Effect of spoilage by lipolytic fungi strains on free fatty acids formation in fermented and dried cocoa beans.

Abstract

Aims : High FFA content of cocoa beans is high lead to serious consequences altering the solidification kinetic and soften of butter fraction and chocolate manufacture. During primary post-harvest processing, cocoa beans harbor extracellular lipase producing mold species that invasion could trigger the accumulation of FFA. This study investigated the improvement of the quality of fermented and dry cocoa beans sourced from Côte d'Ivoire regarding its FFA concentration by identification of lipolytic mold species causing the increase of FFA cocoa raw material.

Methods: Raw cocoa beans were sampled on-farm level in some big cocoa producing regions of Côte d'Ivoire. Contaminating mold strains were isolated on the surface of Sabouraud chloramphenicol agar incubated at 25°C for 7 days. Macroscopic and microscopic characterization was made for cultural and morphological identification of molds' isolates. Their lipolytic properties were studied on the same medium enriched with olive oil as only carbon source and rhodamine B as indicator of extracellular lipase production. Molecular analysis were carried out by extraction, amplification by PCR using a specific transcribed spacer primer (ITS4/ITS5) and sequencing of DNA were performed for fungi identification.

Results: Approximately 154 strains spanning 8 genera were isolated from tested cocoa beans samples. However only *Rhizopus oryzae, Absidia corymbifera, Mucor heimalis, Aspergillus niger, A. tubingensis, A. aculeatus, A. flavus, A. tamari, A. fumigatus, Nigrospora sphaerica, Curvularia geniculate* and *Penicillium chrysogenum* appeared greater importance regarding their lipolytic properties. *A. corymbifera, A. niger, A. fumigatus* and *R. orysae* were the most

frequent and predominant species. Cocoa beans inoculated with *R. oryzae*, *A. corymbifera* or *A. niger* recorded FFA content above 1.75%.

Conclusion: Reduction of the lipolytic fungi species growth during cocoa primary post-harvest processing could strongly reduce the accumulation of FFA in cocoa beans and produce sustainable cocoa raw material.

Key words: Cocoa beans, lipolytic fungi species, inoculation, free fatty acids, primary postharvest processing.

1. Introduction

Cocoa (Theobroma cacao L.) is an important food source in many homes due to the frequent consumption of its processed/finished forms such as biscuits, chocolate, chocolate drinks, cocoa powder and sweets [1]. Primary industrial processing of cocoa beans leads to cocoa butter (CB) as the main by-product from cocoa beans beside cocoa liquor and powder [2]. The components of cocoa butter are constituted of 97% of triacylglycerols, di- and mono-glycerides, phospholipids and glycolipids and unsaponifiable matter in the low proportion [3]. According to Zzaman et al. [4], cocoa butter is reported to be the continuous phase that the variable and interesting rheological, physical and chemical characteristics could be affected during both primary post-harvest and industrial processing. Its hardness including crystallization and melting properties depends on the content of saturated and unsaturated fatty acids bound to triglycerides, and on the of free fatty acids (FFA) content. FFA are carboxylic acids with a long carbon chain that are naturally present in small quantities in cocoa butter but released from triacylglycerides or di- and mono-glycerides under activity of lipase or by oxidation reactions, and accumulated in high concentration inside various agricultural commodities including cocoa beans [5]. However, oxidation risks are negligible in cocoa butter due to its low unsaturated fatty acid content [6]. The Directive 2000/36 /EC of European Union [7] set the legal maximum limit for FFA content of cocoa butter at 1.75%.

FFA content above this limit may be considered as a factor reducing the commercial value of cocoa beans [8] and altering the technological properties of cocoa butter fraction and chocolate manufacture [9]. In addition, according to Briones and Aguilera [10], FFA contents exceed international standard of 1.75% causes financial losses for both cocoa producers and chocolate manufacture industry. Côte d'Ivoire is the first world producer of cocoa beans with up 2.2 million tons in 2020-2021 [11]. Unfortunately, primary post-harvest processing of cocoa beans vary considerably from a producing region to another cocoa producing region [12] in this country. Consequently, Ivorian cocoa beans are currently subject to various quality defects including high FFA content for several years [6]. The resurgence of the problem of free fatty acid content in Ivorian cocoa since 2010-2011 has become alarming that it is urgent to find a sustainable solution while European Commission is considering reducing FFA content of cocoa and by-products to 1%. In Côte d'Ivoire, various abiotic and biotic factors could trigger the increase of FFA content inside fermented and dried cocoa beans. In this country like all tropical and humid regions, climatic and agronomic conditions are conducive to mold growth and food quality deterioration [11]. In addition, many research activities highlighted the effect of inappropriate primary post-harvest processing of cocoa mediated by a dynamic of biochemical reactions catalyzed by a microbial succession contaminated spontaneously cocoa bean mucilaginous pulp including the considerable growth of specific filamentous fungi [13]. Chaves-López et al. [14] have concluded that cocoa fermentation and drying are two major and essential steps of primary post-harvest procession that depended on various factors such as genotype of cocoa plant, microclimate, local practices of fermentation process, level of maturity and sanitary status of pods, mass of cocoa beans submitted to fermentation, etc. Management of these operations is crucial for the quality of the final product before export [15]. During these post-harvest operations, filamentous fungi can also develop especially if cocoa beans are from damaged pods [16] or if

fermentation process is performed without regular turning [17]. In addition, storage of fermented and dried cocoa under high relative humidity values promotes growth of molds [18]. Some of these molds could produce lipase enzyme [19], which could hydrolyzed triacylglycerides of cocoa butter and increase FFA content inside cocoa beans [8]. To date, no complete and comprehensive research integrating both the identification of lipolytic molds and the real role of fungal microflora contaminated fermented and dried cocoa beans by producing lipase activity for promotion of the FFA formation was carried out. This study aimed to improve the quality of fermented and dried cocoa beans regarding the FFA content by the identification of lipolytic molds frequently contaminated this agricultural commodity and could promoted the FFA formation.

2. Materials and methods

2.1. Materials

Fifty fermented and dried cocoa beans samples including twenty peasant samples of 200 g from Akoupé region (6° 23′ N, 3° 54′ W) located in Southeast of Côte d'Ivoire and 30 samples from controlled primary post-harvest processing were collected during October 2018 to isolate to evaluate the FFA content and to identify contaminated molds. Each cocoa beans sample was kept in a sterile biodegradable bag for transportation to the laboratory until analyses.

2.2. Methods

2.2.1. Isolation of molds from cocoa beans

In order to isolate the molds present in the cocoa beans, five fermented and dried cocoa beans from each sample were disinfected by soaking in sodium hypochlorite solution (0.4%) for 2 min [20]. Then, they were aseptically cultured by direct contact technic on the surface of Sabouraud chloramphenicol agar medium in Petri dishes [21]. Cultures were incubated at

25°C for 3-5 days. Molds' isolates were purified by successive sub-culturing onto other Petri dishes containing the same medium.

2.2. Identification of molds' isolates

2.2.1. Polyphasic characterization

The mold isolates from the cocoa bean samples were characterized by cultural and morphological characterization. For morphological identification, molds grown on Sabouraud chloramphénicol medium were checked for macroscopic and microscopic characters as described in appropriate keys [20]. For this purpose, fragment of the colony of each mold isolate was carefully taken from the 7-days old culture and placed fresh state in drop of sterile water between slide and coverslip. Macroscopic characteristics related to shape, appearance, colonies growth rate, production soluble pigments, hyphae colour, spores colour and shape of the colony of each mold isolate were determined by naked eye observation of its cultures on chloramphenicol Sabouraud medium as previously described by Kedjebo et al. [22]. Microscopic characteristics related to hyphae structure, spore-forming organs, vesicle shape, presence or absence metula, shape and mode conidia aggregation were determined [23]. A selection of phenotypically similar isolates were subjected to further identification by a molecular approach [1].

2.2.2. Molecular identification

- **DNA extraction and PCR reaction**

For the extraction and purification of genomic DNA of mold, a portion from the 7-day old culture of each isolate grown on Sabouraud chloramphenicol was used [1]. Total genomic DNA was extracted from this portion and sequenced after the amplification of parts of the ITS, β-tubulin and/or calmodulin genes as to previous protocol described by Houbraken et al. [24] and Samson et al. [20], with some modifications. Genomic DNA quality was checked using electrophoresis and quantification using an ND-1000 spectrophotometer (Thermo

Fisher, Waltham, MA, USA). Polymerase Chain Reaction (PCR) were carried out with pairs of primers ITS4-ITS5 [25] targeting the ITS (Internal Transcription Spacer) region of ribosomal DNA. The PCR reactions were carried out in 50 µL of reaction mixes containing 25 µL of PCR master mix X2 (biotechrabbit™) comprising DNA polymerase (0.625 U per sample), Tris/HCl (30 mM, pH 8.05), KCl (100 mM), each dNTP (400 µM) and $MgCl_2$ (5 mM), 1 µL forward (0.2 µM) and reverse (0.2 µM) primers, 18 µL molecular biology water (Molecular Biology Grade Water, Corning) and 5 µL of 1:10 diluted DNA from different fungal strains. The PCR was carried out with a T100TM thermocycler (Bio-RAD) according to program comprising 10 min of initial denaturation at 94°C followed by 35 cycles consisting of 30 sec of denaturation at 94°C, 30 sec of hybridization at 56° C and 1 min elongation at 72°C followed by final elongation at 72°C for 7 min [22].

● **PCR products sequencing**

The PCR products were sequenced in forward and reverse direction on a Sanger sequencer (Applied biosystems). Obtained sequences were processed using UGene Unipro version 38.1 software via visualization and cleaning of chromatograms and assembly of direct and indirect sequences to obtain a consensus sequence. For the confirmation of species identity, sequences obtained in this study were compared with sequences in the NCBI database and the internal database of the Westerdijk Fungal Biodiversity Institute (WI) [26].

2.2.3. Screening of lipolytic mold isolates

Specific medium contained 10 g of peptone, 3g of yeast extract and 5g of NaCl and 26 g of bacto agar dissolved in 890 mL of distilled water was prepared. Olive oil emulsion (0.1 g. mL-1) prepared as only source of carbon in sterile 2% polyvinyl alcohol (PVA) solution using ultra turax and 10 mL of rhodamine B solution (1 $mg.mL^{-1}$) filterated using a cellulose membrane syringe 0.22 µm were added to sterilized medium (121°C, 15 min) supercooling. Mixture was carefully homogenized and then poured into Petri dishes. Each mold isolate was

cultured on this medium enriched in lipids, as organic nutrients, and incubated at 25°C for from 48 to 96 hours. In event of fungal lipase production, there is a complexation of rhodamine B with fatty acids from olive oil. Final complex is materialized by appearance of orange fluorescent halo around of fungal colony once exposed to UV light at 365 nm [8].

2.3. Effect of lipolytic mold inoculation to cocoa beans on FFA formation.

● Preparation of fungal conidia suspension

Fungal conidia were collected from each lipolytic mold strain grown on chloramphenicol Sabouraud by scraping in sterile water solution. After counting conidia with Thomas slide, final suspensions were calibrated to 10^5 conidia.mL^{-1} for inocula preparation [22].

● Inoculation of fungal conidia to beans inside cocoa pods

Cocoa beans were inoculated by the injection of 1 mL of 10^5 conidia.mL^{-1} inside healthy pods, as previously described by Kedjebo et al. [22]. Negative pod control was treated with sterile distilled water. The inoculated pods were incubated in climate chamber at 25°C, 80% relative humidity for 7 days.

2.4. Determination of FFA contents of cocoa beans

Cocoa beans were dried in oven at 40°C for 5 days to 7-8% moisture. Dried cocoa beans were frozen in liquid nitrogen to be ground (< 500 µm). Ten grams of cocoa powder were placed in Whatman cartridge and then soaked in beaker containing 65 mL of petroleum [22]. The cocoa butter was extracted using Soxtec apparatus at 130°C for 4 h. Approximately 5 g of cocoa butter without solvent was weighed (W) and dissolved in 50 mL of mixture of petroleum ether/absolute ethanol (50:50, v/v) containing phenolphthalein. FFA content of cocoa butter was quantified by ISO 660:2020 method using 0.1 N KOH alcohol solution (V). FFA (% oleic acid) percentage was calculated as previsouly described by Asimah and Sebastian [27]:

$$FFA\ (\%\ oleic\ acid) = \frac{28.2 \times V \times N}{w}$$

2.5. Statistical analysis

Data analysis was performed using SPSS for windows v.17.0 (SPSS® Inc., IL, USA). Means for the FFA content measurements data were calculated and tested for significance by the Students t-test at p = 0.05.

3. Results

3.1. Cultural and morphological identification of the genera of mold isolates

Figures 1-6 show cultural and morphological characteristic of colony and some specific vegetative organs of mold isolates are representative specie of each main genra identified. The identification was based on keys of both macroscopic and microscopic characteristics allow to describ 13 species belonging to 7 fungal genera including *Rhizopus* (Figure 1A-D), *Absidia* and *Mucor* (Figure 1E-F), *Aspergillus* (Figure 2A-L), *Penicillium* (Figure 3A-D), *Cuvularia* (Figure 4A-D), *Nigrospora* (Figure 5A-D) and *Botrytis* (Figure 6A-D). Main cultural charcteristics of all colonies on Sabouraud-chloramphenicol medium showed that they were extensive with fast or moderate growth speed. One hundred fifty four (154) molds were isolated from all fermented and dried cocoa beans samples whatever their post-harvest processing. The examination of these molds showed various characteristics related to the colony and to some specific vegetative organs. Microscopic observations revealed the presence of branched or unbranched hyphes as sporangiophores that constitue mycelia. *Aspergillus* isolates were grouping into 3 sections. Molds' isolates were belonging to the Nigri section presented white or yellow mycelium, black or brown conidia that appeared grainy appearance. Another isolates were grouping into the Fumigati section showed white mycelium with blue conidia. The last mold strains were classifying into Flavi section and were characterized by white or yellow mycelium and green spores. Microscopic examiations showed that molds of *Aspergillus* genus were characterized by their uniseriate or biseriate

conidial head as a smooth hyaline conidiophore which bear spherical or hemispherical vesicle bearing smooth or echinulate globose spores at their end. Mold strain belonging to *Penicillium* genus was distinguished by blue or often green colonies with moderate or slow growth speed. Table 1 resumes some cultural and morphological characteristics of the colonies of fungal microflora contaminating tested fermented and dried cocoa beans samples.

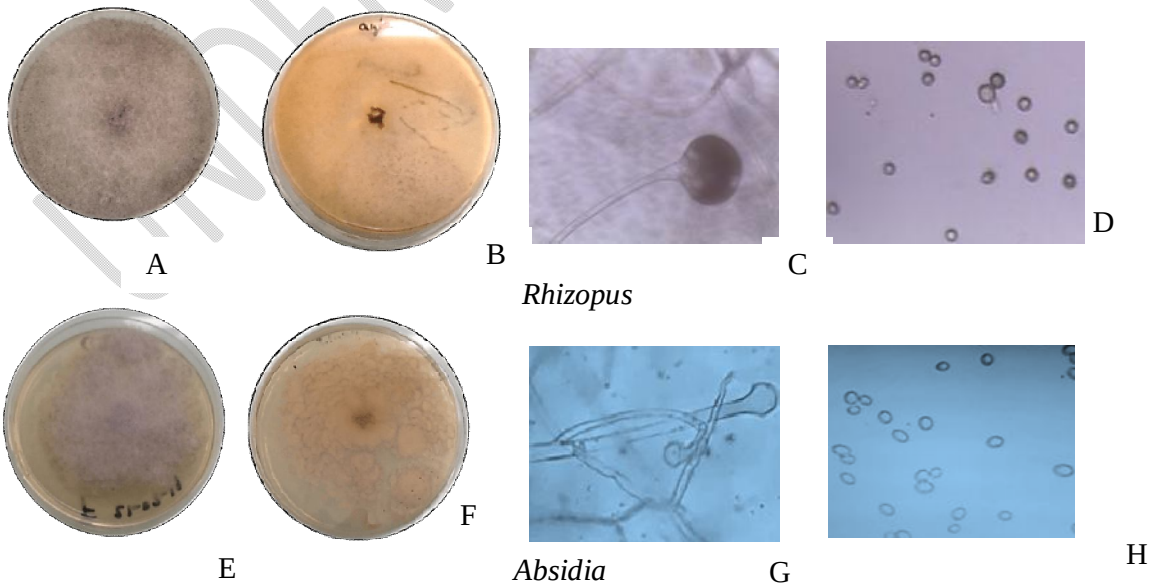
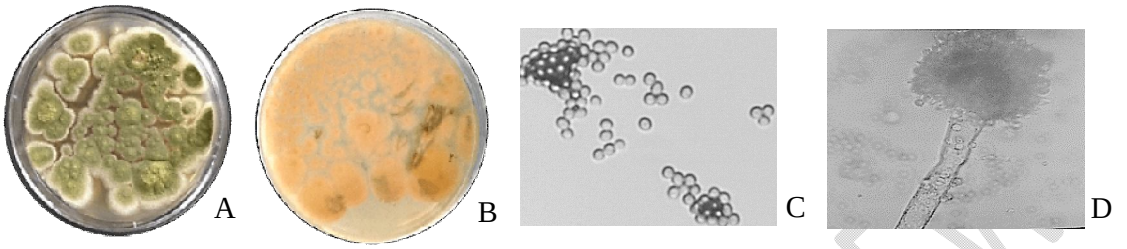


Figure 1. Culture of mould isolates belonging to Mucorales order on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A-E**) Colonies on agar, **B-F**) Reverse side of cultures on the plate, **C-G**) Sporangiophores, **D-H**) Sporangiospores



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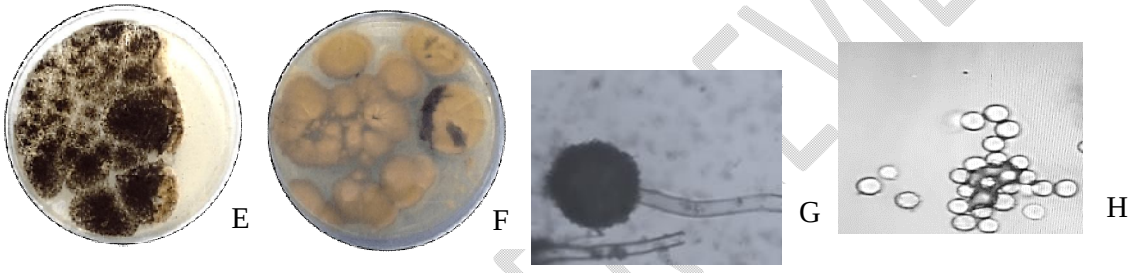

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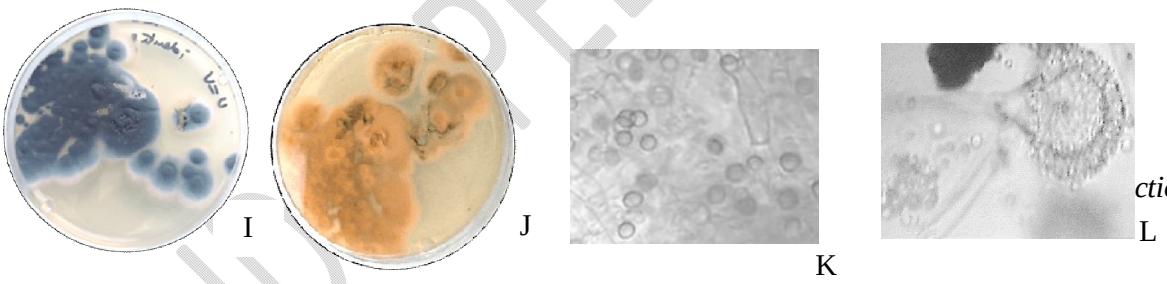


ction Fumigati

figure 2. Culture of mould isolates belonging to *Aspergillus* genus on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A-E-I**) Colonies on agar, **B-F-J**) Reverse side of cultures on the plate, **C-G-K**) Conidiophores, **D-H-L**) Conidiospores.

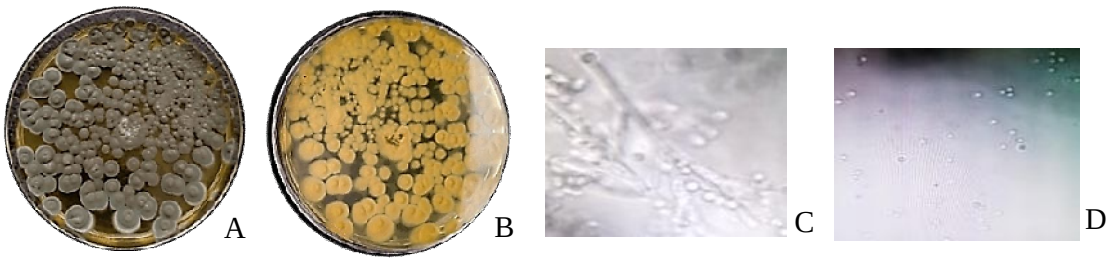


Figure 3. Culture of mould isolates belonging to *Penicillium* genus on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A**) Colonies on agar, **B**) Reverse side of cultures on the plate, **C**) Conidiophores, **D**) Conidiospores.

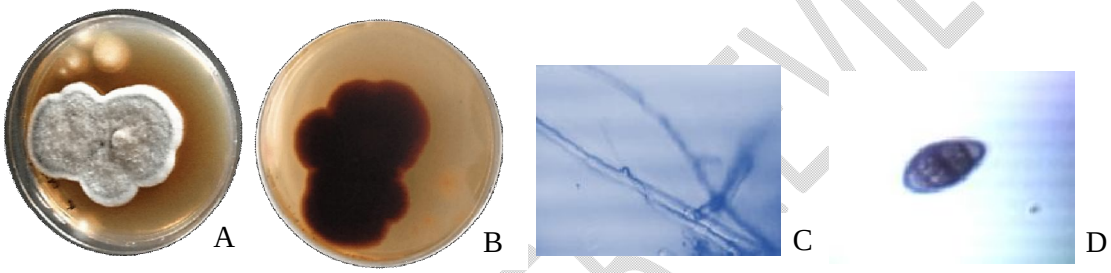


Figure 4. Culture of mould isolates belonging to *Curvularia* genus on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A**) Colonies on agar, **B**) Reverse side of cultures on the plate, **C**) Hyphes, **D**) Scepted spore.

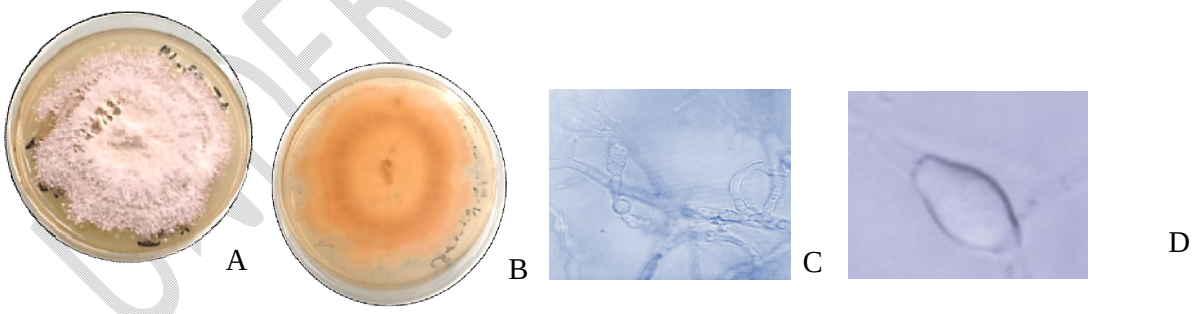
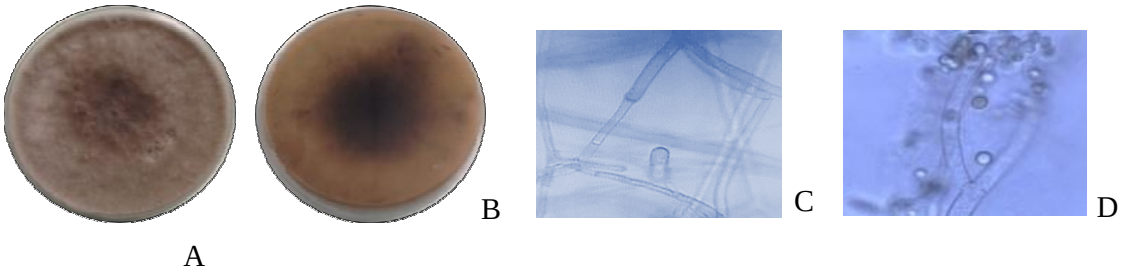


Figure 5. Culture of mould isolates belonging to *Nigrospora* genus on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A**) Colonies on agar, **B**) Reverse side of cultures on the plate, **C**) Hyphes, **D**) Spore.



A B C D

Figure 6. Culture of mould isolates belonging to *Botrytris* genus on Sabouraud-chloramphenicol agar and incubated at 25°C for 5 days. **A**) Colonies on agar, **B**) Reverse side of cultures on the plate, **C**) Hyphes, **D**) Spore.

Table 1. Some cultural and morphological characteristics of some molds isolated from tested fermented and dry cocoa beans samples sourced from Côte d'Ivoire.

Genera	Macroscopic characteristics					Microscopic characteristics		
	Growth speed	**Aspect**	**Hyphae colour s**	**Conidia colour**	**Reverse**	**Hyphae structure**	**Conidia-forming organs**	**Conidia morphology**
Rhyzopus	Fast	Cottony	White and grey	Black	Yellow	Unpartitioned / unbranched	Sporange	Spherical
Absidia	Fast	Cottony	Grey	White	Colourless	Unpartitioned / and branched	Sporange	Spherical
Mucor	Fast	Cottony	Whitish	White	Colourless	Partitioned / branched	Sporange	Spherical
Nigrospora	Slow	Woolly	Dew	White	Orange	Partitioned / branched	-	Ellipsoidal
Botrytis	Fast	Furry	Blackish grey	Black	Brown	Partitioned / branched	-	Ovoid
Cuvularia	Slow	Compact	Grey	White	Black	Unpartitioned	-	Ellipsoidal
Aspergillus (section *Flavi)*	Moderate	Granular	Beige	Green and brown	Yellow	Partitioned / unpartitioned	Vesicle with phialides	Spherical
Aspergillus (section Nigri)	Moderate	Granular	Beige	Brown	Yellow	Partitioned / unpartitioned	Vesicle with metules and phialides	Spherical
*Aspergillus (*section *Fumigati)*	Moderate	Powdery	White	Blue	Yellow	Unpartitioned	Hemispheric vesicle	Spherical
Penicillium	Very slow	Powdery	White	Blue-green	Yellow	Partitioned	Brush shape whorled	Spherical

3.2. Lipolytic molds isolates from cocoa beans

Table 2 shows the ability of molds of each genus isolated from tested fermented and dried cocoa bean samples for production of lipases enzyme. A total of 12 molds strains of 9 genera have exhibited ability for lipolytic activity production: 2 strains of mucorales (*Rhizopus. oryzae, Mucor heimalis* and *Abisidia corymbifera*), 6 *Aspergilli* (*A. niger, A. tubingensis, A. flavus; A. tamari, A. fumigatus and A. aculeatus*), *N. sphaerica, C. geniculate, and P. chrysogenum.* Members of Mucorales appeared to be strongly lipolytic followed the members of *Aspergillus* while *Botrytis* sp. was not able to produce extracellular lipase.

Table 2. Highlighting of lipolytic properties of some mold strains isolated from fermented and dry cocoa beans samples sourced from Côte d'Ivoire.

Genus of fungal moulds	Lipolytic enzyme production	Estimated intensity of lipolytic activity level
Mucorales	yes	++++
Aspergillus	yes	+++
Nigrospora	yes	++
Curvularia	yes	+
Penicillium	yes	+
Botrytis	not	-

3.3. Identification of mold isolates species

Only mold isolates exhibited ability for production of lipase activity were identifying by use molecular tools. Table 3 indicates 12 mold species identified including *Rhizopus oryzae, A. corymbifera, Mucor hiemalis,* Aspergillus *flavus, A. tamari, A. niger, A. aculeatus, A. tubingensis, A. fumigatus, Nigrospora sphaerica, Cuvularia geniculata, Penicillium chrysogenum* with significant alignments of 99 to 100% with the isolated fungal species. Table 4 shows the incidence of each strain among these 12 lipolytic molds isolates: *Absidia sp*. was the most predominantly and frequently strain detected in cocoa beans samples, followed by *Aspergilli* and *Rhizopus* species whereas the species of *Curvularia, Penicillium*

and *Nigrospora* were the least frequent and abundant molds isolates found in our cocoa beans samples.

Table 3. Identity of lipolytic fungal mold strains isolated from fermented and dry cocoa beans samples sourced from Côte d'Ivoire.

Code of fungal molds' isolates	Nc *	% of homology **	Matching species
I	565	100	*Rhyzopus oryzae*
II	561	100	*Absidia Corymbifera*
III	551	100	*Mucor hiemalis*
IV	485	100	*Aspergillus flavus*
V	492	99.9	*A. tamarii*
VI	506	100	*A. niger*
VII	504	100	*A. s aculeatus*
VIII	504	99.8	*A. tubingensis*
IX	503	100	*A. fumigatus*
X	406	100	*Nigrospora sphaerica*
XI	517	99.9	*Cuvularia geniculata*
XII	521	100	*Penicillium chrysogenum*

* number of nucleotides compared

** percentage of sequence homology

Table 4. Incidence and dominance of lipolytic molds isolates from fermented and dry cocoa beans samples sourced from Côte d'Ivoire.

Fungal isolates	Frequency (%)	Predominance (%)
R. oryzae	24	9.38
A. corymbifera	50	19.53
M. heimalis	10	3.91
N. sphaerica	12	4.69
C. geniculata	8	3.13
Aspergillus flavus	14	5.47
A. tamari	10	3.91
A. niger	46	17.97
A. aculeatus	20	7.81
A. tubingensis	14	5.47
A. fumigatus	34	13.28
P. chrysogenum	12	4.69

3.4. FFA content of inoculated cocoa beans with lipolytic mold isolates

Figure 7 illustrates the changes in FFA content of cocoa beans samples inoculated with lipolytic molds according each isolate incubated at 25 °C for 7-days. Cocoa beans inoculated with *R. oryzae* recorded the highest FFA content of 2.05%. FFA content found in those inoculated with *A. corymbifera* showed FFA content of 1.57% while cocoa beans inoculated with *A. niger* exhibited FFA level of 1.41%.Inoculation cocoa beans with *A. fumigatus* led to FFA content of 1.27%. The inoculation of *A. flavus* or *A. tubingensis* to cocoa beans did not promote the FFA formation due to the lowest levels of FFA content below 1%.

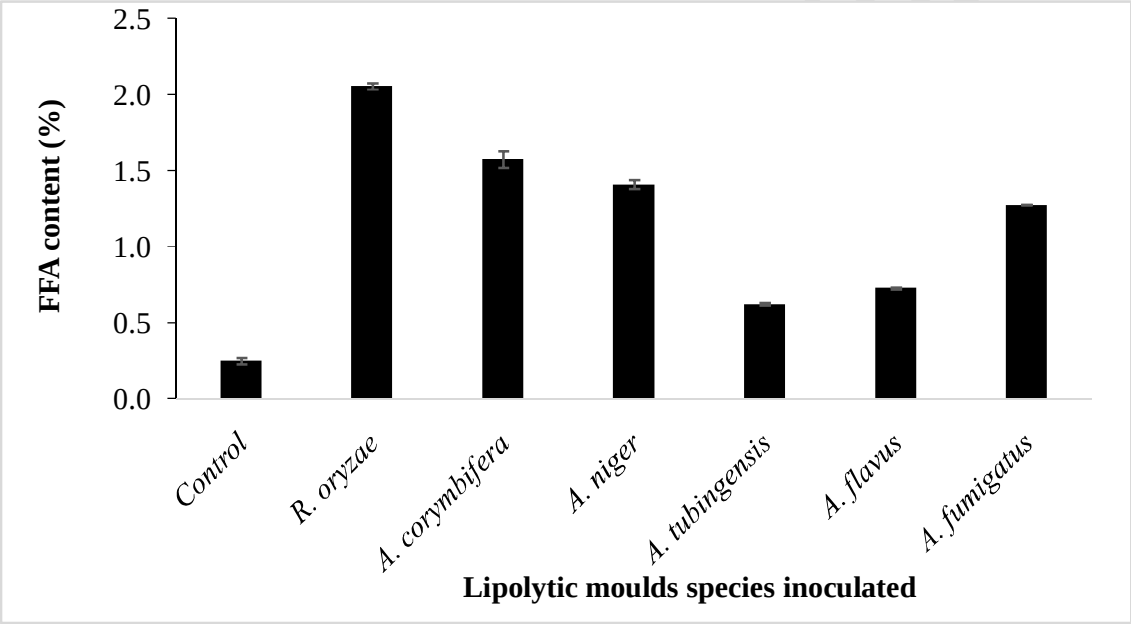


Figure 7. Overall influence of inoculation of lipolytic mold species to fresh cocoa beans inside pods on the FFA formation during incubation at ambient temperature for 7 days.

4. Discussion

A total of 154 fungal isolates spanning 6 genera were recovered from the 50 samples collected during the cocoa processing and the sampling of peasant cocoa beans. These genera are reported to be described elsewhere. Indeed, the diversity of molds detected in this research were in agreement with those found by previous studies related to the mycobiota

contaminated fermented cocoa beans samples and other agricultural commodities [28, 29, 30], except for three genera such as Nigrospora, Curvularia and Botrytiswhich. Among these isolates, 12 lipolytic species spanning 5 genera were identified : *R. orysae, A. corymbifera, M. heimalis, A. niger, A. tubingensis, A. aculeatus*; *A. fumigatus*, A. *flavus, A. tamari, P. chrysogenum, C. geniculata* and *N. sphaerica*. Our results are in agreement with those found previously by Serra et al. [31]. Regarding to the incidence these isolates could be classified in to 2 groups: one of the isolates with high incidence and the second group was constitued by those with low incidence.The incidence of the species were *Mucorales* such *Absidia* and *Rhizopus* (50% <) followed by the members of *Aspergilli*. The second group was constituted of members of *Curvularia, Nigrosporia* and *Penicilium* genera that hace a least incidence. In both fermented and dried cocoa, more than one half of the molds isolates were from peasant cocoa beans while controlled fresh fermented and dried cocoa beans are less contaminated by fungi. Molds species of Mucorales order dominated the other genera as previously shown by Guehi et al. [8]. However, our results are in disagreement with those previously found in other cocoa producing countries such as Sierra Leone, Equatorial Guinea and Ecuador where the predominance of molds of *Aspergillus* genera was reported by Sánchez–Hervas et al. [32]. The non-recovery of *A. ochraceus* or other ochratoxin-producing species from the tested fermented and dried cocoa beans samples and aflatoxin-producing species from dried agricultural commobities was in aggreement with the results founded by Adebo et al. [28] and Akinfala et al. [1]. However, these results differed from many other previous studies [30;32]. These disparities in the occurence of mold may be ascribed to various abiotic factors such as primary post-harvest processing, moisture level which was currentely above the recommended values (7-8%) for the peasant cocoa beans samples [6], local microclimat in each cocoa producing region during the sampling of fermendted and dried cocoa beans. The influence of biotic factors including interactions between filamentous fungi strains due to to

the resilience of certain mold species growing in minimal conditions [33] and to the competitiveness for the space [34;35], for the access to the nutrients such as lipid matrix of cocoa beans [8] play essential role in the presence of certain strains. The filamentous fungi species isolated in present work sourced from environment (air, soil, shelling tools and fermentation) and could contaminate cocoa beans at different steps of primary post-harvest processing [11;36]. The contamination of fermented and dry cocoa beans by molds, as it reduces the quality and may produce mycotoxins which consumption that lead poisoning and death of consumers of cocoa end-products such as chocolate [37]. Among mold isolates found in tested fermented and dry cocoa beans samples, some fungal species such as *Aspergillus niger* contamination could produce mycotoxins, such as ochratoxin A [38;39]. The presence of *A. niger* could increased the microbita of the fungal strains and the OTA contents of fermented and dry cocoa beans [37]. Fortunately, use of some yeasts, lactic and acetic bacteria strains as starter culture could inhibit both the mycelial growth of fungu species and OTA production during cocoa fermentation process [37;40]. Otherwiew, according to Copetti et al. [41], the accumulation OTA in fermented and dry cocoa beans could also be suppressed by the increasing of organic acids contents during fermentation. And the finale dry cocoa beans produced from such as batches are of a good quality and are safe [37]. The results of the tests related tot the ability of the molds isolates from cocoa beans highlighted the extracellular lipase production by all species belonging to all genera found except *Botrytis*. Among these molds, all species belonging to Mucorales order such *Rhizopus*, *Absidia* and *Mucor* genera exhibited the strongest abilties for lipase production as previously described by various studies [42 ;43]. Our results related to the capacity of various mold species belonged to *Aspergillus* for the extracellular lipase production are in agreement with those founded by Colla et al. [44]. *A. niger* [45] and *A. flavus* [46] were reported to be greater producer of the extracellular lipase. The accumulation and the increase of FFA content inside inoculated

cocoa beans are due to the production of extracellular lipase by these mold isolates. The most excessive FFA concentration found from each inoculated fermented and dry cocoa beans with *Rhizopus oryzea, Abisidia corombifera, Aspergillus tubingensis* or *A. fumigatus* confirms that these molds have strong lipase producing ability as previously demonstrated by Guehi et al., [8]. So the presence of these fungi species among microbiota contamiated cocoa beans considerably promote the FFA's occurrence. The combination of high mold contamination level favoured by fungal diseases [19], inadequate primary post-harvest processing such long storage time of the pods prior opening [2] and sanitary condition of beans [6] could promote the formation of FFA and cause excessive FFA content in cocoa beans. In addition, Jonfia-Essien and Tettey [47] have concluded that cocoa beans fermentation duration would be considered to have critically promote the FFA formation because of the cocoa beans harbor fungi during fermentation [11].

Conclusion

The objective of this study is to contribute to the improvement of cocoa beans quality regarding the FFA content by the identification of molds able to produce extracellular lipase and cause the accumulation of FFA in the fermented and dried cocoa beans. We found that molds contaminating fermented and dried cocoa beans included 13 species belonging to 8 genera: *Rhizopus, Absidia, Mucor, Nigrospora, Curvularia, Aspergillus, Penicillium* and *Botrytis*. The incidence of fungi belongind to Mucorales order and Aspergillus genus in cocoa beans are higher than other mold isolates. All mold isolates were reported to produce extracellular lipase except *Botrytis* genus. Fungi species including *A. corymbifera*, R. *orysae, A. niger* and *A. tubingensis* exhibited greatest lipolytic properties. Individual inoculation *R. oryzae, A. corymbifera* or *A. niger* to cocoa beans lead to produce highest FFA concetrations. This study indicated that the reduction of lipolytic fungi growth in cocoa beans could reduce the occurence of FFA and sustainably improve the quality of cocoa raw material.

REFERENCES

[1] Akinfala TO, Houbraken J, Sulyok M, Adedeji AR, Odebode AC, Krska R, Ezekiel CN. Molds and their secondary metabolites associated with the fermentation and storage of two cocoa bean hybrids in Nigeria. International Journal of Food Microbiology, 2020 ;316 :108490. https://doi.org/10.1016/j.ijfoodmicro.2019.108490

[2] Afoakwa EO, Budu AS, Mensah-Brown H, Takrama JF, Akomanyi E. Changes in biochemical and physico-chemical qualities during drying of pulp preconditioned and fermented Cocoa (Theobroma cacao) beans. Journal of Nutritional Health & Food Science, 2014;2(1):1-8.

[3] Servent A, Boulanger R, Davrieux F, Pinot M, Tardand E, Forestier-Chirona N et al. (2018). Assessment of cocoa (Theobroma cacao L.) butter content and composition throughout fermentations. Food Research International, 2018;107:675-682. https://doi.org/10.1016/j.foodres.2018.02.070

[4] Zzaman W, Issara U, Febrianto NF, Yang TA. Fatty acid composition, rheological properties and crystal formation of rambutan fat and cocoa butter. International of Food Research Journal, 2014 ;21(3):983-987.

[5] Hu M, Jacobsen C. Oxidative stability and shelf life of foods containing oils and fats. American oil chemists' society Press, 2016

[6] Guehi ST, Dingkuhn M, Cros E, Fourny G, Ratomahenina R, Moulin G et al. Impact of cocoa processing technologies in free fatty acids formation in stored raw cocoa beans. African Journal of Agricultural Research, 2008;3(3):174-179.

[7] European Union. Directive 2000/36/EC of the European Parliament and of the Council of 23 June 2000 relating to cocoa and chocolate products intended for human consumption. Official Journal L197, 03/08/2000 2000;19–25.

[8] Guehi TS, Dingkuhn M, Cros E, Fourny G, Ratomahenina R, Moulin G et al. (2007). Identification and lipase-producing abilities of molds isolated from Ivorian raw cocoa beans. Research Journal of Agriculture and Biological Sciences, 2007;3(6):838-843.

[9] Müller M, Careglio E. Influence of Free Fatty Acids as Additives on the Crystallization Kinetics of Cocoa Butter. Journal of Food Research, 2018;7(5):85-97. https://doi.org/10.5539/jfr.v7n5p86

[10] Briones V, Aguilera JM. Image analysis of changes in surface color of chocolate. Food Research International, 2005;38(1):87-94. https://doi.org/10.1016/j.foodres.2004.09.002

[11] Delgado-Ospina J, Molina-Hernández JB, Chaves-López C, Romanazzi G, Paparella A. The role of fungi in the cocoa production chain and the challenge of climate change. Journal of Fungi, 2021;7(3):202. https://doi.org/10.3390/jof7030202

[12] Dano SD, Manda P, Dembélé A, Abla AM-JK, Bibaud JH, Gouet JZ et al. Influence of fermentation and drying materials on the contamination of cocoa beans by ochratoxin A. Toxins, 2013;5(12):2310-2323. https://doi.org/10.3390/toxins5122310

[13] Santander 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, 2020;60(10):1593-1613. https://doi.org/10.1080/10408398.2019.1581726

[14] Chaves-López C, Serio A, Grande-Tovar CD, Cuervo-Mulet R, Delgado-Ospina J, Paparella A. Traditional Fermented Foods and Beverages from a Microbiological and Nutritional Perspective: The Colombian Heritage. Compr. Review. Food Science and Food Safety, 2014;13(5):1031–1048. https://doi.org/10.1111/1541-4337.12098

[15] Schwan RF, Wheals AE. The microbiology of cocoa fermentation and its role in chocolate quality. Critical Review in Food Science and Nutrition, 2004;44(4) :205-221. https://doi.org/10.1080/10408690490464104

[16] Copetti MV, Iamanaka BT, Nester MA, Efraim P, Taniwaki MH (2013). Occurrence of ochratoxin A in cocoa by-products and determination of its reduction during chocolate manufacture. Food Chemistry, 2013 ;136(1) :100-104. https://doi.org/10.1016/j.foodchem.2012.07.093

[17] Romanens E, Freimüller Leischtfeld S, Volland A, Steven M, Krähenmann U, Isele D, et al. Screening of lactic acid bacteria and yeast strains to select adapted anti-fungal co-cultures for cocoa bean fermentation. International Journal of Food Microbiology, 2019;290:262–272. https://doi.org/10.1016/j.ijfoodmicro.2018.10.001

[18] Mounjouenpou P, Gueule D, Fontana-Tachon A, Guyot B, Tondje PR, Guiraud J-P. Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon. International Journal of Food Microbiology, 2008;121(2):234–241. https://doi.org/10.1016/j.ijfoodmicro.2007.11.017

[19] Robert A, Boniface W, Humphery G. Effect of reconditioning of discrepant cocoa on the quality of cocoa beans. Food Science and Nutrition Technology, 2018;3:000165. https://doi.org/10.23880/fsnt-16000165

[20] Samson RA, Houbraken J, Frisvad JC, Thrane U, Andersen B. Food and Indoor Fungi. CBS Laboratory Manual Series no. 2, 390p, 2010.

[21] Panchal VH, Dhale DA. Isolation of seed-borne fungi of sorghum (Sorghum vulgare pers.). Journal of Phytology, 2011;3(12).

[22] Kedjebo KBD, Guehi TS, Kouakou B, Durand N, Aguilar P, Fontana A et al. Effect of post-harvest treatments on the occurrence of ochratoxin A in raw cocoa beans. Food Additives & Contaminants : Part A, 2016;33(1):157-166. https://doi.org/10.1080/19440049.2015.1112038

[23] Pitt JI, Hocking AD. Fungi and Food Spoilage, 3rd ed. Springer US: New York, USA, Pp. 1–519., 2009.

[24] Houbraken J, Frisvad JC, Seifert KA, Overy DP, Tuthill DM, Valdez JG et al. New penicillin-producing Penicillium species and an overview of section Chrysogena. Persoonia-Molecular Phylogeny and Evolution of Fungi, 2012;29(1):78-100. https://doi.org/10.3767/003158512X660571

[25] White TJ, Bruns T, Lee SJWT, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications, 1990;18(1):315-322.

[26] Javadi MA, Ghanbary MAT, Tazick Z. Isolation and molecular identification of soil inhabitant. Penicillia. Ann of Biol Res., 2012;3(12):5758-5761.

[27] Asimah H, Sebastian AL. Quality and weight changes in cocoa beans stored under two warehouses' conditions in East Malaysia. The planter, Kuala Lumpur, 2000;76(895):619-637.

[28] Adebo OA, Njobeh PB, Gbashi S, Nwinyi OC, Mavumengwana V. Review on microbial degradation of aflatoxins. Critical reviews in food science and nutrition, 2017;57(15):3208-3217. https://doi.org/10.1080/10408398.2015.1106440

[29] Copetti MV, Iamanaka BT, Pitt JI, Taniwaki MH. Fungi and mycotoxins in cocoa: From farm to chocolate. International Journal of Food Microbiology, 2014;178:13–20. https://doi.org/10.1016/j.ijfoodmicro.2014.02.023

[30] Fapohunda SO, Moore GG, Aroyeun SO, Ayeni KI, Aduroja DE, Odetunde SK. Isolation and characterization of fungi isolated from Nigerian cocoa samples. Curr. Life Sci., 2018 ;4(3):46–52. http://dx.doi.org/10.5281/zenodo.1405083

[31] Serra JL, Moura FG, Pereira GVM, Soccol CR, Rogez H, Darnet S. Determination of the microbial community in Amazonian cocoa bean fermentation by Illumina-based metagenomic sequencing. LWT - Food Sci and Technol, 2019;106:229–239. https://doi.org/10.1016/j.lwt.2019.02.038

[32] Sánchez-Hervás M, Gil JV, Bisbal F, Ramón D, Martínez-Culebras PV. Mycobiota and mycotoxin producing fungi from cocoa beans. International Journal of Food Microbiology, 2008;125(3):336-340. https://doi.org/10.1016/j.ijfoodmicro.2008.04.021

[33] Frisvad JC, Andersen B, Samson RA. Association of moulds to foods. In Food Mycology. CRC Press, pp. 213-254, 2007.

[34] Cvetnić Z, Pepeljnjak S. Interaction between certain moulds and aflatoxin B1 producer Aspergillus flavus NRRL 3251. Arhiv za higijenu rada i toksikologiju, 2007;58(4):429-434. https://doi.org/10.2478/v10004-007-0036-0

[35] Magan N, Hope R, Cairns V, Aldred D. Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. In : Epidemiology of mycotoxin producing fungi. Springer, Dordrecht, pp. 723-730, 2003.

[36] Ruggirello M, Nucera D, Cannoni M, Peraino A, Rosso F, Fontana M et al. Antifungal activity of yeasts and lactic acid bacteria isolated from cocoa bean fermentations. Food Research International, 2019 ;115:519-525. https://doi.org/10.1016/j.foodres.2018.10.002

[37] Rahayu ES, Triyadi R, Khusna RN, Djaafar TF, Utami T, Marwati T et al. Indigenous Yeast, Lactic Acid Bacteria, and Acetic Acid Bacteria from Cocoa Bean Fermentation in Indonesia Can Inhibit Fungal-Growth-Producing Mycotoxins. Fermentation, 2021;7(3):192. https://doi.org/10.3390/fermentation7030192

[38] Alves da Silva S, Pereira, RGFA, de Azevedo Lira N, Micotti da Glória E, Chalfoun SM, Batista LR. Fungi associated to beans infested with coffee berry borer and the risk of ochratoxin A. Food Control, 2020 ;113:107204. https://doi.org/10.1016/j.foodcont.2020.107204

[39] Cabañes FJ, Bragulat MR. Black aspergilli and ochratoxin A-producing species in foods. Curr. Opinion in Food Science, 2018;23:1–10. https://doi.org/10.1016/j.cofs.2018.01.006

[40] Sukmawati D, Arman Z, Hasanah R, Balqis M, Setiawan A, Tafrijiyyah F et al. Application of yeasts isolated from fermented cocoa beans for biocontrol of pathogenic mold in chocolate fruit. Journal of Physics: Conference Series, 2021;1869(1) :012042. IOP Publishing.

[41] Copetti MV, Iamanaka BT, Mororó RC, Pereira JL, Frisvad JC, Taniwaki MH. The effect of cocoa fermentation and weak organic acids on growth and ochratoxin A production by Aspergillus species. International Journal of Food Microbiology, 2012;155(3):158-164. https://doi.org/10.1016/j.ijfoodmicro.2012.01.026

[42] Helal SE, Abdelhady HM, Abou-Taleb KA, Hassan MG, Amer MM. Lipase from Rhizopus oryzae R1: in-depth characterization, immobilization, and evaluation in biodiesel production. Journal of Genetic Engineering and Biotechnology, 2021;19(1):1-13. https://doi.org/10.1186/s43141-020-00094-y

[43] Putranto RA, Budiani A. Isolasi fragmen gen LIPASE dari kapang Absidia corymbifera, Rhizopus oryzae dan Rhizopus oligosporus Isolation of LIPASE gene fragment from Absidia corymbifera, Rhizopus oryzae and Rhizopus oligosporus fungi. Menara Perkebunan, 2009 ;77(1). 10.22302/ppbbi.jur.mp.v77i1.112

[44] Colla LM, Ficanha AMM, Rizzardi J, Bertolin TE, Reinehr CO, Costa JAV. Production and Characterization of Lipases by Two New Isolates of Aspergillus through Solid-State and Submerged Fermentation. BioMed Resear. Int. 2015. https://doi.org/10.1155/2015/725959

[45] Colla E, Santos LO, Deamici K, Magagnin G, Vendruscolo M, Costa JAV. Simultaneous production of amyloglucosidase and exo-polygalacturonase by Aspergillus niger in a rotating drum reactor. Applied biochemistry and biotechnology, 2017;181:627-637. https://doi.org/10.1007/s12010-016-2237-y

[46] Ezema BO, Omeje KO, Bill RM, Goddard AD, Eze SO, Fernandez-Castane A. Bioinformatic characterization of a triacylglycerol lipase produced by Aspergillus flavus

isolated from the decaying seed of Cucumeropsis mannii. Journal of Biomolecular Structure and Dynamics, 2023;41(6):2587-2601. https://doi.org/10.1080/07391102.2022.2035821

[47] Jonfia-Essien WA, Tettey E. Fat content and free fatty acid level of cocoa beans (Theobroma cocoa) relative to fermentation and storage periods. In S. Navarro, D.S. Jayas & K. Alagusundaram (eds.), Proceedings of the 10th International Conference on Controlled Atmosphere and Fumigation in Stored Products (CAF2016). CAF Permanent Committee Secretariat, Winnipeg, Canada, pp. 58–61; 2016