Original Research Article

Screening of anti-fungal *Bacillus* strains andinfluence of their application on cocoa beans fermentation and final bean quality

ABSTRACT

Aims :
Contamination with filamentous fungi reduces the quality of cocoa beans and poses a health risk for consumers due to the potential accumulation of mycotoxins. The aim of this study was to develop antifungal *Bacillus* cultures for cocoa beans fermentation.
Methods and results:
Antifungal activity of 7*Bacillus* isolates was investigated using overlay technique. Solid culture media inoculated with bacterial and fungal isolates were cultivated under aerobic conditions at 30 ºC for 5 days. The *Bacillus* strain with strong antifungal activity was studied and inoculated to 180-kg box fermentations cocoa beans in Cote d'Ivoire in three time-independent replications each including a spontaneous control fermentation. The comparison of inoculated and spontaneous fermentation processes revealed that the *Bacillus thuringiensis*strain ATCC 10792 don't affected the fermentation process and cocoa bean quality. The anti-fungal in vivo assays revealed that the *Bacillus thuringiensis* strain ATCC 10792 completely inhibited growth of all fungi on the surface of cocoa beans. The result showed that sensory evaluation of chocolates is not significant differences for all treatment. The sensory evaluation of control and chocolates made from the inoculated cocoa beans with *Bacillus*showed excellent sensory quality, demonstrating that *Bacillus thuringiensis* strain ATCC 10792 did not affect aroma, texture and appearance of chocolate.
Conclusion :
From this study, it is revealed that the addition of *Bacillus thuringiensis* strain ATCC 10792 during fermentation cocoa beans suppress fungal growth and improve the quality of cocoa beans. Therefore, the anti-fungal culture *Bacillus thuringiensis* strain ATCC 10792 is recommended for future applications and its capacity to limit fungal growth during industrial-scale cocoa bean fermentation should be investigated in further studies.

Keywords: Aspergillus carbonarius, anti-fungal, Bacillus, cocoa beans, chocolate

1. INTRODUCTION

Cocoa beans (Theobroma cacao L.) are the principal raw material of chocolate manufacture [1]. Due to the fact that approximately 80–90% of cocoa produced globally is cultivated on 5–6 million small farms [2], the quality of the resulting cocoa beans is variable. Factors influencing cocoa quality include seasonal variations, changing weather conditions, and different techniques applied during pod-storage, fermentation, and drying [3]. The fermentation of the beans of Theobroma cacao L. is the first step in the post-harvest processing of cocoa. It is a spontaneous process in which microorganisms that drive the fermentation originate from the environment [4]. Several authors have found that good quality in fermented dry cocoa beans was correlated with good on-farm agricultural and post-harvest practices, bean

selection, placenta removal prior to fermentation, and blending of the cocoa bean pulp mass. During the fermentation, there is a well-defined microbial succession of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) [5], however, filamentous fungi can also grow, mainly in the well-aerated and cold outer layers of the fermentation mass and during the drying process [6,7]. Contamination with filamentous fungi has been associated with internal development of mould, off-flavours, increased free fatty acid levels, and the production of mycotoxins [8]. Fungal activity can lead to a contamination with mycotoxins, causing deterioration of the quality of the product, and can pose a health risk to the consumers. Therefore, it is important to reduce or eliminate the fungal contamination in order to keep the leading edge of the production[9]. Reducing or eliminating mold contamination could improve the quality of the cocoa, prevent mycotoxin production, and increase the sales price of cocoa beans [10]. Most of the methods used to fight the fungi include chemicals. However, there are constant increases in microbial infections and in losses of foods and feeds over time, leading to economic losses as well. Microorganisms are of great interest as biocontrol agents to reduce mould growth and the concomitant contamination with mycotoxins [11]. *Bacillus spp*. has a unique ability to replicate rapidly, tolerant to adverse environmental conditions as well as it has broad spectrum of biocontrol ability. For these reasons, *Bacillus spp*. is becoming an attractive candidate for biological control fungal growth [12, 13, and 14].The aim of this study was to selecting antifungal cultures that are well adapted to the cocoa bean fermentation process, reducing the growth of mycotoxigenic filamentous fungi, and do not negatively influence cocoa bean fermentation and the quality of the resulting cocoa beans. The selection of antifungal cultures encompassed three phases: (i) screening of *Bacillus* strains for antifungal activity, (ii) growth inhibition tests in vivo on cocoa beans against potentially mycotoxin-producing filamentous fungal strains, and (iii) the application of selected antifungal *Bacillus* culture to the cocoa bean fermentation.

2. MATERIAL AND METHODS

2.1 Bacterial strains, identification, growth condition and inoculum preparation

Seven strains of the genus *Bacillus*, namely *Bacillusmycoides*, *Bacillusprotéolyticus*, *Bacillusalbus*, *Bacillus subtilis*, *Bacillusparamycoides*, *Bacillustoyonensis* and *Bacillus thuringiensis*strain ATCC 10792 were used in this study. The antagonistic bacteria of the genus *Bacillus* were obtained from spontaneous cocoa beans fermentation. Before identification, the strains were cultivated on Plat Count agar (Bio-Rad, France) at 30 °C for 24 h. Identification of the bacterial strains was performed by 16S rRNA gene sequencing analysis using single colonies of 24 h cultures of each strain according to Hlebaet al[15]. To prepare a bacterial inoculum the bacterial strains were cultivated 24 hours at 30 °C in nutrient broth (Bio-Rad, France). The culture was incubated at 30°C for 24 h and then centrifuged (4000 rpm, 20 min, 4 °C) in order to separate Bacterial biomass of cells from bacterial cell-free extract. The bacterial cell-free extracts was harvested by filtration using 0.22 µm filters (Sartorius®) to remove living cells. Subsequently, the bacterial inoculum was prepared by diluting the bacterial suspension in sterile phosphate saline to a final concentration of 10^8 cells.mL^{-1} at 600 nm using UV–VIS spectrophotometer (UV-1800, Shimadzu, Japan).

2.2 Fungal isolates, identification, growth condition and inoculum preparation

The strain of *Aspergillus carbonarius* voucher IHEM 661 was used in this study. This strain was isolated from fermented and dried cocoa beans samples, marketed in Côte d'Ivoire. The isolate was identified to the species level according to molecular characteristics based on ITS and β-tubulin regions of rRNA gene sequence. *Aspergillus* isolate was tested for ochratoxin A, and their production was confirmed by HPLC-FLD (Shimadzu RF 20A, Japan). To prepare a fungal inoculum the isolate was cultivated on Sabouraud dextrose agar (SDA) (HiMedia, India) for 7 days at 25 °C. Then the conidia of ochratoxigenic*Aspergillus carbonarius*voucher IHEM 661 were aseptically sampled by scratching the pure culture using 10 ml of saline solution containing 1% Tween® 80 (P1754, Sigma-Aldrich, France). The conidia suspension was adjusted to 10^5 conidia.ml^{-1} using usingThoma cell counting chamber according to the method of Kogkaki et al[16].

2.3 Direct Confrontation Test: dual-culture agar overlay methods

Antifungal activity of *Bacillus* strains against *Aspergillus carbonarius*voucher IHEM 661 was tested using a dual culture overlay assay. A 5 µl at 10^8 cell.ml^{-1} of *Bacillus* was inoculated at the center of the plate count agar. Inoculated plates were incubated at 30°C for 24 h. After incubation the above plates were then overlaid with 2.5 ml of Czapek yeast agar (CYA) medium (55 mm in diameter) containing 10^4 conidia.ml^{-1}. All these tests were duplicated. After 5 days of aerobic incubation at 30°C, the zone of inhibition was measured. The inhibition was graded by relating the inhibited growth area per inoculation streak to the total area of the Petridish. The inhibition area was also related to the variation in length of the bacterial streak.

2.4 Inhibition test by indirect confrontation using overlay method

To check the antifungal activity of *Bacillus* strains was carried out by using the "overlay method" described by Ruggirello et al [17]. Starting from microbial cultures stored at -80 °C, 50 µl of *Bacillus* suspension were inoculated and spread uniformly in 2.5 ml of PCA medium. After 24 h of incubation at 30°C, PCA medium previously inoculated were overlaid with 15 ml of CYA and then 5 µl of 10^5 conidia.ml^{-1} of *Aspergillus carbonarius*voucher IHEM 661 were inoculated in the center of the plate. The overlaid plates were finally examined, after 5 days at 25 °C, for mould growth inhibition zone; the antifungal activity was evaluated as strong (++), weak (+), or absent (-) on the basis of the transparency of the inhibition area.

2.5 Procedure of inoculation, fermentation, drying, and sampling

The in vivo anti-fungal activity of *Bacillus* strains was assessed directly on cocoa beans. Beans from cocoa pods (Amelonado) harvested from paysan plantation located at Akoupé (Cote d'Ivoire). A total of 900 cocoa pod were harvested. Two lots of cocoa pods were constituted: healthy pods and decayed or damaged pods. Healthy pods were opened immediately after harvesting, while the decayed or damaged pods were opened after 4 days. Just after pod opening, approximately 108 kg of fresh beans surrounded by mucilage were obtained. This mass of fresh beans was divided in six batch of 18 kg each for fermentation. Each heaps of fresh cocoa beans were placed in wooden boxes measuring 50 × 50 × 50 cm. Spontaneous (control) and inoculated fermentations were carried out. Inoculated fermentations were performed after inoculation of 200 mlcontening 10^8 UFC.ml^{-1} of *Bacillus thuringiensis* strain ATCC 10792. Inoculation of cocoa beans with *Bacillus* strains was carried out at 0 days of fermentation for certain batches and at 4 days for others. The wooden boxes had holes to facilitate the drainage of acidic liquid resulting from liquefaction of mucilaginous pulp and covered with plantain leaves. The wooden boxes were raised above ground level over a drain that carries away the pulp juices liberated by the degradation of the mucilage. 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. For each fermentation method, fresh cocoa beans of one batch (18 kg) were mixed after 48 and 96 h of processing. Each type of fermentation lasted 6 days. Fermented cocoa beans were sun-dried on the racks before microbiological and chemical analysis. All fermentations were performed in triplicate.

2.6 Cut test, chocolates preparation and sensory analysis of dried cocoa beans

The quality of dried beans resulting from each fermentation was assessed by a cut test with 300 dried cocoa beans. The dried beans were sent for chocolate production at CIRAD chocolate factory laboratory (Montpellier, France). The chocolate was prepared with 70% cocoa. The formula of the tested chocolates included cocoa liquor, cocoa butter, sugar and lecithin. The total cocoa liquor content was 70% (w/w), lecithin 0.7% and cocoa butter 3.3%. Filling the recipe (up to 100%) was sugar.Determination of sensorial profile of resulted chocolate was performed with a descriptive analysis performed according to ISO 13,299. For this purpose, twelve judges among 5 women and 7 men who had been previously selected and trained performed sensorial analyses of the chocolates. The consumers evaluated how much they liked each sample. The global quality and the intensity of each attribute were evaluated simultaneously using a scale varying from 0 to 10 and a total score for each sample was assigned.

2.7 Statistical analysis

The statistical analyses were carried out with the XLSTAT (Microsoft) software version 2020. Analyses of variance (ANOVA) with a single factor differences ($P < 0.5$) were performed to indicate the significant differences between the resulted chocolate samples. 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.

3. RESULTS AND DISCUSSION

3.1 *In vitro* screening of *Bacillus*isolates for antagonism by direct confrontation

The results of antifungal activity using the direct confrontation method illustrated in figure 1 show that 6 out of the 7 tested *Bacillus* isolates were active against *Aspergillus carbonarius* voucher IHEM 661 growth. Two *Bacillus* isolates (*Bacillusparamycoides* and *Bacillusalbus*) caused low inhibition levels ranging from 9 to 21mm of diameter while four *Bacillus*isolates (*Bacillusprotéolyticus, Bacillustoyonensis*, *Bacillus subtilis* and *Bacillus thuringiensis* strain ATCC 10792) showed strong inhibition for *Aspergillus carbonarius* voucher IHEM 661 mycelia ranging from 28 to 55 mm in average of diameter. This result corroborates with other works which found that inhibitory effect may be the result of complex interactions between different constituents at the origin of additive, synergistic or antagonistic effect [18, 19]. It may be presumed that antifungal activity is due to competition for nutrients and/or space between *Bacillus* strains and the mycete or to diffuse inhibitory substances produced by *Bacillus* strains that suppressed the growth of the mycete[20, 21].

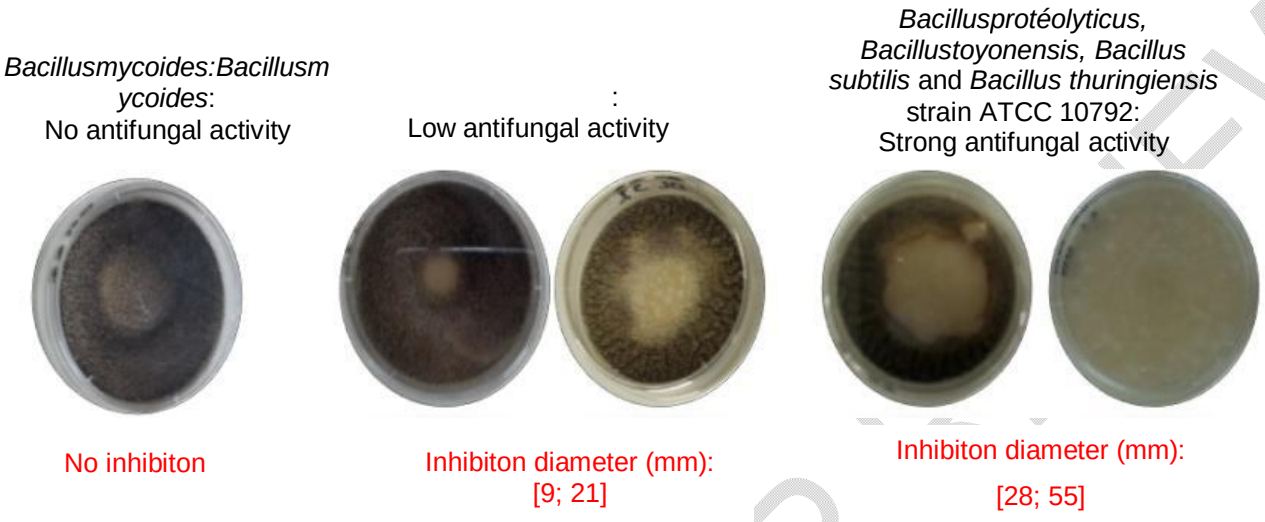
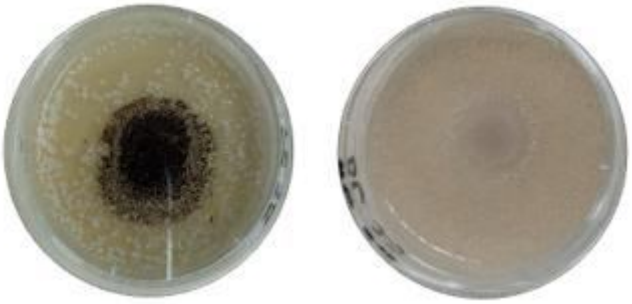


Fig. 1. Antagonistic effects of *Bacillus* sp. against *Aspergillus carbonarius* voucher IHEM 661

3.2 Indirect inhibition assays according overlay method

Four *Bacillus* strains were studied in order to determine their fungistatic and fungicidal activity against *Aspergillus carbonarius* voucher IHEM 661 growth (figure 2). In vitro trials were carried out to study their inhibition activity on fungal spore germination and mycelium growth. *Bacillus protéolyticus, Bacillustoyonensis* and *Bacillus subtilis* showed fungistatic activity while *Bacillus thuringiensis* strain ATCC 10792exhibited fungicidal activity against*Aspergilluscarbonarius* voucher IHEM 661 growth. The fungicidal effect was confirmed when sub-culturing the tested fungi from the agar dilution assays into nutrient broth without *Bacillus* resulted in no further mycelial growth or resumption of spore germination. Antifungal results of *Bacillus* strains demonstrate the inhibitory effects of the diffusible metabolites of *Bacillus* spp. against *Aspergilluscarbonarius* species. This result clearly indicated that the antifungal activity of *Bacillus*strains is due to the production of extracellular bioactive compounds [22, 23]. Anckaertet al[24] have showed also that diffusible metabolites of *Bacillus* strains such as possessing protease, chitinase, β-13-glucanase, iturin, fengycin and surfactin are believed to contribute to their antifungal potential.



Bacillusprotéolyticus, Bacillustoyonensis and *Bacillus subtilis*: Fungistatic activity

Bacillus thuringiensis strain ATCC 10792:
Fungicidal activity

Fig. 2. Effect of diffusible antifungal metabolites secreted by *Bacillus* strainson radial growth of *Aspergillus carbonarius* voucher IHEM 661.

3.3 In situ anti-fungal assay on cocoa beans

The effect of *Bacillus thuringiensis* strain ATCC 10792on growth of mycotoxigenic fungi used to inoculate cocoa beans in this study is shown in figure 3. *Bacillus thuringiensis* strain ATCC 10792showed broad spectrum of activity by inhibiting all the fungal strains contaminate cocoa beans in situ. Moreover results showed development of fungal colonies on beans from untreated cocoa pods. According Rahayu et al[25] cocoa bean fermentation plays an important role in the production of quality cocoa beans. Fungal contamination is the main problem in the fermentation of cocoa beans by cocoa farmers. In addition, it causes fermented cocoa beans to have a low quality, especially if there are fungal mycelia on the surfaces of the cocoa beans. *Bacillus thuringiensis* strain ATCC 10792showed the ability to inhibit the growth of fungi throughout the fermentation and drying of the cocoa beans in this study. Different authors as Ngang et al. [26] and Romanens et al[7] found that the addition of lactic bacteria and yeast as a starter in cocoa fermentation with OTA-producing molds (*Aspergillus niger* and *Aspergillus carbonarius*) had inhibit the growth of the molds until they were almost undetectable. Results of this study are in accordance with what has been conveyed in previous studies.Based on this, it is recommended that *Bacillus thuringiensis* strain ATCC 10792, be used in the fermentation of cocoa beans to improve the quality.

Fig. 3. Reduction of mould contamination by inhibition of their growth on fermented and dried cocoa beans

3.4 Cut test and fermentation degree

The dried beans were cut lengthwise into halves for maximum surface exposed. Both halves of each surface were inspected under artificial light and divided into five groups (fully brown, purple-brown, fully purple, slaty and mouldy) as showed in figure 4. The total of dried beans in each group were counted and expressed in percentage and replicated for three times. Table 1 shows the results of the cut test for the fermented and dried cocoa beans and the mean data after fermentation and dried periods.According to the data, The rate of brown beans varied from 80.33 to 88.67%, the number

of purple-brown beans was 3.33 to 5% and purple beans reaching 8 to 14.67% for A and B batches. Similar data were found by Efraim et al[27] and Afoakwa et al[28] by investigating the effect of fermentation time, the type of drying and the method of storage before fermentation. Changes in the color of the beans, ranging from purple to brown, were reported during cocoa fermentation [27, 29]. Partially brown beans are not defective, as they change to brown upon storage and the trade accepts up to 30–40%; however, samples containing over 50% partially brown beans are unacceptable [30]. Based on the results of cut test, the beans were adequately fermented [31].

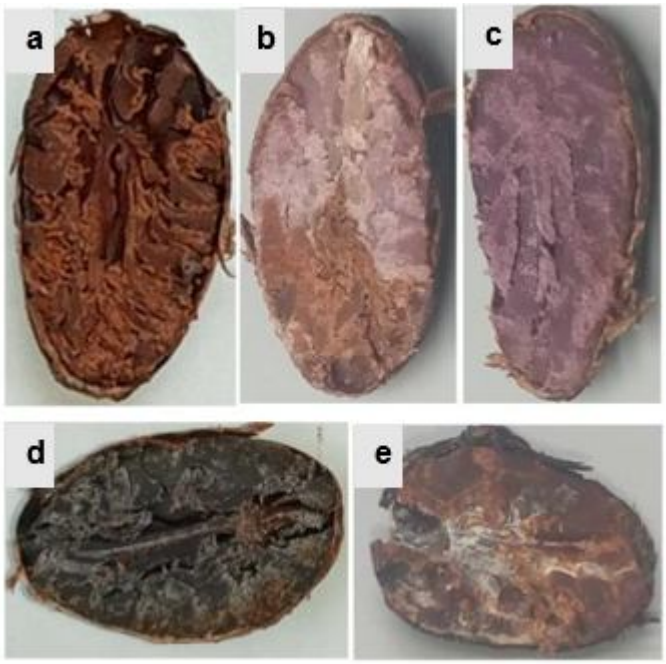


Fig. 4. Color of cut surface of dried cocoa beans. (a) fully brown; (b) purple-brown; (c) fully purple; (d) slaty and (e) mouldy

Table 1. Resultsfrom the Cut Test of cocoabeansfermented and driedsamplesfrominoculated (B) and non-inoculated fermentations (A)

Batches	fully brown (%)	purple-brown (%)	fully purple (%)	Slaty (%)	Mouldy (%)
A	80.33±1.2	5±0.6	14,67±1.4	0	0
B	88,67±2.4	3,33±0.8	8±0.9	1	0,1

3.5 Sensorial analysis

Figure 5 shows the sensory profiles of chocolates produced from inoculated cocoa beans and spontaneously fermented cocoa beans (control). The parameters used descriptive sensorial attributs questionnaire analysis were covered for determining if the flavours of the chocolates were Fruity and roast, Alcoholic, sweet, sour, Animal, bitter, sweet, Astringent. Judges indicated which attributes better described the chocolate samples. The chocolates produced from inoculated (B) and non-inoculated fermentations (A) were more similar in terms of astringent, floral notes, fruity and roast, cocoa aroma, sweet, and chocolate aroma. These chocolates are described to have high scores such as 6.53, 6.68, 6.5 and 6.2 for desirable notes such as Chocolate aroma, global quality, intensity of odor and cocoa aroma attributes,

respectively, whatever the fermentation process. There were no significant differences (P < 0.5, by Fisher's test) between samples from inoculated (B) and non-inoculated fermentations (A). The chocolates made from the inoculated cocoa beans with *Bacillus* strains exhibited no significative difference between the descriptive sensorial attributs compared to those of the chocolate control. The inability of the consumer panel to detect differences between chocolates made from the inoculated beans and spontaneously fermented beans could explain clearly that no link seems to exist between the odor description of aroma compounds detected in cocoa beans and the sensory traits of chocolates produced thereof [32]. Dulce et al[33] showed that spore-forming bacteria of the genus *Bacillus spp*. had produced odor-active compounds such as ethyl acetate and 3-hydroxy-2-butanone, which are associated with the desirable fruity and sweetish sensory notes of chocolates. Although the good scores related to the global quality of chocolates issued from spontaneously fermented beans revealed that despite the innovation brought for the improvement of cocoa aroma quality, the role of complex and multiple microbiota is important in beans fermentation process, the same scores related to the bitter and astringent traits whatever the spontaneous and inoculated fermented beans could be due to the genetics of our cocoa and/or environmental factors of the fermentation [34].

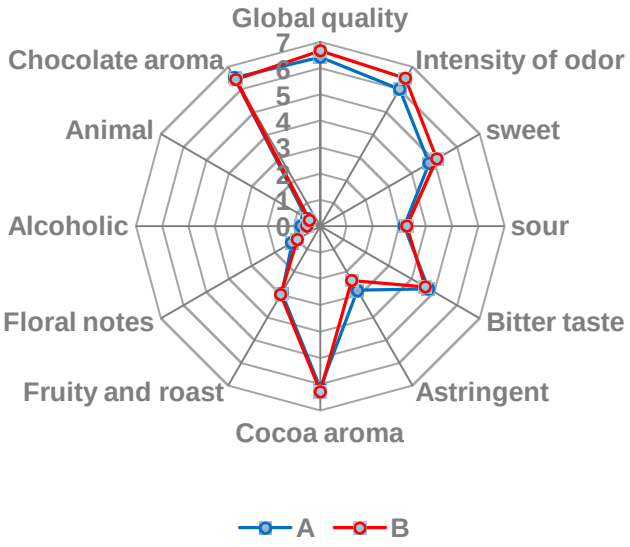


Fig. 5. Sensory profile of chocolate made from each cocoa beans sample inoculated or not with *Bacillus thuringiensis* strain ATCC 10792.

4. CONCLUSION

Bacillus thuringiensis strain ATCC 10792 isolated from cocoa beans showed the highest inhibitory activity against *Aspergilluscarbonarius* voucher IHEM 661 and contributed in a significant suppress fungal growth when it was cultured in combination with cocoa beans contaminated by moulds. About sensory evaluation of chocolates made from the inoculated cocoa beans with *Bacillus thuringiensis* strain ATCC 10792 and non-inoculated control, consumers did not report a significant preference for either chocolate (p < 0.5). Due to an acceptable sensory properties, it can be concluded that *Bacillus thuringiensis* strain ATCC 10792 could be successfully used in the production of dark chocolate. This observation allows to suggest that *Bacillus thuringiensis* strain ATCC 10792 cultures should be investigated in further studies for an effective biological control of mycotoxins during the traditional processing of cocoa.

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