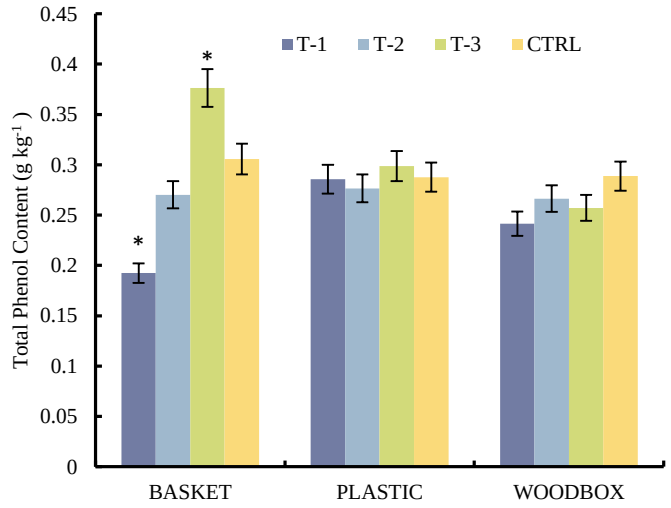

Figure 7. Total phenol content of fermented cocoa beans with different Treatments

*Treatment with significantly different (P<0.05) phenol compared to the control

NB: Treatment-1=Staphylococcus+Pseudomonas+ Bacillus
Treatment-2=Staphylococcus +Pseudomonas + LAB
Treatment-3=Bacillus + Yeast + LAB

Previous studies [24-26] significantly reduced anti-nutrients to improve the quality of plant materials (foods) using fungi as fermenting agent. Similarly, T-1, T-2 and T-3 significantly altered the phytochemical content of fermented cocoa beans. Total phenol content of the fermented cocoa beans showed varying degrees due to different treatments applied. It was obvious that T-3 (Bacillus, yeast and LABs) and basket were the best microbial consortium and chamber of choice when high phenolic fermented cocoa beans are needed for pharmaceutical or cosmetic purposes. Afoakwa et al., [5] inferred that the acceptability and marketability of cocoa products in health sectors, strongly depend on the polyphenol content. This study observed that the porous nature of basket lined with banana leaves provide better enabling environment for T-1 consortium to adequately and rapidly disorient the beans cotyledon to allow the diffusion and draining out of polyphenol, leading to significant reduction of phenolic content. Argument that pod storage, fermentation, drying and roasting processes [6,27,8,28,29] as well as genetic makeup [1,5] can determine the phenolic contents were all standardized by using the same cocoa variety and processing conditions. Significant phenolic content of T-1 and T-3 in basket chamber, suggest that TMBs (Staphylococcus, Pseudomonas and Bacillus) of T-1 prolonged the lactate (major disorienting agent) phase and quantity. This allows more quantity of lactic acid to be smeared into the bean cotyledon and apparent absence of LABs and delay of AABs colonizers, to convert this lactate to acetate for exothermic phenol complexes (flavour precursors), caused most of the phenol to diffuse out of the structurally disoriented beans. Evidently, this phenomenon was not observed in T-2, which has LABs as part of the consortium. Phenolic content of fermented cocoa is a function of the fermentative activities of microbes and the fermenting platforms [24,26,36]. Beans in bucket fermented with T-3 offered ethanol and lactic acid from yeast and LABs respectively, which synergically unlocked more undiffused phenol complexes in the beans cotyledon. Technically, the bucket chamber method offer more aerated environment for TMBs to significantly influence T-1 and T-3 phenolic content, unlike other chambers (plastic and woodbox). Poor aeration of plastic and woodbox encourages acidified environments, limiting TMBs, thereby restoring the system to spontaneous fermentation state. This was evident in the non-significant phenolic content in all plastic and woodbox treatments. Plastic and wood box fermenting chambers may be responsible for non-significant variation of phenolic content of treated and non-treated cocoa beans. As these two chambers trap more heat (temperature) and tannin content which inhibits enzymatic (polyphenols oxidase)

activities that would unshackle more polyphenols for drainage out of fermenting beans [15].

3.3. Tannin content of dried fermented cocoa beans

Tannin contents of all cocoa beans that received T-1 (1.047-1.391g kg^{-1}) in bucket and woodbox chambers were significantly different (P≤0.05) when compared to the controls. Tannin content of fermented cocoa beans with T-3 (Basket) and T-2 (Plastic) were significantly (P≤0.05) different from their control values (1.238 g kg^{-1} and 1.172 g kg^{-1}) respectively (Figure 8). Tannin constitute the major anti-nutrients found in legume, typical cereal and cocoa beans [30]. Direct correlation exists between polyphenols and insoluble tannin (as flavour precursors) during cocoa fermentation, such that phenolic components of cocoa beans biotransform into insoluble tannin. Obviously from the result of this study, a dependent relationship was observed between treatments and chambers that significantly (P≤0.05) varied the tannin content. Microbial consortium as treatments pertains the anaerobic conditions and heat retention capacity (best in plastic chamber) which has better effect on microbial activities that influence bio-transformation of polyphenols to insoluble tannin. Bio-chemically, polyphenols are oxidized to form insoluble tannin during fermentation and insoluble tannin together with other organic acids that constitute the flavour pre-cursors in chocolates processing [31]. Hydrolysable (soluble) and some smaller molecular weight condensed (insoluble) tannins dissolves in water. For this study, total tannin assessed, comprises mainly of condensed tannin and some hydrolysable tannins that were unable to diffuse out alongside cocoa sweating during fermentation. Most diffused tannins were hydrolysable tannins, which are res-ponsible for astringency and bitterness that characterized poor quality fermented cocoa beans for beverage making. However, the presence of these phenolic compounds are hub of interest to confectionary, nutriceutical and pharm-aceutical industries. Metabolites of yeast (ethanol) and LABs (bacteriocins) suppressed TMBs activities in T-3 bucket platform, which significantly reduced tannin content. Only beans in plastic chamber treated with T-2 had significant Tannin content. This is as a result of the heat trapping capacity of plastic that enhanced the exothermic complexes of phenol to insoluble flavor precursors. All other treatments and chamber permutations did not induce any significant variations. Theory of phenol bioconversion to insoluble tannin was strongly supported as one of the benchmarks of quality and successful cocoa fermentation [31-35]. Based on this theory, as a quality benchmark, treatment-chamber interaction would probably influence quality and perhaps, a preference determinant for pro-duction lines (beverages, chocolate, cosmetics or nutraceutical).

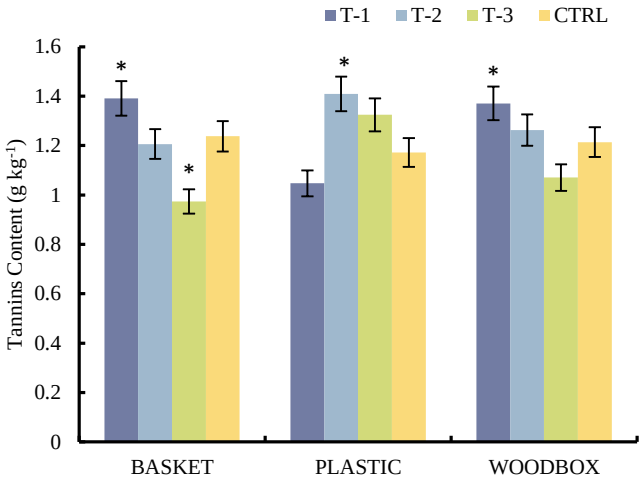

Figure 8. Tannin content of fermented cocoa beans with different treatments

*Treatment with significantly different (P≤0.05) tannis content compared to the control

NB: Treatment-1=Staphylococcus + Pseudomonas + Bacillus
Treatment-2=Staphylococcus +Pseudomonas + LAB
Treatment-3=Bacillus + Yeast + LAB

3.4. Conversion ratio of Polyphenol to Tannin of Dried Fermented cocoa

The conversion ratios of Tannin: Polyphenol were only significantly different (P≤0.05) in Basket T-1 (7.22) and T-3 (2.60) when compared to the control ratio of 4.05. Only plastic fermented beans with T-2 (5.01) was significantly different (P≤0.05) from the control ratio (4.07). Samples from woodbox treated with T-1(5.67) were significantly different (P≤0.05) from the control (4.21). T-2 (4.47) and T-3(4.16) cocoa beans fermented on woodbox were not significant (P≤0.05) as showed in figure. 9.

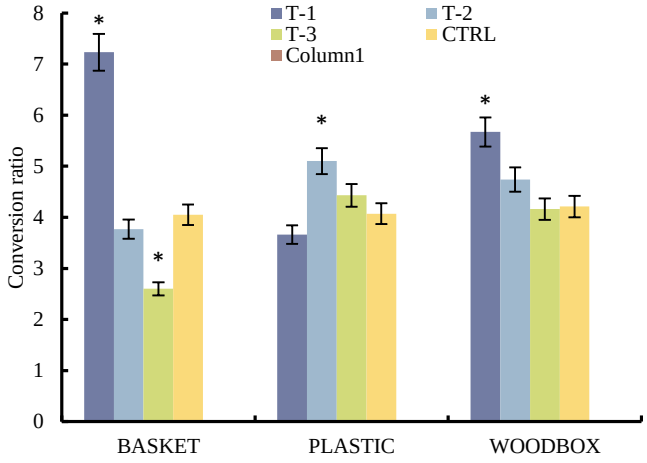

Figure 9. Conversion ratio of polyphenol to tannins obtained from fermented cocoa

*Treatment with significantly different (P≤0.05) ratio compared to the control

NB: Treatment-1 =Staphylococcus + Pseudomonas + Bacillus
Treatment-2 =Staphylococcus +Pseudomonas + LAB
Treatment-3=Bacillus + Yeast + LAB

3.5. Effect of Microbial consortium (Treatments) on quality markers of fermented cocoa beans

Comprehensive review [1,5-8] showed that cocoa beans processed without fermentation would lack the chocolate flavour. Fermentative activities occur both at the external (microbial) and internal (enzymatic) portion of the beans cotyledon. Microbial activities on the cocoa pulp releases requisite metabolites and environment for enzymatic fermentation (inside and within the bean cotyledon), which affects the final quality. Thus, strategic manipulation of successive microbial profile would predetermine the quality of fermented cocoa beans. Studies of Balogu, et al., [23], Afoakwa et al., [24], Muhammad et al., [25] and Muhammad [26] have established progressive microbial dominancy of yeast, LABs, AABs, TMBs and lastly molds. Yeast dominates within 36 h, converting simple sugars from cocoa pulp to secondary metabolites (ethanol and organic acid) and at same time collapsing and aerating the cocoa pulp matrix. LABs precede AABs within 36-84 h, such that LABs releases lactic acids from lactose and AABs exothermically converts lactic acids to acetic acids. TMBs are late colonizers within 84-108 h, scavenging on the leftover (sugar and non-sugar sources) for lactate production. Finally, appearance of mold (as white tint) signals completion of fermentation cycle [2,5,24]. Collectively, these released metabolites (ethanol, lactate, acetate, organic acids and enzymes) disorient beans cotyledon for phytochemical diffusion (reducing astringency and bitterness) and as well as complexed polyphenols to larger molecules (especially insoluble tannins) as flavour precursors [25-28]. In this study, these groups of colonizers were designed and permutated to effectively modulate the final cocoa quality.

The essence of yeast in cocoa fermentation cannot be over emphasized, as it functions both a metabolic pathway modulator and stabilizer of the fermenting system. Yeast metabolizes the simple sugar of the cocoa pulp, with resultant ethanol that serves dual purpose of limiting the spoiling microbial (of flavour and aroma producers) and disorienting the bean cotyledon for easy drainage of polyphenols. Aside the alcohol production, other bioactive metabolites of yeast such as protein and vitamin precursors promotes the growth of later colonizers. As part of the knowledge expansion concept of this study, uncommon yeast specie (Torulopsis) in collaboration with *S. cerevisiae* was used as yeast starter culture consortium for T-3. Since *S. cerevisiae* has been a successful yeast for cocoa fermentation [36], presence of *Torulopsis* sp was not antagonistic rather than synergic to S. *cerevisiae*. Evident in the success achieved by T-3 fermented cocoa beans as the adjudged overall best quality may not be unconnected to the yeast treatment. This means that simple sugars present in the pulp were readily metabolized, availing easy colonization for LABs, AABs and TMBs.

It is important to note that *S. aureus* and *Pseudomons* ssp are among the predominate TMBs that colonizes the last phase of spontaneous cocoa bean fermentation, indicating the ending phase of fermentation [37]. Thus, the objective of introducing TMBs (Bacillus, Staphylococcus and Pseudomonas) as part of starter culture consortium was to assess their competitive interference with the popular early colonizers (yeast and LAB) and perhaps, limit or modulate polyphenol bioconversion to insoluble tannin. This objective was evidently validated in Figure. 10, as T-1 (TMBs only) significantly modulated the quality markers (conversion ratio, tannin and total phenol contents) of fermented beans by 21-30% compared to the control (23-26%). However, T-2 (TMBs + LABs) and T-3 (TMBs + yeast +LABs), having 24-26%were not significantly different when compared to the spontaneous fermentation (control). Interestingly, the ability to metabolize sugar and non-sugar sources to lactate were better in TMB strains of Bacillus than Staphylococcus or Pseudomonas [38,39]. This factor inclusively explains the significant impact of T-1 and T-3 with Bacillus contrary to T-2 with staphylococcus and pseudomonas. Perhaps, the antibacterial metabolites (lactic acids) of LABs present in T-2 would have also limited the impact of TMBs (Staphylococcus and Pseudomonas). This phenomenal effect was countered in T-3 by ethanol from yeast. However, the suppressed quality markers (conversion ratio and tannin contents) of T-3 were attributed to presence of LABs (*L. lactis* + *L. plantarum*) that competitively limited the crucial yeast activities as initial colonizers. The authors opinioned that LABs metabolites (especially lactic acid) did not only interfere with the disorientation of beans cotyledon for phenol diffusion, but also temporarily delayed acetic acid bacteria colonization and consequently limiting complexation of polyphenol to insoluble tannin.

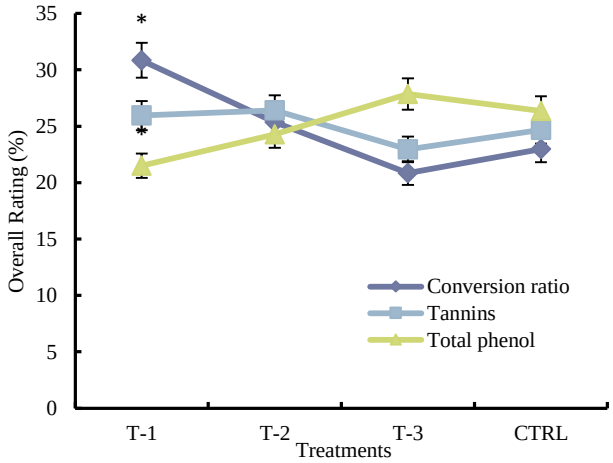

Figure 10. Effect of Treatments on quality markersof fermented cocoa beans

* significantly different (P≤0.05) parameter compared to the control
NB: Treatment-1=Staphylococcus + Pseudomonas + Bacillus
Treatment-2=Staphylococcus +Pseudomonas + LAB
Treatment-3=Bacillus + Yeast + LAB

Therefore, the author concurred that TMBs of T-1 (Bacillus, Staphylococcus and Pseudomonas) effectively limited primary colonizers (yeast) and prolonged the lactic acid phase. This enabled excessive sweating and polyphenol diffusion, and limited enzyme activities. Interaction of LABs and TMBs of T-2 (Staphylococcus and Pseudomonas, lactobacillus) counteracted each other resulting to non-significant impact on the quality markers. This means that LABs limited TMBs but lacks the requisite environment to initiate/moderate the fermentation prior to spontaneous yeast colonization. Similar trend was observed for T-3 (Bacillus, Lactobacillus, Saccharomyces, *Torulopsis* spp) consisting of yeast, TMBs and LABs. As LABs and TMBs limited each other, yeast initiated the fermentation without any significant impact.

AABs are predominant but late colonizers of cocoa fermentation [23,24], even when assessed as starter cultures or natural colonizers during cocoa fermentation [40,41]. This phenomenon justifies the exclusion of AABs in this study. Thus, specific and possible impacts of AABs with the polyphenol profile were not extensively discussed in this study. Imperatively, relative stable AABs profile among all the treatments with exception of T-2, denotes that excessive and prolong lactate phase from T-2 (LABs + TMBs) offered AABs the best conducive environment to proliferate (exothermically metabolizing lactate to acetate). High temperature (up to 50^{o}C) that typifies cocoa fermentation within 72-108 h strongly correlates with AABs activities [23,2]. The short dominancy of AABs within this period enables complexation of flavour precursors, prior successive colonization by TMBs and mold as white tint indicating completion of fermentation. The beans are immediately dried to limit the adverse impact of mold on fermented beans.

3.6. Phytochemical screening of dried fermented cocoa samples

All sampled dried beans regardless of the treatments and fermentation methods revealed that saponins, alkaloid, tannin and total phenol were present (+) at frequency of 100%. However, there were limited number of flavonoid (-) or not detected in all screened sample at all (Table 1).

Qualitative deficiency of flavonoids observed in this study when fermented beans were screened, is in conflicts with the reports of previous studies [41,42], that affirmed the presence of flavonoids in their respective study on chocolate and cocoa powder (both cocoa products undergone roasting process). This contradictory reported, further strengthens the argument that high temperatures (during roasting or drying with high heat) were necessary for transformation of complex polyphenol to flavonoids [43,5-8]. Fermented cocoa beans can be utilized for beverage and confectionery products depending on polyphenol content.

4. Conclusion

Results of this study showed that phytochemical qualities (flavour precursors) were strongly modulated by microbial flora of fermenting beans, either by microbial metabolism or by chemical modification. Early presence of yeast, LAB and bacillus (T-3) during cocoa beans fermentation, significantly ($P \leq 0.05$) limited phenolic biotransformation to insoluble tannin (major flavour precursor) when compared to the spontaneous (control). Synergic effect of microbial flora and fermenting chambers, significantly ($P \leq 0.05$) altered phytochemical quantity of fermented cocoa beans which pertains to cocoa beverage products of good commercial value. However, fermented beans with significantly ($P \leq 0.05$) higher phenolic contents are recommended to pharmaceutical, confectionery and nutraceutical industries. These industries spend huge resources to artificially reconstitute the cocoa butter with the desired polyphenol content for their various products. The results of this study would be valuable alternatives to avert huge expenses and maximize economic benefits in cocoa industry. Added microbial consortia were normal floras and human friendly, and evidently did not support the prevalence of pathogens. Thus, the fermented cocoa beans were considered safe for further processing to finished products.

Table 1. Phytochemical screening of fermented dried Cocoa samples

	Basket				Plastic				Wood box				Frequency (%)
	T_1	T_2	T_3	C_S	T_1	T_2	T_3	C_S	T_1	T_2	T_3	C_S	
Saponin	+	+	+	+	+	+	+	+	+	+	+	+	100
Total phenol	+	+	+	+	+	+	+	+	+	+	+	+	100
Alkaloid	+	+	+	+	+	+	+	+	+	+	+	+	100
Flavonoid	-	-	-	-	-	-	-	-	-	-	-	-	0.0
Tannin	+	+	+	+	+	+	+	+	+	+	+	+	100

T= Treatment; Cs = Control sample; "+" = positive; "-" = negative
NB: A total 12 samples of dried fermented cocoa were used from each treated chambers and control.

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6. Conflict of Interest

Authors declared no conflict of interest. .

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پلی فنل و پروفایل میکروبی دانه کاکائوی تخمیر شده در مزرعه با کنسرسیوم میکروبی انتخابی

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***نویسنده مسئول**

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چکیده

سابقه و هدف: کیفیت و برتری کاکائو به عنوان ماده اولیه برای فرآورده‌های گوناگون کاکائو، در درجه اول به روش تخمیری که طعم و خواص فیتوکمیکال حاصل را تعدیل کند، بستگی دارد. مطالعه حاضر اثر ترکیبی کنسرسیوم میکروبی انتخابی و بیوراکتور بر پروفایل فیتوکمیکال دانه‌های تخمیر شده کاکائو را بررسی کرده است

مواد و روش‌ها: سه کنسرسیوم میکروبی برچسب گذاری شده (T-1, T-2, T-3) به عنوان کشت آغازگر ($\approx 10^{5}$ cells ml^{-1}) و به مدت ۷ روز برای تخمیر در مزرعه کاکائو بر سه محفظه (سبد، جعبه چوبی و پلاستیکی)، مورد استفاده قرار گرفتند. کنسرسیوم‌های جدید T-1، گونه‌های *استافیلوکوکوس*+ گونه‌های *سودوموناس* + گونه‌های *باسیلوس*؛ T-2، گونه‌های *استافیلوکوکوس* + گونه‌های سودوموناس + لاکتوباسیلوس لاکتیس؛ و T-3، گونه‌های *باسیلوس* + گونه‌های لاکتوباسیلوس + گونه‌های ساکارومایسس + گونه‌های ترولوپسیس بودند.

یافته‌ها و نتیجه‌گیری: پروفایل میکروبی به طور معنی داری در تمام تیمارها (T-1, T-2, T-3) تغییر کرد ($P\leq 0/05$) و تناوب میکروبی تا ۲۲/۵-۰/۵درصد افزایش یافت. میزان ترکیبات فنولی تیمارهای T-3 و T-1 در محفظه سبدی به‌طور معنی داری تغییر کرد. در T-1 (سبد و جعبه چوبی و پلاستیک) و T-2 (پلاستیک) میزان تانن به‌طور معنی داری تغییر کرد. نسبت تبدیل تانن: پلی فنل به عنوان معیار کیفی تعیین شده برای دانه کاکائوی تخمیر شده در T-1 (سبد و جعبه چوبی) و T-2 (پلاستیک) به‌طور معنی داری افزایش یافت، اما درT-3 (سبد) به‌طور معنی داری متوقف شد. براساس شواهد این مطالعه می‌توان نتیجه‌گیری کرد که هم‌افزایی مناسب فلور میکروبی و محفظه تخمیر می‌تواند کیفیت خوب کاکائو حاوی مقادیر پایین پلی فنل (بهترین برای نوشیدنی‌های کاکائویی) یا حاوی مقادیر بالای پلی فنول (بهترین برای صنایع دارویی، قنادی و غذا دارو) را نتیجه دهد. این یافته‌ها می‌توانند در یافتن جایگزینی اقتصادی برای بازسازی پرهزینه بازسازی پلی فنول های کره کاکائوی مورد استفاده در فرآورده های صنعتی گوناگون و به حداکثر رساندن منافع اقتصادی کشاورزان و نیز صنعتگران کاکائو مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافعی وجود ندارد.