Original Research Article

ASSESSMENT OF MICROBIAL INTERACTIONS BETWEEN STARTER POTENTIAL LACTIC ACID BACTERIA AND MAJOR MICROORGANISMS DURING *IN VITRO* FERMENTATION IN A MEDIUM SIMILAR TO COCOA PULP IN COTE D'IVOIRE

Comment [AR1]: Assessment of microbial interactions between potential lactic acid bacteria starters and major microorganisms during in vitro fermentation in a medium similar to cocoa pulp in Cote d'Ivoire

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ABSTRACT

Comment [AR2]: Consider simplifying complex sentences and using more concise language for better clarity and understanding

Côte d'Ivoire has been the world's leading producer of cocoa beans for several decades. However, the variability of cocoa beans due to random fermentation and lack of control causes enormous economic losses to the country, estimated at several billion CFA francs. The aim of this work was to analyse the interactions between potential starter lactic acid bacteria and major microorganisms selected during cocoa fermentations in Côte d'Ivoire. Yeast, lactic, acetic and bacillus genus starters were grown in their respective media YPG, MRS, YEPG and BN for 48 h at 30°C before being placed in the similar interaction medium of cocoa pulp (PSM) for the microbial interaction study. In this study, the starters were inoculated alone (monoculture) or together (coculture) and then incubated at 30°C for 5 days. Samples were taken After 0; 48 and 120 hours of incubation and the interaction was monitored by assessing microbial growth using optical density and Thoma cell counts for monocultures and agar media for cocultures. Acid production and the consumption of reducing sugars were also measured. All cultures showed good growth. Growth was greater in cocultures, with values ranging from 4.12 log (cfu/ml) to 9.21 log (cfu/ml). Also, cocktails acidified the medium much more than monocultures, ranging from 0.10 ± 0.00 to 0.21 ± 0.00. In addition to acidity, the amount of sugars in the different broths decreased significantly in the cocktails during the first 48 hours, from 1.08 ± 0.08 to 0.20 ± 0.01. Microbial starters in monocultures and cocultures showed their ability to adapt to the similar environment of the similar medium of cocoa pulp. In addition, the high microbial growth, sugar consumption and acidification of the medium in the cocktails reflected a synergistic interaction between the strains during fermentation when they were together.

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Keywords: Lactic acid bacteria, cocoa fermentation, interaction, starter

1. INTRODUCTION

Ivorian cocoa bean quality remains unpredictable, resulting in massive economic losses for the country. In fact, economic losses, which amounted to 150 billion CFA francs in 2006, have increased over time to around 300 billion CFA francs in 2017 [1]. These losses are mainly related to post-harvest treatment of beans [2-4]. Among these treatments,

fermentation is an indispensable step in the transformation process of cocoa into chocolate and has a major impact on the flavour, colour and aroma of cocoa products [5-6]. During cocoa fermentation, complex biochemical reactions triggered by the microbial flora take place allowing the production of cocoa and chocolate with desirable organoleptic characteristics. However, cocoa fermentation occurs randomly, spontaneously; or uncontrolled under the influence of numerous microbes, resulting in beans of varying chemical and sensory quality from place to place.
To solve this problem, several studies have been carried out to improve and control the fermentation process of Ivorian cocoa [7-13]. These studies have highlighted the microbial diversity involved in cocoa fermentation from 12 major producing regions in Côte d'Ivoire while other studies have highlighted the diversity of biochemical and functional properties such as enzyme production by yeasts and Bacillus, as well as citrate lyase, lactic and acetic acid production by lactic and acetic acid bacteria [7, 8, 9, 14]. The microbial flora responsible for fermentation has been identified in all the studies on Ivorian cocoa. Its Genetic diversity has been analysed and well elucidated [13]. In addition to these studies, the functional properties of strains belonging to the major species have been analyzed, leading to the screening of starter strains that can be used in controlled fermentation and improve the cocoa fermentation process. The use of these microorganisms as starters in cocoa fermentation is a strategy to standardise and improve the sensory quality of chocolate [15]. However, the microbial interactions that occur between species remain unknown. Yet, these interactions may have a significant impact on the microbial development during fermentation and thus influence cocoa quality.
In Côte d'Ivoire, no studies have been carried out on the interactions between the main players involved in cocoa fermentation. The aim of this work was to study the interactions between potential starter lactic acid bacteria and the main microorganisms selected during cocoa fermentations in Côte d'Ivoire.

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2. MATERIAL AND METHODS

2.1. Microbial strains

The microbial material consisted of eight strains, including two yeast strains (D5P12, T7GB10), three lactic acid bacteria (T11G3, T10G5, T8AB6), two acetic acid bacteria (T6HS14, T3G2) and Bacillus (T11I10), previously isolated from fermenting cocoa in Côte d'Ivoire and identified by 16S gene sequencing. These microorganisms were characterised at the level of their functional properties as dominant strains and potential starter for cocoa fermentation [8-11]. The aim of this study was to analyse the interactions between these potential starter microorganisms.

2.2. Study of microbial interactions

2.2.1. Determination of starter viability during microbial interactions

To study the interactions, microbial starters of yeast (D5P12 and T7GB10), lactic acid bacteria (T11G3, T10G5 and T8AB6), acetic acid bacteria (T6HS14 and T3G2) and Bacillus (T11I10) were each cultured in 5 mL of Yeast extract- Peptone-Glucose (YPG), Man Rogosa Sharpe (MRS), Yeast extract- Ethanol- Peptone- Glucose (YEPG) and Bouillon Nutritif (BN) broths respectively and then incubated at 30°C for 24 hours. subsequently 100 µL of each preculture were inoculated in 5 mL of cocoa pulp simulation medium PSM containing 3% fructose; 2% glucose; 1% sucrose; 1% pectin; 1% citric acid; 0.6% yeast extracts; 0.6% soya peptone; 0.2% potassium sulphate; 0.2% magnesium sulphate, 0.04% manganese sulphate and 0.1% Tween 80, adjusted to pH 6.0 [16] and incubated at 30°C for 12 to 18 hours to facilitate strain acclimation to the culture media. This second pre-culture was used to

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determine the microbial load in order to seed monocultures and cocultures for the test. The load of each microorganism was determined using the microscopic counting method with a counting cell proposed by [17]. The counting cell and coverslips were immersed in ethanol (95%) for one minute and wiped with paper towels for disinfection. Then 25 µL of each suspension was added with 25 µL of methylene blue and covered with a coverslip. The whole set was placed under the microscope (X 40) and the white living cells were counted for each pre-culture. After counting the microorganisms, the load in each suspension was adjusted to 10^5 cells/ml for each starter before being used to seed the cultures in the interaction test. The formula below was used to determine the microbial load of each cell suspension:

$$N = n \times 5 \times 10^5$$

N: number of cells per milliliter (cells/mL)
n: average number of live cells counted in 5 Thoma cell squares

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Once the load was determined, 16 Erlenmeyer flasks, of which 8 were 25 mL and 8 were 100 mL, were sterilised at 121°C for 15 min. The first 8 25 mL Erlenmeyer flasks were then filled with 10 mL of PSM broth and each flask received one microorganism from the pre-cultures whose load was determined and fixed at 10^5 cells/mL. A volume of PSM broth ranging from 60 to 80 mL was added to the other 8 100 mL Erlenmeyer flasks, depending on the number of strains present in the coculture. For the cocultures, 8 combinations were made taking into account each microorganism.
These combinations were used to inoculate the PSM broths into the 8 x 100 mL Erlenmeyer flasks. Figure 1 shows the monocultures and cocultures, as well as the volume and number of strains. The study was carried out over 5 days and samples were taken at 0, 48 and 120 hours to study the interactions between the different microorganisms. The study started by monitoring the growth of microorganisms in broths. Microbial growth in monoculture and coculture was first assessed by measuring biomass at 600 nm using a spectrophotometer. One (1) mL of each suspension was placed in a reading cell and read against a blank consisting of 1 mL of sterile, uninoculated PSM medium. All suspensions with readings greater than 0.8 were diluted and read again. Thus, the counting method proposed by [17] was used to enumerate the living cells present in the monocultures. In the case of co-cultures, enumeration was carried out on agar media, and each co-culture suspension was inoculated into MRS, YPG, YEPG and GN agar media to enumerate lactic acid bacteria, yeasts, acetic acid bacteria and Bacillus, respectively [16, 18-19]. Successive decimal dilutions from 10^{-1} to 10^{-8} were made and used to inoculate the respective culture media. Petri dishes were then incubated at 30°C for 24 to 72 hours, depending on the strain. The formula below was used to determine the load of each group of microorganisms.

$$N(UFC/mL) = \frac{\Sigma C}{[(n\,1 + 0{,}1 \times n\,2) \times d \times V]}$$

C: Number of colonies in two successive dilutions; **V**: Volume of inoculum; **n1**: Number of Petri dishes inoculated with the 1st dilution considered; **n2**: Number of Petri dishes inoculated at the 2nd dilution considered; **d**: 1^{s}t dilution used **CFU/g**: Colony-forming unit per range

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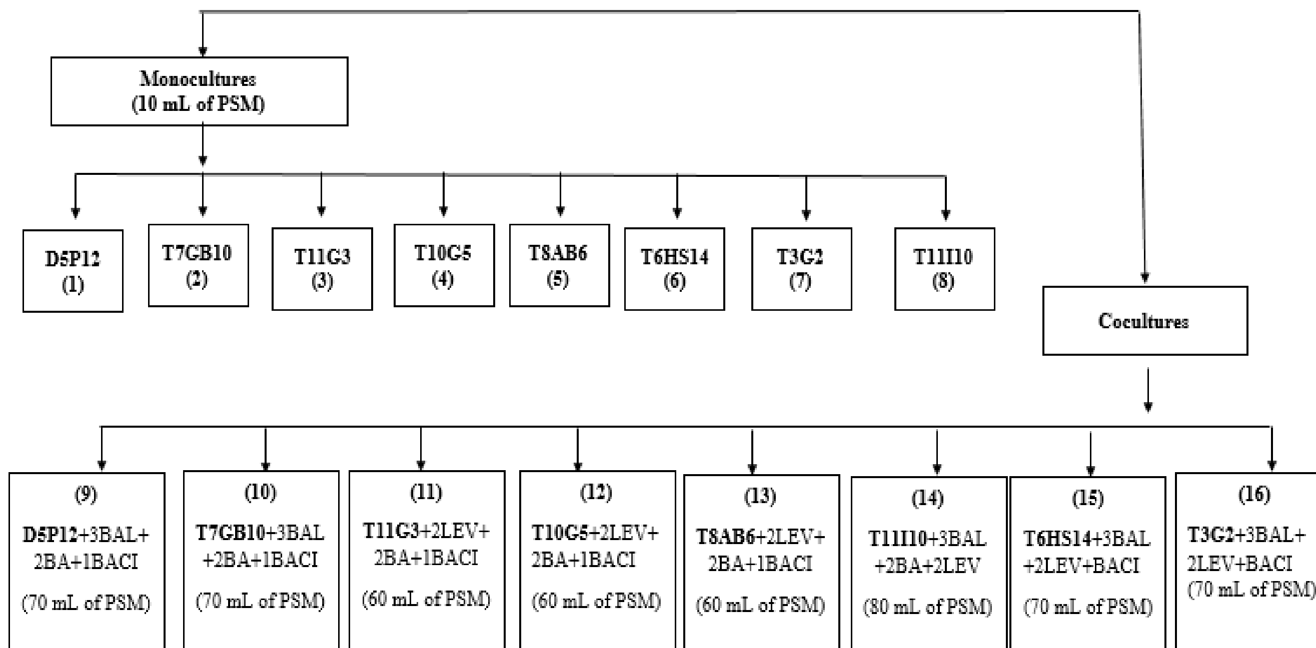

Figure 1: Presentation of the different monocultures and cocultures carried out during microbial interactions between cocoa starters

BA : Acetic acid bacteria ; BAL : Lactic acid bacteria; BACI : Bacillus ; LEV : Yeast
PSM : similar medium of cocoa pulp
D5P12, T7GB10, T11G3, T10G5, T6HS14, T3G2, T11I10 and T8AB6 : Cocoa starters

2.2.2. Measuring pH and acidity during interactions

The method proposed by [20] was used. The pH was measured using a calibrated pH meter. Five (5) mL of each cell suspension was taken separately. The electrode of the pH meter was then inserted into the suspension and the hydrogen potential value displayed on the screen of the meter was noted. The electrode is then rinsed with distilled water for one minute to clean it before reuse. Acidity determination is based on the principle of neutralising the acids present in a medium with a base solution (NaOH). After measuring the pH of the suspensions, two drops of phenolphthalein were added to the various samples to be analysed. The samples were homogenised and then titrated with NaOH (0.1N) in a graduated burette, drop by drop, until they turned light pink. The total acidity was quantified using the following formula.

$$\text{Acidité } (\%) = \frac{\mathbf{V(NaOH) \times N \times 100}}{\mathbf{V(solution\ drawn)}}$$

N: NaOH normality

2.2.3. Determination of the amount of reducing sugars during microbial interactions

Quantification of reducing sugars was performed using the method [21], which is based on the reducing properties of sugars. The reducing function of sugars complexes with DNA under alkaline and hot conditions, reducing it to 3-amino-5-nitrosalylic acid. This phenomenon produces a reddish colour, the intensity of which is proportional to the reducing sugar content of the medium. A volume of 100 µL of each cell suspension was placed in a series of test tubes to which 200 µL of DNS was added. The mixture was homogenised by vortexing and then heated in a boiling water bath at 100°C for 5 minutes. After cooling, 2 mL of distilled water was added and the optical density of the solution was read at 540 nm using a PIOWAY spectrophotometer. The water-soluble sugar-free blank was processed under the same conditions as the assays. A standard range was established under the same conditions using a glucose stock solution at 1 mg/mL. The amount of reducing sugars was determined from the equation of the regression line obtained from the standard range.

2.3. Statistic analysis

Microsoft Excel 2019 was used to enter data, determine means and create graphs and tables. In addition, analysis of variance (ANOVA) was carried out using XSLAT 2016/R software to study the degree of difference between variables. In the event of a significant difference between the parameters studied, the classification of means (homogeneous groups) was carried out using Duncan's test with a threshold of $\alpha = 0.05$. Statistical differences with a probability of less than 0.05 were considered significant.

3. RESULTS

3.1. Microbial biomass during microbial interaction between starters

The results of the optical density of monocultures and cocultures during the 5 days of microbial experiment are shown in Table I. The results show an increase in microbial growth during the experimental days, with values ranging from 0.002 ± 0.00 to 7.59 ± 0.06. In general, monocultures had lower growth rates (between 0.002±0.00 and 2.26 ± 0.05) than

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cocultures (between 0.002 ± 0.00 and 7.59 ± 0.06). Exceptions were the monocultures with the yeast strain T7GB10, which gave a much higher growth rate, within the range of the cocultures, with values oscillating between 0.08 ± 0.00 and 6.19 ± 0.05, and the cocultures with the microbial cocktail containing the only added yeast strain, strain D5P12, whose values were lower than those of the other cocktails, i.e. between 0.01 ± 0.00 and 1.77 ± 0.01. Bacterial monocultures and microbial cocktails containing lactic acid bacteria starters also showed good growth during interactions.

Table I : Evolution of microbial biomass in monocultures and cocultures during interaction

Type of culture	Microbials strains	Biomass at 600nm		
		0 hour	48 hours	120 hours
Monoculture	T8AB6	$0,004 \pm 0,00^{g}$	$1,076 \pm 0,03^{j}$	$2,263 \pm 0,05^{f}$
	T11G3	$0,015 \pm 0,00^{e}$	$1,643 \pm 0,02^{h}$	$1,943 \pm 0,05^{gh}$
	T10G5	$0,002 \pm 0,00^{gh}$	$0,196 \pm 0,02^{l}$	$1,977 \pm 0,06^{g}$
	T11I10	$0,011 \pm 0,00^{f}$	$0,617 \pm 0,02^{k}$	$0,042 \pm 0,00^{k}$
	T7GB10	$0,086 \pm 0,00^{a}$	$4,360 \pm 0,06^{g}$	$6,193 \pm 0,06^{e}$
	D5P12	$0,002 \pm 0,00^{gh}$	$0,046 \pm 0,00^{m}$	$1,807 \pm 0,06^{gh}$
	T3G2	$0,022 \pm 0,00^{c}$	$0,056 \pm 0,00^{m}$	$1,265 \pm 0,12^{i}$
	T6HS14	$0,002 \pm 0,00^{gh}$	$0,064 \pm 0,00^{m}$	$0,238 \pm 0,00^{j}$
Coculture	D5P12+3BL+2BA+1BACI	$0,017 \pm 0,00^{d}$	$1,483 \pm 0,04^{i}$	$1,770 \pm 0,01^{h}$
	T7GB10+3BL+2BA+1BACI	$0,015 \pm 0,00^{e}$	$5,773 \pm 0,07^{c}$	$6,467 \pm 0,11^{d}$
	T11G3+2LEV+2BA+1BACI	$0,037 \pm 0,00^{b}$	$5,726 \pm 0,01^{cd}$	$6,470 \pm 0,35^{d}$
	T10G5+2LEV+2BA+1BACI	$0,010 \pm 0,00^{f}$	$5,057 \pm 0,03^{f}$	$6,433 \pm 0,05^{d}$
	T8AB6+2LEV+2BA+1BACI	$0,001 \pm 0,00^{h}$	$6,286 \pm 0,00^{a}$	$6,836 \pm 0,04^{c}$
	T11I10+3BL+2BA+2LEV	$0,010 \pm 0,00^{e}$	$5,640 \pm 0,02^{e}$	$7,593 \pm 0,06^{a}$
	T6HS14+3BL+2LEV+BACI	$0,012 \pm 0,00^{f}$	$5,690 \pm 0,01^{de}$	$6,443 \pm 0,04^{d}$
	T3G2+3BL+2LEV+BACI	$0,002 \pm 0,00^{gh}$	$5,890 \pm 0,01^{b}$	$7,386 \pm 0,07^{b}$

The values indicated in the table are the means ± standard deviations of tests carried out in triplicate. Values in the same column assigned different alphabetical letters are significantly different according to the Duncan test at the 5% threshold.

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3.2. Enumeration of microbial starters seeded in monoculture in the PSM medium

Figure 2 shows the growth of microbial starter monocultures in culture medium similar to cocoa pulp as a function of time. From 0 hour to 120 hours, the microbial population increased, with values ranging from 5.00 to 7.68 log(Cells/mL). Lactic acid bacteria T8AB6, T11G3 and T10G5 and yeast T7GB10 showed the highest population growth after 48 hours, with values of 7.33; 7.23; 7.24 and 7.04 log(Cells/mL) respectively. This was followed by Bacillus (T11I10), acetic bacteria (T6HS14), yeast (D5P12) and acetic bacteria (T3G2) strains with values of 6.84; 6.63; 6.45; 6.20 log(Cells/mL) respectively. Finally, after 120 hours of culture, microbial populations increased for strains T8AB6 (7.39), T11G3 (7.52), T7GB10 (7.68) and D5P12 (7.22) log(Cells/mL), while the other strains T10G5 (6.83), T3G2 (6.17), T6HS14 (5.98) and T11I10 (5.82) log(Cells/mL) showed a decrease in population.

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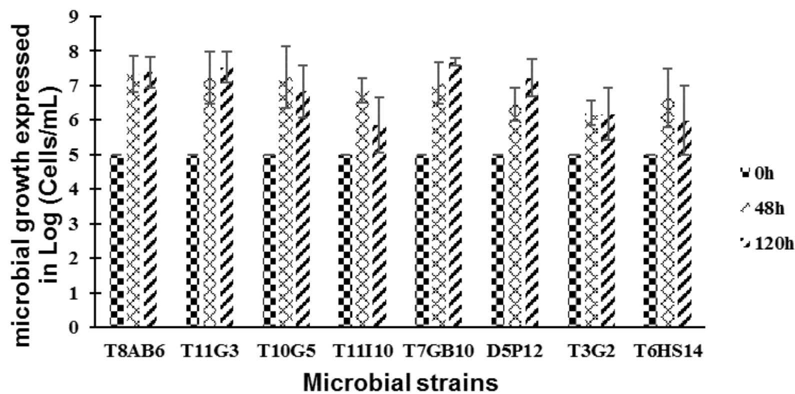

Figure 2: Enumeration of microbial starters in PSM broth monocultures

Strains for the study of monocultures and cocultures were grown in PSM Broth containing 3% fructose; 2% glucose; 1% sucrose; 1% pectin; 1% citric acid; 0.6% yeast extracts; 0.6% soy peptone; 0.2% potassium sulphate; 0.2% magnesium sulphate, 0.04% manganese sulphate and 0.1% Tween 80 and incubated at 30°C for 5 days

3.3. Effect of interactions on the microbial starters growth in coculture

Figure 3 shows the microbial growth of yeast, Bacillus, lactic acid and acetic acid bacteria strains in cocultures after 0, 48 and 120 hours of interaction. In general, all strains grew faster than the initial value. As for the two (2) yeast strains T7GB10 and D5P12, their growth in cocultures showed an increase with values ranging between 4.12 and 8.80 log (CFU/mL) after 48 h before tending towards stability at 120 h. In addition, the population of T7GB10 yeast increased much more than that of D5P12 yeast in their different cocktails. With regard to the Bacillus population, the colony count, which reflects the Bacillus population in the cocktail increased from 5.50 log (CFU/mL) at 0 h to 8.41 log (CFU/mL) and then 8.86 log (CFU/mL) at 48 h and 120 h respectively. Moreover, the population of acetic acid bacteria starters increased much more in the cocktail with strain T3G2 than in that with T6HS14, from 5.42 to 9.21 log(CFU/mL) for T3G2 versus 6.52 to 8.72 log(CFU/mL) for T6HS14 after 48 and 120 hours of incubation. Finally, lactic acid bacteria strains T11G3, T10G5 and T8AB6 increased their numbers by 7.79, 7.02 and 7.44 log(CFU/mL) respectively after 48 hours and 8.04, 8.17 and 8.25 log(CFU/mL) after 120 hours of incubation, with the highest lactic acid bacteria populations recorded in the microbial cocktail with strain T10G5.

Figure 4 shows the microbial growth of yeasts (T7GB10 and D5P12), lactic acid bacteria (T11G3, T10G5 and T8AB6), acetic acid bacteria (T6HS14 and T3G2) and Bacillus (T11I10) in co-culture after 0, 48 and 120 hours of incubation. In general, the population of each major microbial group increased strongly with time, reaching values between 5.50 log(CFU/mL) and 8.86 log(CFU/mL). In addition, yeast and Bacillus populations increased from 5.61 log(CFU/mL) to 8.34 log(CFU/mL) and 5.50 log(CFU/mL) to 8.41 log(CFU/mL) respectively from 0 to 48 hours, before tending towards 8.69 log(CFU/mL) and 8.86 log(CFU/mL). As for lactic acid bacteria, their growth increased steadily over time, from 6.27 log(CFU/mL) at 0 hour, to 7.78 log(CFU/mL) at 48 hours and 8.38 log(CFU/mL) at 120 hours. Acetic acid bacteria showed a slight population increase to 6.94 log(CFU/mL) after 48 hours, compared to 6.40 log(CFU/mL) at 0 h. Their population increased sharply at 120 hours of interaction, reaching 8.50 log(CFU/mL).

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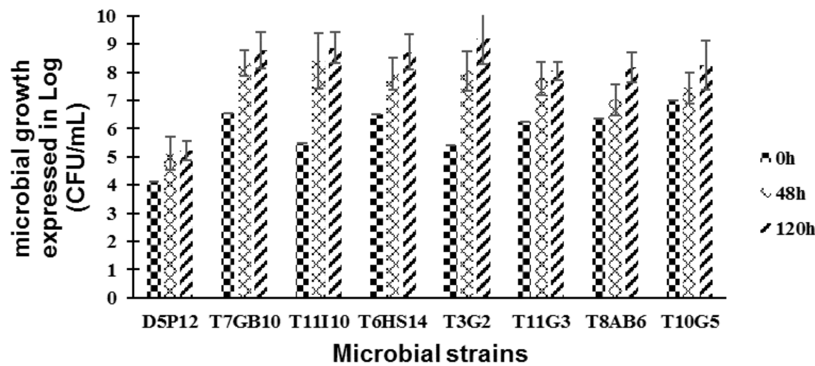

Figure 3: Enumeration of Bacillus, lactic acid, acetic acid bacteria and yeast in the different cocultures

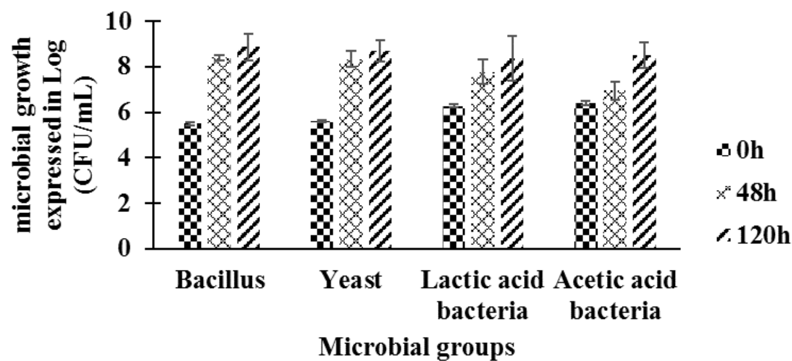

Figure 4: Evolution of different microbial groups during interaction

Strains for the study of cocultures were grown in PSM Broth containing 3% fructose; 2% glucose; 1% sucrose; 1% pectin; 1% citric acid; 0.6% yeast extracts; 0.6% soy peptone; 0.2% potassium sulphate; 0.2% magnesium sulphate, 0.04% manganese sulphate and 0.1% Tween 80 and incubated at 30°C for 5 days

3.4. Impact of monoculture and coculture growth on pH and acidity of PSM medium

Figure 5 shows the evolution of hydrogen potential in monocultures and cocultures after 0, 48 and 120 hours of cultivation. In general, between 0 and 120 hours the pH of the solutions in both monocultures and cocultures decreased, reaching values between 3.42±0.02 and 4.40±0.01. Throughout the study period, the pH of the different solutions did not exceed 5. In monocultures, solutions containing lactic acid bacteria strains T8AB6, T11G3 and T10G5 gave the most acidic pH, followed by those containing yeast strains T7GB10 and D5P12. On the other hand, with cocultures, all cocktails gave pH values in the same range, except for the microbial cocktail containing yeast D5P12 and other microorganisms, which gave a lower pH than the others. With regard to acidity, the results obtained were inversely correlated with those of pH. Overall, the acid concentration ranged from 0.1±0.01 to 0.39±0.07.

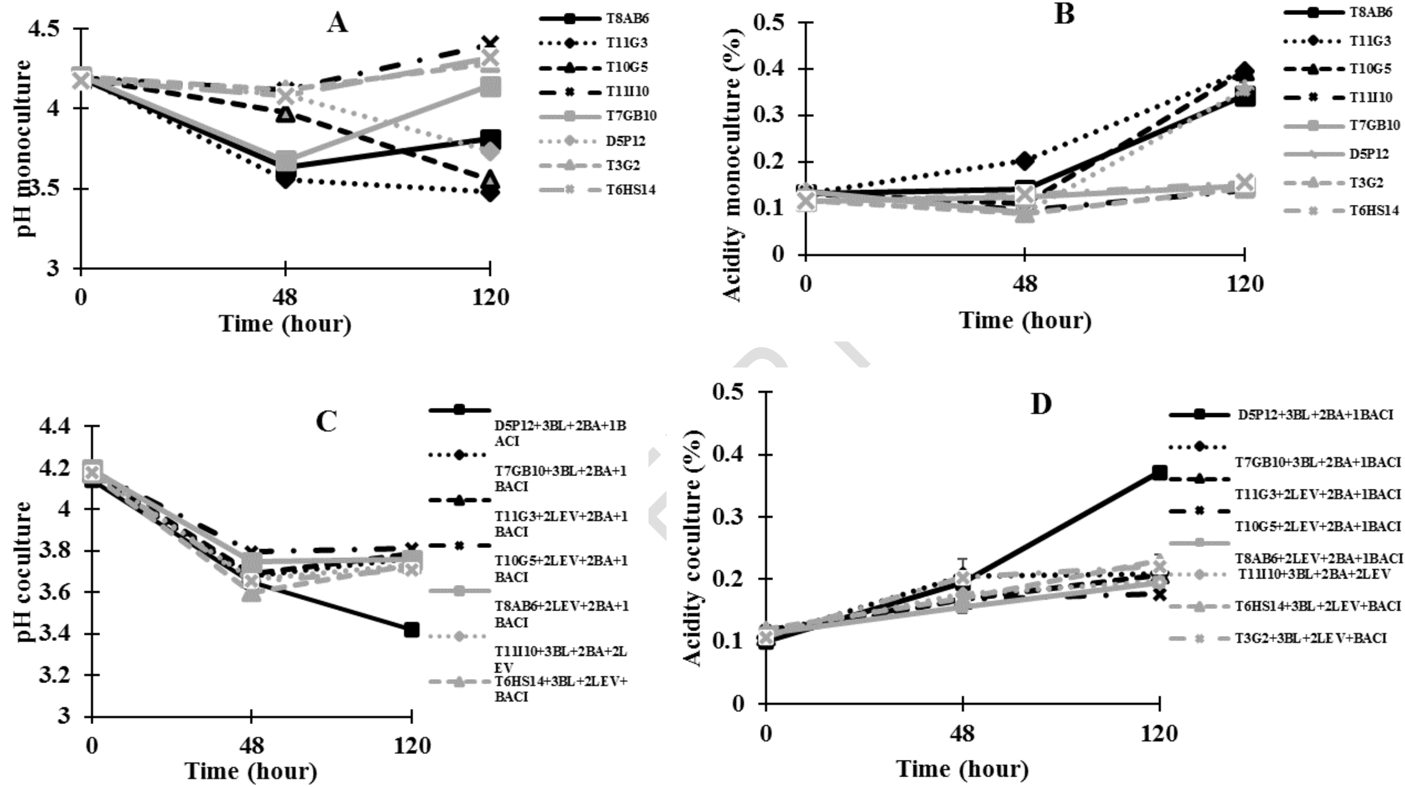

Figure 5 : Variation in pH and acidity in monocultures and cocultures during microbial interactions with cocoa starters

A : pH Monoculture ; **B** : Acidity monoculture ; **C** : pH coculture ; **D** : Acidity coculture

3.5. Evolution of reducing sugars concentration in monocultures and cocultures

The reducing sugar content of the monocultures and cocultures is shown in Figure 6. The reducing sugar concentration in the samples decreased from 1.08±0.01 to 0.006±0.00 from 0 hour to 120 hours. This decrease in sugar concentration is much slower in monocultures than in cocultures. Thus, from 0 h to 48 h, the sugars concentration in monocultures decreased from 1.08±0.01 to 0.77±0.01, with the exception of T11G3, which shows a value of 0.341.08±0.00. On the other hand, with cocultures, except for the cocktail with strain D5P12, which shows a slight decrease (from 1.08±0.01 to 0.96±0.05), the other cocktails show a strong decrease from 1.08±0.01 to 0.20±0.01. From 48 h to 120 h, the decrease in sugars was much greater in the monocultures (0.90±0.01 to 0.18±0.02) than in the cocktails (0.34±0.02 to 0.006±0.00).

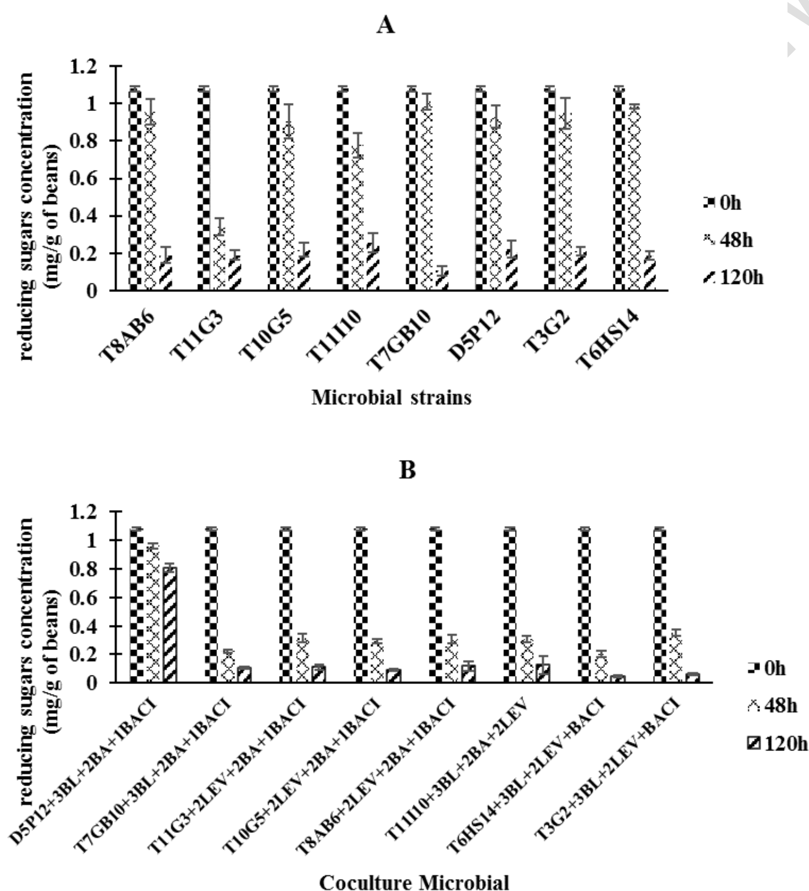

Figure 6 : Variations in reducing sugars content in monocultures and cocultures during microbial

A : Monoculture **B** : coculture

4. DISCUSSION

Fermentation of cocoa beans is a random and uncontrolled process resulting from the temporal succession of the activities of several microorganisms in the pulp: yeasts, lactic and acetic acid bacteria and Bacillus. During this process, exchanges take place between the indigenous microorganisms in the pulp, which influence the microbial growth and the concentrations of ethanol, lactic acid and acetic acid. This process generally takes between 4 and 6 days if carried out correctly [6, 22-24]. Furthermore, during this process, the population of the different autochthonous microorganisms varies as represented by the biomass and load of the mono and cocultures enumerated. Indeed, in this study, the microbial population increased much more in cocultures than in monocultures. This strong increase especially in cocultures could be explained by microbial ecology and succession [5, 16, 23]. The metabolic products of some strains in the PSM medium could serve as substrates for the others. The combined action of the strains could therefore be advantageous in cocoa fermentation. According to [25-28], the cocoa fermentation process is characterised by exchange reactions between the environment and indigenous microorganisms. These interactions in the fermentation process could be different if we take into account the species, number of microorganisms, metabolite concentration and substrate over time. With regard to the number of cocultures, yeast and lactic acid bacteria colonies showed a very good population after 48 and 120 hours of incubation, whereas acetic acid bacteria and Bacillus had to wait until 120 hours to reach the highest population. In the case of yeasts and lactic acid bacteria, the adaptation time and the nutrient richness of the medium could explain the strong growth over 120 hours. Indeed, according to [15, 23], during the first 24 hours, the fermentation is essentially dominated by yeasts, which are joined by lactic acid bacteria which increase when part of the pulp is drained, mainly due to the degradation of pectin by yeasts. These two microbial groups sustain their growth in the pulp by consuming glucose, fructose and sucrose [5, 29-31]. These growth-supporting substrates are gradually replaced by metabolites such as ethanol, lactic and acetic acids, glycerol, mannitol and CO_2. After about 72 hours, the environment becomes less restrictive and more favourable for the development of acetic acid bacteria colonies. These grow in large numbers in the medium, using the ethanol released by the yeast as a carbon source [32]. The consumption of ethanol is followed by the synthesis of acetic acid, their main metabolite. After about 96 hours, the environment becomes much more favourable to Bacillus, which was present in low concentration but now grows massively, producing aromas and pectinolytic enzymes [7, 33,34]. This sequence of microorganisms and metabolites characterises all the main interactions in cocoa pulp during fermentation. In addition, the presence of lactic acid bacteria in cocoa fermentation has long been debated by the scientific community due to their ability to produce lactic acid, a compound that is difficult to evaporate [4]. However, their role is equally laudable, as lactic acid could serve as a substrate for acetic acid bacteria, especially for the genus Acetobacter which is mainly found in all cocoa fermentations carried out worldwide for the production of acetic acid [25]. As appreciated in this study, they have an impact on the growth of certain strains and even on the modulation of the environment. Other studies have also highlighted their role in the bioprotection of beans during fermentation and drying [35].

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With regard to pH and acidity of monocultures and cocultures during interactions, a decrease in pH and an increase in acidity were observed. These two parameters were inversely related. The decrease in pH and increase in acidity during interactions are also quite normal. During normal fermentation, the pH of the pulp increases and the acidity decreases because the acids synthesised by the microorganisms diffuse into the cotyledons. This diffusion of acids into the cotyledons leads to a decrease in pulp acidity and an increase in pH towards basic [34, 36, 37]. In addition, with regard to the unconventional variations in pH and acidity in PSM broths with the D5P15 yeast strain, work by [38,39] reports that this

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strong acidification of the medium with yeast may be related to the fact that certain yeast species can produce organic acids, notably acetic, phosphoric, oxalic, malic and succinic, during fermentation under certain conditions and constraints.

The results showed a decrease in reducing sugar concentration in all samples, with a much greater decrease in cocultures. This more pronounced sugar reduction in cocultures could be due to the fact that cocultures contain several microorganisms, including yeasts and lactic acid bacteria which are able to degrade the sugars present in the PSM medium for their growth. The same decrease in the sugar content of cocoa bean pulp was also observed in the work of [36, 40]. Furthermore, the decrease in reducing sugar in the samples is attributed by [5] to the activity of yeasts and lactic acid bacteria in the different samples. In addition, they break down these sugars to ensure their growth and produce metabolites such as ethanol by yeasts and lactic and acetic acids by lactic acid bacteria [41, 42]. As a result, when both groups of microorganisms are present in the same sugar solution, sugar consumption is much faster than when only one group is present.

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5. CONCLUSION

The three lactic acid bacteria starters used in this study demonstrated their ability to be used in cocktails with the other cocoa fermentation starter microorganisms, i.e. yeast, acetic acid bacteria and Bacillus. All the strains cultivated together in a medium similar to cocoa pulp manage to coexist and exert their fermentative activity. There seems to be a kind of synergy between the different strains when they are cultivated together. These results are promising for a starter cocktail composition containing the different strains in question for controlled fermentation processes.

REFERENCES

1. Douet M. Agroalimentaire: les bonnes récoltes font plonger le cacao. 2017. Consulted 14 Aout 2023. Available : https://www.jeuneafrique.com/mag/398032/economie-entreprises/agroalimentaire-bonnes recoltes-plonger-cacao/.
2. Papalexandratou Z, Camu N, Falony G, de Vuyst L. Comparison of the bacterial species diversity of spontaneous cocoa bean fermentations carried out at selected farms in Ivory coast and Brazil. Food Microbiology. 2011; 28: 964-973.
3. Papalexandratou Z, de Vuyst L. Assessment of the yeast species composition of cocoa bean fermentations in different cocoa producing regions using denaturing gradient gel electrophoresis. FEMS Yeast Research. 2011;11: 564-574.
4. Lefeber T, Papalexandratou Z, Gobert W, Camu, N, de Vuyst L. On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. Food Microbiology. 2012; 30: 379-392.
5. Schwan RF, Wheals AE. The microbiology of Cocoa.Fermentation and its Role in Chocolate Quality. Critical Reviews for Food Science and Nutrition. 2004; 44: 205–221.
6. Lima LJR, Almeida MH, Nout MJR, Zwietering MH. *Theobroma cacao* L., “The food of the gods”: Quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. Critical Reviews in Food Science and Nutrition. 2011; 51: 731-761.
7. Ouattara HG, Koffi BL, Karou GT, Sangaré A, Niamke SL, Diopoh JK. Implication of *Bacillus sp.* in the production of pectinolytic enzymes during cocoa fermentation. World Journal of Microbiology and Biotechnology.2008; 24: 1753–1776.

UNDER PEER REVIEW

8. Samagaci L, Ouattara H, Niamké S, Lemaire M. *Pichia kudrazevii* and *Candida nitrativorans* are the most well-adapted and relevant yeast species fermenting cocoa in Agneby-Tiassa, a local Ivorian cocoa producing region. Food Research International. 2016; 89: 773-780.
9. Ouattara HD, Ouattara HG, Droux M, Reverchon S, Nasser W, Niamke SL. Lactic acid bacteria involved in cocoa beans fermentation from Ivory Coast: Species diversity and citrate lyase production. International Journal of Food Microbiology. 2017; 256: 11-19.
10. Soumahoro S. Typage biochimique et moléculaire de bactéries acétiques à forte capacité de production d'acide acétique isolées de la fermentation du cacao de six régions de Côte d'Ivoire: recherche de potentiels starters et application biotechnologique. Thèse de doctorat, Université Félix HOUPHOUËT-BOIGNY, Abidjan, Côte d'Ivoire. 2017. 190 p.
11. Yao W. Sélection de starters de Bacillus à forte capacité de production d'enzymes pectinolytiques issus de la fermentation de cacao de six régions de Côte d'Ivoire: Impact sur la fermentation contrôlée, le séchage et le stockage des fèves de cacao. Thèse de l'Université Félix HOUPHOUËT–BOIGNY. 2017. 176 p.
12. Koffi O. Sélection de potentielles souches starters de levure à forte capacité de production d'éthanol issues de la fermentation du cacao de Côte d'Ivoire: fermentation contrôlée et aspects qualitatifs des fèves. Thèse de l'Université Félix HOUPHOUËT –BOIGNY d'Abidjan, Côte d'Ivoire. 2018. 197 p.
13. Ouattara HG, Niamké S. Mapping the functional and strain diversity of the main microbiota involved in cocoa fermentation from Cote d'Ivoire. Food Microbiology.2021; 98: 1-12.
14. Adiko CEJR. Bactéries lactiques à faible capacite de production d'acide lactique issues de la fermentation du cacao de diverses régions de la Côte d'Ivoire: propriétés biochimique, moléculaire et conditions de sélection de souches starters d'intérêt fermentaire. Thèse de l'Université Félix HOUPHOUË –BOIGNY d'Abidjan, Côte d'Ivoire. 2018.174 p.
15. Figueroa-Hernández C, Mota-Gutierrez J, Ferrocino I, Hernández-Estrada ZJ, González Ríos O, Cocolin L, Suárez-Quiroz ML. The challenges and perspectives of the Selection of starter cultures for fermented cocoa beans. International Journal of Food Microbiology. 2019; 301: 41–50.
16. Ouattara HG. Caractérisation biochimique et moléculaire des Bacillus fermentaires du cacao et de leurs enzymes pectinolytiques : recherche de potentiel starter, thèse de doctorat unique. Université de Cocody Abidjan Côte d'Ivoire.2010. 153 p.
17. Fossi BT, Achidi AU, Tiencheu B, Bille YE, Takop NG, Akwanwi CM, Ndjouenkeu R, Wanji S. Optimisation of inhibitory activity of lactic acid bacteria against ochratoxin a producing moulds isolated from cocoa using response surface methodology. European Journal of Biotechnology and Bioscience. 2016; 4(12): 17-26.
18. AFNOR NF ISO 15214. Microbiologie des aliments. Méthode horizontale pour le dénombrement des bactéries lactiques mésophiles. Technique par comptage des colonies à 30°C. Juillet 1988, 7 p.
19. NF ISO 21527-1. Microbiologie des aliments. Méthode horizontale pour le dénombrement des levures et des moisissures. Partie 1; technique par comptage des colonies dans les produits à activité d'eau supérieure à 0,95. Juillet 2008, 9 p.
20. Nout MJR, Roabouts FM, Havelaar A. Effect of accelerated natural lactic fermentation of infant ingredients on some pathogenic microorganisms. Journal of Food Microbiology. 1989; 8: 351-360.
21. Bernfeld D. Amylase β et α, In method in enzymology 1, Colowick S.P. and Kaplan N.O., Academic Press. 1955. pp 149-154.

UNDER PEER REVIEW

22. Saltini R, Akkerman R, Frosch S. Optimizing chocolate production through traceability: a review of the influence of farming practices on cocoa bean quality. Food Control. 2013; 29:167-187.
23. De Vuyst L, Weckx S. The cocoa bean fermentation process: From ecosystem analysis to starter culture development. Journal of Applied Microbiology. 2016;121: 5–17.
24. Muñoz SM, Cortina RJ, Vaillant FE, Parra ES. An overview of the physical and biochemical transformation of cocoa seeds to beans and to chocolate: Flavor formation. Critical Reviews in Food Science and Nutrition. 2019; 1-21.
25. Adler P, Bolten CJ, Dohnt K, Hansen CE, Wittmann C. Core fluxome and metafluxome of lactic acid bacteria under simulated cocoa pulp fermentation conditions. Applied and Environmental Microbiology. 2014; 79: 5670-5681.
26. Moens F, Lefeber T, De Vuyst L. Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation. Applied and Environmental Microbiology. 2014; 80: 1848-1857.
27. Illeghems K, De Vuyst L, Weckx S. Comparative genome analysis of the candidate functional starter culture strains *Lactobacillus fermentum* 222 and *Lactobacillus plantarum* 80 for controlled cocoa bean fermentation processes. BMC Genom. 2015; 16: 753-766.
28. Mota-Gutierrez J, Botta C, Ferrocino I, Giordano M, Bertolino M, Dolci P, Cannoni M, Cocolin L. Dynamics and biodiversity of bacterial and yeast communities during fermentation of cocoa beans. Applied and Environmental Microbiology. 2018;84: 1146-1164.
29. Agyirifo DS, Wamalwa M, Otwe EP, Galyuon I, Runo S, Takrama J, Ngeranwa J. Metagenomics analysis of cocoa bean fermentation microbiome identifying species diversity and putative functional capabilities. Heliyon, 2019; 5:e02170.
30. Von Wright A, Axelsson L. Lactic Acid Bacteria. In Lactic Acid Bacteria, 5th ed.; Vinderola, G., Ouwehand, A.C., Salminen, S., Von Wright, A., Eds.; CRC Press: Boca Raton, FL, USA, 2019; pp. 1–16.
31. Abedi E, Seyed MBH. Lactic acid production-producing microorganisms and substrates sources-state of art. Heliyon. 2020; 6(10): 49-74.
32. Dzialo MC, Park R, Steensel J, Lievens B, Verstrepen KJ. Physiology, ecology and industrial applications of aroma formation in yeast. FEMS Microbiology Reviews. 2017; 41: 95-128.
33. Drobniewski FA. *Bacillus cereus* and related species. Clinical Microbiology Reviews. 1993; 6: 324-338.
34. Nielsen DS, Teniola OD , Ban-Koffi L, Owusu M, Anderson TS, Holzapfel WH. The microbiology of Ghanaian cocoa fermentations analysed using culture-dependent and culture-independent methods. International Journal of Food Microbiology. 2007; 114: 168-186.
35. Ouattara HG, Yao W, Ouattara HD, Karou GK, Ahonzo-Niamké LS. "Fungal Inhibition by Lactic Acid Bacteria (LAB) Is Modulated *in Vitro* by Cocoa Fermentation-Related Conditions: Towards a Biocontrol of Fungi in Processing Cocoa". Emirates Journal of Food and Agriculture. 2023; 35(9): 853-861.
36. Ardhana M, Fleet GH. The microbial ecology of cocoa fermentations in Indonesia. International Journal of Food Microbiology. 2003; 86: 87-99.
37. Afoakwa EO, Paterson A. Cocoa fermentation: Chocolate Flavour Quality. In: Encyclopedia of Biotechnology in Agriculture and Food. Taylor & Francis Publishing Inc. Oxford, U.K. 2010; pp 457-468.
38. Nielsen DS, Hønholt S, Tano-Debrah K, Jespersen L. Yeast populations associated with Ghanaian cocoa fermentations analysed using denaturing gradient gel electrophoresis (DGGE). FEMS Yeast Research. 2005; 22: 271–284.

UNDER PEER REVIEW

39. Daniel HM, Vrancken G, Takrama JF, Camu N, de Vos P, de Vuyst L. Yeast diversity of Ghanaian cocoa bean heap fermentations. FEMS Yeast Research. 2009; 9: 774–783.
40. Afoakwa EO, Kongor JE, Takrama JF, Budu AS. Changes in acidification, sugars and mineral composition of cocoa pulp during fermentation of pulp preconditioned cocoa (*Theobroma cacao*) beans. International Food Research Journal. 2013; 20: 1215-1222.
41. Thompson SS, Miller KB, Lopez AS. Cocoa and coffee. In Doyle M. J., Beuchat L. R. & Montville T. Journal of Food Microbiology Fundamentals-Frontiers. 2nd Ed. Washington DC. 2001; pp 721-733.
42. Camu N, De Winter T, Verbrugghe K, Cleenwerck I, Vandamme P, Takrama SJ, Vancanneyt M, De Vuyst M. Dynamics and biodiversity of populations of lactic acid bacteria and acetic acid bacteria involved in spontaneous heap fermentation of cocoa beans in Ghana. Applied and Environmental Microbiology. 2007; 73:1809–1824.

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