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Research Article

Link between the Reduction of Ochratoxin A and Free Fatty Acids Production in Cocoa Beans Using *Bacillus* sp. and the Sensory Perception of Chocolate Produced There of

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Abstract

Ochratoxin A (OTA) is a mycotoxin produced by fungi species causing adverse human and animals health effects. Free fatty acids (FFA) are carboxilic acids from hydrolysis of triacylglycerides of cocoa butter due to lipolytic molds acitivity. This study investigated the ability of two *Bacillus* species to control both the OTA and FFA in dry fermented cocoa beans by inhibiting mold growth. Fourteen *Bacillus* species isolated from both fermented African locust seeds (9) and cocoa beans (5) were identified using molecular tools. Antifungal activity of *Bacillus species* against the growth of a OTA producing *Aspergillus carbonarius* strain were highlighted. The suspensions of *Bacillus strains* cells adjusted to 108 CFU.mL-1 were separately inoculated to 80 kg of fresh cocoa beans from 0-, 4-, 7- and 10-days cocoa pods opening delays. Cocoa fermentation lasted 6] days with two turnings at 48 and 96] hours of the process. Fermented cocoa beans were sun-dried until to 7-8% moisture. FFA and OTA of cocoa beans were quantified by Association of Officical Analytical Chemists (AOAC) and High-performance liquid chromatography with fluorescence detection (HPLC-FD) methods respectively. Main results highlighted that Bacillus halotolerans and B. thuringiensis strain ATCC 10792 were greater antifungal than *B. paramycoides, B. subtilis Subsp subtilis*, *B. subtilis, B. albus, B. thuringiensis* strain IAM 12077. Their inoculation to cocoa beans during spontaenously fermentation totally removed OTA and strongly reduced FFA content. This study indicates that *Bacillus* species could be applied as promising biological agents to improve the microbiological quality, to removal of OTA from dry fermented cocoa beans and to reduce their FFA content.

Keywords: Cocoa Beans; *Bacillus*; Bio-Decontamination; Bio-Detoxifcation; Ochratoxin A; Free Fatty Acids

Abbreviations

OTA: Ochratoxin A; FFA: Free Fatty Acids

Introduction

Cocoa is an important cash crop in many tropical countries [1]. Cocoa beans are mainly consumed as chocolates and widely used in beverages, cosmetics and pharmaceuticals [2]. The quality of cocoa beans is highly dependent on post-haverst technologies processing and particulalrly on the conditions of the storage [3]. Moreover, poor practices of harvest and post-harvest treatments are conducted without sanitary control leading to the amplification of the risk of fungal infections [4]. Contamination by different fungal species during these stages is common and may alter its quality [5]. Development of fungi inside the cocoa beans is a very serious defect [6]. Mold defects are normally irreversible because the fungi grow in the nib of the cocoa beans and produce enzymes (lipases) which increase or facilitate the production of free fatty acids (FFA) in the cocoa beans [7]. Fungi such as *Absidia corymbifera*, *Rhizopus oryzae*, *Penicillium chrysogenum*, *Aspergillus niger*, *A. flavus*, and *A. tamarii* have been reported from cocoa beans with high FFA content [8]. Although FFA play an extremely positive in the immune defense of the human body [9], their generation at the evel about 1.75% degrades beneficial rheological properties and produces rancidity of cocoa butter and chocolate [10]. High FFA content could be due to various factors including high values of RH, level of ripeness of cocoa pods, post-harvest processing on-farm

level and microbial lipolytic activity [8]. Moreover, cocoa is one of major commodities frequently contaminated with ochratoxin A [11]. Ochratoxin A has been reported to be a dangerous, nephrotoxic, and carcinogenic mycotoxin [12]. The main ochratoxin A (OTA) producing molds contaminated cocoa beans sourced from African countries belong to *Aspergillus* (*Aspergillus carbonarius* and *A. niger aggregate*) and *Penicillium* genera [13]. It is well known that defects and anomalies in cocoa pods strongly influence the physicochemical characteristics and occurrence of OTA in the by-products [14]. Contamination by spores that can generate OTA occurs mainly during the cocoa pod's opening process that is done inside the crop without any protection [4]. The presence of this mytoxin in cocoa beans and chocolate products is emerging an important public health issue [15]. Since several years, cocoa beans sourced from Côte d'Ivoire more and more suffered from high FFA [16] and OTA [17] contents. So many stages at risk of contamination make the improvement of the practices, in cocoa processing, difficult to implement satisfactorily [18]. Different strategies including chemicals fungicide application are used in order to reduce the impact of fungi in both food and feed chains [19]. The development of fungicide resistance in many fungal pathogens as well as rising of public concern on the risks associated with pesticide use led to the search for alternative environmentally friendly methods [20]. Biological control is an environmentally-friendly alternative to chemicals fungicides and it is an attractive method protecting from pathogens [21]. Protection of agriculture products against their fungal enemies by using bacterial antagonists is recently became an attractive alternative approach to facilitate sustainable agriculture in many countries [22]. Among different biocontrol agents used worldwide, bacteria from *Bacillus* genus are ideal candidates. Several studies also confirm that *Bacillus* species have exceptional abilities to eliminate various toxins [23]. *Bacillus* species show broad-spectrum antimicrobial activity and have been widely used as agricultural biocontrol agents. In addition, *Bacillus* are part of the major groups of microorganisms involved in cocoa fermentation besides yeast, acetic acid bacteria (AAB) and lactic acid bacteria (LAB) [24]. This paper aimed to determine the effect of some *Bacillus* strains from fermentation of *Parkia biglobosa* seeds on fungal growth, OTA and FFA content in dry fermented cocoa beans, as well as their impact on final chocolate.

Materials and Methods

beans

Cocoa beans from the cultivar ripe pods Amelonado, Ivorian first generation of hybrids (Forastero) and open pollinated progenies were extrated for cocoa pods harvested from farmer plantations in October 2020. 10210 ripe cocoa pods were manually harvested and divided in 2 batches, one of which consisted of healthy cocoa pods and the other of damaged cocoa pods. The pods were opened using a piece of wood billet as a bludgeon Distal portion of the pod falls away and the beans remain attached to the placenta from which they can be easily extracted. The beans were removed carefully by hand from placenta to exclude any germinated or mouldy beans, or pieces of shell [25]. Cocoa pods were stored for 0, 4, 7 and 10 days after harvest at room temperature on the farm level. After harvesting, healthy cocoa pods were immediately opened, while the damaged pods were opened after 4, 7 and 10 days. Just after cocoa pod opening, approximately 1225 kg of fresh cocoa beans surrounded by mucilage were obtained. This mass of fresh beans was divided in 49 batches of 25 kg per batch for the micro-fermentation. The fresh cocoa beans surrounded with pulp were immediately transferred to plastic box measuring 50×50×50 cm^3 [26] for the fermentation. Each plastic box had holes to facilitate the drainage of acidic liquid from liquefaction of mucilaginous pulp. The inner of each plastic box was compltely covered with plantain leaves. Spontaneous (control) and inoculated cocoa beans fermentations were carried out *in situ* on the farm level. Controlled fermentations were performed after inoculation of 200 mL of cellular suepension contening 10^8 $CFU.mL^{-1}$ per tested Bacillus isolate. Inoculation of cocoa beans with *Bacillus* isolate was carried out at 0 days for certain batches and for tother at 4 days of fermentation respectively. The plastic box were raised above ground level over a drain that carries away the pulp juices liberated from the degradation of the cocoa bean mucilageînous pulp. The heap of fresh cocoa beans was then covered in the box with other fresh banana leaves to insulate the top of the box before placing the cover. The fermenting cocoa beans were aseptically mixed every 48 h. The fermentations were terminated at day 6 and fermented cocoa beans were sun-dried on the racks. Cocoa beans samples (2 kg) were taken from each cocoa fermented heaps for microbiological analysis were used immediately while those for chemical analysis were stored at –20 °C until examined. All fermentations were performed in duplicate.

Starter cultures

Isolation of Bacillus strains

Several Bacillus strains isolated from *Parkia biglobosa* seeds fermented were used in this study. 10g of fermented African locust bean seeds and cocoa beans were separately suspended in 90 ml of sterile a 0.9% NaCl solution. Each suspension was heated at 80°C for ten minutes in a shaking water-bath to kill non-spore forming organisms. Serial dilutions were transferred to Nutrient Agar (NA) plates and incubated aerobically at 30°C for 24 hours (h). The inoculated plates were examined for the appearance of sub-cultured colonies on new NA plates. Presomptive colonies of Bacillus species exhibiting cultural characteristics i.e. round or irregular; thick and opaque; cream-colored colonies were identified [27]. Bacillus isolates were grown on Plat Count agar medium (PCA) at 30°C for 24 h until colonies developed and then were stored in Cryobank™ tube for storage in a suitable freezer at -80˚C for further analysis.

Molecular identification of *Bacillus* isolates

DNA extraction

Bacterial DNA was extracted by CTAB-NaCl (10 mM tris, 20 mM EDTA, 1.4M NaCl, 0.2% mercaptoethanol in 2% CTAB solution pH 8.0) procedure according to the modified method [28]. Collected living cells from an overnight grown bacterial culture were pelleted by centrifugation (13,000 rpm for 2 min at 4 °C), washed twice with TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0) and

resuspended in 250 µl of TE buffer containing 20% SDS and 20 mg.ml^{-1} of proteinase K. The final suspension were incubated at 6]5°C for 1 hour and the cells were lysed by adding 100 µl of NaCl, 5 M and 400 µl of CTAB-NaCl solution. At 10 min of incubation, the suspended proteins were avoided by addition of equal volume of mixture of Phenol, Choloroform and Isoamyl alcohol (25:24:1) and then centrifuged (13,000 rpm for 10 min). The DNA was precipitated by addition of two volumes of ice isopropanol preceded with 70% ethanol wash. The air dry pellet was suspended in 50 µl of TE buffer. The DNA extracts were quantified using a nanodrop (ND-1000, Thermoscientific, Labtech) adjusted at a concentration about 20 ng.µL^{-1}. The residual RNA was eliminated by RNase treatment at 30°C for 30 min.

Amplification of 16 S rDNA genes of *Bacillus* isolates

The 16S rDNA gene of *Bacillus* isolates was amplified with the primers 799f (AACMGGATTAGA TACCCKG) and 1492r (GTTACCTT-GTTACGACTT) (Eurofins genomic) as previously described [29]. PCR process was carried out in a reaction volume of 25 µL containing 12.5 µL PCR Master Mix 2x Kit, 9 µL deionized water, 0.5 µL of each primer (10 µM; 0.2 µM) and genomic DNA (2.5µL). The cycling program was started with an initial denaturation step at 94°C for 10 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. The PCR was ended with a final extension at 72°C for 10 min. The amplicons were visualized using a UV transilluminator (Cleaver Scientific, England) after electrophoresis on 1.8% agarose gel at a constant voltage of 75 V in Tris Acetate and EDTA (TAE) 0.05 M running buffer.

Sequencing of PCR products and identification of *Bacillus* species

The amplified fragments were sent to Eurofins (Germany) for sequencing of the 16 S rDNA regions. Sequence data were formatted and analyzed using the Geneious software. Results were considered acceptable if homology was > 99.5% with other entries in the databases used for comparison. Available sequences that were considerably different from the majority of entries for one species were considered outliers and discarded in the analysis.

Inoculation of *Bacillus* isolates to cocoa beans for fermentation

Both *Bacillus* isolates keeped in CryobankTM tube for storage at –80 °C were checked for purity by streak culture on PCA medium before using. Bacterial colonies from these plate cultures were used to inoculate Nutrient Broth (Oxoid). After culture at 30 °C for 24 h, microbial biomass for each microrganism was aseptically harvested and washed with sterilised water by centrifugation at 4000 rpm for 20 min at 4 °C in an EBA 12 Centrifuge (Hettich, Newport Pagnell, Buckinghamshire, UK). The cell pellet was then resuspended in sterilised water prior to being inoculated into the cocoa mass.

OTA quantification

OTA was extracted from the whole (unshelled) dry fermented cocoa beans as previously described [17]. 100 g of cocoa beans collected from sample were frozen at –80°C and then ground. 50 g of ground cocoa beans with 4 g of NaCl were extracted in 200 mL of solvent (acetonitrile/water, 60/40, v/v). Final homogenate solution was filtered using paper Whatman No. 4. Filtered extract (4 mL) was diluted in 44 ml of PBS with 48 µl of Tween 20. An immunoaffinity column (Puri-Fast OTA HCS IAC, Germany) was conditioned with 10 mL of PBS and the extract was purified throughout the immunoaffinity column at a flow rate of 1 mL.min^{-1}. Elution of OTA was performed using 2 vols of 1.5 mL of methanol at a flow rate of 1 mL.min and evaporated till dry in a nitrogen stream at 70°C. The residue was resuspended in 1mL of the mobile phase (water/acetonitrile/acetic acid, 69:30:1, v/v/v). Quantification was performed using the Shimadzu HPLC system (Shimadzu, Japan). Fluorescence detection was set at 333 nm.excitation^{-1} and 46]0 nm emission. The mobile phase was acetonitrile : water : acetic acid (69:30:1, v/v/v) and the flow rate was 1mL.min^{-1}. An OTA standard was used for the five-point calibration curve of peak areas versus concentration (ng.mL^{-1}). The injection volume was 100µL for both standard solution and sample extracts and the LOD was 0.05 µg.kg^{-1}. The determination of OTA concentration of each cocoa beans sample was was triplicated and the average calculated.

FFA quantification

The FFA content of dry fermented cocoa beans was measured using previous method [16]. About 20 g of cocoa beans were manually shelled and finely ground in a kitchen-scale coffee grinding (Moulinex, France) to the smallest particle (size < 500 µm). The final cocoa powder material obtained is heated in a microwave oven (Panasonic, Germany) at a power of 700 watts for 3 minutes. The hot ground material was subjected to a mechanical press (Gelgoog, China) and the cocoa butter was collected in a beaker. About 5 g of extracted cocoa butter was weighed (W) and dissolved in 50 mL of a previous hot petroleum ether/absolute ethylic alcohol mixture (1:1, v/v) neutralized by adding phenolphthalein. The final mixture was then titrated against 0.1 N sodium hydroxide in ethanol solution which volume (V) was noted until pink colour persists for 15 seconds and FFA (% oleic acid) concentration of cocoa butter extracted from each cocoa beans sample was calculated as followed formula

FFA content (%) =

Sensorial analysis of chocolate

Cocoa beans containing OTA amount below 8 µg.kg^{-1} were sampled for sensory analysis. The chocolate was prepared by the classical manufacture processing. The sensory quality of the chocolate samples was assessed twice by 8 expert tasters using 7 sensory criteria: aroma (intensity and quality), acidity, sourness, body, astringency, bitterness and global quality. A hedonic assessment was carried out when the beverage temperature reached 55 °C. Scoring was on a scale of 0 to 10, where a score of 0 corresponded to the total absence of the criterion in the coffee.

Statistical analysis

Statistical analysis was performed with the XLSTAT (Microsoft) software version 2020. The rate of OTA and FFA reduction and the results of chocolate samples were analyzed by one-way ANOVA with a single factor difference ($P < 0.5$). The sensorial analyses results were analyzed using Microsoft Excel Program, 2013 (Microsoft Corporation, Redmond, Washington, USA). The means were separated by the Fisher's test.

Results and Discussion

Results

Bacillus species

All Bacillus isolates from fermented African locust beans and cocoa seeds proved to be creamy-white, opaque or translucent, rough colonies with irregular or circular edges. Molecular analysis of the amplified DNA fragments nucleotide sequences confirmed that all presomptive bacterial strains belonged to *Bacillus* genus (Table 1).

Code of strains	Detected Bacillus isolates	GenBank accession number	Fermented foods
BN14	*Bacillus halotolerans*	NR_115929	*Parkia biglobosa* seeds
BN15	*B. paramycoides*	NR_157734	
BN33	*B. subtilis Subsp subtilis*	NR_102783	
BN36	*B. subtilis*	NR_027552	
BN41	*B. paramycoides*	NR_157734	
BN42	*B. thuringiensis strain ATCC 10792*	NR_043403	
BN43	*B. albus*	NR_157729	
BN45	*B. thuringiensis strain IAM 12077*	NR_043403	
BN46	*B. protéolyticus MCC 1A00365*	NR_157729	
BC35	*B. protéolyticus strain MCC 1A00365*	NR_157735	Cocoa beans
BC46	*B. albus strain MCC 1A02146*	NR_157729	
BC52	*B. toyonensis*	MF445144	
BC53	*B. paramycoides strain MCCC 1A04098*	NR_157734	
BC54	*B. thuringiensis strain ATCC 10792*	NR_114581	

Table 1: *Bacillus* isolates from the both fermented African locust bean seeds and cocoa beans identified by molecular technique using amplification of 799f /1492r (ADNr 16S) and rpoBf /rpoBr genes.

Effect of Bacillus strains on the growth of Aspergillus cabronarius voucher IHEM 661

Figure 1 presents the results about the inhibition of the growth of greater OTA producing molds using living cells of *Bacillus* isolates. The cells of tested *Bacillus* reduced the mycelial growth of the growth of *A. carbonarius* voucher IHEM 661on average from 28.10 to 99.78%. *B. halotolerans* (isolate BN14) from fermented Afrian locust seeds and *B. thuringiensis* strain ATCC 10792 (BC54) exhibited the highest antifungal activity for fungal growth with the percent of 99.78 ± 0.03% and 63± 0.02% respectively for each tested fermented foods.

Effect of the addition of Bacillus strains on the cocoa beans OTA content

The ability of *Bacillus halotolerans* and *Bacillus thuringiensis* strain ATCC 10792 for OTA reduction in dry fermented cocoa beans from various cocoa pods opening delays was evaluated. Figure 2 shows the changes in OTA contents of cocoa beans samples inoculated with *Bacillus* species. When cocoa beans were inoculated with *Bacillus* strains at 0 days of fermentation, OTA content measured was about 810 $\mu g.kg^{-1}$ (Figure 2 A), 740 $\mu g.kg^{-1}$ (Figure 2 B) and 795 $\mu g.kg^{-1}$ (Figure 2 C) in dry fermented cocoa beans from 4-, 7- and 10-days cocoa pod-opening delays respectively. An approximative OTA content about <1 $\mu g.kg^{-1}$ with a rate of OTA reduction (100%) was found in inoculated cocoa beans with *Bacillus* species whatever the cocoa beans fermentation period of bacterial inoculation whereas the uninoculated cocoa beans samples recorded OTA content ranging from 300 to 700 $\mu g.kg^{-1}$ (Figure 2 A-C).

Effect of the addition of Bacillus strains on the FFA content of cocoa beans

Figure 3 showed the influence of *Bacillus* strains on FFA content of dry fermented cocoa beans. FFA content of uninoculated cocoa beans reached about 2.12% and 1.83% when cocoa beans were extracted from 7 and 10 days cocoa pod-opening delay. However, when cocoa beans were inoculated with *B. halotolerans* and *B. thuringiensis* strain ATCC 10792, it decreased from 2.12% to unless 1.23 and to 1.19% respectively whatever the period of fermentation (0, 4 days) of bacterial inoculation (Figure 3 A). The FFA content of cocoa beans from 10 days cocoa pods opening delay inoculated with *Bacillus halotolerans* and *B. thuringiensis* strain ATCC 10792 has fallen from 1.81 to unless 1.11 and 1.22% respectively whatever the period of fermentation (0, 4 days) of bacterial inoculation (Figure 3 B).

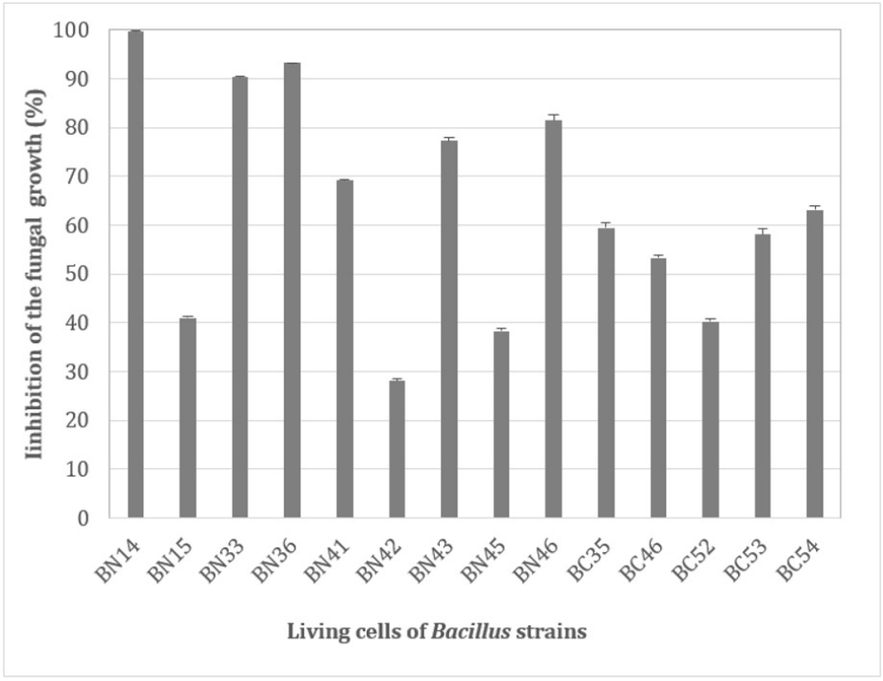

Figure 1: Percent of inhibition of the growth of *A. carbonarius* voucher IHEM 661 by the living cells of various *Bacillus* strains on the CYA medium.

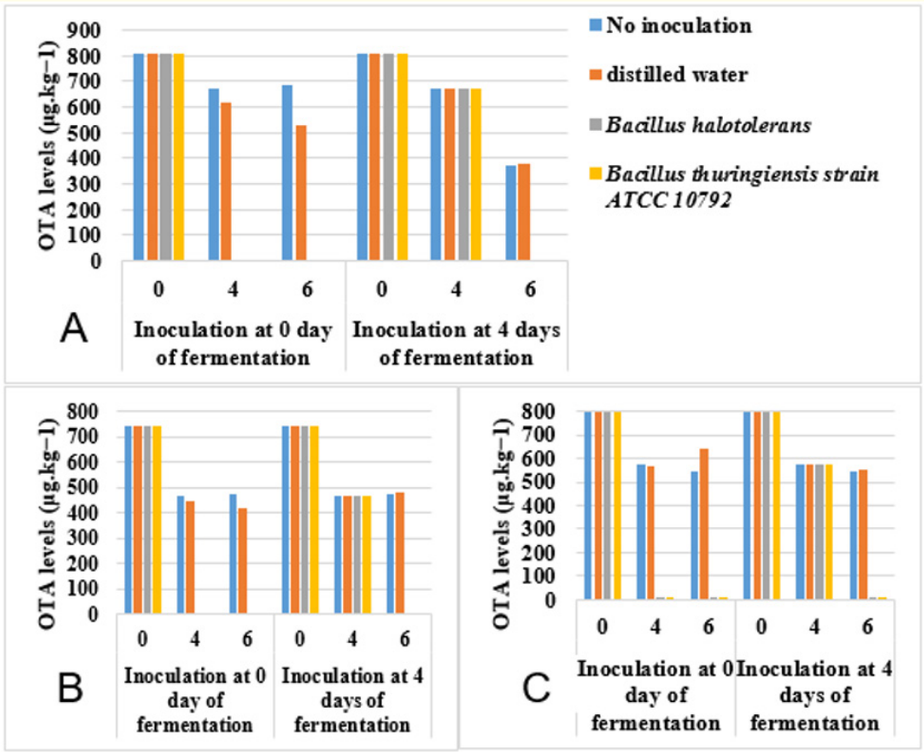

Figure 2: Effect of the inoculation of living of cells of *Bacillus halotolerans* and *B. thuringiensis* strain ATCC 10792 to fresh cocoa beans according to the cocoa pod-opening delay and the period of inoculation during the fermentation process on the OTA contents: A) cocoa beans from 4 days cocoa pods opening delays, B) cocoa beans from 7 days cocoa pods opening delays, C) cocoa beans from 10 days cocoa pods opening delays.

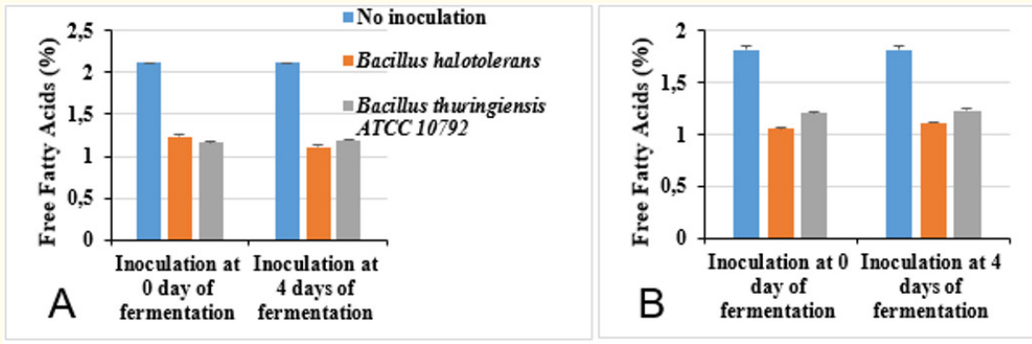

Figure 3: Effect of the inoculation of living cells suspension of *Bacillus halotolerans* and *B. thuringiensis* strain ATCC 10792 on the FFA content of cocoa beans from A) 7 and B) 10 days cocoa pods opening delay.

Effect of the addition of Bacillus strains on the sensory quality of chocolate

Sensory analyses were performed for both chocolates samples produced from spontaneously fermented cocoa beans (C1, C3, C6 and C8) and those produced from inoculated cocoa beans with *Bacillus* strains (C2, C4, C5, C7 and C9). Chocolates produced from inoculated cocoa beans with *Bacillus* and spontaneously fermented cocoa beans from 4 days pods-opening delays (C3, C4 and C5) were judged exhibiting predominantly pleasant sensory traits compared to those from cocoa beans extracted from 0, 7 (C6; C7) and 10 (C8 ; C9) days cocoa pods-opening delays (Figure 4). Few differences were highlighted between some sensory descriptors chocolates samples according to cocoa pod-opening delays. Despite these low differences, there were no significant differences ($p < 0.5$) in the sensory traits between the two types of chocolate whatever *Bacillus* strains inoculation period during cocoa fermentation.

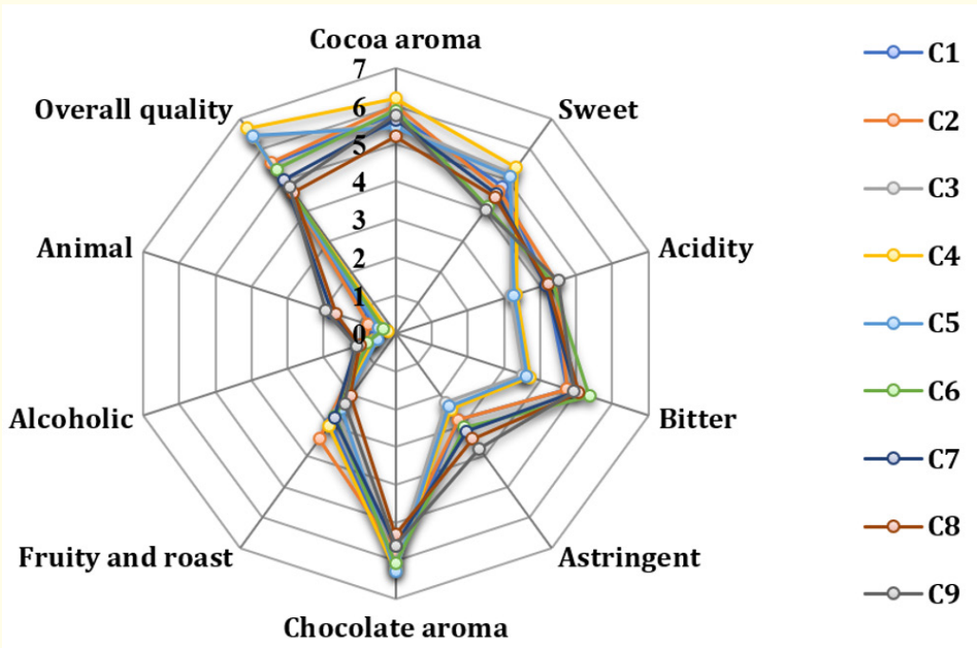

Figure 4: Effect of inoculation of *B. halotolerans* or *B. thuringiensis* strain ATCC 10792 to cocoa beans to reduce both OTA and FFA contents on sensory traits of chocolate produced thereof. C1 : uninoculated cocoa beans from healthy 0 day cocoa pod-opening delay ; C2 : cocoa beans from healthy 0 day cocoa pod-opening delay inoculated with *B. halotolerans* ; C3 : uninoculated cocoa beans from healthy 4 day cocoa pod-opening delay; C4 : cocoa beans from healthy 4 day cocoa pod-opening delay inoculated with *B. halotolerans* ; C5 : cocoa beans from healthy 4 day cocoa pod-opening delay inoculated with *B. thuringiensis* strain ATCC 10792; C6 : uninoculated cocoa beans from healthy 7 day cocoa pod-opening delay; C7 : cocoa beans from healthy 7 day cocoa pod-opening delay inoculated with *B. thuringiensis* strain ATCC 10792; C8 : uninoculated cocoa beans from healthy 10 day cocoa pod-opening delay; C9 : cocoa beans from healthy 10 days cocoa pod-opening delay inoculated with *B. halotolerans.*

Discussion

The influence of individual inoculation of *Bacillus halotolerndans* a *Bacillus thuringiensis* strain ATCC 10792 to the fermenting cocoa beans was tested. Results showed development of fungal colonies on cocoa beans uninoculated with Bacillus during fermentation regardless the cocoa pods opening delay. Cocoa beans fermentation plays an important role in the production of quality cocoa beans [30]. However, contamination by filamentous fungi reduces the quality of dry fermented cocoa beans [6]. The presence of fungi inside dry fermented cocoa beans constitues the serious defect of cocoa marketable quality [4]. However, adding of *Bacillus halotolerans* or *Bacillus thuringiensis* strain ATCC 10792 to fermenting fresh cocoa beans caused the inhibition of the fungal growth and the reduction of the level of fungi contamination of dry fermented cocoa beans. This shows that the addition of *Bacillus* starter to cocoa beans during the fermentation suppressed the development of fungi populations like using lactic acid bacteria [18,31]. The inhibition of fungal growth could be ascribed to the production of antimicrobial agents namely subtilin, bacilysin, mycobacillin and lipopeptides belonging to surfactin, iturin, lichenycin and fengycin families by *Bacillus* strains [32,33]. Bacillomycin F, a member of the iturins family, inhibits the growth of *Aspergillus niger*, while iturin A inhibits the growth *A. flavus* and *Fusarium moniliforme* [34]. Such a relationship may indicate a specific interaction between the pathogen molds and the iturin metabolites. Moreover, the inhibitory effect *Bacillus* strains inoculated may also be ascribed to a competition for space or nutrients and to the lysis of fungal hyphae, vacuolization and granulation in mycelium structures [35,36]. The effect of *B. halotolerans* and *B. thuringiensis* strain ATCC 10792 on the OTA production was evaluated by its quantification in cocoa beans. A significantly higher ($p \leq 0.05$) OTA concentration (>740 $\mu g.kg^{-1}$) was found in the dry fermented cocoa beans from damaged cocoa pods stored for 4, 7 and 10 days as compared to those inoculated with *Bacillus* (< 1 $\mu g.kg^{-1}$). These results showed more than to 99% inhibition of OTA production in dry fermented cocoa beans inoculated by *Bacillus* strains. The drcrease of OTA contents of cocoa beans dur to the inoculation of living cells of *B. halotolerans* and *B. thuringiensis* strain ATCC 10792 is mainly due to the completely inhibition (100%) of the growth of OTA producing fungi strains. However, there are other mechanisms (less likely applicable in the present situation) such as down regulation of OTA biosynthesis genes by molecules produced by antifungal *Bacillus* strains [37]. It was also reported total removal of OTA from grape berries inoculated with living cells or culture supernant of *Bacillus* strains [35]. It is known that lipolytic molds were currently involved in FFA occurrence in cocoa beans [8,38]. As previously showed, high FFA concentration above 1.75% seriously impairs the quality and marketable value of dry fermented cocoa beans [16]]. Our study showed that uninoculated cocoa beans recorded higher FFA content above the maximal than cocoa beans samples inoculated with *B. halotolerans* and *B. thuringiensis*. European guidelines limit the concentration of FFA in cocoa butter to 1.75% [39]. High FFA content of inoculated cocoa beans could be attributed to the activities of the extracellular enzyme lipase produced by lipolytic molds contaminated cocoa beans [40]. The addition of *Bacillus halotolerans* and *Bacillus thuringiensis* strain ATCC 10792 in the cocoa beans improved the fermentation of the cocoa beans and also decreased free fatty acids content from 2.12% to 1.11%. This decrease in FFA content could be explained by the inhibition of the growth of lipolytic molds [41]. This decrease shows that both *B. halotolerans* and *B. thuringiensis* strain ATCC 10792 does not produce lipase contrary to *B. altitudinis* [42] and to *B. amyloliquefaciens* [43]. Additionnaly, inoculated *Bacillus* strains could use the FFA from lipase activity of lipolytic mold as organic susbtrate to produce energy for their growth [44,45]. Sensory profile of chocolates made from each cocoa beans sample inoculated or not with *B. halotolerans* or *B. thuringiensis* strain ATCC 10792 were evaluated in this research. The chocolates made from the inoculated cocoa beans with *Bacillus* strains exhibited no significant difference between the sensory descriptor compared to those of the chocolate made from uinoculated dry fermented cocoa beans in function of any cocoa pod-opening delay. The results showed that chocolates produced from spontaneously fermented cocoa beans extracted from 4 days cocoa pod-opening delay was preferred than chocolate made from other cocoa bean samples previously obtained [25]. This positive results indicated that adding of *Bacillus halotolerans* or *Bacillus thuringiensis* strain ATCC 10792 to fermenting cocoa beans probably could have positive effetc on rheological, physical and chemical characteristics during processing of cocoa butter and chocolate produced thereof [46]. According to previous research, *Bacillus* strains are essential for the development of chocolate flavor and their inoculation may accelerate and control the fermentation process due to its pectinolytic enzymes production [47]. Moreover, other authors have also found that the synergy between *Bacillus* and yeast strain for ethanol production may play a crucial role since, this metabolite is highly required for a well processed cocoa fermentation [48]. That make them candidate as starter for cocoa fermentation control as these properties are very relevant and necessary for a fine and well-performed process of cocoa fermentation [49,50] and for the generation of volatile organic compounds [51].

Conclusion

This study highlighted that the individually addition of *B. halotolerans* and *B. thuringiensis* strain ATCC 10792 to fermenting cocoa beans allowed to suppress the growth of molds, to totally control the OTA production and to reduce FFA occurrence in dry fermented cocoa beans. Hence, using of these two strains of *Bacillus* improved microbiological quality, safety and marketable quality of dry fermented cocoa beans. *B. halotolerans* and *B. thuringiensis* strain ATCC 10792 could be used as biological agents to molds bio-decontaminate, to control the OTA and FFA production of cocoa in the same time.

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Conflict of Interest

Declare if any financial interest or any conflict of interest exists.

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