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Impact of New Fermentation Systems on the Health Quality of Cocoa Beans (*Theobroma cacao* L. 1753) in the Main Cocoa-Growing Regions of Côte D'ivoire: Nawa, Bas-Sassandra and Haut-Sassandra

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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

Aims: Cocoa cultivation plays a crucial role in the Ivorian economy, requiring rigorous post-harvest processes to ensure the quality of the chocolate produced. Fermentation is a fundamental step in these processes, directly influencing the development of the flavor and aromatic characteristics of cocoa beans. This study focuses on evaluating the impact of various fermentation supports on the microbiological quality of cocoa beans after drying.
Study Design: The beans were fermented on six types of supports: palm leaves, cocoa pods, polypropylene tarps, polypropylene bags, jute bags, and banana leaves (control), using two varieties of cocoa, *Forasteros* and *Mercedes* in the San Pedro, Soubré and Daloa regions.
Methodology: At the end of the drying process, 2 kg of dried beans from each fermentation support were collected. The analyses focused on moisture, pH, and the level of microbiological contamination, including fungal flora and *Bacillus* (spore-forming bacteria). The analyses were performed in triplicate. The fungal flora was identified through macroscopic and microscopic observations, while biochemical tests were used to identify presumptive *Bacillus cereus*.
Results: The results indicate that beans fermented in pods showed a pH above 5.5, and those fermented on polypropylene bags had moisture exceeding 8%. *Bacillus* bacteria were more frequent in the beans fermented in cacao pods. Molds, primarily *Aspergillus ochraceus* and *Aspergillus niger*, were more prevalent on jute bags and in the pods compared to the control. The presence of *Bacillus* spores and ochratoxigenic molds in the fermented beans, in the pods, and on jute bags poses a risk to the quality and safety of the cocoa beans.
Conclusion: In contrast, polypropylene bags, polypropylene tarps, and palm leaves are identified as suitable supports for maintaining the sanitary quality of the beans, similar to banana leaves (control support). These results show that, despite the gradual decline in banana plantations, cocoa producers will benefit from adequate support for the fermentation process, thereby guaranteeing the quality of cocoa beans and enabling them to obtain a competitive purchase price in the field, while offering entrepreneurs the opportunity to acquire higher quality products to increase their turnover.

Keywords: Cocoa; fermentation; quality; aspergillus; ochratoxin; bacillus.

1. INTRODUCTION

The transformation chain of cocoa beans (*Theobroma cacao L.*) for cocoa powder production begins in tropical countries, where farmers, after harvesting the cocoa pods, subject the seeds to a natural fermentation process [1]. Without this process, the raw and dried cocoa beans do not develop the characteristic cocoa flavor during [2]. This operation is dominated by various microbial activities, including fungal flora and bacteria of the genus *Bacillus*, which sometimes remain active after the fermentation process. The ability of members of this group to form endospores resistant to extreme heat implies that they can survive industrial processes and pose spoilage and safety issues [3,4]. Indeed, bacteria of the genus *Bacillus cereus* are the third leading cause of foodborne illness outbreaks, capable of forming spores under unfavorable environmental conditions. These spores can remain dormant for many years, even millennia, awaiting the return of favorable conditions [5].

Moreover, fungal activity can lead to contamination by mycotoxins, posing a health risk to consumers [6]. According to [7], poorly conducted post-harvest treatments in cocoa can result in the spread of molds such as *Aspergillus* and *Penicillium*, which are responsible for the production of ochratoxin A (OTA), for example. Fungal contamination is possible at many critical points, including temperature, humidity, endogenous fungal species, as well as the conditions and duration of storage in the cocoa production chain [6]. Additionally, fungal proliferation compromises the commercial quality of food products, leading to significant economic losses [8]. Several previous studies have reported the presence of ochratoxin A in cocoa and various by-products [9]. In Côte d'Ivoire, the world's leading producer and exporter, this mycotoxin (OTA) has been found in cocoa from two production areas and Ivorian ports [10].

The objective of the present study is to evaluate the impact of new fermentation supports on the physicochemical parameters and microbiological quality (OTA-producing fungal flora and bacteria of the genus *Bacillus*) of the final cocoa beans.

2. MATERIALS AND METHODS

2.1 Biological Material and Study Area

The biological material consists of fermented and dried cocoa beans from *Forastero* cocoa varieties in the San Pedro and Daloa regions, and the hybrid variety "Mercedes," proposed by CNRA/Divo, for the Soubré region (Fig. 1).

2.2 Shucking and Fermentation

Pod breaking was carried out three days after harvest, primarily under traditional conditions using knives. Six fermentation techniques (Fig. 2) were spontaneously performed by the same farmer, sheltered from weather and drafts. These include: fermentation on banana leaves (F1; control); on palm leaves (F2); inside cocoa pods (F3); on a polypropylene sheet (F4); in polypropylene bags (F5); and in jute bags (F6) without separating the beans from the placenta. The industrial protocol was applied to all experiments, involving a fermentation duration of 144 hours (6 days) with turning intervals at 48, 96, and 120 hours. For each fermentation method, a minimum of 250 kg of beans and at least 1,000 pods, equivalent to 250 kg of fresh beans, were stored for fermentation.

2.3 Drying

Drying was carried out in thin layers on polypropylene sheets, which are commonly used for storing dried beans before transporting them to the factory. The daily drying duration was 7 hours, taking place from 9:00 AM to 4:00 PM.

2.4 Sampling

At the end of the fermentation process, 2 kg of dried beans from each of the fermentation supports were collected during the months of September, October, and November 2022 and transported to the laboratory. These beans were then shelled, finely ground (using an IKA A11 basic grinder; Germany), placed in 100 g containers, and stored at a temperature of minus 80°C for further analyses.

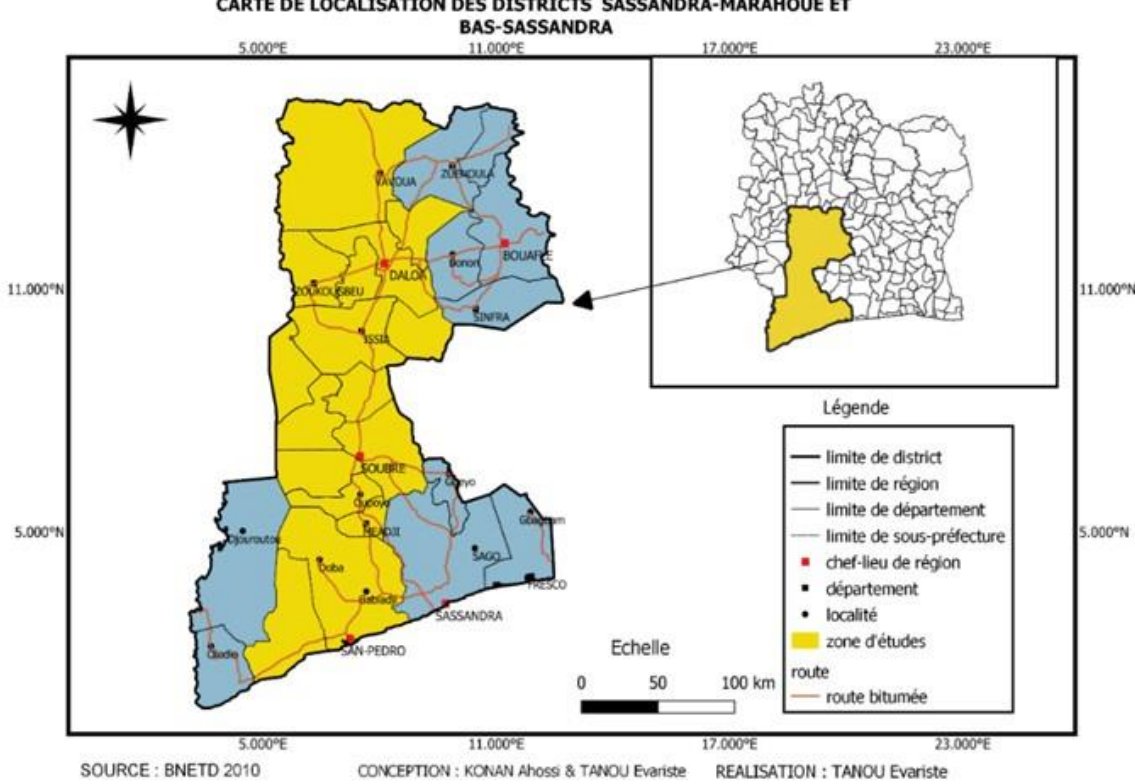


Fig. 1. Map of the study area

Fig. 2. Different fermentation supports

a: Banana leaves (control; F1); b: Palm leaves (F2); c: Cocoa pods (F3); d: Polypropylene sheeting (F4); e : Polypropylene bags (F5); f : Jute bags (F6).

2.5 Physico-chemical Characteristics

Moisture content was determined by measuring 500 grams of beans from each fermentation medium in a mini GAC (Dickey John multigrain) using the method of [11]. The pH was determined using the AOAC method [10].

2.6 Testing for Spore-Forming *Bacillus bacteria* and Fungal Flora in Cocoa Powder Samples

2.6.1 Preparation of the stock solution and decimal dilutions

Sample preparation began with the preparation of the stock solution. Thus, 10 g of cocoa powder from each sample were added to 90 mL of Buffered Peptone Water (BPW) in flasks and left at room temperature for 30 minutes. About 1 mL of the flask's content was added to 9 mL of sterile distilled water to carry out decimal dilutions. The 10^1 and 10^2 dilutions were selected for inoculation.

2.6.2 Sporulation test

The treatment of spores is carried out in test tubes using the previously prepared stock suspension. A quantity of 10 mL is taken and introduced into the test tube. The tubes are then placed in a water bath at 80°C for 10 minutes and immediately cooled in an ice-water bath to select sporulated forms. 0.1 mL of the different dilutions were inoculated onto Mosel agar medium supplemented with emulsified egg yolk for the detection of lecithinase.

2.6.3 Incubation and enumeration

All the plates were incubated at 30°C for seven days. Daily observations were made until mycelium appeared to check for fungal flora. For Bacillus bacteria, the inoculated plates were placed in an incubator for 24 hours at 37°C.

2.6.4 Enumeration

Counting, followed by isolation and macroscopic and microscopic identification of the fungal flora, was carried out according to ISO 21527-1 (2018), and the number of colonies was expressed using the following equation:

$$N\ (UFC/g) = \frac{\Sigma Ci}{(n_1 + 0{,}1 n_2) d.V}$$

N (CFU/g): number of germs. ∑Ci: colony sums; V: inoculum volume; n1: number of Petri dishes counted at 1st dilution; n2: number of Petri dishes counted at 2nd dilution; d: dilution rate at the first dilution retained.

2.6.5 Isolation and purification

After counting, the plates showing isolated colonies were selected. Only the reddish and yellowish isolates were retained for purification. The isolates were then subcultured on Lysogeny Broth (LB) medium using the quadrant streaking technique. For the fungal flora, daily observations were made until mycelium appeared. Each developed mycelium underwent several successive inoculations until pure strains were obtained. The colonies were subcultured three times until identical colonies were achieved.

2.7 Identification of Bacteria of the Genus Bacillus Spore Form

2.7.1 Gram staining

The morphology, cellular organisation and parietal type of the isolates were assessed on juvenile cultures grown using the Gram staining technique (1884). The smears were examined under a light microscope equipped with an immersion objective (x100 objective).

2.7.2 Identification of enzyme tests

The detection of catalase was performed according to the method of Wysocki et al.[12] while the detection of lecithinase was performed according to the method of Guiraud et al. [13].

2.7.3 Identification of fungal flora

The identification of the fungal flora was carried out according to the method of Verscheure et al. [14] with some modifications. Briefly, a portion of the colony to be identified was taken with forceps and placed in a drop of methylene blue on a microscope slide. The preparation was then covered with a coverslip and observed under an optical microscope at different magnifications (x10, x40, and x100). The frequency of isolation (Fr) of fungal species was calculated according to Yapo-Kouadio et al. [15].

$$Fr(\%) = \frac{Number\ of\ samples\ per\ species}{Total\ number\ of\ samples} x100$$

3. RESULTS

3.1 Physico-Chemical Composition of Fermented and Dried Beans

The pH of the dried beans, across all fermentation supports, ranged from 4 to 6, while the moisture content varied between 6.5% and 8.85%, with significant differences (P < 0.05) (Fig. 3). The variety of cocoa had no significant influence on the pH and moisture content. However, in the three study areas, beans fermented on support F3 recorded pH values significantly higher than the recommended threshold (pH 5.5), with averages of 6.26 ± 0.04 in San Pedro, 6.36 ± 0.0 in Vavoua, and 6.23 ± 0.04 in Soubré. The homogeneity test performed with Dunnett (α = 0.5) showed that beans fermented on F2 (5.16 ± 0.00; 5.09 ± 0.01) in San Pedro and Soubré, as well as those on support F4 (5.42 ± 0.02) in Vavoua, were closer to the beans fermented on the control support. Regarding moisture content, beans fermented on support F5 in all three areas (San Pedro, Vavoua, and Soubré) with 8.76 ± 0.20%, 9.03 ± 0.15%, and 9.1 ± 0.26%, respectively, were above the 8% standard, along with those fermented on supports F4 (8.85 ± 0.13%) in San Pedro and F6 (8.23 ± 0.3%) in Vavoua. Additionally, Dunnett's similarity test indicated that the values recorded in beans fermented on support F6 (7.6 ± 0.17%) in San Pedro; F2 (7.83 ± 0.3%), F3 (6.8 ± 0.26%), and F4 (7.86 ± 0.11%) in Vavoua, as well as F6 (7.9 ± 0.4%) and F4 (7.83 ± 0.66%) in Soubré, were closer to those of beans fermented on the control support F1.

3.2 Bacillus Cereus Species of Fermented and Dried Beans

After 24 hours of incubation at 30°C on MYP medium, the majority of observed colonies are pink, indicating mannitol-negative fermentation, and are frequently surrounded by a precipitation halo, suggesting lecithinase activity. Gram staining followed by microscopic observation reveals that most isolates are Gram-positive bacilli (Fig. 4). Biochemical tests were conducted on 51 isolates from all fermentation media, with 18 from San Pedro, Soubré, and 15 from Vavoua. Based on this, 37 out of the 51 isolates were positive for lecithinase, representing a proportion of 69.81%. Specifically, 22.64% of the samples from San Pedro were positive, 24.52% from Vavoua, and 22.64% from Soubré.

The bacterial load of the genus Bacillus (Table 1) in commercial beans of the Forastero variety ranged from 33 to 69 × 10^2 CFU/g in San Pedro and from 0 to 60 × 10^2 CFU/g in Vavoua. Among all the samples analyzed, the contamination of fermented beans in the cacao pod (F3) was more

pronounced in these two regions, with 69.7 ± 5.259 × 10² CFU/g in San Pedro and 66.67 ± 13.89 × 10² CFU/g in Vavoua, showing significant differences (Dunnett; α = 0.05) compared to the control medium F1 (33.3 ± 18.9 × 10² CFU/g) in San Pedro and 42.42 ± 10.50 × 10² CFU/g in Vavoua. Similarly, samples of the Mercedes variety taken in Soubré showed that fermented beans on F3 media (106.06 ± 10.50 × 10² CFU/g) were more contaminated with a significant difference ($P < 0.05$) compared to those on the control medium (F1, 42.42 ± 10.50 × 10² CFU/g), with a higher bacterial population than that of the Forastero variety.

3.3 Fungal Contamination

3.3.1 Enumeration of fungal flora

Table 1 presents the level of fungal contamination in fermented and dried cacao beans. The total fungal colony count observed on the cacao beans indicates a high level of contamination (×10² CFU/g). There is no significant difference in contamination levels from one area to another. The highest contamination was observed in beans fermented on F3 and F6 media across all three study areas, with significant differences compared to beans fermented on the control medium F1 (Dunnett; α = 0.05). Specifically, in the San Pedro area, the fungal population was 30.3 ± 3.19 × 10² CFU/g on F3, followed by F6 (23.03 ± 0.52 × 10² CFU/g) compared to 12.12 ± 1.38 × 10² CFU/g on F1. A similar trend was observed in Vavoua, with a population of 22.42 ± 1.38 × 10² CFU/g on F3 and 14.85 ± 2.29 × 10² CFU/g on F6, compared to 6.97 ± 1.05 × 10² CFU/g on F1. Beans of the Mercedes variety in Soubré also showed a higher degree of contamination on beans fermented on F3 (26.06 ± 1.38 × 10² CFU/g) and F6 (11.21 ± 1.89 × 10² CFU/g) compared to 5.76 ± 1.38 × 10² CFU/g on F1.

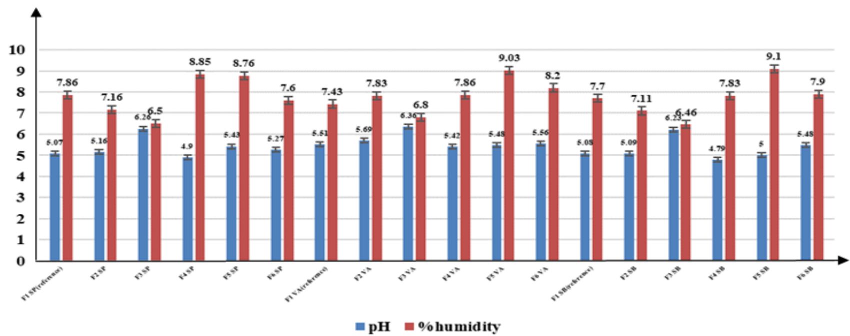


Fig. 3. Variation in pH and water content on different fermentation support
Banana leaves (control; F1); Palm leaves (F2); Cocoa pods (F3); Polypropylene sheeting (F4); Polypropylene bags (F5); Jute bags (F6); SP: San Pedro;VA: Vavoua and SB: Soubré

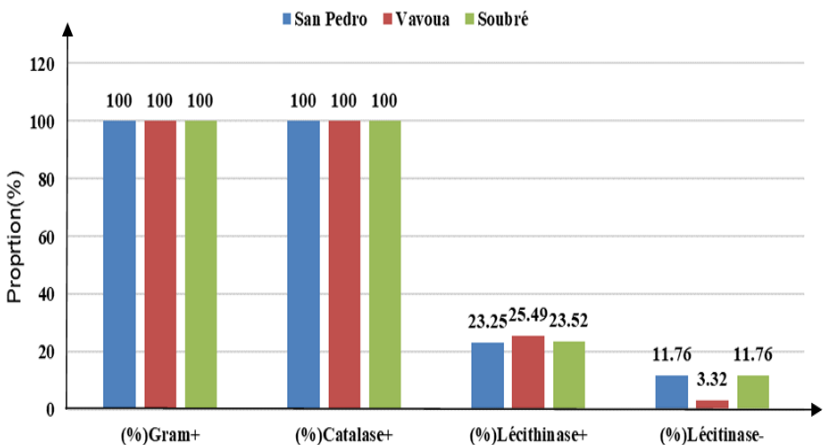


Fig. 4. Biochemical tests carried out on 51 isolates

Table 1. Contamination of fungal flora and *Bacillus bacteria* in spore form on dried cocoa beans

Supports	FF.(UFC/g)10^2	*Bacillus* N(UFC/g)10^2
F1 SP (Reference)	**12,12 ± 1,38 [a]**	**33,30 ±18,9 [a]**
F2 SP	10,00 ± 2,73 [a]	48,48 ± 5,25
F3 SP	30,30 ± 3,19	69,70 ± 5,25
F4 SP	13,64 ± 0,00 [a]	63,64 ± 9,09
F5 SP	07,88 ±1,38 [a]	63,60 ± 18,2
F6 SP	23,03 ± 0,52	60,61 ± 5,25
F1 VA (Reference)	**07,88 ± 1,38 [a]**	**45,45 ± 9,09** [a]
F2 VA	06,97 ± 1,05 [a]	00, 00 ± 0,00
F3 VA	22,42 ± 1,38	66,67 ± 13,89 [a]
F4 VA	07,58 ± 1,38 [a]	63,64 ± 9,09 [a]
F5 VA	04,55 ± 0,90 [a]	60,61 ± 5,25 [a]
F6 VA	14,85 ± 2,29	48,48 ± 13,89 [a]
F1 SB (Reference)	**05,76 ± 1,38** [a]	**42,42 ± 10,50** [a]
F2 SB	04,85 ± 1,38 [a]	75,76 ± 13,89
F3 SB	26,06 ± 1,38	106,06 ± 10,50
F4 SB	06,36 ± 0,90 [a]	84,85 ± 5,25
F5 SB	05,15 ± 1,05 [a]	97,00 ± 22,9
F6 SB	11,21 ± 1,89	81,80 ± 39,6

Mean ± S.E.M = Mean values ± Standard error of means; FF: fungal flora
Banana leaves (control; F1); Palm leaves (F2); Cocoa pods (F3); Polypropylene sheeting (F4); Polypropylene bags (F5); Jute bags (F6); SP: San Pedro;VA: Vavoua and SB: Soubré
In the columns, the averages not labelled with the letter 'a' are significantly different from the control level average.

3.3.2 Mould species isolated and identified in cocoa powder

The study of macroscopic characteristics, such as color, colony appearance, and the underside of the plates, as well as microscopic characteristics, including the shape of the thallus and spores of the isolated fungal strains, allowed for the identification of the genera *Aspergillus, Mucor*, *Rhizopus*, and *Rhizomucor*. Using the identification key, strains of *Mucor sp.*, *Rhizopus*, and *Rhizomucor pusillus* were identified. For the genus *Aspergillus*, the species *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus ochraceus* were detected. Table 2 presents some of the mold strains isolated from the different samples, observed under a microscope at a magnification of ×100.

3.3.3 Frequency of isolation of fungal species in market beans

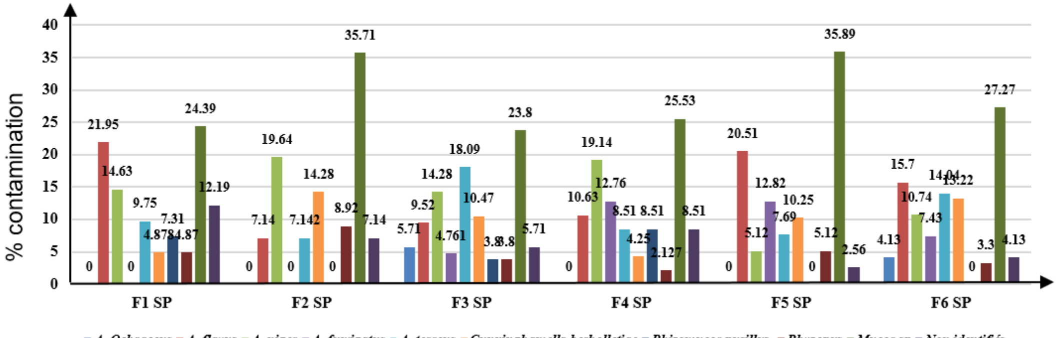
In terms of diversity of isolated strains, no significant difference was observed between the cocoa bean samples analyzed in the three areas. In the San Pedro area, a total of 13 strains were isolated, of which nine were identified. In Vavoua, 11 strains were isolated, with seven identified. In contrast, the Soubré area recorded a lower isolation frequency with the *Mercedes* variety, where a total of eight strains were isolated, all identified.

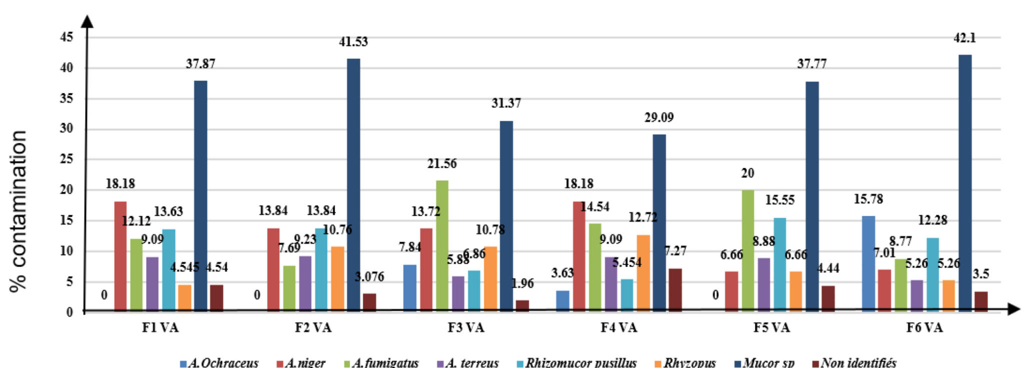
Fig. 5 presents a graphical representation of the contamination of commercial beans from different fermentation sources in the three study areas. In all study areas, the genus *Mucor* showed a higher dominance in the various analyzed samples, with a percentage ranging between 24% and 30% in San Pedro, between 29% and 41% in Vavoua, and between 19% and 54% in Soubré, where the *Mercedes* variety was slightly more dominant compared to the *Forasteros* variety beans.

For the genus *Aspergillus, Aspergillus flavus* and *Aspergillus niger* had the highest contamination percentages in the three study areas. In the San Pedro area, *Aspergillus flavus* was more abundant on the control support F1 and support F5, at 21.95% and 20.51% respectively, while *Aspergillus niger* was observed in beans fermented on supports F4 (19.14%) and F2 (19.64%). In the Vavoua area, the population of *Aspergillus niger* was dominant in beans fermented on supports F1 (18.18%) and F4 (18.18%), while *Aspergillus fumigatus* was more abundant in beans fermented on supports F3

(21.56%) and F5 (20%). Aspergillus flavus was not identified in samples from the Vavoua area. However, in the Soubré area with *Mercedes* variety beans, *Aspergillus flavus* was identified with a higher population in beans fermented on supports F6 (16.88%) and F1 (15.25%). Conversely, *Aspergillus niger* was more prevalent in beans fermented on supports F1 (16.94%) and F3 (24.17%).

The species *Aspergillus ochraceus* was identified only in *Forastero* variety beans, specifically in beans fermented on supports F3 and F6, at 5.71% and 4.13% in San Pedro, and at 7.84% and 15.78% in Vavoua. Beans fermented on support F4 in Vavoua also showed the presence of *Aspergillus ochraceus*, but in a lower proportion, at 3.63%. No trace of *Aspergillus ochraceus* was observed in the Soubré area.





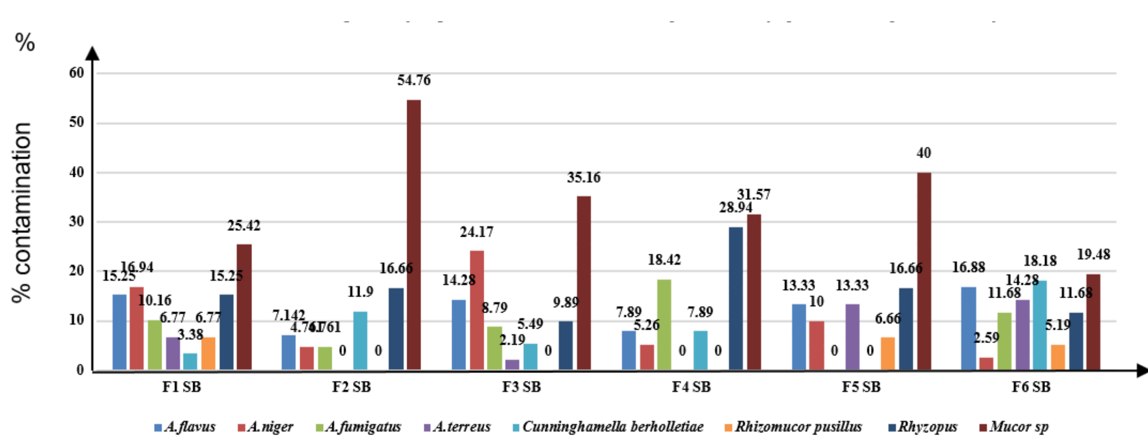


Fig. 5. Fungal flora isolated from fermented and dried cocoa beans

Banana leaves (control; F1); Palm leaves (F2); Cocoa pods (F3); Polypropylene sheeting (F4); Polypropylene bags (F5); Jute bags (F6); SP: San Pedro;VA: Vavoua and SB: Soubré

Table 2. Mould species isolated in cocoa powders

Species	Macroscopic aspects	Recto(R) & Verso(V)	Microscopic appearance x40	Description	Reference image
Aspergillus flavus	Colour: small green spot Appearance: powdery Reverse: no pigment			Hyphae septate; Conidiophore long, not septate, hyaline; Phiales directly inserted on the vesicle; Conidia globular, pale green, echinulate; Head aspergillate; uniseriate, radiate	A.cryzae
Aspergillus fumigatus	Rapid growth on Sabouraud chloramphenicol; downy appearance; green to blackish mycelium			Rapid growth on Sabouraud chloramphenicol; downy appearance; green to blackish mycelium	
Mucor sp	Blackish colour with a velvety, powdery appearance and water droplets on the surface. No pigment on reverse side			Conidia produced by phiales inserted at the dilated end of a wide, non-partitioned conidiophore.	
Rhyzopus sp	Rapid growth on Sabouraud chloramphenicol; cottony colony; extensive, aerial mycelium of a whitish to greyish colour; white colour with a bulging cottony appearance. no pigment on the underside.			Presence of chlamydospores; sporocysts strangulated under the columella; smooth conidiophore ending in spores densely grouped at the tip.	

Species	Macroscopic aspects	Recto(R) & Verso(V)	Microscopic appearance x40	Description	Reference image
Cunninghamella bertholletiae	Rapid growth on Sabouraud chloramphenicol; cottony colony; extensive, aerial mycelium of a whitish to greyish colour; white colour with a bulging cottony appearance. no pigment on the underside.			Rhizoids and short sprocystophore with subterminal branching; after rupture of the sporocyst, a well-developed columella without apophysis is visible.	
Aspergillus clavus	Downy to powdery colonies, white at first then yellow and yellow-green. The underside is colourless..			Conidial head uniseriate, radiate, then divided into several columns of poorly individualised spores. The conidiophores are hyaline and verrucose. The vesicles are subglobose and the phiales are inserted directly onto the vesicle	
Aspergillus-nigerr	Colonies are black on the front, cottony white on the back and pale yellow. The growth is rapid and the shape is rode			Unpartitioned mycelium, the head bears numerous conidiophores, phiales formed on the vesicle..	
Aspergillus terreus	Black colony on front with white halo. The reverse side is grey. Rapid growth and round shape			Thallus with cloisonné mycelium, bearing numerous conidiophores not branched into vesicles; phiales formed directly on the vesicle or on millstones.	

Species	Macroscopic aspects	Recto(R) & Verso(V)	Microscopic appearance x40	Description	Reference image
Aspergillus flavus	Powdery colonies, white at first, then yellow or ochre-yellow to buff. The reverse of the colonies is colourless to pale yellow, growing rapidly.			Conidia produced by phiales inserted at the dilated end of a wide, unpartitioned conidiophoree (aspergillate head arrangement).	
Aspergillus ochraceus	Colour: darker black Appearance: woolly Reverse: no pigment			Unpartitioned hyaline mycelium; short, erect, unbranched conidiophore; radiating aspergillate heads; conidia in diverging chains.	
Rhizomucor pusillus	Rapid growth on Sabouraud chloramphenicol; cottony colony; extensive, aerial, whitish to greyish mycelium.;			Branching sporocystophore ending in a columella projecting into the globular sporocyst	

4. DISCUSSION

Data on the hydrogen potential (pH) of cocoa beans after fermentation and drying revealed variations among the different samples analyzed, depending on the fermentation support. This variation may be attributed to a change in the biochemical heterogeneity of the cocoa beans during the fermentation process. Beans fermented on supports F1 (pH: 5.506 ± 0.02), F2 (pH: 5.69 ± 0.01), and F6 (pH: 5.56 ± 0.02) in the Vavoua region showed higher pH values after drying. However, these values are comparable to those reported by Romanens et al. [16]. However, all beans fermented on support F3 in the three areas, namely 6.26 ± 0.04 in San Pedro, 6.23 ± 0.04 in Soubré, and 6.36 ± 0.01 in Vavoua, were significantly different from those on the control support and were higher than 5.5, in accordance with the international Codex standard for cocoa (86-1981, Rev.1-2001). These results indicate that the cocoa variety does not impact the final pH of the dried beans. However, the high pH values observed in all beans from support F3 could also be explained by a relatively low fermentation temperature (T < 40 °C). According to Lashermes et al. [17], at temperatures below 40 °C, dried beans are likely to develop more mold, as most molds prefer high moisture levels. Additionally, more or less acidic solutions could inhibit microorganism proliferation in the final product [18]. However, the values recorded on supports F2 and F6, as well as on control support F1 in Vavoua, might be explained by poor mixing, leading to heterogeneity in the beans.

The moisture content of commercial cocoa beans is crucial as an indicator of their preservation state and degree of contamination. It is essential to reduce this moisture to less than 8% during the drying process to prevent any spontaneous growth of molds and bacteria during transport and storage, as noted in the study by Niikoi et al. [19]. Overall, the moisture content of fermented and dried beans was below 8%, except for those fermented on supports F4 (8.85 ± 0.13%) and F5 (8.76 ± 0.20%) in San Pedro, F5 (9.1 ± 0.26%) in Soubré, and F5 (9.03 ± 0.15%) in Vavoua, which had values significantly higher than 8% compared to beans fermented on the control support (Fig. 3). These differences could be attributed to the design of these fermentation supports. Indeed, supports F4 and F5 are primarily made of polypropylene, an impermeable material that hinders juice drainage during fermentation while slowing down the fermentation process. Our results are consistent with those obtained by Bankoff, et al. [20], who also reported moisture levels ranging from 7.53% to 8.10%. According to Calvo et al. [21] cocoa beans lose their moisture during fermentation due to the diffusion of moisture outward from the bean, and then during the turning process, facilitated by the low relative humidity of the atmosphere.

The evaluation of the fungal flora and *Bacillus* genus bacteria in crushed commercial cocoa beans showed results that varied from one fermentation support to another.

The load of Bacillus genus bacteria (Fig. 5) in commercial cocoa beans from the Forastero variety ranged between 33 and 69.10^2 CFU/g in San Pedro and between 0 and 60 × 10^2 CFU/g in Vavoua, which was lower than the contamination rate observed with Mercedes variety beans in the Soubré area, ranging between 42 and 106.10^2 CFU/g, with significant differences ($P<0.05$). These observations of *Bacillus* contamination are generally related to the fact that these microorganisms are known to be abundant in soil. Furthermore, these microorganisms showed higher contamination in the later days of fermentation and could easily infiltrate the beans if they are broken. Their ability to be thermotolerant by forming spores indicates their presence in these beans.

Considering the contamination rate based on fermentation supports, among all fermentation supports, beans fermented in cacao pods (F3) were more contaminated than those from the control support and others, with a significant difference according to the Dunnett test. The results revealed a population of 69.7 ± $5.259.10^2$ CFU/g in San Pedro, 66.67 ± $13.89.10^2$ CFU/g in Vavoua, and 106.06 ± $10.50.10^2$ CFU/g in Soubré. The higher contamination observed in beans fermented in cacao pods is likely related to the higher rates of damaged beans caused by elevated germination rates during fermentation, as well as the acidity of the final beans obtained after fermentation and drying.

Indeed, after the drying process, beans fermented inside the cacao pods generally reached a pH approaching 7 overall. According to the work of Merzougui et al. [22], pH has a significant influence on the thermal resistance of microorganisms. An acidic environment with a pH of 4 during treatment can lead to lower thermal resistance of spores compared to a

neutral environment. Experiments have shown that B. cereus spores were less resistant when the pH of sporulation was 5.5 compared to a pH of 7.

For the identification and characterization of Bacillus cereus colonies isolated from cocoa powder, a total of 51 colonies were isolated from all samples. Initially, we confirmed the genus Bacillus of our strains [23] by studying certain morphological and biochemical characteristics, including examining colony features (shape, appearance, and size) on 48-hour nutrient agar cultures. The results of the morphological characterization showed that the isolated strains were either short or long rods, either singly or in chains, with rounded ends. Gram staining revealed that all isolated colonies were Gram-positive bacilli. The biochemical study of the strains was based on catalase and lecithinase tests. Based on this, 37 out of the 51 isolates were positive for lecithinase, representing a proportion of 69.81%, with 23.52% from samples collected in San Pedro and Soubré.

In the Vavoua area, 25.49% of the isolates were positive for lecithinase. According to the Bergey's Manual of Systematic Bacteriology, the previously described characteristics represent the typical traits of species belonging to the genus Bacillus, specifically Bacillus cereus. It has been observed that in the case of UHT (Ultra High Temperature) milk, the spores present can be activated by thermal shock, leading to their germination and the production of extracellular enzymes [24]. Indeed, according to Stenfors Arnesen et al. [25], spore-forming bacteria such as Bacillus cereus have been identified as responsible for food poisoning. Additionally, these bacteria can withstand high temperatures, including the major thermal treatments used in the food industry. However, the obtained load is significant. According to Stenfors Arnesen et al.[25], in the case of diarrheal foodborne illnesses, the quantity of pathogenic B. cereus cells or spores found in the implicated foods is generally equal to or greater than 10^5 CFU per gram or milliliter, and in very rare cases equal to or greater than 10^3 per gram or milliliter.

According to Schwan et al. [26], molds play a significant role in the ecology of cocoa beans during fermentation and have the ability to produce mycotoxins, compounds responsible for the unpleasant odor of cocoa. The evaluation of fungal contamination levels in commercial cocoa beans in this study revealed a real influence of the fermentation support on fungal population growth. However, the differences in contamination rates from one area to another are not statistically significant. Nevertheless, among all supports analyzed in the three study areas, it appears that beans fermented on jute sack supports (F6) and inside cacao pods (F3) had higher contamination rates. In San Pedro, the levels were 23.03 ± 0.52. 10^2 CFU/g for F6 and 23.03 ± 0.52.10^2 CFU/g for F3, in Vavoua with F3 (22.42 ± 1.38.10^2 CFU/g) and F6 (14.85 ± 2.29.10^2 CFU/g), and in Soubré where contamination was more pronounced in beans fermented on support F3 (26.06 ± 1.38.10^2 CFU/g).

The higher mold population observed on support F6 in all study areas could be explained by the nature of the support itself. Indeed, these supports (jute sacks) are primarily containers that have been used to store dried cocoa beans and transported over long distances, sometimes on dusty roads during the dry season or muddy roads during the rainy season. All these conditions could favor pre-existing contamination of these sacks even before the fermentation process begins. According to Benyagoub et al. [27], some fungi with a sexual reproduction mode can easily colonize food products when storage conditions are not optimal. Additionally, during fermentation, white mold spots were observed as early as the 4th day on beans that were fermented with all the jute sacks.

Regarding the beans fermented inside cacao pods, the observed level of contamination seems to be directly related to the percentage of sprouted and moldy beans exceeding 3%, as observed during the cutting test in previous studies. Our results are similar to those of Yao et al. [28], who recorded fungal contamination of cocoa beans at 5.8. 10^3 CFU/g in Alépé, 2.7.10^3 CFU/g in Duékoué, and 1.7.10^3 CFU/g in Soubré. However, our results are significantly lower than those of Mounjouenpou et al.[29], who, in studies conducted during the 2007 cocoa season in Cameroon, observed a maximum mold population ranging from 2.02 ± 1.3.10^4 to 1.72 ± 0.10.10^8 CFU/g. This disparity may be influenced by various factors such as climatic conditions, storage (humidity, temperature, and ventilation system), and the presence of a significant fungal load, which can lead to qualitative and quantitative changes in the microflora (Le Bars, 1987). Nevertheless, despite this contamination, our results suggest that the commercial beans analyzed in this study meet quality criteria and do

not pose a health risk. Indeed, according to Gourama et al.[30], the mold load in commercial bean samples should be below 10^6 CFU/g.

The main molds isolated and identified in this study are *Mucor* species (the majority), Rhizomucor pusillus, Rhizopus, and the Aspergillus genus, which includes Aspergillus flavus, *Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus ochraceus*. These identified molds are consistent with those already reported in the literature regarding cocoa beans [29], [31]. The presence of *Aspergillus flavus*, black Aspergillus species (A. *carbonarius* and A. *niger*), and especially A. *ochraceus*, indicates a risk of mycotoxin production. Furthermore, among the assessed *fungal flora*, *Aspergillus niger* was observed in beans fermented on supports F4 (19.14%) and F2 (19.64%) in San Pedro, on support F1 (18.18%) and F4 (18.18%) in Vavoua, and on support F1 (16.94%) and F3 (24.17%) in Soubré. *Aspergillus flavus* was identified in beans fermented on supports F1 (21.95%) and F5 (20.51%) in San Pedro, and in beans fermented on supports F1 (15.25%), F3 (14.28%), F6 (16.88%), and F5 (13.33%) in Soubré. Conversely, *Aspergillus ochraceus* was identified only in *Forasteros* variety beans, specifically in beans fermented on supports F3 (5.71%) and F6 (4.13%) in San Pedro, and in Vavoua with F6 (15.78%) and F3 (7.84%).

This disparity may be attributed to climatic conditions, storage factors (humidity, temperature, and ventilation system), soil organic matter composition, soil texture, and pH [32]. According to Gwlady and Tap (2004), A. *ochraceus* and A. *niger* are potentially capable of producing ochratoxin A (OTA). The high contamination observed in beans fermented inside cacao pods (F3) could result from the fact that when pods are partially open, cocoa beans come into direct contact with the air and soil, which are potential sources of *Aspergillus niger* and *Aspergillus ochraceus*. Additionally, the final pH of commercial beans, ranging from 5 to 6, promotes mold proliferation, along with a high germination rate in the beans during fermentation, which exceeds 3%. According to Duron (1999), fungi can proliferate in environments with a pH ranging from 3 to 8, with optimal growth observed between 5 and 6. The contamination rate observed in beans fermented on support F6 after fermentation and drying is likely related to visible white mold contamination observed on the bean shells before drying. Withlow et al. (2001) also demonstrated that *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Absidia*, and *Rhizopus* are indigenous strains typically isolated from most field and soil environments.

Our results show differences compared to those of de [15], who observed higher contamination rates in cocoa bean samples from Soubré and Yamoussoukro. They reported that 78.91% of the contaminations were due to Mucor species, 54.05% to *Aspergillus*, 30.4% to *Penicillium,* and 21.81% to the *Fusarium genus.*

5. CONCLUSION

At the end of the drying process, the influence of cocoa variety on the physico-chemical characteristics of the final beans was not observed. However, in the three study areas, beans fermented in the cocoa pods exhibited pH and moisture content values that were higher than those defined by standards. Additionally, beans fermented on polypropylene sacks also showed elevated pH and moisture content values. The assessment of the microbiological quality of cocoa beans fermented on different substrates revealed significant diversity in contamination by populations of spore-forming Bacillus bacteria and fungal flora. Spore-forming *Bacillus* bacteria were more frequently detected in beans of the Mercedes variety compared to the *Forasteros* variety. This trend was more pronounced in beans fermented inside cocoa pods for both varieties. Furthermore, although there was no significant difference in fungal contamination levels between the two varieties, contamination was higher in beans fermented on jute sacks and in cocoa pods. Identification of the fungal flora revealed a significant predominance of *Aspergillus ochraceus* and *Aspergillus niger* species in beans fermented on jute sacks and in cocoa pods compared to the other group. Thus, the detection of spore-forming Bacillus bacteria and molds such as Aspergillus ochraceus and *Aspergillus niger*, which produce *ochratoxin A* (OTA), in cocoa beans fermented inside cocoa pods and on jute sacks presents a risk to their quality and safety.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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