International Journal of Food Microbiology 178 (2014) 13–20

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Review

Fungi and mycotoxins in cocoa: From farm to chocolate

Marina V. Copetti [a,*], Beatriz T. Iamanaka [b], John I. Pitt [c], Marta H. Taniwaki [b]

[a] Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
[b] Instituto de Tecnologia de Alimentos, Campinas, SP 13070-178, Brazil
[c] CSIRO Animal, Food and Health Sciences, North Ryde, NSW 2113, Australia

ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 13 February 2014
Accepted 27 February 2014
Available online 11 March 2014

Keywords:
Ochratoxin A
Aflatoxin
Chocolate
Cocoa by-products
Food safety

ABSTRACT

Cocoa is an important crop, as it is the raw material from which chocolate is manufactured. It is grown mainly in West Africa although significant quantities also come from Asia and Central and South America. Primary processing is carried out on the farm, and the flavour of chocolate starts to develop at that time. Freshly harvested pods are opened, the beans, piled in heaps or wooden boxes, are fermented naturally by yeasts and bacteria, then dried in the sun on wooden platforms or sometimes on cement or on the ground, where a gradual reduction in moisture content inhibits microbial growth. Beans are then bagged and marketed. In processing plants, the dried fermented beans are roasted, shelled and ground, then two distinct processes are used, to produce powdered cocoa or chocolate. Filamentous fungi may contaminate many stages in cocoa processing, and poor practices may have a strong influence on the quality of the beans. Apart from causing spoilage, filamentous fungi may also produce aflatoxins and ochratoxin A. This review deals with the growth of fungal species and formation of mycotoxins during the various steps in cocoa processing, as well as reduction of these contaminants by good processing practices. Methodologies for fungal and mycotoxin detection and quantification are discussed while current data about dietary exposure and regulation are also presented.

Contents

* Corresponding author at: Departamento de Tecnologia e Ciência de Alimentos, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil. Tel.: +55 55 3220 8254x209.
E-mail address: mvc@smail.ufsm.br (M.V. Copetti).

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.023
0168-1605/

1. Introduction

Cocoa is an important crop, as it is the raw material from which chocolate is manufactured. Cocoa trees thrive best in very humid tropical climates, and are grown mainly in West Africa although significant quantities also come from Asia and Central and South America. Primary processing is carried out on the farm. Pods are harvested and opened by hand. The beans are piled in heaps or in wooden boxes, and allowed to undergo a natural fermentation. Microorganisms contaminate the beans from the outer surfaces of pods, workers' hands and tools, plant leaves, collection baskets, insects or residual mucilage in equipment. The desirable types are yeasts, and lactic or acetic acid bacteria, which secrete enzymes, alcohol and lactic and acetic acids (Ostovar and Keeney, 1973; Schwan and Wheals, 2004). These metabolic products lead to embryo death and production of important precursors of chocolate aroma (Voigt, 2013). After fermentation, which lasts several days, the beans are transferred to wooden sun drying platforms, or sometimes are dried on cement or on the ground, where a gradual reduction in moisture content and volatile acid production occurs, eventually stopping microbial growth and enzyme production. When the beans are fully dry, they are transferred to storage rooms, then later bagged and marketed. In processing plants, the dried fermented cocoa beans are roasted, shelled and ground, then two distinct processing lines produce powdered cocoa or chocolate.

Cocoa beans are susceptible to fungal contamination during many of these processing steps. Microbial growth is affected by intrinsic parameters of cocoa beans such as pH, by water activity and by the various organic acids produced during fermentation. Besides causing deteriorative alteration of sensorial properties, the presence of filamentous fungi in cocoa and chocolate is also a cause for concern due to the possibility of mycotoxin formation. Both aflatoxin and ochratoxin A have been reported from cocoa and chocolate.

The discovery of ochratoxin A in cocoa and by-products prompted international discussions. In 2012 the Codex Committee on Contaminants in Food elaborated a discussion paper on ochratoxin A in cocoa (Codex Alimentarius Commission, 2012). Information was gathered on the occurrence of ochratoxin A in cocoa and by-products to determine levels of contamination, the contribution of these products to ochratoxin A in the human diet, to elucidate the main factors responsible for ochratoxin A synthesis in cocoa and to reduce it during processing. A code of practice was formulated and is under discussion (Codex Alimentarius Commission, 2013).

This review focuses on the factors affecting the development of filamentous fungi and the potential for formation of aflatoxins and ochratoxin A at all processing stages. An update on international reports of mycotoxins in cocoa and chocolate is also provided.

2. Fungi and mycotoxins in farm processing of cocoa

2.1. Fermentation

Beans and pulp inside an intact, healthy cocoa pod are microbiologically sterile (Fig. 1, 1A–C) but when opened soon become contaminated with microorganisms, especially those that will contribute to the subsequent natural fermentation process.

During fermentation, the microbial population is dominated by yeasts in the first hours after which their level is surpassed by those of lactic acid bacteria, that in turn decline after 48 h of fermentation after which acetic acid bacteria develop, in a well established pattern of succession (Schwan and Wheals, 2004; Nielsen et al., 2013). Lima et al. (2011) present in detail the importance of fermentation and environmental factors in the development of a good quality product. The products of microbial metabolism strongly influence the microbial population that sequentially dominate this micro-environment. High amounts of alcohol are produced by yeasts and lactic and acetic acids by bacteria. Together with environmental factors including low pH, the action of the organic acids, elevated temperatures due to exothermic reactions and microaerophilic conditions, these fermentation products restrict the growth of filamentous fungi. Studies reported the presence of filamentous fungi especially in the last days of fermentation, in the surface (Fig. 1, 2A) (Schwan and Wheals, 2004; Copetti et al., 2013a) or when the cocoa mass is not turned regularly (Nielsen et al., 2013). Filamentous fungi can sometimes be seen in the cocoa inside the fermentation boxes when the mass is turned (Fig. 1, 2B).

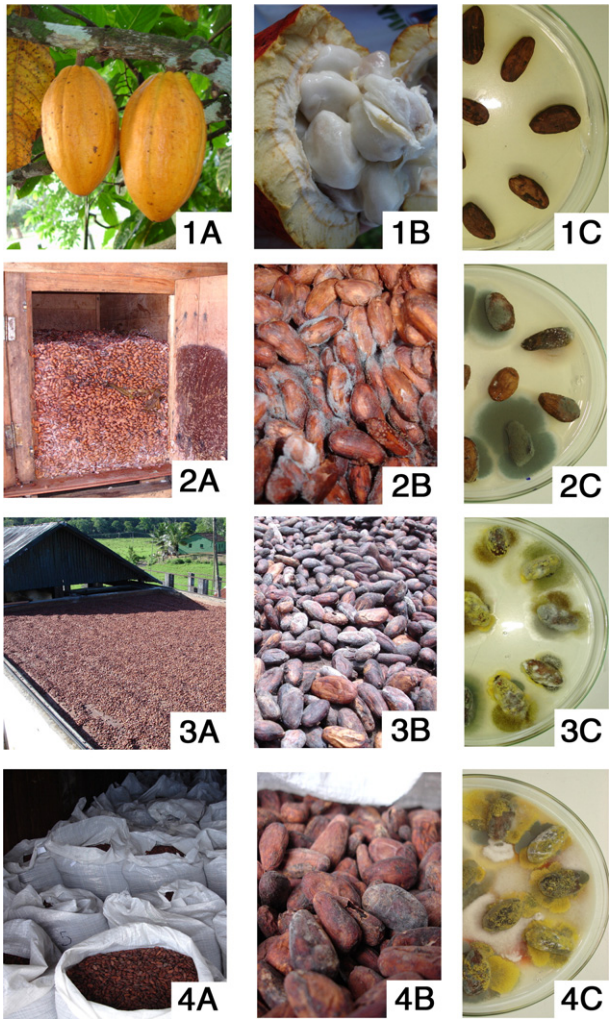

Fig. 1. 1A, cocoa pod; 1B, cocoa beans surrounded by pulp; 2A, 2B, fungal presence during fermentation; 3A, sun drying of cocoa beans; 3B, mouldy cocoa beans at drying; 4A, storage of cocoa beans; 4B, mouldy cocoa beans in storage; 1C, 2C, 3C, 4C, mycological evaluation of cocoa beans by direct plating in DG18, 1C, before fermentation; 2C, during fermentation; 3C, during drying; 4C, during storage.

The role of filamentous fungi during cocoa fermentation is not well understood. It is known that some species can cause hydrolysis of the pulp, produce acids or off flavours and so alter the taste of the cocoa beans (Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Extensive fungal development at the end of fermentation may cause increased deterioration in the drying phase (Gilmour and Lindblom, 2008).

After studying the microbial ecology of cocoa fermentation in wooden boxes in Indonesia, Ardhana and Fleet (2003) observed the presence of *Penicillium citrinum* and an unidentified basidiomycete in the first 36 h of fermentation. Both fungi showed strong polygalacturonase activity, suggesting their role in the degradation of pulp in the early stages of fermentation. The presence of *Aspergillus versicolor*, *Aspergillus wentii* and *Penicillium purpurogenum* was also reported (Ardhana and Fleet, 2003).

The occurrence of filamentous fungi, especially species potentially producing ochratoxin A, was compared between heap and box fermentation in Cameroon by Mounjouenpou et al. (2008). No significant differences were seen between the two methods in relation to the fungal species found (*Aspergillus fumigatus*, *Aspergillus tamarii*, *A. versicolor*, *Aspergillus carbonarius*, *Aspergillus niger*, *Penicillium sclerotiorum*, *Penicillium paneum*, *Penicillium crustosum*, *Mucor* spp., *Rhizopus* spp., *Fusarium* spp. and *Trichoderma* spp.). However, pod integrity and, to a lesser degree, a delay in pod opening, affected fungal diversity. Damaged pods often showed proliferation of toxigenic fungi including *A. carbonarius*, *A. niger*, with the potential to produce ochratoxin A, and *Fusarium* species.

In a study of cocoa fermentation in Brazil (Copetti et al., 2011a), 18 species of filamentous fungi were isolated. However, only *Monascus ruber*, *P. paneum* and *Geotrichum candidum* were present in more than 20% of the 51 samples examined, 19.6, 23.5 and 25.5%, respectively, with an average of beans infected of 14.5, 13.7 and 46.6%, respectively (Fig. 1, 2C). Existing physiological information (Pitt and Hocking, 2009) provided suggestions that might explain the higher prevalence of these species. *G. candidum* is able to grow only at high water activities, but under microaerophilic conditions, while *M. ruber* and *P. paneum* are capable of growth under low oxygen tension. *P. paneum* is closely related to *Penicillium roqueforti*, known to tolerate high levels of CO_2 and weak acid preservatives similar to those found in cocoa fermentation. Other acetic acid tolerant species, *Paecilomyces variotii* and *Thielaviopsis ethaceticus*, have been reported from cocoa fermentations on Brazilian farms (Ribeiro et al., 1986).

Species producing aflatoxins, *Aspergillus flavus* and *Aspergillus parasiticus*, have been isolated in samples from fermentations (Copetti et al., 2011a), and also *A. niger* and *A. carbonarius*, species producing ochratoxin A (Mounjouenpou et al., 2008; Copetti et al., 2010). In general mycotoxin producing species were present in less than 5% of samples during fermentation (Copetti et al., 2010, 2011a), but these initial inocula could contribute to elevated populations when competition diminishes in subsequent processing steps.

Only a few samples of cocoa collected during fermentation have been analysed for mycotoxins: three studies for ochratoxin A (Gilmour and Lindblom, 2008; Mounjouenpou et al., 2008; Copetti et al., 2010), and one for aflatoxins (Copetti et al., 2011a). While mycotoxin formation was found to be possible during fermentation, reported concentrations were low, on average lower than 0.02 μg/kg for aflatoxins and about 0.05 μg/kg for ochratoxin A when healthy pods were sampled (Gilmour and Lindblom, 2008; Mounjouenpou et al., 2008; Copetti et al., 2010, 2011a).

2.2. Drying

At the end of fermentation, cocoa beans contain 40–60% moisture and should be dried to 6–7% moisture for microbial stability. Drying may be carried out in the sun on wooden platforms (Fig. 1, 3A), in mechanical driers: sometimes both methods are used under changeable weather conditions. Sun drying, the common method, usually takes about 7 days under sunny conditions but can take 2 to 4 weeks if the weather is adverse. As would be expected, prolonged drying increases the chance of fungi growth and spoilage (Nielsen et al., 2013).

As the water activity is reduced during drying, from a water activity of 0.99 down to 0.85, first the growth of bacteria ceases, then that of the yeasts, which have a higher tolerance of low water availability (Beuchat, 1987). Xerophilic fungi become dominant in the later stages of drying, as water activity continues to decrease (Fig. 1, 3B–C). The final water activity of the beans is about 0.50 (Copetti et al., 2010). Wooden drying

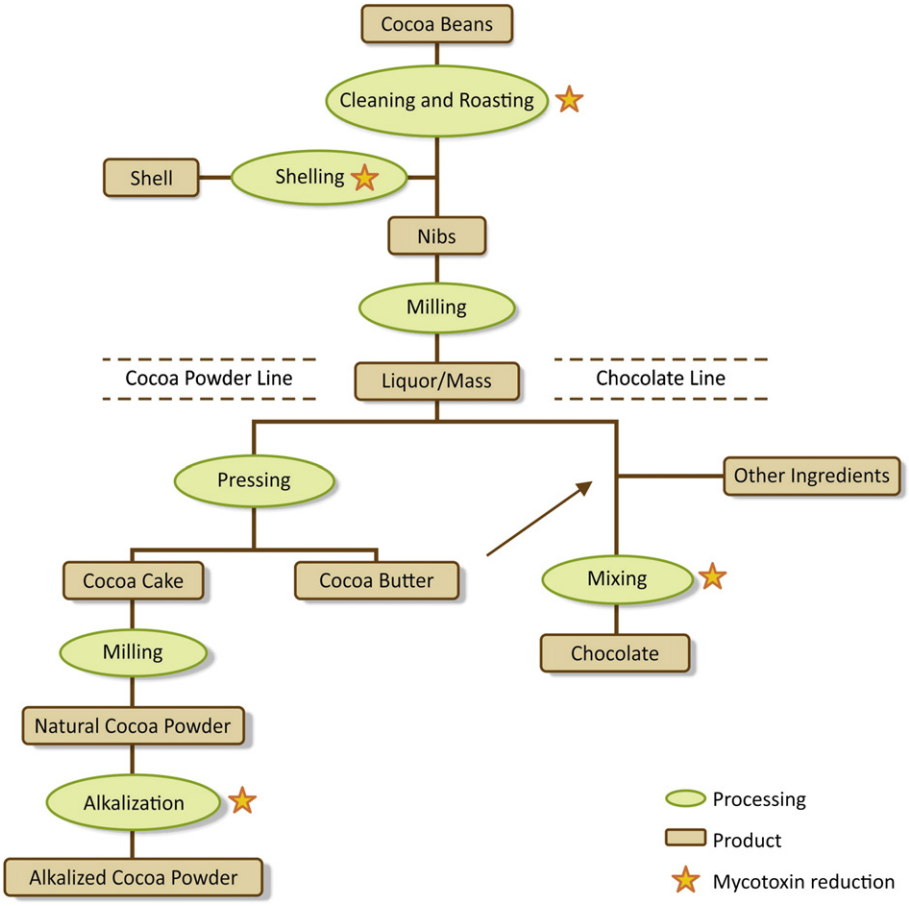

Fig. 2. The industrial processing of cocoa beans and main steps where the reduction of mycotoxin contamination occurs.

platforms are a source of fungal spore inoculum. Sun drying in thin layers also increases the oxygen tension, enhancing filamentous fungal growth and at the same time reducing the concentration of inhibitory acids produced during the fermentation, especially acetic acid, due to volatilisation (Copetti et al., 2012a).

Yeasts and fungal species established during fermentation dominate during the first days of drying, but are overtaken by genera adapted to lower moisture, especially *Aspergillus* and *Penicillium* (Copetti, 2009). Numbers of potentially toxigenic species, including *A. flavus*, *A. parasiticus*, *A. niger* and *A. carbonarius*, increase during the later stages of drying (Fig. 1, 3C) (Copetti et al., 2011b).

Frequent isolation of species potentially producing aflatoxin from samples during drying is a cause for concern (Copetti et al., 2011a). Water activities often remain high for long enough to permit mycotoxin production (Copetti et al., 2011b). However, concentrations of aflatoxins found in samples evaluated were very low (mean 0.13 μg/kg), suggesting the existence of inhibitors in cocoa. Inhibition could be related to the presence of high levels of polyphenols reported in oilseeds by Molyneux et al. (2007), who observed up to 99.8% inhibition of aflatoxin synthesis in the presence of these antioxidants.

About 50% of samples taken during sun drying were positive for ochratoxin A (Copetti et al., 2010), but levels were generally low, with a mean of only 0.13 μg/kg. A correlation was observed between the occurrence of ochratoxin A in the samples and the presence of *A. carbonarius*, indicating that it is the major species producing ochratoxin A contamination in cocoa.

2.3. Storage

Dried beans are typically stored in bags at farms until marketed (Fig. 1, 4A–B) (Minifie, 1999). As spores of the fungi present at the end of drying (Fig. 1, 4B) remain viable for long periods, good storage conditions are crucial to maintaining the quality of the beans. As cocoa beans are low in soluble solids, storage at high humidity may cause rapid increases in water activity, providing suitable conditions for spore germination, fungal growth and spoilage (Raters and Matissek, 2003). Wood (1985) recommended that only under carefully controlled conditions should cocoa storage in tropical countries exceed 2–3 months.

Studies have reported toxigenic fungal species, usually *A. flavus* and *A. niger*, in dried cocoa beans from all growing areas (Niles, 1981; Aroyeun et al., 2007; Mounjouenpou et al., 2008; Rahmadi and Fleet, 2008; Sanchez-Hervas et al., 2008; Copetti et al., 2011b). Xerophilic species, especially *Eurotium amstelodami*, *Eurotium chevalieri*, *Eurotium rubrum* and *Aspergillus penicillioides* will also grow when cocoa beans are stored under poor conditions (Rahmadi and Fleet, 2008; Copetti et al., 2011a) (Fig. 1, 4C). These species grow under conditions of reduced water activity and are responsible for large economic losses in stored grains, nuts, spices and cereal products (Pitt and Hocking, 2009). However, these species produce no significant mycotoxins.

Aflatoxins were first reported in cocoa by Campbell (1969). Most stored samples show low contamination (Raters and Matissek, 2003; Aroyeun et al., 2007; Copetti et al., 2011a). Concentrations of ochratoxin A in cocoa beans appear to vary according to origin, with higher levels in samples coming from Ivory Coast (Raters and Matissek, 2003; Gilmour and Lindblom, 2008), and sometimes Nigeria (Dongo et al., 2008). A high proportion of cocoa bean samples have been reported to be contaminated with ochratoxin A but in most studies fewer than 20% of samples had concentrations above 2 μg/kg (Amezqueta et al., 2004; Bonvehi, 2004; Gilmour and Lindblom, 2008; Dembele et al., 2009; Copetti et al., 2010; de Magalhaes et al., 2011).

3. Fungi and mycotoxins in cocoa manufacturing

Cocoa beans are manufactured into powdered cocoa or chocolate by a series of processing steps, involving heat treatment or segregation of fractions (Fig. 2), which impact on fungal and mycotoxin contamination in finished products. The influence of the various factors leading to mycotoxin formation and reduction in cocoa fermentation, drying and processing are shown in Fig. 3. Mycotoxin concentrations reported from cocoa and cocoa products are shown in Table 1.

3.1. Roasting

Roasting completes the chemical reactions responsible for the development of chocolate aroma and is a critical step for ensuring cocoa quality (Voigt, 2013). At the same time this process reduces microbial contamination. Roasting nibs, with treatments of 15 min to 2 h at 105–150 °C, is considered to be the only step in chocolate production that destroys all microorganisms (ICMSF, 2005).

Roasting decreased ochratoxin A concentrations by 24–40% in experiments conducted by Manda et al. (2009), while the reduction was about 17% under conditions evaluated by Copetti et al. (2013b).

3.2. Shelling and winnowing

The cocoa bean testa (shell) makes up about 12% of the weight in fermented and dried cocoa beans. A maximum of only 1.0–1.5% of testa residues is generally allowed in the nibs (beans), so shelling is followed by winnowing (Minifie, 1999). Most contaminant fungi are present in the testa, especially *Aspergillus*, *Eurotium* and *Absidia* species (Copetti et al., 2010) and are almost completely removed by winnowing (Minifie, 1999; Copetti et al., 2010).

It has been reported that ochratoxin A is concentrated in the testa fraction, so that only a small part of the toxin contaminates the nibs (Bonvehi, 2004; Amezqueta et al., 2005; Gilmour and Lindblom, 2008; Manda et al., 2009; Copetti et al., 2013b). Mechanical shelling removed an average of 48% (range 27–72%) of ochratoxin A (Gilmour and Lindblom, 2008), while shelling by hand reduces between 50 and 100% (Amezqueta et al., 2005; Manda et al., 2009). The efficiency of winnowing is therefore a critical point for reducing the level of contaminants present in the nibs before the subsequent processing steps (Copetti et al., 2013b).

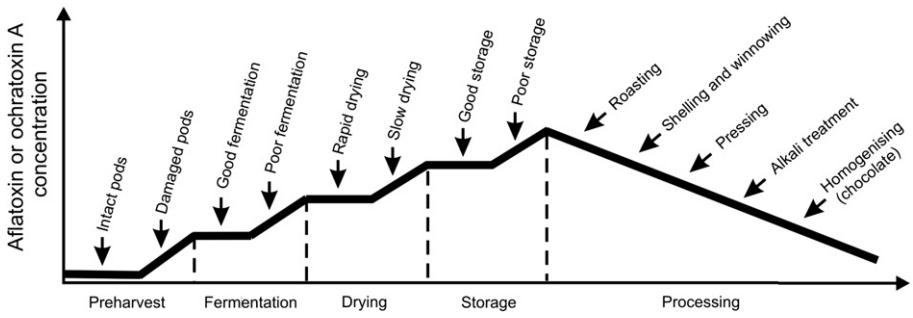

Fig. 3. Schematic of formation and reduction in aflatoxin and ochratoxin A during stages of production of cocoa powder and chocolate from cocoa beans. The diagram is qualitative.

Table 1
Occurrence of ochratoxin A in samples of cocoa products.

Cocoa product	Ochratoxin A					Origin	Reference
	Number of samples evaluated	Positive	>2 μg/kg	Mean (μg/kg)	Maximum (μg/kg)		
Beans	33	24	5	1.85	14.8	Ivory Coast	Amezqueta et al. (2004)
Beans	7	3	–	1.55	3.88	Cameroon	Amezqueta et al. (2004)
Beans	6	2	0	0.26	0.42	Equatorial Guinea	Amezqueta et al. (2004)
Beans	29	24	0	0.1	0.38	Brazil	Copetti et al. (2013a,b)
Beans	21	16	1	0.45	3.5	West Africa	Bonvehi (2004)
Butter	4	0	0	<0.1	<0.1	West Africa	Bonvehi (2004)
Butter	25	20	0	0.03	0.05	Brazil	Copetti et al. (2013a,b)
Butter	5	–	0	0.03	0.08	–	Turcotte et al. (2013)
Cake	80	74	41	2.79	9	Various	Bonvehi (2004)
Cake	26	26	–	0.97	3.18	Brazil	Copetti et al. (2013a,b)
Liquor/mass	8	4	2	1.07	3.5	West Africa	Bonvehi (2004)
Liquor/mass	25	25	0	0.34	1.09	Brazil	Copetti et al. (2013a,b)
Liquor/mass	5	5	0	0.43	0.56	–	Turcotte et al. (2013)
Nibs	2	0	0	<0.1	<0.1	West Africa	Bonvehi (2004)
Powder (alkalized)	28	28	–	0.9	3.59	Brazil	Copetti et al. (2013a,b)
Powder (alkalized)	21	21	0	1.06	1.88	–	Turcotte et al. (2013)
Powder (natural)	16	16	–	1.42	5.13	Brazil	Copetti et al. (2013a,b)
Powder (natural)	15	15	2	1.17	4.72	–	Turcotte et al. (2013)

3.3. Grinding or conching

Nibs are then ground using large oscillating stones known as conches at temperatures of 50–70 °C to form cocoa liquor, also called cocoa mass when cool. Grinding requires 2 to 72 h, depending on the equipment type, cocoa quality and desired chocolate type (Beckett, 2008). At this point the processing can follow two different ways, to produce either powdered cocoa or chocolate.

3.4. Powdered cocoa manufacture

Powdered cocoa is manufactured by pressing cocoa mass under high pressure and temperatures between 95 and 105 °C. Separation out of cocoa butter leaves a residual cake, which has relatively little fat (12–22%) and high solids (Minifie, 1999). The cake is then milled to produce "natural" cocoa powder (Fig. 2).

In the pressing step, ochratoxin A remains bound to the cocoa cake (Table 1), so levels found in the butter are very low. Higher levels of cocoa solids non fat in a final product tend to be correlated with higher concentrations of ochratoxin A in it (Miraglia and Brera, 2002; Bonvehi, 2004; Gilmour and Lindblom, 2008; Copetti et al., 2013b; Turcotte et al., 2013). The same correlation has been observed for aflatoxins (Copetti et al., 2012a; Turcotte et al., 2013).

The final step in producing powdered cocoa is an alkali process, usually using potassium carbonate in combination with heat. This process influences the dispersibility of the cocoa particles in liquids and is an important influence in the final colour of the cocoa powder (Minifie, 1999).

The alkali process produces a sterile product, nevertheless fungi can be isolated from cocoa products sometimes. These fungi are likely to be postprocessing contaminants (Copetti et al., 2011a).

Mycotoxin levels in alkalized cocoa powder tend to be lower than in untreated fractions (Copetti et al., 2011b, 2013b; Turcotte et al., 2013), although the alkali process appears to be more effective in reducing aflatoxin concentrations than those of ochratoxin A.

3.5. Chocolate Manufacturing

Chocolate is the homogeneous combination of cocoa materials (liquor and butter) with milk products, sugars and/or sweeteners, and other additives (Codex Alimentarius Commission, 2003). The addition of these other ingredients dilutes the mycotoxin content of the final product but may also introduce new contaminants. The homogenising process is carried out at temperatures between 45 and 100 °C, causing a decrease in acidity and moisture content, enhancement of the Maillard reaction and a reduction in microbial contamination.

Chocolate is regarded as a microbiologically stable product because of its low water activity. However, extremely xerophilic fungi such as *Bettsia alvei*, *Chrysosporium xerophilum* and *Xeromyces bisporus* can cause deterioration in chocolate and chocolate confectionery (ICMSF, 2005; Kinderlerer, 1997). Storage under high humidity may be partly responsible. The xerophilic yeast *Zygosaccharomyces rouxii* sometimes causes leaker spoilage in filled chocolates. The water activity in chocolates is not sufficiently low to prevent growth of this yeast, so chocolate fillings must be free of it during chocolate manufacture (Pitt and Hocking, 2009). Mycotoxigenic fungal species have been not reported in chocolate.

Ochratoxin A in chocolate was first reported by Engel (2000). Since that time, a considerable number of chocolate samples have been evaluated and the majority have been reported as positive (Table 2). Concentrations are generally low. As noted above, the concentration of ochratoxin A tends to be directly related to the amount of cocoa solids used in the chocolate formulation.

The literature has few reports on aflatoxins in chocolate; these are summarised in Table 2. Kumagai et al. (2008) reported that 50% of samples of bitter chocolate analysed were contaminated with aflatoxin B_1. The occurrence of both aflatoxins and ochratoxin A in at least 80% of chocolate samples was reported by Copetti et al. (2012b) in Brazil and by Turcotte et al. (2013) in Canada (Table 2). It is possible that cocoa butter substitutes may be an additional source of aflatoxins in chocolate (Kershaw, 1982).

Overall, it has been reported that processing, from the unroasted bean to manufactured chocolate, results in more than 90% reduction in ochratoxin A concentrations, mostly as the result of shelling.

4. Methodology for detection of fungi and mycotoxins in cocoa and cocoa products

4.1. Fungi

The choice of plating technique and medium will influence results of mycological analyses in all types of foods, including cocoa and cocoa products. The International Commission on Food Mycology (ICFM) recommends direct plating as the preferred technique for solid products such as chocolate. Surface disinfection of pieces in 0.4% freshly prepared chlorine (household bleach) before direct plating is considered essential (Hocking et al., 2006). However, analyses are more commonly carried out on powders, and here ICFM recommends dilution plating. Initial dilution 1:9 in 0.1% peptone with homogenisation in a Stomacher is

Table 2
Occurrence of total aflatoxins and/or ochratoxin A in chocolate bars.

Product	Aflatoxin, total					Ochratoxin A				Origin	Reference
	Number of samples	Positive	>1 μg/kg	Max. (μg/kg)	Mean (μg/kg)	Positive	>1 μg/kg	Max. (μg/kg)	Mean (μg/kg)		
Bitter/dark	78	78	–[a]	1.8	0.59[b]					Germany	Engel (2000)
Bitter	42	22	0	0.60	0.18	–	–	–	–	Japan	Kumagai et al. (2008)
Bitter	41	–	–	–	–	27	0	0.94	0.35	Japan	Kumagai et al. (2008)
Bitter	25	25	4	1.65	0.66	25	0	0.60	0.31	Various	Copetti et al. (2012b)
Dark	35	–	–	–	–	35	–	–	0.25[b]	Spain	Burdaspal and Legarda (2003)
Dark	52	–	–	–	–	52	–	–	0.27[b]	Various	Burdaspal and Legarda (2003)
Dark	536	–	–	–	–	536	4	–	0.26	Europe	Gilmour and Lindblom (2008)
Dark	25	25	0	0.91	0.43	25	0	0.87	0.34	Brazil	Copetti et al. (2012b)
Dark	120	–	–	–	–	92	0	0.74	0.2	Italy	Brera et al. (2011)
Dark	20	16	0	0.91	0.23	20	0	0.65	0.39	Canada	Turcotte et al. (2013)
Milk	39	–	–	–	–	36	0	0.41	0.08	Germany	Engel (2000)
Milk	47	–	–	–	–	47	–	–	0.12[b]	Spain	Burdaspal and Legarda (2003)
Milk	122	–	–	–	–	122	–	–	0.10[b]	Various	Burdaspal and Legarda (2003)
Milk	228	–	–	–	–	228	2	–	0.16	Europe	Gilmour and Lindblom (2008)
Milk	25	18	0	0.32	0.08	25	0	0.45	0.15	Brazil	Copetti et al. (2012b)
Milk	78	–	–	–	–	21	0	0.26	0.15	Italy	Brera et al. (2011)
Milk	10	7	0	0.53	0.15	10	0	0.33	0.19	Canada	Turcotte et al. (2013)
White	5	–	–	–	–	5	–	–	0.03[b]	Spain	Burdaspal and Legarda (2003)
White	9	–	–	–	–	8	–	–	0.03[b]	Various	Burdaspal and Legarda (2003)
White	25	5	0	0.1	0.01	23	0	0.05	0.03	Brazil	Copetti et al. (2012b)

[a] Data not available.
[b] Median.

recommended (Hocking et al., 2006). ICFM recommends Dichloran Rose Bengal Chloramphenicol agar (DRBC; King et al., 1979) as a general purpose isolation and enumeration medium for foods of high water activity, i.e. >0.95 and Dichloran 18% Glycerol agar (DG18; Hocking and Pitt, 1980) for analyses of food with water activities <0.95 (Hocking et al., 2006). However, for analyses of chocolate, the medium of choice is malt yeast 50% glycerol agar (Pitt and Hocking, 2009), as only extreme xerophiles are able to cause spoilage of chocolate.

Sanchez-Hervas et al. (2008) analysed cocoa beans by direct plating, using DRBC as medium, while Amezqueta et al. (2008) and Mounjouenpou et al. (2008) used potato dextrose agar and dilution plating. The most recent study by Copetti et al. (2011a,b) used direct plating and DG18.

4.2. Mycotoxins

Levels of mycotoxins in cocoa and chocolate are usually very low, so sensitive methods should be used for their detection. Sampling and analysis are both of critical importance to avoid unacceptable consignments being accepted or satisfactory loads being unnecessarily rejected (Turner et al., 2009). For ochratoxin A and aflatoxins in cocoa and by-products, the common technique is high performance liquid chromatography (HPLC) with fluorescence detection (FLD), because these mycotoxins fluorescence naturally, enabling very low detection limits.

The combination of the HPLC-FLD method with cleanup by immunoaffinity columns has been used for the detection of ochratoxin A in various studies on cocoa beans (Amezqueta et al., 2004; Bonvehi, 2004; Copetti et al., 2010; de Magalhaes et al., 2011), cocoa products (Brera et al., 2003, 2011; Manda et al., 2009; Bonvehi, 2004; Turcotte et al., 2013) and chocolate (Bonvehi, 2004; Manda et al., 2009; Brera et al., 2011; Copetti et al., 2012b; Turcotte et al., 2013). This methodology has also been the choice for aflatoxin analyses (Kumagai et al., 2008; Copetti et al., 2011a, 2012b; Turcotte et al., 2013). The values found during protocol validation were similar for ochratoxin A and aflatoxins, showing recoveries of about 90% and limit of detection close to 0.05 μg/kg in most reports.

Recently the use of tandem mass spectrometry (LC/MSMS) to analyse mycotoxins in foods has increased, the main advantage being the detection and quantification of mycotoxins simultaneously. However methodologies developed to evaluate mycotoxins in cocoa or cocoa products are not available. Besides that the cost of the equipment and maintenance are still high, so HPLC-FLD is still the most commonly used instrument for analysing mycotoxins in cocoa and cocoa products.

5. Mycotoxin exposure and regulation in cocoa and cocoa products

A survey of dietary intake of ochratoxin A concluded that cocoa represents about 5% of ochratoxin A intake in the diet of the European population (Miraglia and Brera, 2002). Chocolate was reported to contribute about 6% of the total dietary exposure to ochratoxin A (Codex Alimentarius Commission, 2012).

Few countries have set regulatory limits for ochratoxin A and/or aflatoxins in cocoa beans and cocoa products. An expert panel of the European Union recommended setting a maximum limit for ochratoxin A of 1 μg/kg in chocolate, chocolate powder and drinking chocolate and 2 μg/kg in cocoa beans, cocoa nibs, cocoa mass, cocoa cake and cocoa powder (Tafuri et al., 2004). However, the European Commission has stated that a maximum limit for ochratoxin A in cocoa and cocoa products does not appear necessary (European Commission, 2010).

On the basis of significant consumption of cocoa products and chocolate by children and results published by Copetti et al. (2010, 2011a), the Brazilian Sanitary Surveillance Agency (ANVISA) set limits of 10 μg/kg for cocoa beans and 5 μg/kg for cocoa products and chocolate sold in Brazil, for both ochratoxin A and total aflatoxins (ANVISA, 2011).

6. Prevention of mycotoxin formation in cocoa

Mycotoxins are stable compounds in storage, and are more or less resistant to chemical and physical treatments, so the best approach to limiting mycotoxin contamination in foods is reduction of formation. A code of practice to assist in preventing the formation and improve the reduction of ochratoxin A in cocoa is under discussion (Codex Alimentarius Commission, 2013).

In a study focused on the Ivory Coast, the main factors involved in the occurrence of ochratoxin A in cocoa were elucidated by Gilmour and Lindblom (2008). They reported that ochratoxin A formation may commence between harvest and fermentation, especially with small holders, and that damaged pods appear to be critical for ochratoxin A accumulation, an observation confirmed by Mounjouenpou et al. (2008). To minimize the problem, the Codex Alimentarius Commission (2013) has

recommended keeping the cocoa plantation as free of mould infection as possible, to separate out diseased pods in the field and discard mummified pods. The healthy pods should be harvested as soon they are ripe, avoiding damage to prevent inoculation by fungal spores. Damaged pods should not be stored longer than one day before opening and fermenting.

Experiments carried out in Brazil (Copetti et al., 2012a) demonstrated the importance of organic acids, especially acetic acid, produced by fermentative bacteria in suppressing the growth of fungi with the potential to produce ochratoxin A, highlighting the importance of an adequate fermentation step. They also showed that fermentation that occurred during drying of partially depulped beans can increase ochratoxin A production, so, a traditional fermentation of 4–7 days should be adopted, and the mass should be turned frequently. Fermentation beyond 7 days should be avoided as this could lead to fungal proliferation (Codex Alimentarius Commission, 2013).

As toxigenic fungi can grow as fermentation ceases, drying should start immediately (Copetti et al., 2011b). The code of practice (Codex Alimentarius Commission, 2013) recommends that layers of drying cocoa beans should not exceed 6 cm thick to avoid slow or inadequate drying, and beans should be dried to a moisture content of 6–8%. The drying area should be located away from contaminant sources and receive maximum sun exposure and air circulation during the day. At night or during rainy weather, the cocoa beans should be heaped and covered to avoid re-wetting.

Dried cocoa beans are hygroscopic, so cocoa will absorb moisture from the environment under high humidity conditions. Wood (1985) recommended a maximum 2–3 months storage in tropical countries. If storage is longer term, humidity should be controlled below 70% RH. The moisture content of the stored cocoa beans should be periodically checked and kept below 8% (Codex Alimentarius Commission, 2013).

The formation and reduction of ochratoxin A and aflatoxins during processing of cocoa beans to cocoa powder and chocolate are shown diagrammatically in Fig. 3. The figure is purely qualitative, as insufficient data exists to quantify most stages in formation and reduction. Aflatoxins and ochratoxin A are shown on the same graph, as qualitative effects are similar for both toxins, though quantitative effects are known to vary quite widely.

7. Final considerations

The concentrations of aflatoxins and ochratoxin A found in cocoa, cocoa derivatives and chocolate indicate that these products are responsible for a relatively low contribution to human exposure. However, the increase of chocolate consumption with high levels of cocoa in recent years could elevate exposure to these food contaminants, as products with a high cocoa content tend to have the higher concentrations of mycotoxins.

Chocolate appears to be a minor source of ochratoxin A and aflatoxins in the diet, although the fact that products containing chocolate are widely consumed by children is a concern, so monitoring of their occurrence in these products is important.

Knowledge of factors influencing contamination of cocoa by mycotoxins has increased in recent years, as well as the processing steps which may result in a decrease. However, further studies are needed, particularly on the influence of fermenting microorganisms and the organic acids they produced on the subsequent inhibition of fungal growth and mycotoxin production.

References

Amezqueta, S., Gonzalez-Penas, E., Murillo, M., Lopez de Cerain, A., 2004. Validation of a high-performance liquid chromatography analytical method for ochratoxin A quantification in cocoa beans. Food Addit. Contam. 21, 1096–1106.

Amezqueta, S., Gonzalez-Penas, E., Murillo, M., Lopez de Cerain, A., 2005. Occurrence of ochratoxin A in cocoa beans: effect of shelling. Food Addit. Contam. 22, 590–596.

Amezqueta, S., Gonzalez-Penas, E., Dachoupakan, C., Murillo-Arbizu, M., Lopez de Cerain, A., Guiraud, J.P., 2008. OTA-producing fungi isolated from stored cocoa beans. Lett. Appl. Microbiol. 47, 197–201.

ANVISA (Agência Nacional de Vigilância Sanitária, Brazil), 2011. Resolução RDC no. 7, de 18 de fevereiro de 2011 — dispõe sobre limites máximos tolerados para micotoxinas em alimentos. http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2011/res0007_18_02_2011_rep.html (Accessed on 24/10/2013).

Ardhana, M.M., Fleet, G.H., 2003. The microbial ecology of cocoa bean fermentations in Indonesia. Int. J. Food Microbiol. 86, 87–99.

Aroyeun, S.O., Adegoke, G.O., Varga, J., Kocsube, S., Pal, K., Vagvolgyi, C., 2007. Effect of fermentation and storage on mycotoxigenic fungi, ochratoxin A and aflatoxin B_1 in cocoa beans from southwestern Nigeria. Malays. Cocoa J. 3, 35–46.

Beckett, S.T., 2008. The Science of Chocolate, 2nd edn. Royal Society for Chemistry, London.

Beuchat, L.R., 1987. Influence of water activity on sporulation, germination, outgrowth, and toxin production. In: Rockland, L.D., Beuchat, L.R. (Eds.), Water Activity: Theory and Applications to Food. Marcel Dekker, New York, pp. 153–172.

Bonvehi, J.S., 2004. Occurrence of ochratoxin A in cocoa products and chocolate. J. Agric. Food Chem. 52, 6347–6352.

Brera, C., Grossi, S., De Santis, B., Miraglia, M., 2003. High performance liquid chromatography method for the determination of ochratoxin A in cocoa powder. J. Liq. Chromatogr. 26, 585–598.

Brera, C., Debegnach, F., De Santis, B., Iafrate, E., Pannunzi, E., Berdini, C., Prantera, E., Gregori, E., Miraglia, M., 2011. Ochratoxin A in cocoa and chocolate products from the Italian market: occurrence and exposure assessment. Food Control 22, 1663–1667.

Burdaspal, P.A., Legarda, T.M., 2003. Ocratoxina A en distintos tipos de chocolate y preparados de cacao en polvo comercializados en España y en otros países extranjeros. Alimentaria 347, 143–153.

Campbell, T.C., 1969. 83rd annual meeting of the AOAC. Report presented to the Joint AOAC-AOCS Committee on Aflatoxins, October, 13–16-1969, Washington DC. Codex Alimentarius Commission. 2003. Codex Standard for Chocolate and Chocolate Products. CODEX STAN 87-1981, Rev. 1 ((www.codexalimentarius.org/input/download/standards/67/CXS_087e.pdf). Accessed on 24/10/2013).

Codex Alimentarius Commission, 2012. Discussion paper on ochratoxin A in cocoa. Joint FAO/WHO Food Standards Program, FAO, Rome ((ftp://ftp.fao.org/codex/meetings/cccf/cccf6/cf06_15e.pdf). Accessed on 24/10/2013)).

Codex Alimentarius Commission, 2013. Proposed draft code of practice for the prevention and reduction of ochratoxin A contamination in cocoa. Joint FAO/WHO Food Standards Program, FAO, Rome ((ftp://ftp.fao.org/codex/meetings/cccf/cccf7/cf07_09e.pdf). Accessed on 24/10/2013).

Copetti, M.V., 2009. Micobiota do cacau: fungos e micotoxinas do cacau ao chocolate. (PhD Thesis) State University of Campinas, Campinas SP, Brazil.

Copetti, M.V., Pereira, J.L., Iamanaka, B.T., Pitt, J.I., Taniwaki, M.H., 2010. Ochratoxigenic fungi and ochratoxin A in cocoa during farm processing. Int. J. Food Microbiol. 143, 67–70.

Copetti, M.V., Iamanaka, B.T., Pereira, J.L., Fungaro, M.H., Taniwaki, M.H., 2011a. Aflatoxigenic fungi and aflatoxin in cocoa. Int. J. Food Microbiol. 148, 141–144.

Copetti, M.V., Iamanaka, B.T., Pereira, J.L., Frisvad, J.C., Taniwaki, M.H., 2011b. Mycobiota of cocoa: from farm to chocolate. Food Microbiol. 28, 1499–1504.

Copetti, M.V., Iamanaka, B.T., Pereira, J.L., Frisvad, J.C., Taniwaki, M.H., 2012a. The effect of cocoa fermentation and weak organic acids on growth and ochratoxin A production by *Aspergillus* species. Int. J. Food Microbiol. 155, 158–164.

Copetti, M.V., Iamanaka, B.T., Pereira, J.L., Lemes, D.P., Nakano, F., Taniwaki, M.H., 2012b. Co-occurrence of ochratoxin A and aflatoxins in chocolate marketed in Brazil. Food Control 26, 36–41.

Copetti, M.V., Iamanaka, B.T., Taniwaki, M.H., 2013a. Fungi and mycotoxin occurrence in cocoa. In: Watson, R.R., Preedy, V.R., Zibadi, S. (Eds.), Chocolate in Health and Nutrition. Humana Press, London, pp. 61–71.

Copetti, M.V., Iamanaka, B.T., Nester, M.A., Efraim, P., Taniwaki, M.H., 2013b. Occurrence of ochratoxin A in cocoa by-products and determination of its reduction during chocolate manufacture. Food Chem. 136, 100–104.

de Magalhaes, J.T., Sodre, G.A., Viscogliosi, H., Grenier-Loustalot, M.F., 2011. Occurrence of ochratoxin A in Brazilian cocoa beans. Food Control 22, 744–748.

Dembele, A., Coulibaly, A., Traore, S.K., Mamadou, K., Silue, N., Abba, T.A., 2009. Determination du niveau de contamination de l'ochratoxine A (OTA) dans les faves de cacao a l'exportation. Tropicultura 27, 26–30.

Dongo, L., Bandyopadhyay, R., Kumar, M., Ojiambo, P.S., 2008. Occurrence of ochratoxin A in Nigerian ready for sale cocoa beans. Agric. J. 3, 4–9.

Engel, G., 2000. Ochratoxin A in sweets, oil seeds and dairy products. Arch. Leb. 51, 81–128.

European Commission, 2010. Commission Regulation (EU) No. 105/2010 of 5 February 2010 amending Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. Off. J. Eur. Union 35, 7–8.

Gilmour, M., Lindblom, M., 2008. Management of ochratoxin A in the cocoa supply chain: a summary of work by the CAOBISCO/ECA/FCC working group on ochratoxin A. In: Leslie, J.F., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxins: Detection Methods, Management, Public Health and Agricultural Trade. CABI, Wallingford, pp. 231–243.

Hocking, A.D., Pitt, J.I., 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low moisture foods. Appl. Environ. Microbiol. 39, 488–492.

Hocking, A.D., Pitt, J.I., Samson, R.A., Thrane, U., 2006. Advances in Food Mycology. Springer, New York.

ICMSF (International Commission on Microbiological Specifications for Foods), 2005. Cocoa, chocolate, and confectionery. Microorganisms in Food, 6: Microbial Ecology of Food Commodities. Blackie Academic and Professional, London, pp. 467–479.

Kershaw, S.J., 1982. Occurrence of aflatoxins in oilseeds providing cocoa-butter substitutes. Appl. Environ. Microbiol. 43, 1210–1212.

Kinderlerer, J.L., 1997. *Chrysosporium* species, potential spoilage organisms of chocolate. J. Appl. Microbiol. 83, 771–778.
King, A.D., Hocking, A.D., Pitt, J.I., 1979. Dichloran rose bengal medium for enumeration and isolation of molds from foods. Appl. Environ. Microbiol. 37, 959–964.
Kumagai, S., Nakajima, M., Tabata, S.E., Ishikuro, E., Tanaka, T., Norizuki, H., Itoh, Y., Aoyama, K., Fujita, K., Kai, S., Sato, T., Saito, S., Yoshiike, N., Sugita-Konishi, Y., 2008. Aflatoxin and ochratoxin A contamination of retail food and intake of these mycotoxins in Japan. Food Addit. Contam. 25, 1101–1106.
Lima, L.J.R., Almeida, M.H., Nout, M.J.R., Zwietering, M.H., 2011. *Theobroma cacao* L., "The food of the gods": quality determinants of commercial cocoa beans, with particular reference to the impact of fermentation. Crit. Rev. Food Sci. Nutr. 51 (8), 731–761.
Manda, P., Dano, D.S., Kouadio, J.H., Diakite, A., Sangare-Tigori, B., Ezoulin, M.J.M., Soumahoro, A., Dembele, A., Fourny, G., 2009. Impact of industrial treatments on ochratoxin A content in artificially contaminated cocoa beans. Food Addit. Contam. 26, 1081–1088.
Minifie, B.W., 1999. Chocolate, Cocoa, and Confectionery: Science and Technology, 3rd edn. Van Nostrand & Reinhold, New York.
Miraglia, M., Brera, C., 2002. Reports on tasks for scientific cooperation, task 3.2.7,(http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf (Accessed on 24/10/2013)).
Molyneux, R.J., Mahoney, N., Kim, J.H., Campbell, B.C., 2007. Mycotoxins in edible tree nuts. Int. J. Food Microbiol. 119, 72–78.
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.
Nielsen, D.S., Crafack, L.J., Jakobsen, M., 2013. The microbiology of cocoa fermentation. In: Watson, R.R., Preedy, V.R., Zibadi, S. (Eds.), Chocolate in Health and Nutrition. Humana Press, London, pp. 39–60.
Niles, E.V., 1981. Microflora of imported cocoa beans. J. Stored Prod. Res. 17, 147–150.
Ostovar, K., Keeney, P.G., 1973. Isolation and characterization of microorganisms involved in the fermentation of Trinidad's cacao beans. J. Food Sci. 38, 611–617.
Pitt, J.I., Hocking, A.D., 2009. Fungi and Food Spoilage, 3rd edn. Springer, New York.
Rahmadi, A., Fleet, G.H., 2008. The occurrence of mycotoxigenic mould in cocoa beans from Indonesia and Queensland, Australia. Proceeding of International Seminar on Food Science, University of Soegiyapranata, Semarang Indonesia ((http://www.researchgate.net/publication/235935636_The_Occurrence_of_Mycotoxigenic_Moulds_in_Cocoa_Beans_from_Indonesia_and_Queensland_Australia/file/d912f5147b8e179f4d.pdf). Accessed on 24/10/2013).
Raters, M., Matissek, R., 2003. Neue Studien zur Analytik und zum Vorkommen von Ochratoxin A in Kakao und kakaohaltigen Erzeugnissen Abschlussbericht Projektzeitraum: 01.07.2000–31.12.2002 Projekt Nr. 33 der Stiftung der Deutschen Kakao- und Schokoladenwirtschaft,(http://www.yumpu.com/de/document/view/5973570/neue-studien-zur-analytik-und-zum-vorkommen-von-ochratoxin-a-in-(Accessed on 13/08/13)).
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.
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.
Schwan, R.F., Wheals, A.E., 2004. The microbiology of cocoa fermentation and its role in chocolate quality. Crit. Rev. Food Sci. Nutr. 44, 1–17.
Tafuri, A., Ferracane, R., Ritieni, A., 2004. Ochratoxin A in Italian marketed cocoa products. Food Chem. 88, 487–494.
Turcotte, A.M., Scott, P.M., Tague, B., 2013. Analysis of cocoa products for ochratoxin A and aflatoxins. Mycotoxin Res. 29, 193–201.
Turner, N.W., Subrahmanyam, S., Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: a review. Anal. Chim. Acta. 632, 168–180.
Voigt, J., 2013. Chocolate and cocoa aroma. In: Watson, R.R., Preedy, V.R., Zibadi, S. (Eds.), Chocolate in Health and Nutrition. Humana Press, London, pp. 89–101.
Wood, G.A.R., 1985. From harvest to store. In: Wood, G.A.R., Lass, R.A. (Eds.), Cocoa. Longman Scientific and Technical, New York, pp. 444–504.