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Post-Fermentation *Standard* and *Premium*TypesDryCocoa Beans (*Theobroma cacao* L.)Quality: living organisms and toxins

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Abstract:*Living organisms (insects, mites, fungi) and toxin (aflatoxin B_1 - AFB_1) contamination in post-fermentation dry cocoa beans (Theobroma cacao L.) of Standard and Premiumquality Typeswere evaluated. A total of 60 samples (30 each quality Type) was collected in farms (48) and warehouses (12) during five production months and analyzed for light filth (Wildman with stereo and scanning electron microcopies identification), fungi (total load, genera and species identification), AFB_1(reverse phase liquid chromatography / fluorescence detection) and humidity (moisture content – mc and water activity – aw). Out of the total samples, living organisms presence (insects, larvae, eggs fragments) were detected in 53.3% (32/60) of them, being 68.7% (22/32) and 31.3% (10/32) of the positive from theStandard and Premium Types, respectively. The biological contaminants detected, apart from insects and mites, were also fungi, with the totalload ranging from 10 to $3.5x10^2$andthestrains identified ofAspergillus, Penicillium and Fusarium genera(16.7, 16.8 and 1.7%of the total samples,respectively)which43.3% (13/30) and 26.6% (8/30) of Standard and Premium Types, respectively. AFB_1 was detected in 33.3% (20/60) of the cocoa beans (levels ranging from 6.61 to 15.03 µg/Kg) being 50.0% (15/30) and 16.7% (5/30) of the positivefromStandard andPremiumTypes, respectively.Indeed, the dry cocoa beans surveyed werehighlyhumid(mc: 4.71-15.68%, mean: 10.19±1.06% and aw:0.59-0.88)thusfavorable conditions for fungi and mycotoxin production. Regarding mc limit,35(58.3%) of cocoa beans samples had mc values higher than the described in the literature fungi wise safe.These results may serve as subsidies to chocolate industries in order to select quality cocoa beans to ensure safety that continues from the raw materials to the chocolate processing.*

Keywords:*cocoa, fungi, light filths, insect, stereoscopy, electron scanning microscopy, Theobroma cacao, aflatoxin B_1*

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I. Introduction

Chocolate quality can be influenced by a variety of environmental, agricultural and technological factors to which the cocoa beans (*Theobroma cacao L.)*are subjected from the opening of the cocoafruituntil the end of the industrial process. Among these factors, it is known that the microorganisms (bacteriaandyeast) present at the fermentation steps play an essential role on the development of the sensorial characteristics of chocolate (Schwan and Wheals, 2004; Afoakwa et al., 2008, Kreibich et al., 2014, 2015, 2016a).Their desirable types are the lactic and acetic acid bacteria, which producetheir related alcohols and acids (Schwan and Wheals, 2004; Kreibich et al., 2016). That process causes beans swelling and abrownish color development (enzimes reaction on polyphenols content as pH reduces)in which the moisture content (mc) is still high,around 60% (Ribeiro, 1986; Barel, 1997; Nachtigall, 1999; Kreibich et al., 2016).From that stage, they are sent to sun drying on cement platforms (mc: around 7.5%) (Hii et al., 2006; Efraim, 2009; Kreibich et al.,2014, 2016a). Next, they are packaged in bags and sold for the internal market or exported. Despite those actions, difficulties maybe be faced which need to be controlled, such as, fungi proliferation andinsects/mites infestation specially if storage environment is not controlled (Faleiro et al., 2004; Rubini et al., 2005; Scarpari et al., 2005; Mounjouenpou et al., 2008; Kreibich et al., 2014, 2016).

The presence of biological contaminants (insects, mites, rodents) in the cocoa paste(chocolate preliminary step) is indication of lack of environment control during the phases involving its initial production, i.e., from seed to bean transformation (pulp extration / fermentation / drying / storage) (Kreibich et al., 2014, 2016a).They are important carries of contaminants microorganisms (bacteria, fungi, viruses) and of their metabolites (toxins) (Scussel et al., 2011; Koerich et al., 2013; Kreibich et al., 2014; Savi, et al., 2014). In addition, mites, which develop in stored products (especially those with high fat / protein content) can trigger allergic reactions (Lorini, 2012) and inappropriate packaging with bad storage conditions can enable them

passing to the final product, including external odors (Lorini and Schneider 2004; 2008). The infestation leads to increase of temperature for humidity, fungi spores dissemination and proliferation (Kreibich et al., 2014; 2015a).

Apart from their deteriorative influence and consequent sensory quality reduction of cocoa bean and chocolate, the presence of toxigenic fungi strains is also of health concern (regarding toxins formation). *Aspergillus*, *Penicillium* and *Fusarium* are the main genera producing toxic secondary metabolites in food, the mycotoxins. Several of them are mutagenic, teratogenicity, and carcinogenic in humans and animals(IARC 1993; Scussel 2002, 2004).

Regarding the presence of aflatoxins (AFls) in cocoa beans, there are only a few reports in the literature. A survey conducted in Japan to assess levels of mycotoxins in products sold in retail outlets,Kumagai et al. (2008) evaluated samples of bitterchocolatefor AFLs presence. Authors found 22 positive for those toxins out of 42 sampletested, with an average contamination of 0.18 µg/Kg (maximum of 0.60µg/Kg. Another studyinvestigatedthe effect of some fermentation practices on AFLs and also ochratoxinAinchocolate marketed in Brazil. A total of 125 samples of powdered, bitter, dark, milk andwhite chocolate were evaluated and 80% were positive at mean levels of0.53, 0.66, 0.43,0.08 and 0.01µg/Kg, respectively (Copetti et al., 2012).

Considering that the quality of different commercial Types of post-fermentation dry cocoa beans can be spoiled by insects (fungi spores vectors), thusimproving environment conditions for fungi development and possibletoxinsproduction,this work reportsan investigation on cocoa beans (*Premium* and *Standard* Types)living organisms (insects, mites), fungi and possible AFB_1contamination.

II. Materials and Methods

2.1. Material

2.1.1. ***Samples:*** dry cocoa beans (60) post-fermentation of *Premium**and*Standard***Types (n=30 each), from the 2014 harvest season (March / July). They were from different cocoa producers farms and warehouses located in the Itabuna-Ilheus region of Bahia state, Northeastern Brazil.*Cocoa of selected varieties /high quality/ characteristic, **Cocoaof not high / controlled & sensory standards quality (Kreibich et al., 2016a).

2.1.2. ***Chemicals and culture media:****(a) chemicals* - potassium hydrogen phosphate, sodium nitrate, potassium chloride, magnesium sulfate, ferrous sulfate, sodium lauryl sulfate,ethanolandheptane,all from Vetec (Duque de Caxias, RJ, Brazil); hydrochloric acid, methanol, trifluoroacetic acid (TFA), o-phthalaldehyde (OPA), sucrose, glycerol, chloramphenicol, all from Kyma (Americana, SP, Brazil); lactophenol cotton blue, Laborclin(Pinhais, PR, Brazil); ultrapure distilled water (MilliQ),AFB_1standard, Sigma-Aldrich (St. Louis, USA)and *(b) culture media* - potato dextrose agar (PDA), malt extract agar (MEA) and bacteriological peptone, all from Himedia (Curitiba, PR, Brazil);Czapek-dox, 25 % glycerol nitrate (G25N), and Czapek yeast extract (CYA), Vetec (Duque de Caxias, RJ, Brazil) and nitrogen, White Martins (Rio de Janeiro, RJ, Brazil).

2.1.3. ***Equipments:***healtingplates,Dist (Florianópolis, SC, Brazil); heating block and vaccum pump, Tecnal (Piracicaba, SP, Brazil); microwave oven, Philco, (São Paulo, SP, Brazil);stomaker, MA440/CF, Marconi (Piracicaba, SP, Brazil); vortex andautoclave, from Phoenix (Araraquara, São Paulo, Brazil); microbiological oven, Quimis (Diadema, SP, Brazil); colony counter, CP608, Phoenix (Araraquara, SP, Brazil); laminar flow hood, Veco(Campinas, SP, Brazil); aqualab 4-TE, Decagon (Pullman, WA, USA); microscopes[*stereo* - with stereo-light source, MZ16, Leica Microsystems - Heerbruhh, Switzerland& *scanning electron* -5000x, JSM-6390LV, Jeol- Peabody, Mass., USA]; gold coating machine, EM-Scd500, Leica (Leider, Ill., USA); stubs of 9x 10 mm for diameter x height, respectively), Leica (Leider, III., USA); stainless steel scalpel, GSMtronix (São Paulo, SP, Brazil);cientific camera OPT 10.0 MP - OPTICAM; microscope imaging software - OPT HD 3.7 - OPTICAM (São José dos Campos, SP, Brazil.Liquidchromatography (LC) system with fluorescence detector (FLD), Gilson(Middleton, WI, USA).

2.1.4. ***Others*:**wildman filth bottle trap (Erlenmeyer-2 L with metal rod and rubber cap – 250 and 60/30/50 mm for length and lower/upper/height, respectively), Dist (Florianopolis, SC, Brazil); Buchner funnel (13 cm diameter);glass bottles with polyethylene cap (200 mL); granulometric sieves (200 mesh), Bertel (Sao Paulo, SP, Brazil) and filter paper,n4, Whatman (Maidston, England).The chromatographic column used was a C18, 250 x 4.60 mm reversed-phase, 4µmparticle size,SynergiFusion-RP 80, Phenomenex (Torrance, USA).The immunoafinity columns (IAC) from Neocolumn Aflatoxin DR, AOAC RI 081002 (2010), Neogen Corporation (Lansing, MI).

2.2. Methods

2.2.1. ***Sampling:*** cocoa beans (500 g portions) of S*tandard* and *Premium*Types (30 and 30, respectively), were collected (after passing through the drying step)fromthebatches (pilled bags) in the farms (48) and warehouses (12), located in different cities (n=17) of Bahia state (Kreibich et al., 2016). After that, they were sent to the Food Microscopy; Mycotoxicology& Food Contaminants and Electron Microscopy laboratories at the UFSC to carry out the investigation on the presence and identification of *living organisms* (rodents / insect / mites / fungi),*fungi* (total count / genera & species identification) and *toxins*(AFB_1). They had also moisture contentand water activity (mc / aw) checked.

2.2.2. ***Light filth analysis:*** was carried out according to the AOAC (2005) method, art. 965.38 (by stereoscopy, for cocoa, chocolate and candies). Briefly, *(a) fat extraction* -a portion (100 g)of the grind sample had sodium lauryl sulfate 2% (500 mL) added, followed by sieve cleaning with hot water then the content was transferred to a Wildman filth bottle trap, under heating (10 min). Next, the flask was cooled (room temperature) and liquid petrolatum added, followed by water (until completion-1L). The flask trap stirring rod was held with tweezers above the liquid surface (2 immiscible phases) and added a magnetic stirring bar to accelerate separation, with further heating (5 min). At the end of this step, the flask was allowed to stand (30 min); *(b) light filth separation* - petroleum phase (with light filths) was removed by vacuum filtration and dried at 105°C followed by*(c)* ***SM****identification* - the filter paper was transferred to the stereo microscope, where light filths reading (counting) and image identification (filths characterizations) were carried out according to Flint (1996) and (*d)* ***SEM*** - they had also their characteristic identified by SEM (Manfioet al., 2012; Scussel et al., 2014) at different magnifications (10 to 2000x).

2.2.3. ***Mycology tests****:(a) total fungi count* - samples (25g) were transferred into sterile polyethylene bags and added 0.1% peptone water (225 ml), followed by homogenization in a stomacher. Each diluted samplewasinoculated (100 µL) on PDA medium surface (n = 2) containing chloramphenicol (100 mg/L) in a laminar flow hood and incubated at 25°C±1°C for 7 days (Silva et al., 2010). The colonies developed were counted and expressed as colony forming unit per gram (CFU / g). *(b) colonies isolation* - the colonies were then transferred to MEA, G25N & CYA, incubated (25°C±1°C for 7 days) and observed macroscopically (diameters and individual characteristics) followed by light microscopy(LM)for fungi genera identification and *(c) microcultive*(genera/species)- a support was added on a glass slide in a Petri dish, then cubes (5 cm) of G25N grown colony were placed in the middle and lid covered. Inside the plate, was added a piece of moist cotton. After incubationat 25°C±1°C for 3 days, the stained slides were viewed under LM and identified according to Raper and Fennell (1965); Pitt (1979); Pitt and Hocking (2009); Barnett and Hunter (1986).

2.2.4. ***Aflatoxin B_1determination:***eachground sample was analyzed using IAC for clean-up step and LC/FLD for detection, according to Neogen protocol AFB_1DR HPLC (Neogen, 2013), with some modifications. Briefly, sample (25 g)was ground in an industrial blender jar with NaCl(5.0 g)and methanol 70% (125 mL). The mixture was blended (2 min), followed by filtration. The filtrate (15.0 mL) was diluted in phosphate buffered saline 10 mM(30 mL)PBS) and then filtered. Next,the filtrate (15.0 mL) was cleaned by anIACcolumn in a flow rate of one drop per second. After washing the column with a ultrapure grade water (20 mL), the toxin was slowly eluted with 100% methanol (1.0 mL)and water (1.0 mL). The eluate was evaporated to dryness using a heating block (40^oC) with gentle nitrogen stream and the dry residue was then redissolved with 100 µL of mobile phase (water: methanol: acetonitrile [60:20:20] added potassium bromide (119 mg) and nitric acid (47.6 µL). The extract (20 µL) was injected into the LC/FLD system(λ:ex.335 and em. 440 nm.). The mobile phase was delivered at a constant flow rate (1 mL/min). AFB_1 level quantification was performed by measuring the peak area at AFLs retention time compared with the standard solutions used for calibration curve (0.2-2.5 µg/mL for AFB_1); with a correlation factor equal to 0.985. The mean recoveries (%) of cocoa beans samples fortified with AFB_1 at concentrations levels of 4.0 and 8.0 µg/Kgwere 83 and 87%, respectively.

2.2.5. ***Humidity:***the*(a) mc* - was determined by the AOAC (2005) gravimetric method, art.31.1.02. Each homogenized sample (2g) was dried (n=3) in an oven at 105^oC up to constant weight; *(b) aw*- was obtained by measuring (2 g) of each sampleinthea_w meter device (at 25^oC).

III. Results and Discussion

The living organisms (mites, insects and their fragments), AFB_1 and humidity data showed variation among the quality *Premium* and *Standard* Types of post-fermentation dry cocoa beans and are presented in the Figures 1,2,3 and Tables 1,2.

***3.1. Premium and Standards Types light filth*:**as expected**,** a total of 53.3% (32/60) of the samples surveyed,

present some type of biological impurities that usually are not visible in the samples, being 12.5% (4/32) of insects, 3.1% (1/32) of the mites, 31.3% (10/32) of insects fragments, 6.3% (2/32) of larvae and 46.8% (15/32) with more than one light filth per sample (2 to 4 types). The light filth detected were of whole or in fragments (ground during processing), and are considered indicators of cocoabad quality and/or handling conditions. In the current study, insects, either whole or fragments and so their larvae and pupa (growing stages) were identified. Figure 1 and Table 1 show the positive samples percentage and each light filth type detected in total dry cocoa beans post-fermentation, of *Premium* (selected cocoa varieties - i.e., beans with high standard of tissue structure and characteristics aroma) and *Standard*(beans of not highly controlled standards and sensory quality)Types.As expected the *Premium* type of cocoa beans had lower rates of biological contaminants with 10 of the 32 contaminated samples (31.3%) while 22 (68.7%) were of *Standard* Type, suggesting an efficient control on the *Premium*Type beansprocessing.*The cocoa beans larvae*, which are commonly found (accepted by regulation though - FAO, 2014), they are resistant and persistent to the mild processing temperatures and commercialization storage at selling stores. The total moth life cycle is 35 to 50 days.Important to emphasize that adult phase lasts 10 to 20 days; the eggs delivery take 4 to 7 days to hatching; has 4 to 8 weeks and pupa has 5 to 10 days(Figure 3) (Lorini and Schneider 2004; Lorini 2008).***SEM*** - the detected ones were (a) *whole mites (Aceriaanthocoptes*) showing the main parts of its body(Figure 2): (a.1) *proterosoma* (at the top, in the head, formed by gnatosoma and propodosoma), and *hysterosoma* (left, formed by *metapodosoma*and*opisthosoma*); (a.2) *opisthosoma, gnathosoma* ((left), and four *pairs of legs*); (b) and (c) insects (b.1) whole and (b.2, c.1, c.2) fragments of ants (*Camponitusconsobrinus*L.; *Linepithemahumile*) showing: (b.1) the head, *mesosome* (chest), *petiole* (waist), *gaster* (responsible for digestion, elimination of waste), *legs* and *antennae*; (b.2) *head,* compound *eyes*, *jaw* and *stock up on food*); (c.1) *legs* (formed by *thigh, trochanter, femur, tibia* and *tarsus*); (c.2) *legs (tibialspurs,tarsus and tarsal claw*).On the other hand, *mites* (allergy promoters) were detected in 3.1% of the total samples. Fortunately, rodents' hair was not detected in any of the samples, which indicates that both processing plants and storage are applied rodent control programs, thus preventing several mouse transmitted diseases (Lorini, 2008; Kreibichet al., 2014). The light filths are considered vectors of several diseases that may affect consumers and also can cause alteration to the final products (reduction of dry matter / nutritional value/ sensory characteristics) leading to fermentation and/or deterioration. Under the Brazilian Resolution14/2014, it is considered harmful to human health different matters detected either *macro* or *microscopically*, thus apart from insects (at any development stage, living or dead, whole or in fragments); also parasites; insects excrement; as well as hard or sharp objects that may cause injury to the consumer.

3.2. Cocoa beans fungi load and identification:the cocoa beans samples showed total fungi load from $1x10^1$ to $3.5x10^2$ (mean: $5x10^2 \pm 1.4x10^2$CFU/g). Table 2shows the fungi quantitative data and the genera/species isolated. A high percentage of the samples (65 %) did not present fungi growth (NG) i.e., no spores contamination that could allow them to grow on the mycological media utilized.Only 35% of the total samples had them grown, whoever at rather low count ($1x10^1$ to $3.5x10^2$ CFU/g).Despite of the low fungi load, the presence of toxigenic fungal species in cocoa beanscan be indicative of potential mycotoxin accumulation. From the 35% (21/60) of the samples fungi strains isolated 16.7, 16.8 and 1.7% were of *Aspergillus*, *Penicillium* and *Fusarium*, respectively.The *Premium*Type cocoa beans had fungi detected in 26.6% (8/30) of the samples and the strains isolated were of*Aspergillus*, *Penicillium* and *Fusarium*(13.3, 10.0, 3.3%). On the other hand, the commercial type showed in 43.3% (13/30) of the *Standard* Type samples with fungi strain isolated (20.0 and 23.3 % were *Aspergillus*and *Penicillium* genera).Important to emphasize that one isolated strain was aflatoxigenic, indicating the importance to control the fungi growth, storage conditions as well as to have safe/high quality raw materials (aflatoxin free) to avoid final product contamination (Figure 3). Studies have reported high fungi growth, on cocoa beans due to heterogeneous/high mc during the drying process (Samson et al., 2002; Pitt and Hocking, 2009; Copettiet al., 2011; Genovese, 2009). In addition to beans fungi deterioration and consequent influence on the quality of cocoa paste and chocolate, their metabolites (mycotoxins) can remain stable in the final products due to whole processing mild temperatures / conditions applied (Scussel, 2002; Netto, 2009).

One of the recommendations to contaminants biological control is the fumigation. It is an effective insecticide treatment although it is not quite simple to apply.That procedure/step depends on the cocoa bean buyers decisiontoapply (Kreibich et al., 2016a). It may be carried out in the warehouses or in the cocoa own containers. Fumigation consists on distributing tablets (2 cm diameter) beneath the sacks (for as long as 20 sec). The amount is calculated per m^2 bags. After this step, the beans are covered with a canvas sheet during 72 hours.Afterthat, it can be removed and the gases vented off to the air. Other methods of insect/fungi control could be also by applying modified atmosphere (dioxide carbon, ozone-O_3) or keep silos hermetic or sealed (as long as innovative facilities - no air entrance are adopted/developed) and so also preserving the contact with environment moist.Kreibichet al. (2016b) investigated O_3effect, by utilizing gas concentrations of 20/40/60 µmol/mol, time of exposure of 30/105/180 min and 30 days storage. *A. flavus*was efficiently destroyed by the O_3 gas at 60 µmol/mol and 180 min (100% of spores did not germinate).

3.3. Aflatoxins:the limits of detection and quantification (LOD and LOQ) obtained of the methodology applied for AFB_1were of 0.26 and 3.1 µg/Kg, respectively. Linearity was confirmed with a correlation coefficient of R2 = 0.985 for AFB_1. The calculation of recovery reached87%, which was adequate for the study purposes. In the current study, the percentage of total samples contaminated obtained was 33.3% (20/60), being 20.0%(12) of themhigher than the maximum tolerated levels (MTL) set by the National Health Surveillance Agency (10µg/Kg)(Brasil 2011). The total AFB_1contamination levels werefrom6.61 to 15.03µg/Kg(Table 2).The *Premium* Type cocoa beans present some AFB_1levels (6.61-12.41µg/Kg) in 5/30(16.7%) of the samples surveyed, being 3/30 (10.0%) of them above the LMT. On the other hand, the *Standard* Type showed AFB_1levels (6.69-15.05 µg/Kg) in 15/30(50%) samples, being that 9/30 (30%) above the LMT. These results show the higher quality and control in the cultivation, processing and storage of *Premium*Type, ensuring a chocolate greater flavor and quality.AFLs are highly toxic metabolic(mutagenic and carcinogenic)(IARC, 1993) compounds produced by *Aspergillus* section *Flavi* mainly by *Aspergillusflavus* and *A. parasiticus*(Frisvad et al., 2005). Recent publications report the occurrence of fungi Section *Flavi* in cocoa beans (Copetti et al., 2011; Sanchez-Hervaz et al., 2008), includingChilean raisins and peanuts (Zorzete et al., 2013).Despite evidence found, the European Community (EC) Scientific Committee on Food,has not set MTL for AFLs in cocoa and cocoa products.The presence of mycotoxins in foods is a risk to human health, requiring control measures and monitoring. The best way to prevent contamination in cocoa beans is through good agricultural practices, proper storage,transportand processing, preventing mycotoxin contamination in cocoa.

3.4. Cocoa beans humidity conditions: *(a) moisture analysis* -the samples of cacao beans showed mc variation (Table 2), probably influenced by the region weather conditions (relative humidity and environment temperature). Mc ranged from 4.71 to 15.68% (mean 10.19 ± 1.06%). Two samples showed high humidity (13.57 and 15.68%), thus extremely favorable conditions for fungi and possible mycotoxin production. A total of 35 cocoa beans (58.3%) samples had values higher than the safehumidity limit (7.0%) described in the Ministry of Agriculture,Normative Instruction 38(MAPA, 2008). The cocoa beans *Premium* Type, the mc rangedfrom 4.71 to 10.16%. A total of 12/30 (40%) of the samples analyzed had values higher than 7.0%. On the other hand, the commercial type showed a mc range of 6.39 to 15.68% and 23/30 (76.7%) samples had the value higher than the upper limit described in the literature *(b) water activity*-aw range of 0.59 to 0.88 (mean of 0.73±0.0095%. Among these, five samples (8.3%) showed high levels of aw (0.88, 0.86, 0.85, 0.83, 0.83), favorable conditions for the production of toxins by toxigenic fungi present in the samples (Beauchat, 1981). The *Premium* Type cocoa beans aw ranged from0.59 to 0.83 and 1/30 (3.3%) samples analyzed had the value higher than 0.83. On the other hand, the *Standard* Type showed aw range of 0.68 to 0.88 and 4/30 (13.3%) samples had the values higher than the upper limit described in the literature.

IV. Conclusions

From the total samples studied (*Premium*&*Standard*), different biological contaminants were detected in 53.12% (17 samples) and 46.87 % (15) of samples had either only one or more than one type (2 to 4) of light filth per sample. Despite of the low fungi load, from the 35% of the samples fungi strains isolated, 16.7, 16.8 and 1.7 % were of *Aspergillus, Penicillium* and *Fusarium*, respectively.

Dry cocoa beans showed aw ranging from 0.59 to 0.88, and a mc of 4.71 to 15.68%, favorable conditions of the production of toxins by toxigenic fungi present. AFB_1 was detected in 33.3% (20 of 60) of the samples analyzed, with levels ranging from 6.61 to 15.03µg/Kg for AFB_1,which 50.0% and 16.7% of this samples were from*Standard* and*Premium*Types, respectively. The cocoa bean was shown to be quite prone to living organism proliferation after fermentation. Therefore moisture control is a must to prevent and/or control their proliferation and so deterioration. Knowledge on cocoa beans safety (living organisms contamination)are of interest to help chocolate industries to improve final products quality.

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References

[1]. Afoakwa, E. O., Paterson, A., Fowler, M. 2008.Factors influencing rheological and textural qualities in chocolate – a review.*Trends in Food Science &Technolology*,18, 290-298.

[2]. AOAC. 2005. *Official Methods of Analysis of AOAC International*, 18th Ed., (H. Willian and W.L. George, eds.) Current through revision 3, 2010, Gaithersburg, MD.APHA. 1999. *Standard Methods for the Examination of Water and was and Wastewater*, 16th Ed., American Public Health Association,Washington.

[3]. Barel, M.,1997. Fermentation of cocoa: the way of its estimation and control.*Revuedes Industries AlimentairesetAgricoles,* 14, 211-214.

[4]. Barnett, H. L.; Hunter, B.B.; 1986.Ilustrated genera of imperfect fungi. New York: Macmillan Publishing Company, 218p.

[5]. Beauchat, L.R. Microbial stability as effected by water activity. *Cereal Food World*, 26, 345-349, 1981.

[6]. BRASIL.National Health Surveillance Agency - ANVISA. It provides for maximum permissible levels (LMT) for mycotoxins in food. Resolution - RDC No. 7, February 2011. Available in: <http://www.brasilsus.com.br/legislacoes/anvisa/107378-7.html>. Accessed on: 04 abr. 2015.

[7]. BRASIL. National Health Surveillance Agency - ANVISA. It provides for macroscopic and microscopic foreign matter in food and beverages, their tolerance limits and other measures. Resolution of the Collegiate Directorate - RDC No. 14 of March 28, 2014. Available in:<*portal.anvisa.gov.br*>.Accessed on: 04 abr. 2015.

[8]. Copetti, M. V.,Iamanaka, B.T., Pitt, J.I., Taniwaki, M.H. 2011.Aflatoxigenic fungi and aflatoxin in cocoa.*Intl J Food Microbiol.*148, 141–4.

[9]. Copetti, M.V.,Iamanaka, B.T., Pereira, J.L.,Lemes, D.P.,Nakano, F.,Taniwaki, M.H.2012. Co-occurrence of ochratoxina and aflatoxins in chocolate marketed in Brazil. *Food Control***.**26, 36-41.

[10]. Efraim, P., 2009. Contribution to quality improvement of cocoa in Brazil, through the characterization of oil–resistant broom–witchand seeds damaged by fungus cultivars.Thesis PhD in Food Technology, Campinas University.

[11]. FAO. 2014.Food and Agriculture Organization of the United Nations.FoodOutlok – Global Market Analysis, Wheat, 1997. Available: <htpp://www.fao.org>. Access in: 05/05/2015.

[12]. Faleiro F. G., Niella G. R., Cerqueira, A. R. R.N., 2004. DamacenoV. O.; Gomes L. M.C;FaleiroA. S.G. Produção de Micélio de *Crinipellis perniciosa* em Quatro Meios de Cultura,VisandoExtração de DNA. *Revista Fitopatologia Brasileira*, n. 29.

[13]. Flint, O. 1996. FoodsPreparation for StereoMicroscopy. Chap3, Acribia, Zaragosa ed., Spain, 9-12.

[14]. Frisvad, J. C.; Skouboe, P.; Samson, R. A.; 2005. Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B_1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov..*SystAppl Microbiol.*28, 442–453.

[15]. Genovese, M. I., Lannes, S. C. S.,2009.Comparison of total phenolic content and antiradical capacity of powders and chocolates from cocoa &cupuaçu. Ciência e Tecnol. Alimentos, 29, 810-814.

[16]. Hii, C.L., R.A. Rahman, S. Jinap, Y.B. Che Man., 2006. Quality of cocoa beans dried using adirect solar dryer at different loadings. *J. Sci. Food Agr*., 86,1237-1243.

[17]. IARC.1993. Monographs on the evaluation of the carcinogenic risks to humans: Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon, France: Int.*Agency Res. Cancer*, 56,489–521.

[18]. Koerich, K. S., Tonon, K. M. &Scussel, V. M.,2013.Labels layout of cats and dogs food sold in Brazil and their national regulation adequacy. *Cienc.Rural*, 43, 366-369.

[19]. Kreibich, H.H., Savi, G.D., Moecke, E., Scussel, V. M. 2014. Easter eggs and other cocoa (*Theobroma cacao* L.) products quality: insects, mites, fungi and packaging versus critical control points. *Intl J. Applied Sci.Technol.*, 4.,5.

[20]. Kreibich, H.H., Moecke, E., ScusselL, V. M. 2015. Biological contaminants control strategies for cocoa beans better quality. Int. Coffee, Cocoa and Tea Conf. Aveiro, Portugal.

[21]. Kreibich, H.H. 2016. Qualidade e segurança das amêndoas de cacau (*TheobromacacaoL*.) e seus produtos com relação aos contaminantes biológicos e a descontaminação de fungos toxigênicos com ozônio gasoso. Dissertação de mestrado em Ciência dos Alimentos, 168p., Universidade Federal de Santa Catarina, Brasil.

[22]. Kreibich, H.H., Moecke, E.,Scussel, V. M. 2016a. Cocoa beans processing and storage conditions control for safe chocolate products. *J. Chem. Bio. Phys.Sci.,6, 513-526.*

[23]. Kreibich, H.H., Christ, D., Silva, J., Savi, G.D., Scussel, V. M. 2016b. Decontamination of Cocoa Beans (*Theobroma Cacao* L.)Inoculated with *Aspergillusflavus*by Ozone Gas. *J. Chem. Bio. Physical Sci., 6,560-570.*

[24]. Kumagai, S., Nakajima, M., Tabata, S. E.,Ishikuro, E., Tanaka, T., Norizuki, H., et al.(2008). Aflatoxin and ochratoxinA contamination of retail food and intake ofthese mycotoxins in Japan.*FoodAddit.Contam.*, 25, 1101-1106.

[25]. Lorini, I.; Schneider, S.; 2004.Pragas de grãos armazenados: resultados de pesquisa. Passo Fundo: EMBRAPA-CNPT, 47p.

[26]. Lorini, I.; 2008. Manejo Integrado de Pragas de Grãos de Cereais Armazenados. Passo Fundo: Embrapa Trigo, 72p.

[27]. Lorini, I.; 2012. Insetos que atacam grãos de soja armazenados.In: Hoffmann-Campo, C. B., Correa Ferreira,B. S.; MoscardiI, F. **Soja**: manejo integrado de insetos e outros artrópodes-praga. Brasília, DF. Embrapa.421-444p.

[28]. Manfio, D., Beirao, L.H., Damian, C., Savi, G.D &Scussel, V.M. 2012.Brazil nut (*Bertholettia excels* H.B.K.) brown skin characterization—a waste product generated from shelled dry nut factories of Amazon region. *Agric. Sci Res. J.* 2, 253-60.

[29]. MAPA - Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa nº 38, de 23 de junho de 2008. Estabelece o Regulamento Técnico da Amêndoa de Cacau, definindo o seu padrão oficial de classificação, com os requisitos de identidade e qualidade, a amostragem, o modo de apresentação e a marcação ou rotulagem, nos aspectos referentes à classificação do produto. Available in: <http://sistemasweb.agricultura.gov.br/sislegis>. Acessed on: october 2015.

[30]. Mounjouenpou, P.,Gueule, D., Fontana-Tachon, A., Guyot, B., Tondje, P.R., Guiraud, J.P. 2008.Filamentous fungi producing ochratoxin A during cocoa processing in Cameroon.*Int.J Food Microbiol.*128, 234–241.

[31]. Nachtigall, A. M., 1999. Processamento de chocolate.Bacharelado em Químicade Alimentos, 25 p. Universidade Federal de Pelotas, Pelotas.

[32]. Neogen. 2013. Aflatoxins (AFLs) TestingSolutions. AFLs - Test HPLC.Indaiatuba, São Paulo, Brazil.Available at: <http://neogen.com/>.Acessed on: october13, 2015.

[33]. Netto, C. G.; 2009.Pesquisa mostra como micotoxinas migram do cacau para o chocolate.Availablein:<http://www.unicamp.br/unicamp/unicamp_hoje/ju/setembro2009/ju443pdf/Pag05.pdf>.Acessed on:august 22, 2015.

[34]. Pitt, J.I. 1979. The genus *Penicillium* and its teleomorphics states *Eupenicillium*and*Talaromyces*. London: Academic Press, 634p.

[35]. Pitt, J.I., Hocking, A.D. 2009. Fungi and Food Spoilage, third ed. Springer, New York.

[36]. Raper, K. B.; Fennell, D.I.; 1965.The genus Aspergillus. Baltimore: The Williams &Wilkings Company, 686p.

[37]. Ribeiro, N.C.A., Bezerra, J.L., Lopez, A. 1986. Micobiota na fermentação do cacau no estado da Bahia, Brasil. *Rev. Theobroma* 16, 47–55.

[38]. Rubini, M. R., Silva-Ribeiro, R. T., Pomella, A. W. V., Maki, C. S., Araújo, W. L., Santos, D. R., Azevedo, J. L., 2005. Diversity of endophytic fungal community of cacao (*Theobroma cacao* L.) and biological control of *Crinipellisperniciosa*, causal agent of Witches' Broom Disease.*Int. J. Biol. Sci*., 1, 24–33.

[39]. Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O., 2002. Introduction to food-and airborne fungi. CBS ed., The Netherlands.389p.

[40]. Savi, G.D., Piacentini, K.C., Bittencourt, K.O.,Scussel, V.M. 2014. Ozone treatment efficiency on Fusarium graminearum and deoxynivalenol degradation and its effects on whole wheat grains (*Triticum aestivum* L.) quality and germination. *J. Stored Prod. Res.*59, 245-253.
[41]. Scarpari, L. M.; Meinhardt, L. W.;Mazzafera, P.; Pomella, A. W. V.; Schiavinato, M. A.; Cascardo, J. C. M.; Pereira, G. A. G.; 2005. Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellisperniciosa*. *J. Exp. Bot.*, 56, 865–877.
[42]. Sanchez-Hervas, M., Gil, J.V., Bisbal, F., Ramon, D., Martinez-Culebras, P.V. 2008.Mycobiota and mycotoxin producing fungi from cocoa beans.*Int. J. Food Microbiol.* 125, 336-340.
[43]. Schwan, R.F., Wheals, A.E. 2004.The microbiology of cocoa fermentation and its role in chocolate quality.Critical Reviews in *Food Sci.Nutr.*44, 1-17.
[44]. Scussel, V.M. 2002. Mycotoxins in Storage Grains. In: Lorini, I.; Miike, L.H.; Scussel, V.M., Armazenagem de Grãos Chap 9.2, ed. Bio Geniziz, 693-737, Campinas, SP, Brazil
[45]. Scussel, V.M. 2004. Aflatoxin and food safety: recent South American perspectives. *J. Toxicol.* 23, 179-216.
[46]. Scussel, V.M., Giordano, B.N., Simao, V., Manfio, D., Galvao, S., Ferreira Rodrigues, M.N. 2011. Effect of oxygen-reducing atmospheres on the safety of packaged shelled brazil nuts during storage. *Intl. J. Anal. Chem.1-9.*
[47]. Scussel, V.M., Manfio, D., Savi, G.D. &Moecke, E.H.S. 2014. Stereoscopy and scanning electron microscopy of Brazil nut (*Bertholletiaexcelsa* H.B.K.) shell, brown skin and edible part: part one - Healthy Nut. *J. Food Sci.* 79, 1443-1453.
[48]. Silva, N.D.A., Junqueira; V.C.A., Silveira; N.F.A., Taniwaki, M.H.; Santos, R.F.S.; Gomes, R.A.R. 2010. *Métodos de Análise Microbiológica de Alimentos e Água*, 4th Ed., p. 624, Varela, Sao Paulo.
[49]. Zorzete, P., Baquião, A.C., Atayde, D.D., Reis, T.A., Gonzales, E., Correa, B. 2013.Mycobiota, aflatoxinsandcyclopiazonicacid in storedpeanutcultivars. *Food Res.Int.*52, 380-386.

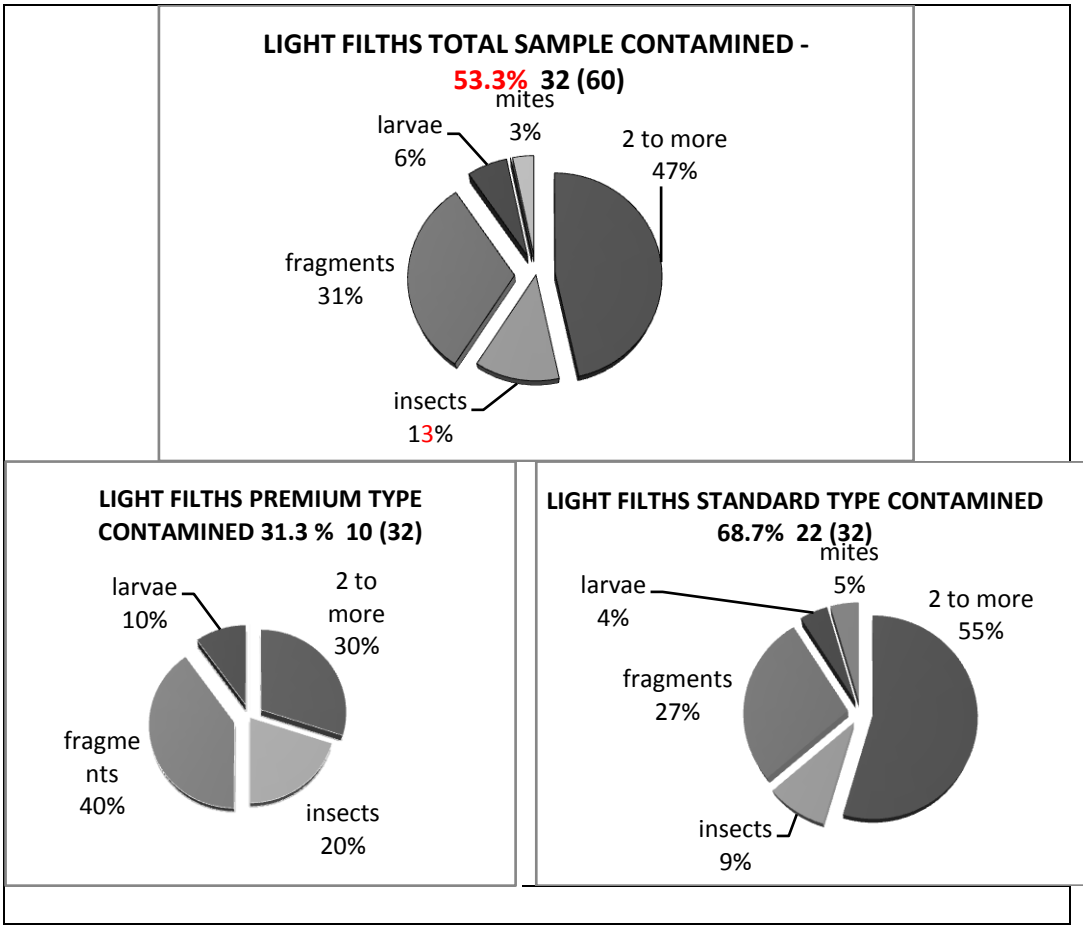


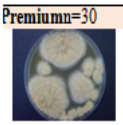
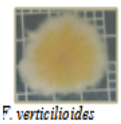
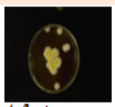
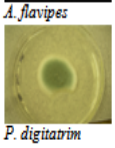
Figure 1. Cocoa beans (*Theobroma cacao* L.) light filth percentage total samples detected in Bahia state.

Table 1. Light filths detected in different post-fermentation dry cocoa beans *Premium* and *Standard* Type and your percentage from the 2014 harvest season (March / July) in Bahia State

Cocoa beanssamples			Light filths									
Type	Contaminated		Insects		Mites		Fragment		Larvae		2to 4 light filths	
	number /total	(%)										
Premium	10/32	31.3	2(10)	20.0[P]	ND	ND	4(10)	40.0[P]	1(10)	10.0[P]	3(10)	30.0[P]
*Standard**	22/32	68.7	2(22)	9.09[P]	1(22)	4.54[P]	6(22)	27.27[P]	1(22)	4.54[P]	12(22)	54.54[P]
Total(n=60)	32/60	53.3	4(32)	12.5[P]	1(32)	3.1[P]	10(32)	31.3[P]	2(32)	6.3[P]	15(32)	46.8[P]

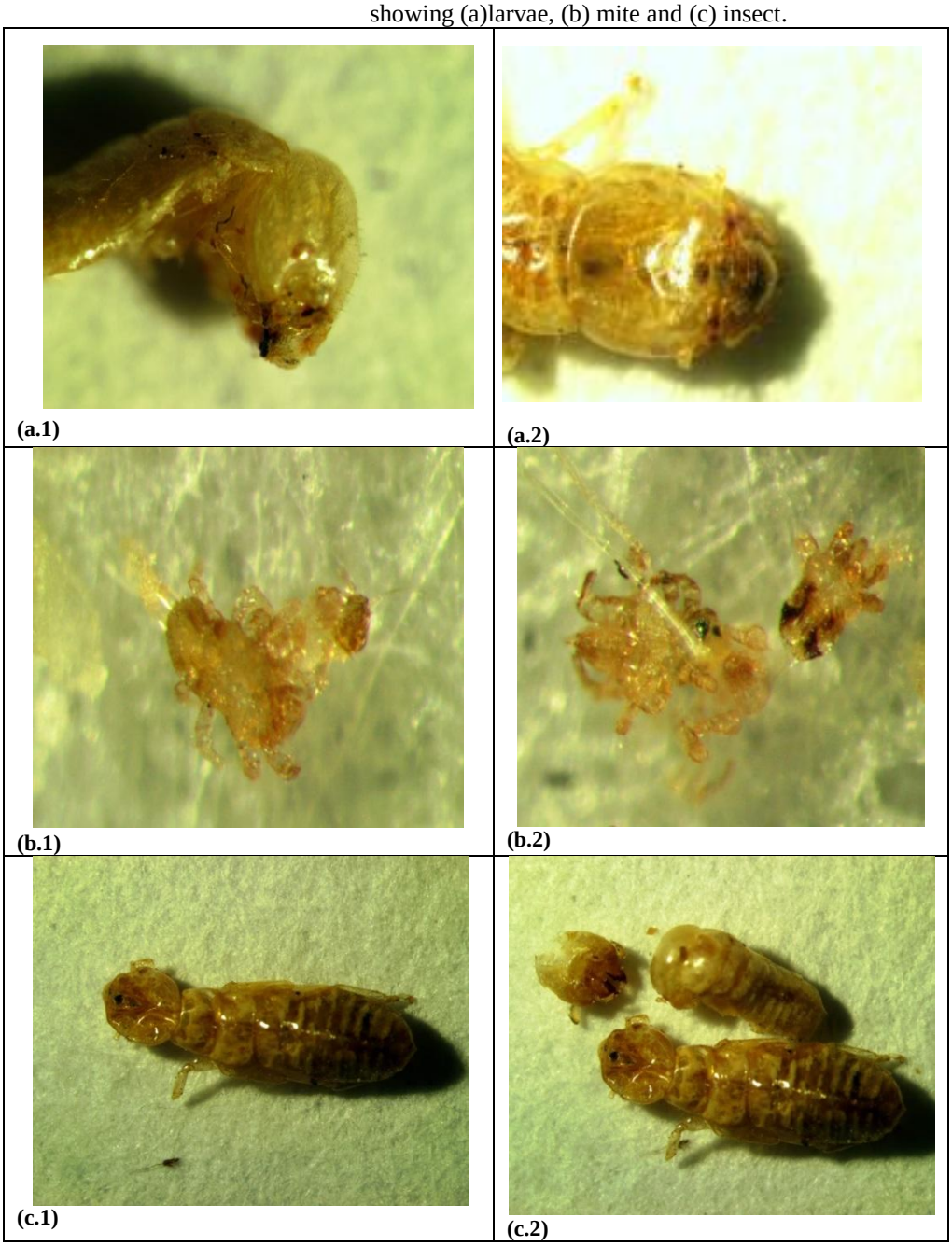
ND: No detected *also called Commercial[P]:percentage

Table 2. Results of fungal species isolated in cocoa beans (*Theobroma cacao* L.), aflatoxin B1 and humiditydetected in cocoa beans (*Theobroma cacao* L.) duringthe study (2014 season)**

Cocoabeans	Mycologytest(µg/Kg)	Aflatoxintest(µg/Kg)				Humidity			
SampleType	NS[c]*Fungi[P]isolated*	NS[c]levels		AFB1*mean		mc[a]*meanCS[b]mc[a c]*		aw[b]meanCS[b]aw[bc]*	
Total n=60	21/60(35)	8(60)	6.61-9.63	13.3	7.79	4.71-15.6810.19±1.0635(60)58.3		0.59-0.88 0.73±0.0095(5/60)8.3	
 *A. flavus*	*Aspergillusflavus*4/60(6.7)	6.61<MTL				4.71		0.59	
	*A. flavipes*3/60(5.0)	6.69				5.70		0.63	
	*A. ochraceus*3/60(5.0)	6.94				6.52		0.66	
		7.92				6.99		0.67	
		8.93				7.00		0.73	
 *P. chrysogenum*		12(60)11.01-15.03		20.0	13.08	7.60		0.78	
	*Penicilliumdigitatum*1/60(1.7)	11.01>MTL				8.65		0.80	
	*P.citreonigrum*1/60(1.7)	12.41				9.40		0.81	
	*P. chrysogenum*7/60(11.7)	13.34				13.57		0.82	
	*P. purpurogenum*1/60(1.7)	14.94				15.68		0.88	
	*Fusariumverticillioides*1/60(1.7)	15.03							
Premium n=30	8/30(26.6)	2(30)	6.61-6.94	6.7	6.78	4.71-10.167.43±0.8612(30)	40.0	0.59- 0.830.71±0.0081(1/30)	3.3
 *P. ocharaceus*	*Aspergillusflavus*2/30(6.66)	6.61<MTL6.94				4.71		0.59	
	*A. flavipes*1/30(3.33)					5.77		0.60	
	*A. ochraceus*1/30(3.33)	3(30)11.01-12.4110.0			11.71	6.11		0.62	
						6.39		0.65	
	*P. chrysogenum*2/30(6.66)					6.57		0.68	
 *F. verticilioides*	*P. purpurogenum*1/30(3.33)	11.01>MTL				6.91		0.70	
	*Fusariumverticillioides*1/30(3.33)	11.98				7.69		0.74	
		12.41				7.75		0.75	
						8.93		0.77	
						10.16		0.83	
Standard n=30	13/30(43.3)	6(30)	6.69-9.0820.0		7.88	6.39- 15.6811.03±1.14 23(30)	76.7	0.68-0.880.78±0.0094(4/30)	13.3
 *A. flavipes*	*Aspergillusflavus*2/30(6.66)	6.69<MTL				6.39		0.68	
	*A. flavipes*2/30(6.66)	7.13				6.61		0.71	
	*A. ochraceus*2/30(6.66)	7.92				7.99		0.72	
		8.17				8.18		0.76	
		8.93				8.46		0.78	
		9.08				8.93		0.79	
 *P. digitatrim*	*Penicilliumdigitatum*1/30(3.33)	9(30)11.92-15.05		30.0	13.48	9.40		0.83	
	*P. citreonigrum*1/30(3.33)	11.92>MTL				10.16		0.85	
	*P. chrysogenum*5/30(16.6)	12.65				12.47		0.86	
		12.90				15.68		0.88	
		13.26							
		14.18							

a: moisture content b: water activity CS: contaminated samples c:contaminatedg: genera s:specie *:percentage**: March/July NS: Number of the samplesLOD/LOQ= 0.26 e 3.1 µg/KgR^2 = 0.985

Figure 3. Different light filth detected in cocoa beans samples SM micrographs images in size 40 to 100x – showing (a)larvae, (b) mite and (c) insect.



(a.1) **(a.2)**

(b.1) **(b.2)**

(c.1) **(c.2)**

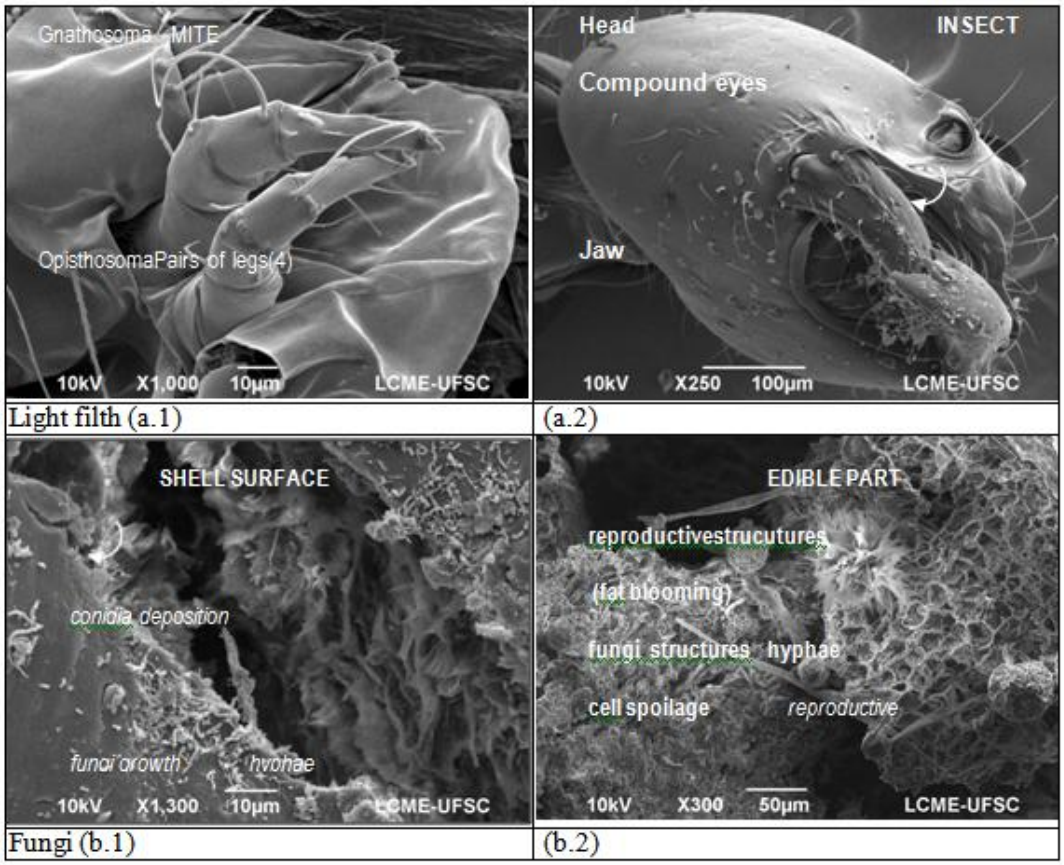


Figure 2. Scanning electron micrographies of (a) DIFFERENT LIGHT FILTH:(a.1) mite and (a.2) insect fragment; (b) FUNGI GROWTH (b.1) mycelia on shell surface and (b2) hyphae with conidia [reproductive structures] onediblepartinpost-fermentation dry cocoa beans (*Theobroma cacao* L.) samples

Kreibich,H.H. " Post-Fermentation Standard and PremiumTypesDry Cocoa Beans (Theobroma cacao L.)Quality: living organisms and toxins." IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS) 11.9 (2018): 58-67.