

Microbial Dynamics Associated with Spontaneous Fermentation of Cocoa (*Theobroma cacao* L.) in Cameroon and Evaluation of the Quality of Marketable Beans

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Abstract: Cocoa fermentation is essential for the production of marketable beans for chocolate manufacturing. It is a microbial process that affects the marketable quality of the beans. The microbial dynamics associated with cocoa fermentation as well as the quality of marketable beans were evaluated in a couple of towns in Cameroon. After plating on selective agar plates, the growth of microflora named yeast, acetic acid bacteria, lactic acid bacteria, bacillus and molds associated with the different fermentations was monitored by enumeration using decimal dilution. The quality of the beans was assessed by the fermentation index, pH and grain size. Cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja were sampled after 0, 24, 48, 72, 96, 120, and 144 hours of spontaneous fermentation. It was found that the order of emergence and the time of appearance of the different microbial genera varied between locations and the fermentations process were generally dominated by yeasts followed by lactic acid bacteria and bacillus which were found during all the fermentation stages except in Elogbatindi where bacillus appear after 48h. Bean pH decreased from 5.88 ± 0.02 - 6.52 ± 0.01 to 4.34 ± 0.02 - 5.68 ± 0.06 . The fermentation index of the beans ranged between 1.00 and 1.40 at the end of the process of fermentation and the seeds obtained had consistent specific weight since the value of their weight were greater than 1. The microbial strains would have a high technological potentiality leading to a good quality of fermented cocoa beans and may be use as starter in improvement program of cocoa beans fermentation process.

Keywords: Cocoa, Fermentation, Merchant Beans, Microbial Dynamics

1. Introduction

Fermentation of fresh cocoa beans is the first step in the chocolate manufacturing chain, it is a key step for flavor formation. and an entirely microbial process [1]. During fermentation, the pulp surrounding the seed is degraded in a series of biochemical and enzymatic reactions involving microorganisms like yeast, lactic acid bacteria (LAB), acetic acid bacteria (AAB), and bacillus [2]. Metabolites are formed that modify the parameters of fermentation, temperature and

pH in particular, leading to the establishment in the fermentary medium, of an order of emergence and succession of microorganisms involved in the fermentation of cocoa [3]. Microbial dynamics during fermentation also vary depending on the geographical origin of the pods. This variation relates to the order of appearance of different microbial types, their numbers, and the different species that develop [4]. Microbial succession, as well as the sequences of enzymatic reactions and metabolites formed during fermentation impact the quality of marketable beans [5]. Microbial activity during fermentation remains uncontrolled such that the quality of

marketable beans varies randomly [6]. Well-fermented beans are brown [7], with an average weight of 1g or more. Their aromatic potential can be measured by the fermentation index, a much more objective criterion than the cutting test [8]. Marketable bean quality has been the subject of many studies. A study conducted in Cameroon by revealed that marketable beans from the Southern and Central Regions of Cameroon were of lower quality, a defect attributed to unsuccessful fermentation [9]. Moreover, while Cameroonian cocoa was once prized for its high quality and specific color, almost all Cameroonian production is now exported as "fairly fermented," corresponding to average quality [10]. To solve this problem, several studies have been carried out, including the use of starters. Such studies have been carried out in all the main cocoa producing countries, but not in Cameroon. It would be of interest to undertake such a study in order to improve the physico-chemical quality of Cameroon's commercial cocoa beans. The objective of this work is to monitor the dynamics of the microflora associated with the spontaneous fermentation of cocoa in Cameroon and to evaluate the quality of marketable beans.

2. Materials and Methods

2.1. Postharvest Processes and Sampling

The biological material consisted of fresh seeds from pods harvested at maturity in four localities of Cameroon: Bafia (4° 45' 00" North, 11° 14' 00" East), Bertoua (4° 34' 30" North, 13° 41' 04" East), Elogbatindi (3° 27' 0 North, 53.10° 7' 60 East) and Penja (4° 38' 00" North, 9° 41' 00" East). Harvesting was done between September and December 2020. After 5 days of storage and hulling, the fresh seeds, weighing about 11 kg, were fermented in 0.27m³ wooden box. Fermentation lasted 4 to 6 days, with stirring every 24 hours. Samples were taken from the center of the fermenting mass at 24-hour intervals.

2.2. Analysis of Temperature and pH

The temperature of the fermenting mass was determined at 24-hour intervals by introducing the industrial range thermometer into the core of the fermenting mass [11]. The pH was determined separately in the pulp and cotyledons according to the method of Lopez et al. [12]. Twenty grams of pulp and cotyledons were immersed in 100 ml of deionized water for 5 to 10 minutes. After filtration through a whatman No. 4 filter paper, the pH was measured in the filtrate using a pH meter (Hanna) calibrated with pH 7 buffer solution.

2.3. Microbiological Analysis

2.3.1. Isolation and Microbial Count

Microorganisms Isolation and count were done following the modified method of [13]. 5g of fresh seeds were mixed and steamed for 5 minutes in 45ml of physiological solution (0.9% NaCl, m/v). 2ml of the suspension was then diluted in 18ml of physiological solution and serial dilutions from 10⁻¹

to 10⁻⁵ were prepared. 0.1ml of each dilution was introduced into the culture media for isolation of the desired microbial type. Yeasts and molds were isolated by plating on sabouraud dextrose agar (Biolife). Lactic acid bacteria were isolated on MRS agar (De Man Ragosa Sharp, Biolife) supplemented with nystatin (400mg/l) to inhibit growth of yeasts and molds, acetic acid bacteria on GYC agar (glucose-yeast extract-calcium carbonate, Condalab) supplemented with 100mg/l of nystatin to inhibit the growth of yeasts and molds and 100 mg/l of penicillin G to specifically inhibit the growth of lactic acid bacteria and bacilli on nutrient agar (Biolife). After incubation at 28 - 30°C for two to four days, the microorganisms were counted in terms of colony forming units (CFU).

2.3.2. Identification of the Isolated Microflora

The identification of the different microbial was done on the basis of their key morphological and biochemical characters. Yeasts were identified under the microscope as large rounded or oval cells. Molds were identified and characterize according to their filamentous mycelium [14]. Lactic acid bacteria were non sporulating Gram-positive bacilli negative to catalase test [15]. AAB are small gram-negative, aerobic and catalase positive bacilli [16]. *Bacillus* is a gram-positive, sporulation, catalase positive bacteria. [17].

2.4. Evaluation of the Marketability of the Seeds

2.4.1. Fermentation Index

The fermentation index was determined according to the method described by [18]. In this method, 0.5g of cocoa bean was soaked in a mixture of methanol/HCl (97:3, v/v) homogenized for 15 minutes and stored in a refrigerator at 4°C for 16 hours. The mixture was filtered using cellulose acetate paper. The volume was adjusted to 50 ml with methanol/HCl. The fermentation index was calculated using the ratio of the absorbance at 460 nm to the absorbance at 530 nm read in a UV-visible spectrophotometer.

2.4.2. Beans Count

Beans count was done according to the method described by [19]. The method takes into account the average number of healthy and normal beans contained in 100g healthy seeds.

2.5. Statistical Analysis

All analysis were performed in triplicate. Results are expressed as mean ± standard error. Analysis of variance and LSD were performed to discriminate between means that differed significantly. All analyses were performed using Statgraphics Centurion software version 17.1.12. Probability was estimated at the traditional 5% threshold.

3. Results

3.1. Changes in Temperature and pH During the Fermentations

Figure 1 shows changes in temperature during the process

of fermentation. The starting temperature, ranged between 25 - 27°C. It then significantly ($p < 0.05$) increased and reached

a maximum value (40.00 - 44.67°C) between 3 and 4 days. After the 3rd and 4th days.

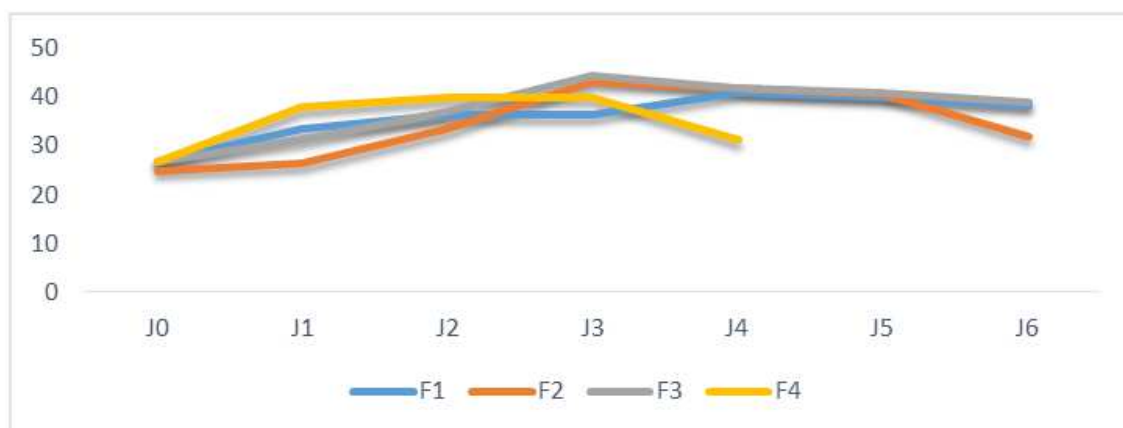


Figure 1. Changes in temperature during fermentations.

F1, F2, F3, F4 are fermentation of cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja respectively.

J₀, J₁, J₂, J₃, J₄, J₅, and J₆ are respectively the Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, of the fermentation process.

Fermentation caused a significant ($p < 0.05$) increase in pulp pH and a significant ($p < 0.05$) decrease in cotyledon pH (Table 1). The pH value of the pulp increased significantly ($p < 0.05$) from 3.26 - 4.23 to 5.25- 6.52 at the end of

fermentation. It rather significantly ($p < 0.05$) decreased in cotyledons from 5.57 - 6.52, to 4.86 and 5.69 at the end of fermentation.

Table 1. pH change in pulp and nibs during the fermentation stage.

Pulp and cotyledon pH		J ₀	J ₁	J ₂	J ₃	J ₄	J ₅	J ₆
Pulp	F1	4.23±0.07 ^a	4.14±0.04 ^a	4.15±0.03 ^a	4.23±0.05 ^a	4.65±0.02 ^b	5.87±0.06 ^c	5.87±0.06 ^c
	F2	3.85±0.03 ^a	4.34±0.48 ^{abc}	4.47±0.00 ^{cd}	4.22±0.10 ^b	4.57±0.15 ^d	5.30±0.02 ^e	6.89±0.04 ^f
	F3	4.10±0.25 ^a	4.92±0.05 ^b	5.17±0.03 ^c	4.29±0.01 ^d	4.48±0.05 ^c	4.75±0.03 ^b	5.49±0.02 ^f
	F4	3.27±0.01 ^a	2.83±0.01 ^b	3.24±0.01 ^a	3.48±0.01 ^c	5.25±0.01 ^d		
Nibs	F1	6.00±0.05 ^a	5.88±0.20 ^{ab}	4.30±0.01 ^c	5.87±0.03 ^{ab}	5.79±0.01 ^b	5.69±0.05 ^d	5.68±0.06 ^d
	F2	6.52±0.01 ^a	6.45±0.02 ^b	6.30±0.01 ^c	5.53±0.02 ^d	4.94±0.06 ^e	5.12±0.02 ^f	5.25±0.02 ^g
	F3	6.03±0.21 ^a	5.96±0.06 ^a	5.68±0.10 ^b	4.32±0.08 ^c	4.13±0.05 ^d	4.02±0.01 ^e	4.34±0.02 ^c
	F4	5.58±0.02 ^a	5.45±0.01 ^b	5.36±0.02 ^c	4.64±0.03 ^d	4.88±0.02 ^c		

Data are Mean ± SE. For each fermentation, means values with the same letter in the same line are not significantly ($P > 0.05$) F1, F2, F3, F4 refer to fermentation of cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja respectively.

J₀, J₁, J₂, J₃, J₄, J₅, and J₆ are respectively the Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, of the fermentation process.

3.2. Variation of Microorganism Population

Figure 2 (a, b, c, and d) below shows changes in microbial population during fermentation F1, F2, F3 and F4. For the F1, F2 and F4, the result of microbial growth showed that yeast increased at the first two days, respectively from 2.99 log₁₀ CFU/ml to 4.55 log₁₀ CFU/ml, 3.85 log₁₀ CFU/ml to 5.57 log₁₀ CFU/ml and 3.59 log₁₀ CFU/ml to 4.96 log₁₀ CFU/ml, then decrease till the end of process. However, at the end of fermentation, the number of yeast in F2 fermentation increase to 5.59 log₁₀ CFU/ml. In the case of F3, yeast population increased from 3.79 log₁₀ CFU/ml to 4.85 log₁₀ CFU/ml and decreased gradually to 3.79 log₁₀ CFU/ml at the end of fermentation. Number of LAB population increased during the first two days of F3

and F4 fermentation and until the fourth and fifth day for F2 and F1 before decreasing. Population of *Bacillus* seems to be constant for F1 fermentation and increased for F2, F3 and F4 fermentation with the respectively values of 4.03 log₁₀ CFU/ml to 4.18 log₁₀ CFU/ml, 4.19 log₁₀ CFU/ml to 4.93 log₁₀ CFU/ml, and 4.10 log₁₀ CFU/ml to 5.32 log₁₀ CFU/ml. AAB was present at the beginning of the fermentation for F4 fermentation but occurred from the third day for others as followed: 3.14 log₁₀ CFU/ml to 3.57 log₁₀ CFU/ml for F1 and 2.78 log₁₀ CFU/ml to 4.55 log₁₀ CFU/ml for F2. Decreased values were observed for F3 (4.18 log₁₀ CFU/ml to 4.03 log₁₀ CFU/ml) and F4 (3.91 log₁₀ CFU/ml to 4.04 log₁₀ CFU/ml). Molds were observed by the end of F1, F2, and F3 fermentation; there were not observed in F4 fermentation.

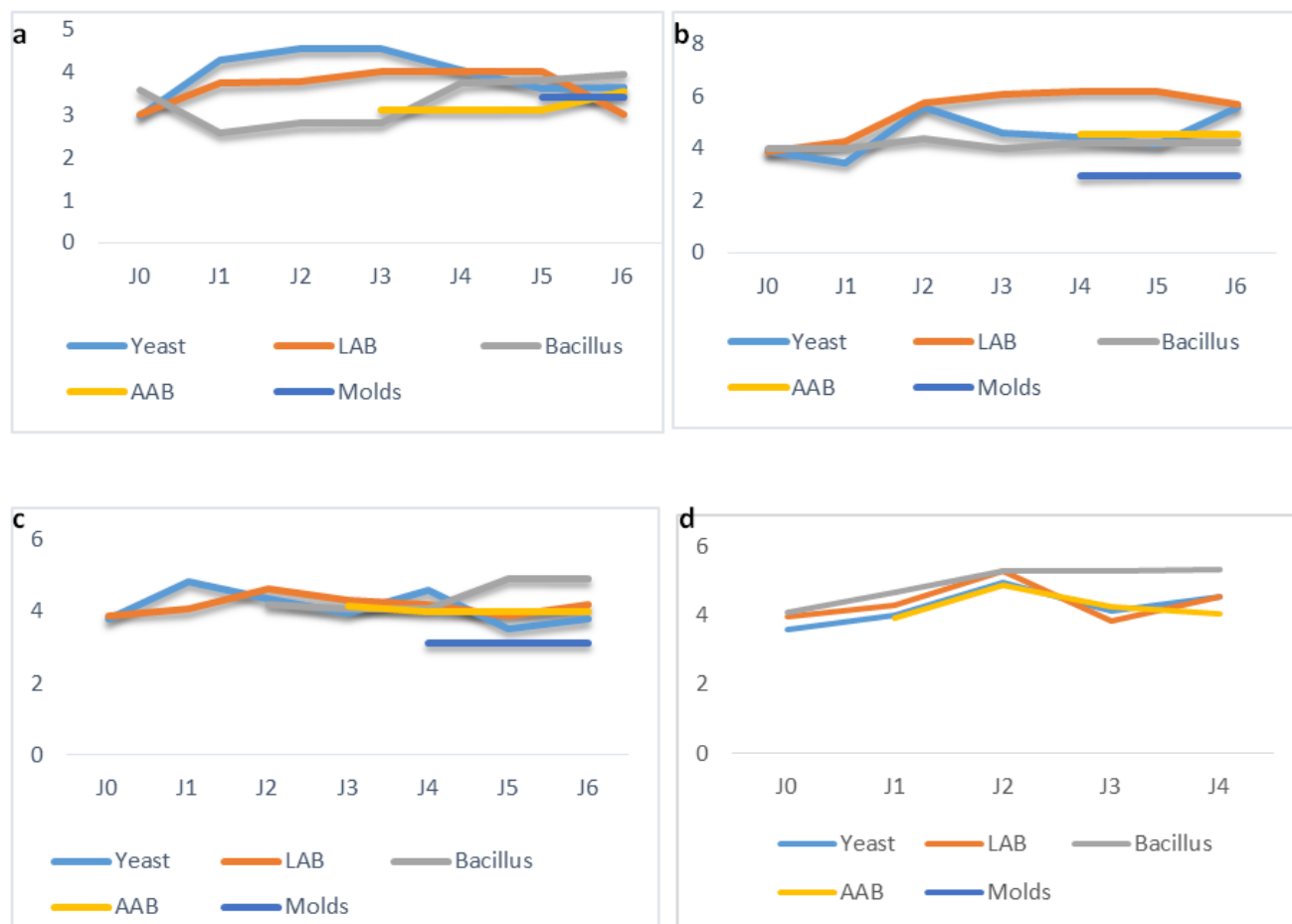


Figure 2. Changes in the microbial population during the different cocoa beans fermentation.

a: Bafia; b: Bertoua; c: Elogbatindi; d: Penja

J₀, J₁, J₂, J₃, J₄, J₅, and J₆ are respectively the Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, of the fermentation process

LAB= Lactic acid bacteria; AAB= Acetic acetic bacteria.

Microbial dynamics changed from one town to another as observed in Table 2. Among the main microbial genera isolated during the different fermentations, AAB had the

lowest growth level. A joint variation in time and order of occurrence of microorganisms during the different fermentations was observed.

Table 2. Growth and occurrence order of the microbial population during the different fermentations.

		Active fermentation microflora					Order of occurrence
		Bacillus (Ba)	AAB	LAB	Yeast (Y)	Mold (M)	
F1	Onset time [Days]	6	4	3	2	6	Y-LAB-AAB-Ba-M
	Population size [log ₁₀ CFU/ml]	3.57	3.14	4.02	4.55	3.42	
F2	Onset time [Days]	2	4	4	2	6	Y-Ba-LAB-AAB-M
	Population size [log ₁₀ CFU/ml]	4.39	4.55	6.17	5.57	5.03	
F3	Onset time [Days]	5	3	2	1	4	Y-LAB-AAB-M-Ba
	Population size [log ₁₀ CFU/ml]	4.94	4.18	4.64	4.85	3.15	
F4	Onset time [Days]	3	2	2	2	/	Y-LAB-AAB-Ba
	Population size [log ₁₀ CFU/ml]	5.32	4.90	5.29	4.96	/	

F1, F2, F3, F4 refer to fermentation of cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja respectively

Y= Yeast; LAB= Lactic acid bacteria; AAB= Acetic acetic bacteria, Ba= Bacillus; M= Molds.

3.3. Quality of Marketable Beans

3.3.1. Fermentation Index

Table 3 below shows changes of the fermentation index during fermentations. This parameter increased

significantly ($p < 0.05$) with fermentation time from 0.40 - 0.62, to 1.00 - 1.40. The same effect was previously described by others authors [21; 22]. Fermentation index decreased between the last two days of F1 and F2 fermentations.

Table 3. Changes in fermentation index during the fermentation stages.

Fermentation index				
	F1	F2	F3	F4
J0	0.40±0.01 ^a	0.40±0.00 ^a	0.62±0.01 ^a	0.56±0.01 ^a
J1	0.57±0.03 ^b	0.42±0.01 ^a	0.72±0.01 ^b	0.77±0.01 ^b
J2	0.59±0.01 ^b	0.46±0.00 ^b	0.94±0.02 ^c	0.97±0.02 ^c
J3	1.00±0.00 ^c	0.78±0.01 ^c	1.13±0.01 ^d	1.12±0.01 ^d
J4	1.08±0.00 ^d	0.83±0.01 ^d	1.37±0.01 ^e	1.10±0.02 ^d
J5	1.08±0.01 ^d	1.04±0.01 ^e	1.38±0.01 ^{ef}	
J6	1.00±0.00 ^c	1.04±0.01 ^e	1.40±0.01 ^f	

Data are Mean ± Standard Error. For each fermentation, Mean values with different letter in the same column are statistically different ($p < 0.05$).

F1, F2, F3, F4 refer to fermentation of cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja respectively.

J₀, J₁, J₂, J₃, J₄, J₅, and J₆ are respectively the Day 0, Day 1, Day 2, Day 3, Day 4, Day 5, Day 6, of the fermentation process.

3.3.2. Beans Count

Table 4 below shows that on the last day of fermentations, the beans count ranged from 76.67 ± 2.08 to 81.67 ± 5.51 . The results showed no significant difference [$p > 0.05$]. These results suggest that seeds weight was greater than 1g.

Table 4. Bean count values at the end of fermentation.

Beans count				
F1	F2	F3	F4	
76.67 ± 2.08 ^a	81.67 ± 5.51 ^a	77.33 ± 3.05 ^a	77.00 ± 3.00 ^a	

Values are mean ± Standard Error. For each fermentation, means with the same letter are not statistically different ($p > 0.05$).

F1, F2, F3, F4 refer to fermentation of cocoa beans from Bafia, Bertoua, Elogbatindi, and Penja respectively.

4. Discussion

Temperature risen significantly ($p < 0.05$) from 25 -27°C to 40 – 44°C within the 3 – 4 days of the fermentation. This rise in temperature might be related to the production of ethanol and acetic acid during fermentation. Production of ethanol from citric acid by yeast and oxidation of ethanol to acetic acid by acetic bacteria often occurred with elevated temperature in the fermenting material [20, 23]. Overall pH of the pulp significantly increased ($p < 0.05$) between the beginning and the end of fermentation. This could be a consequence of citric acid degradation in the pulp. Indeed, during fermentation, citric acid in the pulp is oxidized to ethanol by yeasts [24, 25], bacillus [26], causing an increase in pulp pH. However, a decrease in pH is observed during earlier for F1 and F4 fermentations, and could be due to the oxidation of sugars to lactic acid by lactic acid bacteria [27]. In contrast to the pulp, pH of cotyledons decreased significantly ($p < 0.05$) between the beginning and the end of fermentations. The same effect was observed by Apriyanto and al [21]. Decrease in cotyledon pH is thought to be related to organic acids diffusion into the seed produced in the pulp by microorganism's activity. In fact, during fermentation, acetic acid and lactic acid produced in the pulp by microbial activity diffuse within the cotyledons, thus leading to the lowering of the pH of the cotyledons [28].

During the cocoa fermentations the microflora differ from time to time according to the population of microbes and their order of emergence. Yeasts emerged first and are favored by elevated levels of citric acid and the low pH values (3.26 - 4.23) of pulp pH [29, 24]. Yeast activity is necessary for oxidize of citric acid and rising of pH, creating favorable living conditions for bacteria [1]. This is observed in the case of the F1, F3 and F4 fermentations, where occurrence of LAB on the 2nd day stood with decline of the yeast population which in turn produce ethanol and pectin degradation thus favoring emergence of AAB. The appearance of AAB on day 2 of the F4 fermentation, day 3 of the F3 and F1 fermentations coincided with the temperature peak on days 3 and 4 respectively. Indeed, AAB use ethanol as a substrate to produce acetic acid [30] in an exothermic reaction [20, 23]. Present at the start of F1 fermentation and the third day of F3 fermentation Bacillus count is maximum after the day fourth of F1 and F3 fermentations. Bacillus can be isolated from the early stages of fermentation and towards the end of the process, their population can increase to the point of dominating the microbial population [31] as was observed between days 5 and 6. As for molds, they appeared last in all fermentations, except in F4 where they were not present at all. It has been reported that when the fermentation lasts longer than four days, molds can be involved in the process [20]. In the F2, the order of emergence is different. Indeed, unlike other fermentations were LAB have succeeded yeasts, here, it is *bacillus* that have reached their peak after yeasts. In addition, except in F2 fermentation, *bacillus* was isolated in the beginning of the F1, F3 and F4 as was found by Ouattara and al [17]. This suggests that the bacillus isolated in F1, F3 and F4 would play a key role in the early stages of the process. This microorganism produces pectinolytic enzymes [2], which are responsible of the hydrolysis of pectin which covers and protects seed. In addition, it has been reported that bacillus degrade citric acid in the pulp and therefore increase its pH [26]. Finally, it was observed that during the F4 fermentation, bacillus were more present at each phase of the process. Rooijackers and al [32] showed that bacillus can be present throughout the fermentation phases at a higher level than yeast, LAB and AAB.

We observed an increase in the fermentation index from the beginning to the end of fermentation process. This could be due to the oxidation of polyphenols found in the seeds during fermentation. Indeed, polyphenols such as anthocyanins are responsible for the purple color of beans [33] and are involved in the bitterness of the seeds. During fermentation, anthocyanins are hydrolyzed to form anthocyanidins, which in turn go through a polymerization reaction with catechins to form tannins. As a result, the amount of polyphenols decrease and the color of the seeds will change from purple to brown, synonymous with well-fermented beans [34]. Thus, polyphenoloxidases would have oxidized the polyphenols [35]. The results show that the number of healthy beans in 100 grams of healthy seeds was less than 100, suggesting that seeds were compliant by specific weight [36].

5. Conclusion

This work shows that during cocoa fermentation, microbial dynamics varied from one place to another and is generally dominated by yeasts, followed by lactic acid bacteria and bacillus. The pH of the pulp decreased during the process and that of the seed increased. Also by the end of the process, fermentation index and specific weight of beans were greater than 1. In respect to nib's pH, fermentation index and bean count, beans obtained are of good qualities and have good aromatic potential. Thus, the isolated microorganisms are of good technological potentiality and may be use for the development of fermentative strain.

Conflicts of Interest

The authors declare that they have no competing interests.

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