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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 Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

However, uncontrolled or random fermentations carried out in the country by farmers producing marketable cocoa beans of variable quality. The problem of variability in the quality of beans has led to significant economic losses in the country estimated at several billion CFA Francs. The objective

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of this work was to analyze the interactions between potential starter lactic acid bacteria and the major microorganisms selected during cocoa fermentations in Côte d'Ivoire. The work methodology began with the cultivation of yeast, lactic acid bacteria, acetic acid bacteria and *Bacillus* strains in their respective YPG, MRS, YEPG and BN media for 48 hours at 30°C. Then, the microbial load of each culture was determined and set at 10^5 cells/mL. The strains were subsequently placed in the similar interaction medium of cocoa pulp (PSM) alone (monoculture) or together (coculture) for the study of microbial interactions. This study took place over 5 days and samples were taken for analyzes after 0, 48 and 120 hours of incubation at 30°C. The interaction was monitored by assessing microbial growth using optical density and Thoma cell counts for monocultures and agar media for cocultures. Additionally, acid production and reduction in sugar intake were also measured over the 5 days. All crops showed good growth. Growth was greater in cocultures with values ranging from 4.12 log (CFU/mL) to 9.21 log (CFU/mL). In addition, the cocktails acidified the environment much more than the monocultures with values oscillating between 0.10 ± 0.00 and 0.21 ± 0.00. Also, the quantity of sugars in the different broths decreased significantly in the cocktails during the first 48 hours, going from 1.08 ± 0.08 to 0.20 ± 0.01. Microbial starters in monocultures and cocultures have shown their ability to adapt to the environment like cocoa pulp. 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. In short, these strains could be used for a starter composition to control fermentation and obtain good quality beans.

Keywords: Lactic acid bacteria; cocoa fermentation; interaction; starter.

ABBREVIATIONS

GN	*: Nutritious Agar*
BN	*: Nutritious Broth*
MRS	*: Man Rogosa Sharpe*
YPG	*: Yeast peptone Glucose*
PSM	*: Similar medium of cocoa pulp*
YEPG	*: Yeast extract- Ethanol- Peptone- Glucose*
CFA Francs	*: Franc from the French Colonies of Africa*
Nm	*: Nanometer*
N	*: Normality*
ANOVA	*: Analysis of variance*
AAB	*: Acetic Acid Bacteria*
LAB	*: Lactic Acid Bacteria*

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 caused 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 [7].

Several studies have been carried out to improve and control the fermentation process of Ivorian cocoa [8-14]. These previous studies have highlighted the microbial diversity involved in the fermentation of cocoa from 12 major producing regions of Côte d'Ivoire. These microorganisms were identified and the genetic diversity was analyzed to identify the main large microorganisms encountered in these

fermentations [8,9,13-15]. Then, other work was carried out to search in this microflora for bacteria and yeasts with exceptional properties capable of producing metabolites of essential interest for good cocoa fermentation. Among these metabolites we can cite pectinolite enzymes, ethanol, citrate lyase and lactic and acetic acids. All these led to a screening of strain that can be used as a starter to improve the cocoa fermentation process [16]. However, the microbial interactions that occur between species remain unknown. Yet, these interactions may have a significant impact on microbial development during fermentation and thus influence cocoa quality.

The studies carried out on interactions do not relate to the use of polymicrobial cocktails involving all the players involved in the proper fermentation of cocoa, namely yeasts, lactic (LAB) and acetic (AAB) bacteria and *Bacillus*. 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.

2. MATERIALS AND METHODS

2.1 Microbial Strains

The microbial material consisted of eight strains, including two yeast strains (D5P12, T7GB10), three lactic acid bacteria or LAB (*Lactobacillus plantarum (*T11G3), *Lactobacillus casei* (T10G5) et *Leuconostoc mesenteroides* (T8AB6)), two acetic acid bacteria or AAB (T6HS14, T3G2) and *Bacillu*s (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 [9-12]. 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), LAB (T11G3, T10G5 and T8AB6), AAB (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 Nutritious broth (BN) broths respectively and then incubated at 30°C for 24 hours. Subsequently, 100 µL of each Pre-culture carried out in their respective environment 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 [17] and incubated at 30°C for 12 to 18 hours to facilitate strain acclimation to the culture media. Pre-culture carried out in an environment similar to cocoa pulp was used to 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 Fossi et al. [18]. 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:

$$\mathbf{N = n \times 5 \times 10^5}$$

N: number of cells per milliliter (cells/mL)

n: average number of live cells counted in 5 Thoma cell squares ((n1+n2+n3+n4+n5)/5)

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 Erlenmeyer 25 mL 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 Erlenmeyer 100 mL 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. Fig. 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 (Pioway, Chine). 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 Fossi et al. [18] was used to enumerate the living cells present in the monocultures. In the case of cocultures, enumeration was carried out on agar media, and each coculture suspension was inoculated into MRS, YPG, YEPG and GN agar media to enumerate LAB, yeasts, AAB and *Bacillus*, respectively [17,19-20]. 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.

$$N(UFC/mL) = \frac{\Sigma C}{[(n1 + 0,1 \times n2) \times d \times V]}$$

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**: 1st dilution used **CFU/g**: Colony-forming unit per range.

2.2.2 Measuring pH and acidity during interactions

The method proposed by Afnor [21] 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 was then rinsed with distilled water for one minute to clean it before reuse. Acidity determination was 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.

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

N: NaOH normality

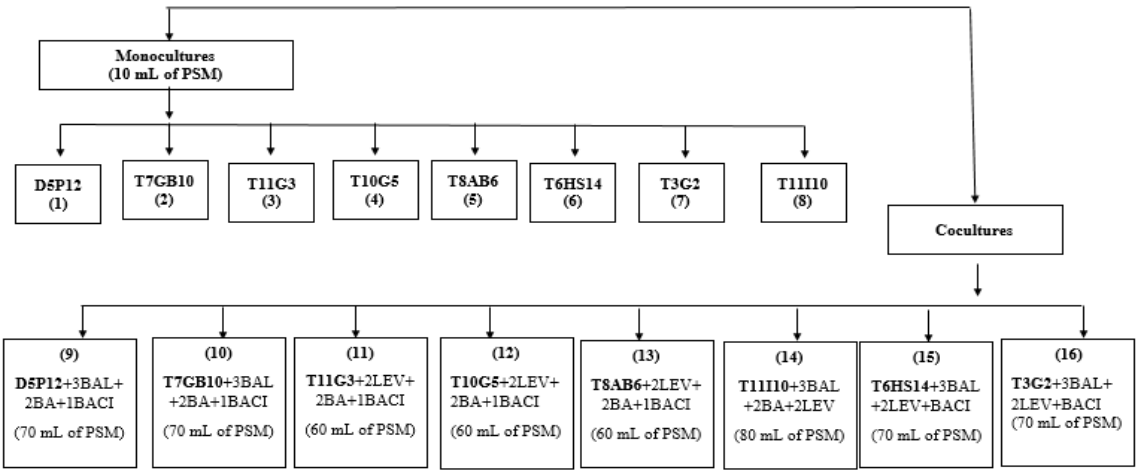


Fig. 1. Presentation of the different monocultures and cocultures carried out during microbial interactions between cocoa starters

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

2.2.3 Determination of the amount of reducing sugars during microbial interactions

Quantification of reducing sugars was performed using the method [22], 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 spectrophotometer (Pioway, Chine). The white without soluble sugar was treated under the same conditions as the tests. 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 software was used to enter data, obtain the mean and plot graphs and tables. In addition, Analysis of variance (ANOVA) was performed with XSLAT 2016/R software to investigate the degree of difference between variables using Duncan's test with a threshold of $\alpha = 0.05$.

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 1. 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 cocultures (between 0.002 ± 0.00 and 7.59 ± 0.06). However, in the monocultures with T7GB10 yeast, which showed higher values than the other monocultures, 0.08 ± 0.00 and 6.19 ± 0.05. In the cocultures, the cocktail with the addition of yeast D5P12 gave lower values than the other cocktails (0.01 ± 0.00 and 1.77 ± 0.01). Monocultures and cocultures containing LAB starters showed good growth over the course of the interactions.

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

The values indicated in the table are the means ± standard deviations of the tests carried out in triplicate. Significant differences are presented within the same column by different alphabetical letters according to the Duncan test at the 5% threshold

3.2 Enumeration of Microbial Starters Seeded in Monoculture in the PSM Medium

Fig. 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). LAB 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), AAB (T6HS14), yeast (D5P12) and AAB (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), yeast T7GB10 (7.68) and D5P12 (7.22) log(Cells/mL), while the other strains LAB T10G5 (6.83), T3G2 (6.17), AAB T6HS14 (5.98) and *Bacillus* T11I10 (5.82) log(Cells/mL) showed a decrease in population.

3.3 Effect of Interactions on the Microbial Starters Growth in Coculture

Fig. 3 shows the microbial growth of yeast, *Bacillus*, lactic acid and AAB 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 AAB 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, LAB 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 LAB populations recorded in the microbial cocktail with strain T10G5.

Fig. 4 shows the microbial growth of yeasts (T7GB10 and D5P12), LAB (T11G3, T10G5 and T8AB6), AAB (T6HS14 and T3G2) and *Bacillus* (T11I10) in coculture 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

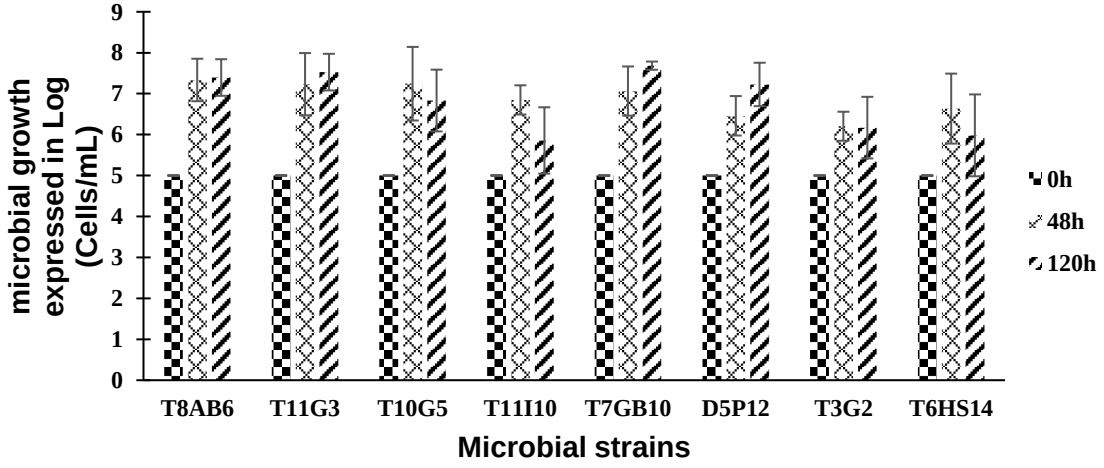


Fig. 2. Enumeration of microbial starters in PSM broth monocultures

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

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 LAB, 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. AAB 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).

3.4 Impact of Monoculture and Coculture Growth on pH and Acidity of PSM Medium

Fig. 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 monocultures decreased with values between 3.5 ± 0.03and 4.40 ± 0.01. While with the cocktails, pH decreased to 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 LAB 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.

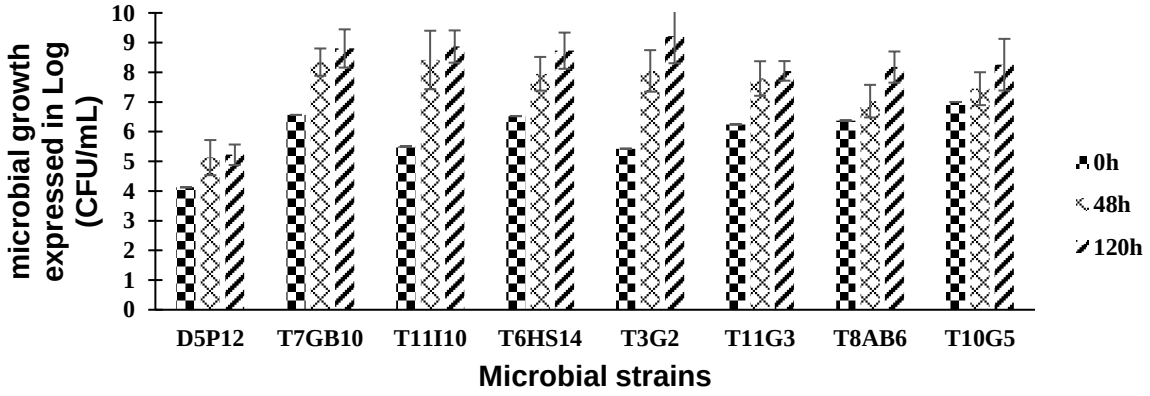


Fig. 3. Enumeration of *Bacillus*, LAB, AAB and yeast in the different cocultures

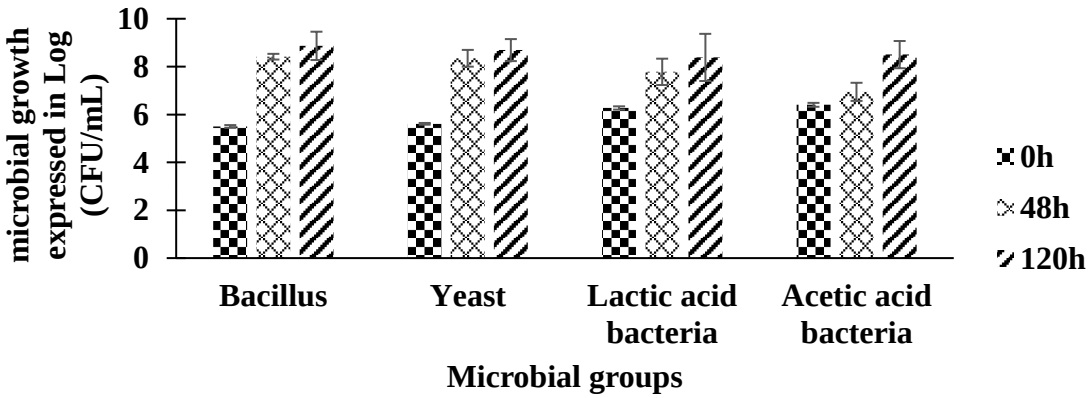


Fig. 4. Evolution of different microbial groups during interaction

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

3.5 Evolution of Reducing Sugars Concentration in Monocultures and Cocultures

The reducing sugar content of the monocultures and cocultures is shown in Fig. 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).

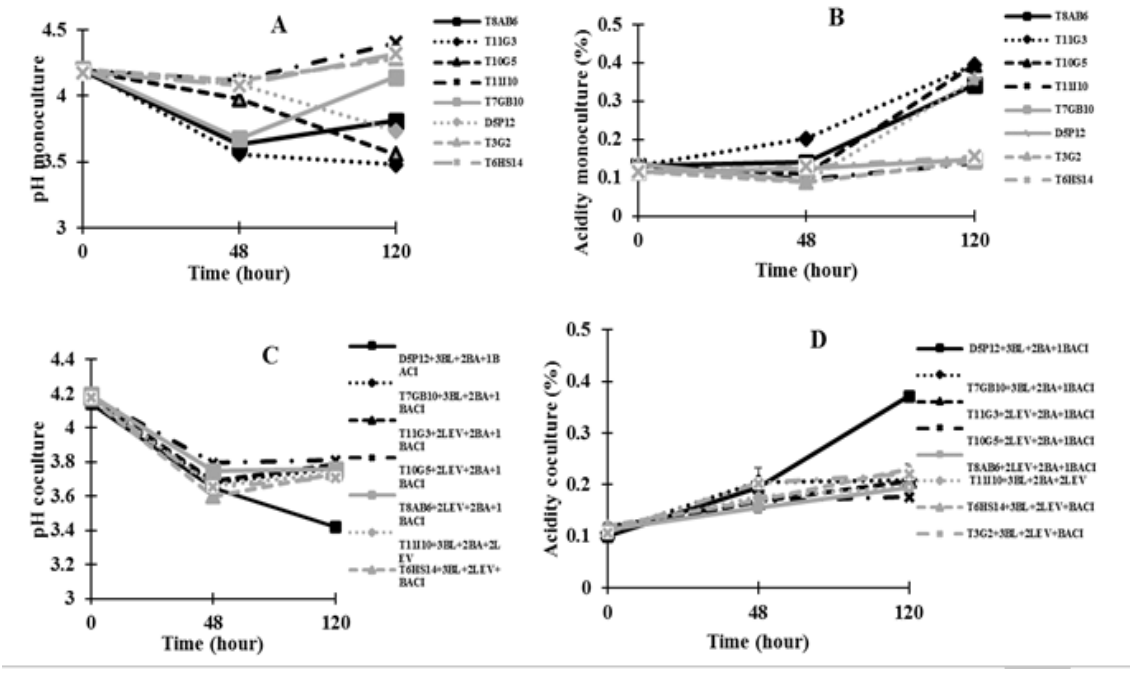
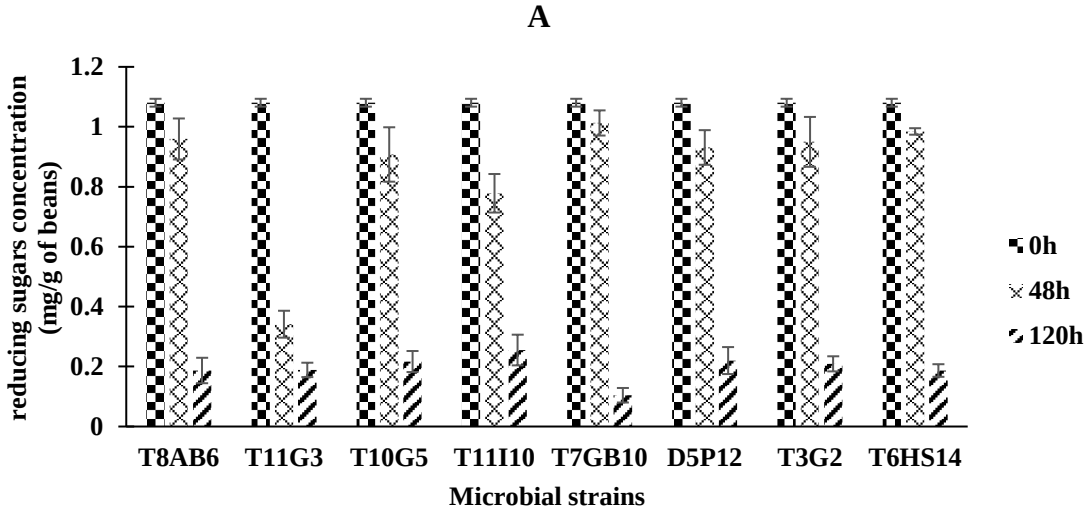


Fig. 5. Variation in pH and acidity in monocultures and cocultures during microbial interactions with cocoa starters

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



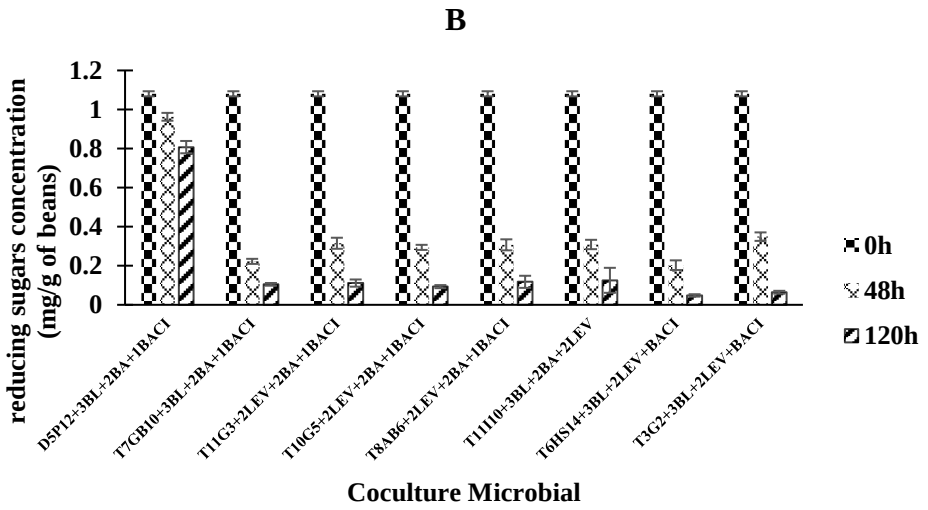


Fig. 6. Variations in reducing sugars content in monocultures and cocultures during microbial interactions

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, LAB and AAB 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 [6,23-25]. Furthermore, during this process, the population of the different autochthonous microorganisms (yeast, BAL, AAB, *Bacillus*) 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 [5,17, 24]. 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 Adler et al. [26-29], 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 LAB colonies showed a very good population after 48 and 120 hours of incubation, whereas AAB and *Bacillus* had to wait until 120 hours to reach the highest population. In the case of yeasts and LAB, the adaptation time and the nutrient richness of the medium could explain the strong growth over 120 hours. Indeed, according to Figueroa-Hernández et al. [16,24], during the first 24 hours, the fermentation is essentially dominated by yeasts, which are joined by LAB 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 [5,30-32]. 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 AAB colonies. These grow in large numbers in the medium, using the ethanol released by the yeast as a carbon source [33]. 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 [8,34,35]. This sequence of microorganisms and metabolites characterises all the main interactions in the cocoa pulp during fermentation. Furthermore, the LAB used in this study are heterofermentative microorganisms [10]. These LAB produce acetic acids, which

ensure fermentation of the beans, and lactic acids, which have an inhibitory action on the moulds responsible for spoilage during fermentation and conditioning of cocoa beans [36]. This lactic acid could serve as a substrate for AAB, in particular the *Acetobacter* genus, which is mainly found in all cocoa fermentations worldwide for the production of acetic acid [26].

About 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. This is because the acids produced during this process do not diffuse into the cotyledon as they should in a normal reaction. During normal fermentation, the pH of the pulp rises and acidity falls, as the acids synthesized by the micro-organisms diffuse into the cotyledons. This diffusion of acids into the cotyledons leads to a decrease in pulp acidity and an increase in pH towards the basic range [35,37,38]. Furthermore, with regard to the unconventional pH and acidity variations in PSM broths with the D5P15 yeast strain, the work of Nielsen et al. [39,40] reports that this strong acidification of the medium with yeast may be linked 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 (defence).

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 because cocultures contain several microorganisms, including yeasts and LAB which can 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 Ardhana and Fleet [37,41]. Furthermore, the decrease in reducing sugar in the samples is attributed by Schwan and Wheals [5] to the activity of yeasts and LAB 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 LAB [42,43]. 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.

5. CONCLUSION

The three LAB starters used in this study have demonstrated their ability to be used in cocktails with the other micro-organisms that initiate cocoa fermentation, namely yeast, AAB 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 grown together. Also, LAB T11G3 showed good growth, sugar consumption and acid production both in monocultures and cocultures. The cocktail of microbial starters with strain T11G3 could be used to improve cocoa bean fermentation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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