

## Microbial and metabolite profiles of spontaneous and adjunct-inoculated cacao (*Theobroma cacao* L.) fermentation

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### Abstract

The succession of the dominant microbial population during cacao fermentation with or without adjunct inoculation of yeast and lactic acid bacteria (LAB) were monitored on a laboratory scale using culture-dependent and culture-independent methods. Yeasts and acetic acid bacteria (AAB) population throughout a five-day fermentation process showed no significant differences but the LAB population increased through adjunct inoculation. Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) identification method showed the dominance of only *Lactobacillus plantarum*, one of the species used as the adjunct inoculum, which resulted in higher lactic acid production. On the other hand, *Acetobacter* spp. and *Gluconobacter* spp. were markedly observed in the spontaneously fermented set-up resulting in increased acetic acid production, significantly different ( $p > 0.05$ ) at three to five days of fermentation. LAB and yeast inoculation resulted in a more desirable temperature and pH of the fermenting mash which may result in better product quality.

## 1. Introduction

The increase in local and international demand for cacao led to surging world prices, reviving interests in cacao growing and processing. The geographic location, climatic conditions and soil characteristics in the Philippines are ideal for cacao production. However, local annual production of only approximately 10,000-12,000 metric tons is far less than the estimated local consumption of more than 50,000 metric tons, hence more efforts are needed to increase production and processing into good quality cacao products (Department of Agriculture, Philippines, 2017).

Preliminary processing of cocoa beans into better-tasting products involves good fermentation and adequate drying. The fermentation process is carried out with the usual practice of settling the beans in a container or in a heap and letting the natural microflora of the beans handle the fermentation process which leads to the release of flavor precursors and inhibition of bean germination. Yeasts, lactic acid bacteria (LAB) and

acetic acid bacteria (AAB) are the important microorganisms in cacao fermentation (Ho *et al.*, 2018). Yeasts and heterofermentative LAB convert pulp sugars to ethanol which is then converted into acetic acid by AAB. Some yeasts and LAB also convert pulp sugars and citric acid into lactic acid which, together with the acetic acid, inhibit growth and activity of putrefactive microorganisms. The acetic acid and lactic acid can easily penetrate cacao seed which leads to the formation of precursors for the defined chocolate flavor (Schwan and Wheals, 2004; Castro-Alayo *et al.*, 2019), thus providing safer and good quality end products.

The application of various inoculants in the fermentation of cacao has been tried by some studies with different observations (Schwan 1998; Leal *et al.* 2008; Ali, 2011; Ho *et al.*, 2018). The use of an inoculant for cocoa fermentation in the Philippines is yet to be tested and determined whether its application would lead to improved cocoa bean quality. This study aimed to determine and compare the succession of LAB

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and AAB during laboratory-simulated cacao bean fermentation through culture-dependent methods, identify the dominant species through culture-independent method, i.e. Polymerase Chain Reaction–Denaturing Gradient Gel Electrophoresis (PCR-DGGE), and to determine the organic acids and ethanol content of the cacao beans upon inoculation with LAB and yeast starter culture with that of non-inoculated spontaneously fermentation.

## 2. Materials and methods

### 2.1 Preparation and fermentation of cacao beans

Mixed varieties of commonly grown mature cacao in the Philippines namely, UF18, PBC123, BR25, K1, K2 and K9, were purchased from various locations in the municipality of San Antonio, Batangas and Tiaong, Quezon, Philippines through coordination with Quezon Agricultural Experimentation Station (QAES) in Tiaong, Quezon and the local government units. The beans were obtained by pod breaking using a clean machete followed by manual scooping of the beans from the pod into 10-kg capacity rattan baskets lined with banana leaves. Each of the four baskets, two for uninoculated and another two for the inoculated set-ups, contained 3 kg of matured beans of randomly mixed varieties. The baskets were covered with banana leaves and placed inside Styrofoam chests (1' L × 0.8' W × 1' H) to simulate a controlled environment, avoiding temperature fluctuations and protecting from pest's infestation. After 72 hrs, the beans were manually mixed to incorporate air into the fermenting mash. The baskets were then placed inside an incubator, set at 45°C, to simulate the increase in temperature of the beans after turning. The beans were kept at 45°C for 48 more hours prior to harvest. The experiment was carried out in duplicate.

The inoculant mix was obtained from the Biotechnology for Industry, Energy and Environment Program, BIOTECH, UP Los Baños, Philippines, containing a microbial cocktail with 10<sup>8</sup> cells LAB and 10<sup>7</sup> cells yeasts per gram of powder. The LAB species are *Lactobacillus plantarum* DIL-8 and *Lactobacillus pentosus* SOL-6 and yeast species are *Saccharomyces cerevisiae* DIY-13 and *Hanseniaspora uvarum* DOY30 which were selected after isolating and screening from dominant species found in fermenting cocoa beans in Tiaong, Quezon and Davao (Elegado et al., 2015). The powdered inoculants were prepared using a low-temperature vacuum spray dryer (YK-100, True Ten Ind. Co., Ltd, Taiwan) operated at an inlet temperature of 45°C and outlet temperature of 50°C with maltodextrin as carrier material. AAB was not included in the cocktail mix being labile at 45-50°C. The inoculant was applied

in powdered form at 1% (w/w) inoculation rate at the start of the cacao fermentation. The incubation conditions during fermentation, sampling and analyses were similar for both the inoculated and uninoculated (naturally-fermenting) set-ups.

### 2.2 Determination of microbial population

A total of 10 g of the fermenting cacao bean samples were obtained every 24 hrs from the middle of the fermenting mass and placed in 90 mL sterile 1.5% (w/v) peptone water. The sample was serially diluted using sterile peptone water and plated in duplicate for each dilution using the spread plate technique. Maltose Extract Agar (MEA) amended with 20 ppm tetracycline was utilized for plate count of the total yeasts count. LAB count was determined using De Mann, Rogosa, and Sharpe (MRS) agar treated with sodium azide (0.02 g/L). For the AAB, Glucose Yeast Extract Calcium Carbonate Agar (GYCA) treated with 20 ppm cycloheximide was used. The LAB plates were placed in candle jars. All plates were incubated at ambient room condition (≈28°C) for 48 hrs and colonies were counted and reported in CFU/g.

### 2.3 PCR-DGGE method

With the assumption that microbial growth would be uniform all over the fermenting mash, only three (3) beans were collected randomly, placed in 40 mL TE (Tris-EDTA) buffer and vortexed thoroughly. Sampling was done every 24 hrs for both set-ups (inoculated and non-inoculated). DNA was isolated from 500 µL of liquid obtained from the shaken tube using Zymo Soil Microbe DNA Mini Prep Kit® (Zymo Research, USA). The DNA obtained was then visualized and quantified through gel electrophoresis using 1% agarose gel. Denaturing Gradient Gel Electrophoresis (DGGE) was performed using DCode™ (Bio-Rad, Hercules, Calif., USA) Universal Mutation Detection System using 8% polyacrylamide initially at 100 V for 10 mins and then at 60 V for 15 hrs. The DGGE primer pairs used were NL1GC and LS2 which target the D1-region of the 26S rRNA, LAC1 and LAC2GC which target the V3-V4 region of the 16SrRNA gene-specific for LAB, and WBAC1 and WBAC2GC which target the V7-V8 region of the 16S rRNA genes for both LAB and AAB. The primer sequences are shown in Table 1. The gel was stained with Gel Red in order to view separated bands using Syngene U: Genius3 Gel Imaging System®. The remarkably visible PCR bands, representing the bacteria or yeast that grew dominantly, were then excised using sterile plastic forceps through the guidance of Quantityone™ Image Production Software. The obtained bands were transferred to 50 µL HPLC water, macerated

Table 1. PCR-DGGE primers used in the study

| Primer Name | Target  | Sequence  | References   |
|-------------|---|---|--|
| NL1F-GC     | D1-region of the 26S rRNA (Yeast)               | 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG<br>GCA TAT CAA TAA GCG GAG GAA AAG-3'            | Kurtzmann and Robnett (1998);  |
| LS2         |   | 5'-ATT CCC AAA CAA CTC GAC TC-3'  | Cocolin <i>et al.</i> (2000) as cited by Nielsen <i>et al.</i> (2005)              |
| LAC1        | V3-V4 region of the 16S rRNA gene (LAB)         | 5'- AGC AGT AGG AAT CTT CCA-3'  | Walter <i>et al.</i> (2000) and Lopez <i>et al.</i> (2003) as cited by Camu (2007) |
| LAC2GC      |   | 5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG<br>CCC CCG CCC CAT TTC ACC GCT ACA CAT G-3'     |  |
| WBAC1       | V7-V8 region of the 16S rRNA gene (LAB and AAB) | 5'- GTC GTC AGC TCG TGT CGT GAG A-3'  | Lopez <i>et al.</i> (2003) as cited by Camu (2007)                                 |
| WBAC2GC     |   | 5'- CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG<br>CCC CCG CCC CCC CGG GAA CGT ATT CAC CGC G-3' |  |

using a sterile yellow tip and centrifuged for 30 s. The samples were incubated in a 37°C water bath for 30 mins and then used as a template for PCR using the Primer sets, in duplicate. The PCR samples, presumed as clean and pure, were sent to AIT-BIOTECH (Singapore) for sequencing and identification. Only one good PCR sample amplicon for a particular dominant bacterium was sent for sequencing. Selected markers of identified bacteria were also run through PCR for additional identification.

### 2.3 Physicochemical properties determination

#### 2.3.1 Temperature

The temperature of the fermenting mass was measured every 24 hrs using a digital thermometer in random points and reported as average value. It was monitored throughout the whole fermentation period.

#### 2.3.2 pH

A total of 5 g of the fermenting beans were placed into 100 mL distilled water in a beaker and shaken very well. Approximately 30 mL of the mixture was placed in a separate beaker and pH was measured using a calibrated digital pH meter (Horiba Navi h F-51, Japan).

#### 2.3.3 Sample preparation for chemical analysis

A total of 10 g of fermenting beans was added with 50 mL distilled water and then minced or crushed. The decanted liquid was filtered through a 0.45 µm membrane filter and used as the sample for analysis of ethanol and organic acids.

#### 2.3.4 Ethanol analysis

An aliquot (1 mL) of sample or standard (analytical grade absolute ethanol) was added to 1 mL of 2% isopropyl alcohol (internal standard) and 3 mL of distilled water. A gas chromatograph (Shimadzu GC-2014, Japan), equipped with a flame ionization detector and glass column (3.2 mm I.D. × 2 mm L) packed with Porapak Q and operated at a column temperature of 190°C and injection temperature of 230°C, was used for

the determination of ethanol concentration in the sample. Nitrogen was used as the carrier gas and was set at a flow rate of 60 mL/min while hydrogen pressure was set at 50 kg per cm<sup>3</sup>. The concentration of standards ranged from 0.001% to 0.20%.

#### 2.3.5 Analyses of organic acids

High Performance Liquid Chromatography equipped with Refractive Index Detectors (RID) was used to determine concentrations of acetic, citric and lactic acids in the samples, using Supelcogel C-610H column (7.8 mm I.D. × 30 cm L), operated at 30°C. Sulfuric acid (H<sub>2</sub>SO<sub>4</sub>), at 0.1% concentration, was utilized as the mobile phase with a flow rate of 0.7 mL/min. A standard stock solution was prepared for each organic acid. Acetic acid and citric acid standard concentrations ranged from 0.005 to 0.01%. For the lactic acid, the concentrations of standards ranged from 0.001 to 0.1%

### 2.4 Data Analysis

Megastat Version 10.2 Release 2.1 (Microsoft Excel 2007) was used for the Analysis of Variance (ANOVA). T-test assuming unequal variances was used to separate and compare the means with an alpha value of 0.05.

## 3. Results and discussion

### 3.1 Yeast growth profiles

The initial yeast population of the inoculated sample was relatively higher due to the addition of the inoculant with a count of 2.21×10<sup>6</sup> CFU/g as compared to the naturally-fermented with a count of 4.80×10<sup>4</sup> CFU/g (Figure 1A). However, during the course of fermentation, there was no significant difference (p<0.05) in yeast count between the two set-ups. Although there was a steady decline of yeast counts thru time, they were still quite high at 3.68×10<sup>7</sup> CFU/g and 9.63×10<sup>7</sup> CFU/g for the naturally-fermented and inoculated setups, respectively, at the end of fermentation. Yeasts that are naturally found in cacao, particularly *Pichia* spp. are generally tolerant to low pH.

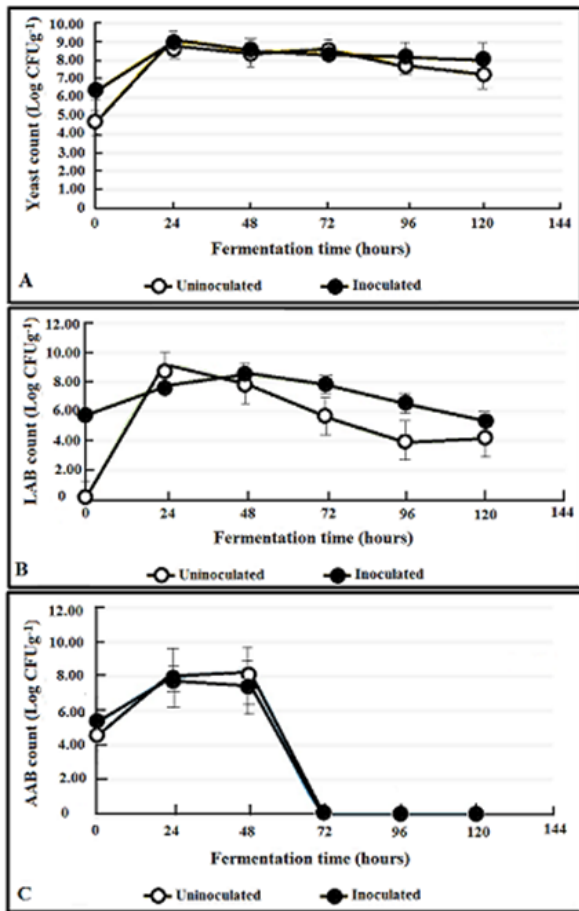


Figure 1. Comparative growth of yeasts (A), lactic acid bacteria (B) and acetic acid bacteria (C) in a simulated cacao fermentation with or without inoculation

### 3.2 Lactic acid bacterial growth profiles

The LAB counts are shown in Figure 1B. The initial count of LAB on samples inoculated at 1% (w/w) rate was  $5.7 \times 10^5$  CFU/g which is remarkably different from the uninoculated set-up. The uninoculated set-up had an initial population of  $<1$  CFU/g but it increased to  $4.7 \times 10^8$  CFU/g after 24 hrs, interestingly higher than the inoculated set-up. However, from 48 hrs onwards, a drastic decrease in the LAB population was observed more prominently in the uninoculated set-up. This could be attributed to the inhibitory effects of ethanol and other metabolites produced by yeasts and other microorganisms. For the inoculated set-up, the LAB population peaked after 48 hrs with a value of  $5.20 \times 10^7$  CFU/g and maintained a higher LAB population than the uninoculated set-up from 48 - 120 hrs, at a slower declining rate. This observation was in agreement with the findings of Kresnowti *et al.* (2013). The LAB inoculants have been previously selected for their acid, ethanol and temperature tolerance. For the naturally-fermented beans, a similar trend was reported by Schwan and Wheals (2004) wherein the LAB population was also observed to peak at 16 - 48 hrs of fermentation followed by a decrease in counts afterwards perhaps due to nutrient depletion and formation of inhibitory compounds. However, there was no statistical difference

( $p > 0.05$ ) on the LAB counts for the uninoculated set-up with that of the inoculated setup at 60 hrs.

### 3.3 Acetic acid bacterial growth profiles

AAB was not present in the inoculant mix, thus almost very similar AAB population profiles were observed for both the inoculated and uninoculated set-ups throughout the fermentation (Figure 1C). There was no significant difference ( $p > 0.05$ ) between the AAB populations of the inoculated and the uninoculated set-ups all throughout the duration of fermentation. However, there was a drastic decline in the counts of AAB in both set-ups from 48 hrs onwards, resulting in counts of less than 1 CFU/g. Such observation could be attributed to the depletion of substrates, formation of AAB inhibitory metabolites and increase in temperature to  $45^\circ\text{C}$ . AAB is known to be much less thermotolerant than LAB (Ho *et al.*, 2018).

### 3.4 Identification of dominant yeasts using PCR-DGGE

PCR-DGGE analysis using NL1F-GC and LS2 primers showed the presence of *Hanseniaspora uvarum*, *Candida spp.* and *Pichia kudriavzevii* for the inoculated set-up while only *Candida spp.* and *Pichia kudriavzevii* were found in the uninoculated set-up (Figure 2). Based on band thickness, the presence of yeasts was minimal from 0 - 48 h of fermentation as bacteria are fast grower than yeasts. *Candida spp.* was dominant for both set-ups. *P. kudriavzevii* was very dominant in the inoculated set-up from 72 - 120 hrs but in the naturally-fermented setup, it only appeared at the 120<sup>th</sup>. *H. uvarum* was observed only after 24 hrs in both set-ups. Band for *Saccharomyces cerevisiae*, one of the yeasts utilized as the inoculant, was barely visible for either set-ups during this particular fermentation although *H. uvarum*, the

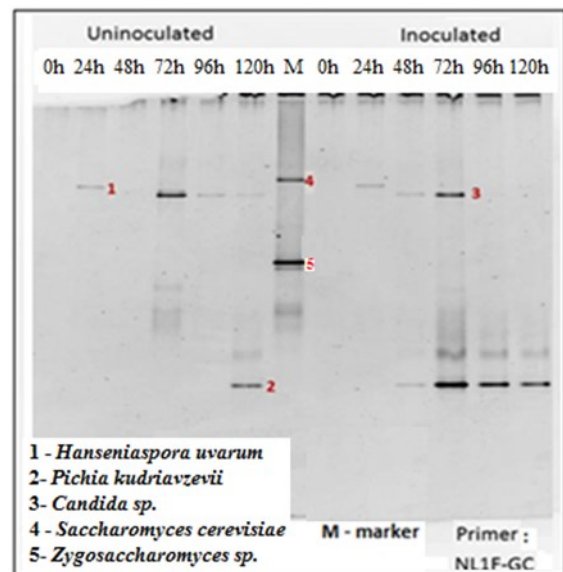


Figure 2. PCR-DGGE gel labeling for yeasts obtained from 120 hrs simulated fermentation of cacao beans using NL1F-GC primer



other inoculant was markedly visible after 72 hrs. It persisted until 120 hrs in the uninoculated set-up but seemed to disappear in the inoculated set-up, perhaps because of the presence of LAB. The dominance of *P. kudriavzevii* was observed in the inoculated set-up due to its acid tolerance. *P. kudriavzevii* and *H. uvarum* were also observed to predominate during cacao fermentation in another study (Moreira et al., 2013). *Candida* spp. and *Pichia* spp. were also reported as the primary yeasts for the fermentation of cocoa (Schwan and Wheals, 2004).

### 3.5 Identification of dominant lactic acid bacteria using PCR-DGGE

PCR-DGGE showed that only *Lactobacillus plantarum* band was visible, particularly in the inoculated set-up (Figure 3). Though *Lactobacillus pentosus* was utilized as an inoculant for the setup, no specific marker was obtained for *L. pentosus* which could have also been enumerated similar to *L. plantarum*. *L. pentosus* is reportedly, genotypically, very closely related to *L. plantarum* and are also not easily differentiated phenotypically. Only the *recA* gene was successfully used to differentiate them through PCR (Torriani et al., 2001). The primer set (LAC1 and LAC2-GC) used for DGGE was unable to differentiate them and *L. pentosus* gave similar band to *L. plantarum*.

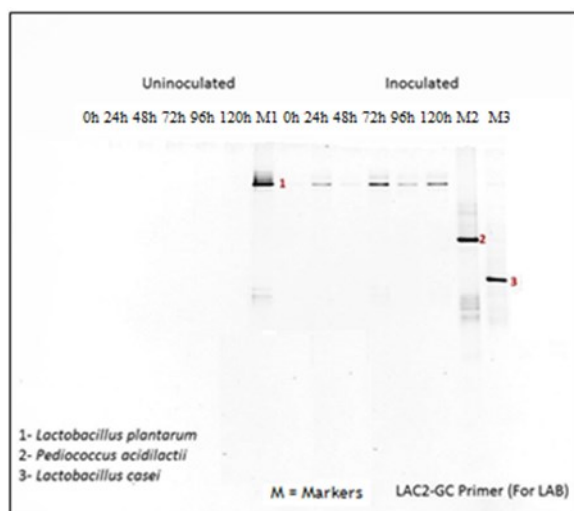


Figure 3. PCR-DGGE gel labeling for acidogenic bacteria obtained from 120 hours simulated fermentation of cacao beans using WBAC-GC primer.

### 3.6 Identification of dominant acidogenic bacteria using PCR-DGGE

Analysis of the DGGE gel (Figure 4) with the WBAC1 and WBAC-GC primer set shows that during fermentation, *Acetobacter* spp., *Acinetobacter* spp., *Gluconobacter* spp., and *L. plantarum* were present in the naturally-fermenting setup. Thick bands were observed for the *Acetobacter* spp., and *Acinetobacter* spp. during the course of fermentation. *Gluconobacter*

spp. and *L. plantarum* were also observed throughout the period of natural fermentation but were observed to have relatively weaker bands. Various species of *Acetobacter* were observed throughout the fermentation process. For the inoculated set-up, the same microorganisms as that in the naturally-fermented set-up were observed at the start of fermentation. However, these became less dominant as fermentation progressed while the *L. plantarum* was observed to become very dominant throughout the fermentation process, primarily because it was added as inoculum. *Acetobacter* spp. was also observed throughout the fermentation, however, relatively weaker bands were observed in comparison to *L. plantarum*. This observation is suggestive of the inhibitory effect of *L. plantarum* versus most of the AAB.

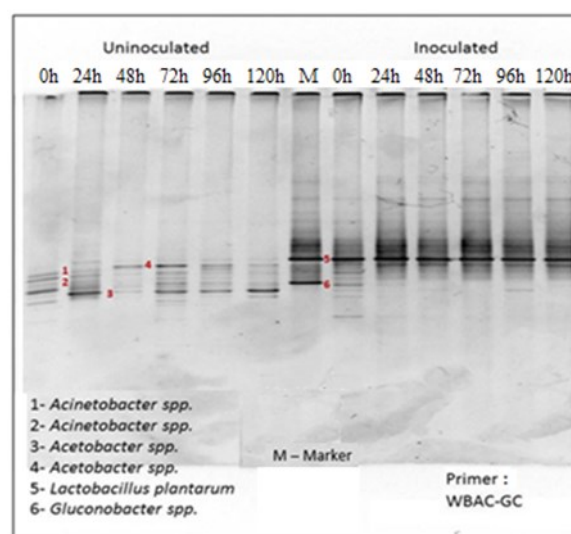


Figure 4. PCR-DGGE gel labeling for acidogenic bacteria obtained from 120 hours simulated fermentation of cacao beans using WBAC-GC primer.

It was a limitation of this study that only the remarkably visible bands were selected for PCR amplification and sequencing. Faint DGGE bands and those that were not clearly separated were not anymore excised nor subjected to PCR amplification. It should be assumed though that more bacterial and yeast species are existing, more diverse in the naturally-fermenting cacao. Table 2 summarizes comparative data on the profiles of dominant microorganisms in adjunct inoculated with that of non-inoculated or spontaneously-fermenting cacao. *L. plantarum* and *P. kudriavzevii* were persistent in the inoculated set-up while in the spontaneously fermenting set-up, more acetic acid bacteria and *Candida* spp. were dominant.

### 3.7 Physicochemical properties

#### 3.7.1 Temperature

For the naturally-fermenting set-up, the temperature increased from 26 to 30.75°C for the first 24 hrs. This temperature was maintained until 72 hrs before reaching 40.83°C at 96 hrs upon artificial incubation. The daily

Table 2. Enumeration of dominant microorganisms identified through PCR-DGGE

| Fermentation Time (hr) | Fermentation Set-up         |                                |
|------------------------|-----------------------------|--------------------------------|
|                        | Uninoculated                | Inoculated                     |
| 0                      | <i>Acetobacter</i> spp.     | <i>Lactobacillus plantarum</i> |
|                        | <i>Gluconobacter</i> spp.   | <i>Gluconobacter</i> spp.      |
|                        | <i>Acinetobacter</i> spp.   | <i>Acinetobacter</i> spp.      |
| 24                     | <i>Hanseniaspora uvarum</i> | <i>Hanseniaspora uvarum</i>    |
|                        | <i>Acetobacter</i> spp.     | <i>Lactobacillus plantarum</i> |
| 48                     |                             | <i>Candida</i> spp.            |
|                        | <i>Acetobacter</i> spp.     | <i>Pichia kudriavzevii</i>     |
|                        |                             | <i>Lactobacillus plantarum</i> |
| 72                     | <i>Candida</i> spp.         | <i>Candida</i> spp.            |
|                        | <i>Acetobacter</i> spp.     | <i>Pichia kudriavzevii</i>     |
|                        | <i>Gluconobacter</i> spp.   | <i>Lactobacillus plantarum</i> |
| 96                     | <i>Candida</i> spp.         | <i>Pichia kudriavzevii</i>     |
|                        | <i>Acetobacter</i> spp.     | <i>Lactobacillus plantarum</i> |
|                        | <i>Gluconobacter</i> spp.   |                                |
| 120                    | <i>Candida</i> spp.         | <i>Pichia kudriavzevii</i>     |
|                        | <i>Pichia kudriavzevii</i>  | <i>Lactobacillus plantarum</i> |
|                        | <i>Acetobacter</i> spp.     |                                |

changes in the temperature of the inoculated set-up showed a similar trend from the start of fermentation up to 24 hrs but a higher temperature was observed during from 48 - 72 hrs even without the incubator yet (Figure 5A). This could be due to increased microbial activities by the inoculant strains, particularly the exothermic processes such as ethanol production. The temperature rise could be the main reason why the AAB population decreased upon yeast and LAB inoculation. From 96-120 h, upon placing the samples at 45°C incubation, the temperature of the fermenting mass increased above 40°C. T-test results show that the observed difference between the means of the temperature profiles of the inoculated and uninoculated set-up do not differ significantly during this period. An increase in temperature was also observed in a study conducted by Ardhana and Fleet (2003) wherein they observed a temperature increase from 25 to 48°C.

### 3.7.2 pH

The daily changes in pH during cacao fermentation in the inoculated and uninoculated set-ups are shown in Figure 5B. A low initial pH can be attributed to the presence of citric acid in the beans. After 24 hrs, the pH of the fermenting cacao samples was observed to be lower (pH 3.68) in the inoculated set-up than the uninoculated set-up (pH 3.77) perhaps due to the enhanced conversion of sugars into lactic acid by the LAB inoculant. But after turning the beans, the pH of the sample gradually increased from 48 hrs onwards for the inoculated set-up. On the other hand, pH increased only after 72 hrs for the naturally-fermenting set-up. Though

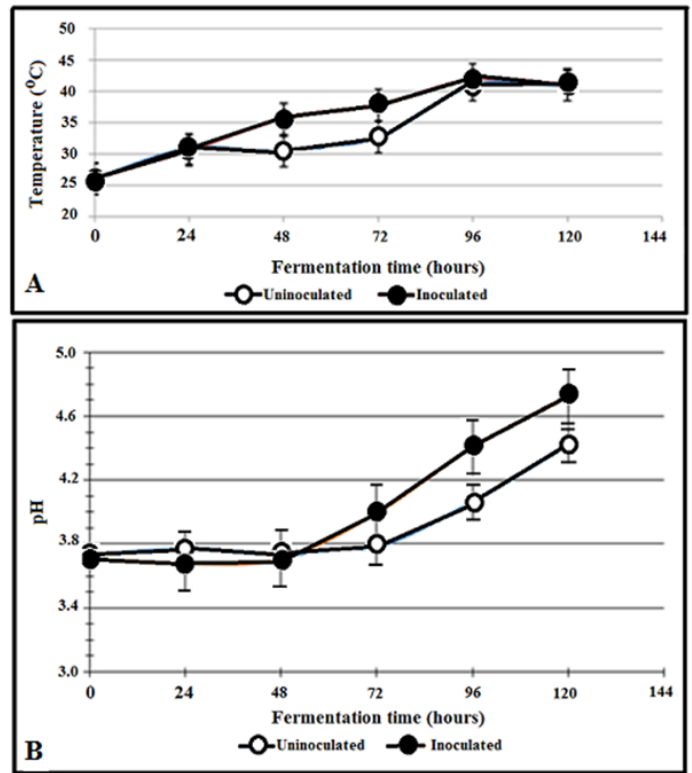


Figure 5. Changes in temperature (A) and pH (B) of inoculated and uninoculated fermenting cacao beans

the increase was visibly higher in the inoculated set-up, no significant difference ( $p > 0.05$ ) was monitored. A higher pH at the end of fermentation is preferred for better-quality fermented cacao (Afoakwa *et al.*, 2013). During cacao fermentation, the more acidulant citric acid is converted to lesser acidulant acetic and lactic acid. Moreover, upon turning the beans, the penetration of these acids into the beans and release them into the environment lead to a pH increase. Lactic acid and acetic acid penetrating the cacao seeds would facilitate the formation of flavor precursors during further processing (Castro-Alayo *et al.*, 2019).

### 3.7.3 Ethanol and organic acid

The results of ethanol, acetic acid, citric acid and lactic acid analyses of the fermenting mass are shown in Figure 6. A higher initial ethanol concentration was observed in the uninoculated fermenting cacao beans (0.054%) as compared to the inoculated fermenting samples (0.003%). As expected, yeast inoculation resulted in a higher and earlier peak (0.131% at 24 hrs) as compared to the uninoculated set-up (0.094% at 48 hrs). Through time, ethanol concentration decreased due to vaporization or conversion into acetic acid. It was only during the 24<sup>th</sup> hour whereby a marked difference in ethanol concentrations in the two set-ups was observed. But from 48 hrs onwards, results showed that the differences between the ethanol contents of the inoculated and uninoculated set-ups were not significant ( $p > 0.05$ ).

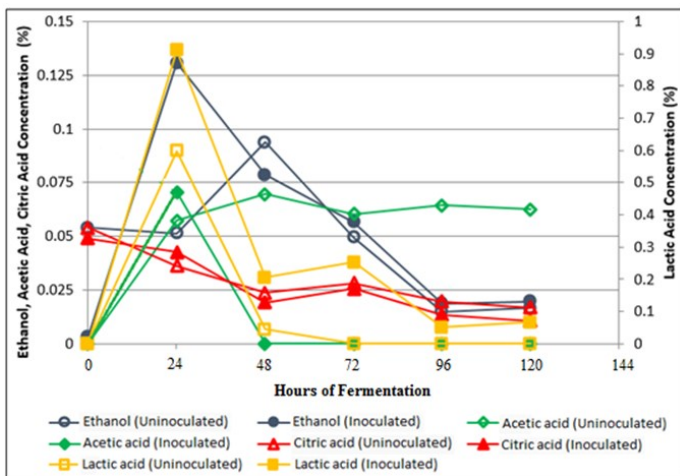


Figure 6. Changes in ethanol and selected organic acids concentrations of uninoculated and inoculated cacao pulp

For the acetic acid assay results, a sharp increase from an undetectable amount to an increase at 24 hrs was observed for both set-ups. Acetic acid production continued throughout the fermentation to an almost constant quantity of around 0.07% for the uninoculated setup due to the continued presence of *Acetobacter* spp. and *Gluconobacter* spp. (Table 2). However, for the inoculated set-up there was a drastic decrease after 24 hrs to an undetectable level. Apparently, in the inoculated set-up, more lactic acid was produced instead of the acetic acid, due to the predominance of inoculated *L. plantarum* and *L. pentosus* and inhibition of AAB growth by the lowered pH and increased temperature. Statistical analysis between the inoculated and uninoculated setups showed a significant difference ( $p < 0.05$ ) at 48–120 hrs.

As for the lactic acid assay results, it was not detected during the start of fermentation but was observed to reach the highest concentration after 24 hrs for both set-ups, markedly higher in the inoculated (0.91%) set-up than the uninoculated set-up (0.63%). A sharp decrease was observed thereafter for both treatments, with the uninoculated setup reaching undetectable levels from 72 hrs onwards. There was a sharp decrease (0.91% to 0.21%) of lactic acid followed by a slow but continuous decrease (0.25% to 0.05%) for the inoculated set-up. Moreira *et al.* (2013) also reported the same trend wherein the lactic acid was observed to peak after 24 hrs. They also describe the low undetectable amounts of lactic acid observed at the beginning of the fermentation due to the lack of initial metabolism of citrate wherein lactic acid production requires the breakdown of citrate through microbial metabolism. Lactic acid is a by-product of the citric acid metabolism of certain microorganisms such as *Lactobacillus*. Hastened pulp breakdown may have been observed due to the application of the inoculant as facilitated by the presence of more yeasts and LAB leading to the ready conversion of sugars into ethanol

and lactic acid. Moreover, the data showed an almost similar trend on the decrease in citric acid concentrations for both the adjunct inoculated and non-inoculated cacao beans, suggestive of almost adequate microbial actions on the inherent citric acid.

#### 4. Conclusion

The plate count method showed no recognizable differences in the growth of yeasts and acetic acid bacteria (AAB) observed during the course of laboratory-simulated cacao fermentation for both the uninoculated and inoculated setups. On the other hand, lactic acid bacteria (LAB) dominated in the inoculated set-up as compared to the uninoculated set-up, suggesting that the *L. plantarum* inoculant was successful in increasing the microbial load for the LAB in the fermentation process throughout the course of fermentation. It was observed that yeasts and LAB dominated in the fermentation while AAB declined in counts after 48 hrs of fermentation. Identification of the dominant microbial species was determined through the use of PCR-DGGE. *L. plantarum* was confirmed as the dominant acid-forming bacteria in the inoculated set-up which was observed throughout 120 hours of the fermentation period. On the other hand, PCR-DGGE showed that *Acetobacter* and *Gluconobacter* were the dominant acid-forming bacteria for the naturally-fermented setup, but which seemed to have been already killed by plate count observation. For PCR-DGGE of yeasts, *Hanseniaspora uvarum* was observed in both set-ups during after 24 hrs while *Pichia kudriavzevii* was very dominant for *L. plantarum* inoculated set-up. However, *Saccharomyces cerevisiae* although used as inoculum was not seen through PCR-DGGE. In terms of the production of organic compound, an increased lactic acid concentration was observed with inoculation of LAB while acetic acid was significantly high for the uninoculated set-up which is in agreement with the dominance of AAB as shown by DGGE results.

Inoculation of carefully selected yeast and LAB strains as adjunct inoculant at the start of fermentation may enhance sugar conversion into ethanol and lactic acid for faster cacao fermentation, minimize contamination of undesirable microbial contaminants by attaining the desired temperature and pH and thus resulting in more uniform microbial load and product quality. This could be advantageous for small-scale cacao farmers. Spontaneous fermentation may need a higher amount of beans to attain a desirable temperature and pH for a good quality product, suited for medium to large-scale cacao producers.

## Conflict of interest

There is no conflict of interests of any nature on the part of the authors.

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