The Occurrence of Mycotoxigenic Moulds in Cocoa Beans from Indonesia and Queensland, Australia

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Abstract

The presence of mycotoxins in cocoa beans and chocolate products is emerging as an important public health issue and has created a need for more information about the occurrence of mycotoxigenic fungi in cocoa beans. This project has surveyed the presence of filamentous fungi on dried cocoa beans from Indonesia and Queensland, Australia. Fungi were isolated by placing chlorine and non-chlorine disinfected beans onto the surface of plates of DG-18 agar, and also their populations were determined by spread plating onto DG-18 and DRBC agar. Fungal population in Indonesian beans varied between 10^4 – 10^6 CFU.g^{-1}, while the populations on Queensland beans were consistently low (< 100 – 2.5 x 10^2 CFU.g^{-1}). However, there was a high incidence of potentially mycotoxigenic filamentous fungi on all bean samples. The main species were *Aspergillus flavus, Aspergillus niger, Aspergillus wentii, Aspergillus clavatus, Penicillium citrinum, and Penicillium spinolosum*. Chlorine treatment of the beans decreased the incidence and diversity of fungal species detected. The beans from Queensland gave high counts of *Bacillus* species and lactic acid bacteria and it is suggested that they served as natural bio-control agents against the filamentous fungi.

1 Introduction

Chocolate is a popular and exotic product made from cocoa beans. This raw material is commonly traded as fermented and dried beans. The trading volume is more than 3 million metric tones per year since 2002 (Galvez et al., 2007; ICCO, 2007) with the top three producers being the Ivory Coast, Ghana and Indonesia (ICCO, 2007). Cocoa beans from Indonesia, Malaysia, Papua New Guinea and other Pacific countries are often sold at lower price because of lower aromatic properties, higher astringency and bitter taste compared with beans from Africa (Natsume et al., 2000; Othman et al., 2007). Recently, Queensland, Australia has emerged as another potential region for cocoa bean production (Lemin, 2005; Fleet and Dircks, 2007).

Regardless of the country of origin, cocoa beans are susceptible to spoilage by filamentous fungi (Minifie, 1980; Minifie, 1999; Ardhana and Fleet, 2003; Schwan and Wheals, 2004). Currently, there is an increasing concern that fungal contamination could also result in the production of mycotoxins, in particular aflatoxins and ochratoxin A, which have significant public health consequences since they could be carcinogenic (Pitt and Hocking, 1997; Samson et al., 2004; Richard et al., 2007). Some studies suggest the presence of mycotoxins were Chaytor and Saxby (1981), Serra-Bonvehi (2004), and Mounjouenpou et al. (2007).

With current practises, there is no cost-effective solution to reduce the level of toxins in agricultural products once they have been produced. Therefore, good production practises are required to control bean properties and made them less favourable for mould growth (Minifie, 1999; Tran-Dinh et al., 1999; Tafuri et al., 2004; ICMSF, 2005).

In summary, there is significant potential for filamentous fungi to grow in cocoa beans and compromise their growth by causing spoilage and producing mycotoxins. Recent, it has suggested that mycotoxins presence will be increasing concern to the chocolate industry. More information is needed about the occurrence of filamentous fungi, especially mycotoxigenic species, in dried cocoa beans. This paper aims to report the occurrence of filamentous fungi in sample of dried cocoa beans from Indonesia and Queensland, Australia.

2 Material and Methods

2.1 Cocoa bean samples

Three samples were obtained from East Kalimantan, Indonesia. The other samples were obtained from Sulawesi (Indonesia) and Irian Jaya (Indonesia). The East Kalimantan beans were collected at the farm level from three plantations dated 12-14 August 2007, while samples of the Sulawesi, Irian Jaya cocoa beans were commercial samples provided by Cadbury-Schweppes, Australia dated July, 2005. Nine samples from Queensland, Australia were obtained as part of a research project on the fermentation of cocoa beans. The North Queensland beans were obtained from Mr Hugh Dircks, PhD student in Chemical Sciences and Engineering, UNSW. The beans were pre-treated with mixed yeast cocktail prior to fermentation as described in Fleet and Dircks (2007). The beans were stored in plastic bags in a dark room and under ambient temperature prior to their analysis.

2.2 Media

Media used were Dichloran Rose Bengal Chloramphenicol Agar, DRBC (Oxoid, Basingstoke), Dichloran 18% Glycerol Agar, DG-18 (Oxoid, Basingstoke), and Malt Extract Agar, MEA (Oxoid, Melbourne). Aspergillus Flavus Parasiticus Agar, AFPA (Oxoid, Basingstoke) was used to confirm the *Aspergillus flavus* and *A. parasiticus* colonies. Chloramphenicol (Oxoid, Basingstoke) was added (100 mg/L) to the media as a selective agent to inhibit the growth of bacteria. Standard plate count agar (Accumedia, Michigan) was used to determine the total microbial count of bean samples. De Mann Rogosa Sharpe Agar, MRS (Oxoid, Basingstoke) was used to selectively grow and estimate the presence of lactic acid bacteria. Bacillus Cereus Agar (Oxoid, Basingstoke) was used to assess the presence of *Bacillus* species found in the beans. The media were prepared as described by the manufacturer recommendations and sterilized by autoclaving at 121°C for 20 minutes.

2.3 Frequency of mould occurrence

2.3.1 Frequency of mould occurrence of surface disinfected beans

Ten (10) beans were soaked in 30-40 mL of 0.4% chlorine for 2 minutes. After soaking, the solution was decanted and the beans washed with sterile distilled water for another 2 minutes. The water was decanted and the beans were directly used for analysis. An

individual bean was placed in the centre of a petri plate of DG-18 agar, which was incubated at 25°C for 3-7 days (Batista et al., 2003).

2.3.2 Frequency of mould occurrence of non-surface disinfected beans

Another set of ten (10) beans were wetted in sterile 0.1% peptone water for 2 minutes, after which the water was decanted. The beans were individually placed onto plate of DG-18. The plates, then, were incubated at 25°C for 3-7 days (Batista et al., 2003) and observed for colony development over seven days.

2.4 Fungal population by dilution plating

Samples (10 g) of beans were aseptically weighed. These were mixed with 90 mL of 0.1% sterile peptone water (Oxoid, Melbourne) in a Stomacher bag. After soaking for 30 minutes of 25°C until beans became soggy, and the mixture was homogenized in a Stomacher 400 for 3 min, followed by vigorous hand shaking and massaging for 3 minutes to give a uniform homogenate. Serial dilutions of the homogenates were performed in 0.1% of sterile peptone water. The dilutions were uniformly mixed by a vortex mixer prior to transferring and spread plating. Aliquots (0.1 mL) from the dilutions were spread-inoculated in duplicate over the surface plates of DG-18 and DRBC agar for isolation of the moulds (Ardhana and Fleet, 2003; Batista et al., 2003).

The plates were incubated aerobically at 25°C for 3-7 days. Fungal colonies obtained from the plates were subcultured onto plates DG-18 and DRBC and incubated for seven days at 25°C. The purified cultures were stored at 5°C prior to identification.

2.5 Identification

Detailed characterization and identification of isolates were conducted at the University of New South Wales, Sydney. The identification was based on observation of colony and cell morphology according to characteristics described in Pitt and Hocking (1997) and was assisted by observation conducted by Dr. Ailsa D. Hocking and Mr Nick Charley, Food Science Australia, CSIRO, North Ride, NSW.

2.6 Aspergillus flavus and Aspergillus parasiticus confirmation

A loop of suspected *A. flavus* or *A. parasiticus* isolate was inoculated onto a plate of AFPA. The plate was incubated for 1-2 days at 30°C (Pitt and Hocking, 1997). A

positive result was obtained when a bright yellow to orange colour appeared surrounding the colony (Pitt and Hocking, 1997).

2.7 Bacterial counts

2.7.1 Total count, lactic acid bacteria, and *Bacillus* species count

Samples (10 g) of beans were aseptically weighed and were mixed with 90 mL of 0.1% sterile peptone water (Oxoid, Melbourne) in a Stomacher bag. The samples were macerated in a Stomacher 400 for 3 min, followed by vigorous hand shaking and massaging for 3 min to give a uniform homogenate. Serial dilutions of the homogenates were performed in 0.1% of sterile peptone water. The solutions were mixed in a vortex mixer prior to transferring and spread plating. Aliquots (0.1 mL) from the dilutions were spread-inoculated in duplicate over the surface of Standard Plate Count Agar (Accumedia, Michigan), de Mann Rogose Sharpe Agar (Oxoid, Basingstoke), and Bacillus cereus Agar (Oxoid, Basingstoke). Dilutions, temperature and time of incubation are described in Table 1**Error! Reference source not found.**. Bacteria were isolated and purified by re-streaking on the respective media.

Table 1 Dilutions, incubation time and temperature parameters for bacterial counts of cocoa beans

Assessment	Medium	Dilutions prepared	Incubation	
			Temperature (°C)	Time (days)
Bacillus species	PEMBA	10^{-5}, 10^{-6},10^{-7},10^{-8}	37	1
Total microbial count	SPC	10^{-6}, 10^{-7},10^{-8},10^{-9}	37	2
Lactic acid bacteria	MRS	10^{-5}, 10^{-6},10^{-7}	37	2

2.7.2 Colony confirmation

A single loop of biomass from a representative colony was used to prepare a microscope slide, where was examined using a phase contrast microscope (Nikon, Japan) at 1000 magnification. Colony shape, Gram staining and the presence of endospores were recorded. A catalase test was performed on colony biomass by addition of a drop of H_2O_2. Gas production indicated a catalase positive reaction. Lactic acid bacteria were catalase negative (Emma et al., 2000).

3 Results and Discussion

3.1 Fungal diversity in cocoa beans

On prolonged storage, dried agricultural product can be infected with high populations of *Aspergillus niger* and *Eurotium* species (ICMSF, 2005; Pitt, 2006). The results revealed that *Aspergillus niger* was the second most frequent species in non-surface-disinfected Indonesian beans, while it was the third in untreated North Queensland beans. *Eurotium chevaleri* was observed only from Penajam (Indonesia) bean sample.

Filamentous fungi isolated from fermented and dried cocoa beans in Indonesia and Queensland were *Aspergillus flavus, A. niger, A. wentii, A. clavatus, A. fumigatus, A. ochraceus, A. carbonarius, A. versicolor. Eurotium chevaleri, Penicillium citrinum, P. spinolosum, P. corylophilum, Eupenicillium cinnamopurporeum, Mucor pyriformis, Stemphylium sp., Cladosporium sp., Chaetomium globosum, Epicoccum nigrum, Fusarium sp., Geotrichum candidum,* and *Phoma sp*. Some of the key species, especially *Aspergillus* and *Penicillium* genera, were also reported from previous research (Hansen and Welty, 1970; Ogundero, 1983; Ardhana and Fleet, 2003; Schwan and Wheals, 2004; Camu et al., 2007). Nonetheless, *Cladosporium sp., Eupenicillium cinnamopurporeum, Stemphylium sp, Chaetomium globosum* and *Phoma sp.*, which were sporadically found in surface disinfected bean samples, have not been associated with cocoa bean spoilage before. These species are recognized as air and soil fungi (Williams et al., 2006).

3.2 Frequency of occurrences

The direct plating results suggest that chlorine treatment had effectively reduced the diversity of fungal species isolated from in cocoa beans. Batista et al (2003) reported a reduction of 52% of fungal diversity between non-chlorine treatment and chlorinated coffee beans. A similar result was recorded in cocoa beans, where in Table 2, the frequency of occurrence of *Aspergillus* and *Penicillium* species in Indonesian cocoa beans, showed 63% and 36% survival from chlorinated beans (Table 3) compared with from non surface disinfected beans (Table 2), respectively. *Aspergillus* and *Penicillium* populations from chlorine treated Queensland bean samples were reduced 40% and 41%, respectively compared with those from untreated bean samples (Table 3 and Table 2). The reductions of *Aspergillus* and *Penicillium* species from all samples with the treatments are compiled in Table 6.

The results suggested that surface contamination is a major source of fungi in fermented and dried cocoa beans, even though *Aspergillus* species such as *A. flavus*, *A. niger,* and *A. wentii* are able to invade the kernel of beans during fruiting stage (ICMSF, 2005).

In general, from all sample origins, *Aspergillus* species were higher in populations than *Penicillium* species. *Aspergillus* requires higher temperature but lower water activity compared with *Penicillium*, and it grows more rapidly as well (Hocking, 2006). The latter genus requires longer time and more light to sporulate, but produce more chemicals resistant spores than other genera (Hocking, 2006; Pitt, 2006).

Penicillium species in North Queensland samples were remarkably higher in population than their population in Indonesia. At the same time, the number of *Aspergillus* species from Queensland cocoa samples was lower than that from Indonesia. According to Pitt and Hocking (1997), species of *Aspergillus* are the predominant spoilage fungi in tropical areas and *Penicillium* species occur in more temperate zones.

3.2.1 Frequency of species occurrence in Indonesian samples

Aspergillus flavus was the most frequent species isolated from Indonesia and the Solomon Islands cocoa beans, followed by *A. niger, A. clavatus,* and *A. wentii* (Table 2). Samples from Penajam, Malinau (Indonesia) and the Solomon Islands were suffering 100 % of *A. flavus* infection. *Penicillium* species were also prominent in he bean samples. *Penicillium citrinum* was the most frequent isolates followed by *P. spinolosum*. Samples from Malinau and Irian Jaya were the source of the highest incidence of *Penicillium*, with four isolates were recovered from each of the locations (Table 2).

Surface disinfection of beans using chlorine altered the isolation of fungal species from the previous table (Table 3), where a reduction in incidence of fungi from samples was observed. For example, whereas all 10 bean samples from Malinau gave isolation of *A. flavus* previously (Table 2). *A. flavus* was no recovered from any of the beans after surface disinfection (Table 3). The total isolation of *Aspergillus* from all bean samples decreased from 90 to 33. The total number of all *Penicillium* species isolates decreased from 11 to 7.

However, *Aspergillus flavus* was still predominant compared with other *Aspergillus* species. *A niger* was found in samples from three different plantations, namely Penajam,

Malinau, and Samarinda. This *Aspergillus* species along with *A. wentii* and *A. clavatus* were found at the highest populations after *A. flavus* (Table 2).

Penicillium species were affected by chlorine treatment as well. Only two isolates of each *P. spinolosum* and *P. citrinum* were recovered. *Mucor pyriformis* was also inhibited by chlorine as shown in Table 2. On the contrary, the treatment gave an advantage for lesser competitive species to grow, such as *Eupenicillium cinnamopurporeum*.

3.2.2 Frequency of moulds in Queensland samples

Within the *Aspergillus* genus, *A. flavus* and *A. niger* were the most frequent isolates from North Queensland cocoa beans. Samples processed with natural fermentation and oven dried gave the highest number of *A. flavus*, while *A. niger* occurred in all bean samples (Table 2).

Penicillium species were the predominant moulds after *Aspergillus* species. Within the genus, two species were isolated, *P. citrinum* and *P. spinolosum*. However, *P. citrinum* was observed slightly more frequently compared with any of the *Aspergillus* species (Table 2). Table 2 indicates that *Mucor pyriformis, Stemphylium sp., Epicoccum nigrum,* and *Phoma sp.* were sporadically found in North Queensland samples but with low occurrences (1-3 isolates).

Chlorine treatment of beans decreased the frequency of fungal species isolation (Table 3). Almost all predominant species, *A. niger , A. clavatus, A. wentii, P. spinolosum,* and *P. citrinum* were decreased in total population compared with the non-chlorine treated beans. *A. flavus* population, in particular, was reduced from 17 to 7 isolates. *Penicillium citrinum* was also reduced more than 50% compared with non disinfected beans (Table 2).

However, *A. niger, P. citrinum, P spinolosum,* and *A. flavus* were still predominant from chlorine treated samples (Table 3). *A. ochraceus* was detected in a low number (3 isolates) from the bean samples, while this species was not recorded from non-chlorinated beans.

3.3 Populations of fungi on DRBC and DG-18 agar

The highest populations of fungi were obtained from Penajam bean samples with 2.1 x 10^6 and 7.2 x 10^6 CFU.g^{-1} on DRBC and DG-18, respectively. Samarinda gave the second highest population with 2.0 x 10^5 CFU.g^{-1} on both agar media. The lowest populations were recorded from Irian Jaya beans with less than 100 CFU.g^{-1}, while the population of fungi in samples from Sulawesi was slightly higher than those from Irian Jaya (Table 4).

The beans were not surface disinfected beforehand. The diversity of genera isolated from these plates included *Aspergillus, Penicillium, Geotricum, Cladosporium, Mucor, Stemphylium* and *Fusarium*. However, *Aspergillus* and *Penicillium* were recorded as the predominant microflora from the samples. Total fungal counts on DRBC agar for all samples were not much different with those on DG-18 agar (Table 4).

3.4 Total bacterial count

A consistent low result for total fungal counts from three repeated experiments suggests an established endogenous control of fungi on those beans. During these experiments, some of the beans gave slime formation on the plates, which led to bacterial activity. Further, the total colony of population of these presumptive *Bacillus* species and lactic acid bacteria were quantified. These numbers, at the lowest 10^6 and at the highest 10^9 CFU g^{-1}, are unexpectedly high, which was never been reported before. The total microbial counts were more than 10^6 CFU.g^{-1}, which is regarded as a significant population (Fleet, 1999; Adams and Moss, 2000).

The highest bacterial count was reported during fermentation by Ardhana and Fleet (2003) at 10^7-10^8 CFU g^{-1} for *Bacillus* species. Calvo et al (2007) reported bacterial contamination at 2 x 10^4 CFU.g^{-1}, which are predominantly thermo-resistant spore forming bacteria (3 x 10^3 CFU.g^{-1}).

Among the spore forming bacteria, Bacillus species is the predominant microflora after 72 hours of cocoa fermentation. The significant species were *B. pumillus, B. licheniformis, B. substilis* and *B. cereus* (Ardhana and Fleet, 2003). These bacteria survive at concentrations of 10^4-10^6 CFU g^{-1} (Ardhana and Fleet, 2003) and remain viable after drying and roasting (Schwan and Wheals, 2004). Currently, the role of

Bacillus species in the fermentation of cocoa beans is not well studied (Ardhana and Fleet, 2003).

Other species were able to grow on MRS agar and showed typical characteristics of lactic acid bacteria: Gram positive, rod, non spore forming, negative catalase and capable of fermenting lactose (Adams and Moss, 2000; Schillinger and Holzapfel, 2006). However, the last characteristic test was not done in this research, which was beyond the scope of study. Plating onto de Man Rogosa Sharpe (MRS) agar is the standard method to count lactic acid bacteria (Adams and Moss, 2000). The genera of *Leuconostoc, Pediococcus, Lactococcus,* and *Lactobacillus* are the most commonly isolated lactic acid bacteria from cocoa bean fermentation (Camu et al., 2007). Ardhana and Fleet (2003) reported *Lactobacillus plantarum* and *L. hilgardii* were able to survive until the end of fermentation, and most probably exists during storage.

4 Conclusion

This research has demonstrated the frequent presence of mycotoxigenic fungi such as *Aspergillus flavus, A. niger, A. clavatus, A. wentii,* and *A. ochraceus* in dried cocoa bean samples obtained from Indonesia and Queensland. The frequency of occurrence of *Aspergillus flavus* and *Aspergillus niger* was 100% in some samples. The incidence of isolation of *Aspergillus* and *Penicillium* species from chlorinated beans from Indonesia was decreased by 70% and 40%, respectively and by about 40% for the Queensland beans. The population of fungi on Indonesian cocoa beans was 2.3×10^4 - 7.2×10^6 $CFU.g^{-1}$, while from Queensland beans, the counts were less than 100 $CFU.g^{-1}$.

High populations of Bacillus and lactic acid bacteria were found in cocoa beans from North Queensland (10^6-10^9 $CFU.g^{-1}$). It is suggested that these bacteria may be antagonistic toward fungi and account for the low populations of fungi found on these beans. They could serve as natural fungal bio-control agents but further research is needed to investigate the possibilities.

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Table 2 Frequency of mould occurrence from non-chlorine treated cocoa beans.

Species	Frequency of isolation from 10 beans Indonesia					**Total Population (Indonesia)**	Frequency of isolation from 10 beans North Queensland									**Total population (Queensland)**
	1A	1B	1C	1D	1E		2A	2B	2C	2D	2E	2F	2G	2H	2I	
Aspergillus & teleomorphs																
A. flavus	10	10	5	1	2	**28**	1	8	1	2	2	1	1	0	1	**17**
A. parasiticus	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
A. niger	10	1	3	1	1	**16**	1	2	2	1	4	1	0	1	1	**13**
A. carbonarius	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
A. clavatus	0	10	3	0	7	**20**	1	2	3	0	2	0	0	0	1	**9**
A. wentii	10	2	4	6	0	**22**	1	4	1	1	0	0	0	1	0	**8**
A. ochraceus	1	0	1	0	0	**2**	0	0	0	0	0	0	0	0	0	**0**
A. versicolor	1	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	**0**
Eurotium chevalieri	1	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	**0**
Total Aspergillus & teleeomorphs	**33**	**23**	**16**	**8**	**10**	**90**	**4**	**16**	**7**	**4**	**8**	**2**	**1**	**2**	**3**	**47**
Penicillium																
P. spinolosum	2	0	0	0	2	**4**	1	1	2	3	3	0	0	0	0	**10**
P. citrinum	2	1	1	1	1	**6**	0	7	6	4	1	0	1	0	0	**19**
P. corylophilum	0	0	0	0	1	**1**	0	0	0	0	0	0	0	0	0	**0**
Eupenicillium cinnamopurporeum	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Total Penicillium	**4**	**1**	**1**	**1**	**4**	**11**	**1**	**8**	**8**	**7**	**4**	**0**	**1**	**0**	**0**	**29**
Others																
Mucor pyriformis	1	0	0	0	0	**1**	0	2	0	0	0	0	1	0	0	**3**
Stemphylium	0	2	0	0	0	**2**	0	0	1	0	0	0	0	0	0	**1**
Cladosporium	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Chaetomium globosum	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Epicoccum nigrum	0	0	0	1	0	**1**	0	0	0	0	0	0	0	0	2	**2**
Phoma sp.	0	0	0	0	0	**0**	0	0	0	0	1	0	0	0	0	**1**
Total others	**1**	**2**	**0**	**1**	**0**	**4**	**0**	**2**	**1**	**0**	**1**	**0**	**1**	**0**	**2**	**7**

1A = naturally fermented, sun dried (Indonesian cocoa beans, Penajam)
1B = naturally fermented, sun dried (Indonesian cocoa beans, Malinau)
1C = naturally fermented, sun dried (Indonesian cocoa beans, Samarinda)
1D = naturally fermented, sun dried (Indonesian cocoa beans, Sulawesi)
1E = naturally fermented, sun dried (Indonesian cocoa beans, Irian Jaya)

2A = naturally fermented, sun dried (Queensland cocoa beans)
2B = naturally fermented, oven dried (Queensland cocoa beans)
2C = naturally fermented, sun and oven dried (Queensland cocoa beans)
2D = box fermented using mixed yeast cocktail, sun dried (Queensland cocoa beans)
2E = box fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2F = barrel fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2G = heap fermented using mixed yeast cocktail, sun dried (Queensland cocoa beans)
2H = washed, fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2I = box with long fermentation using mixed yeast cocktail, oven dried (Queensland cocoa beans)

Table 3 Frequency of mould occurrence from chlorine treated cocoa beans.

Species	Frequency of isolation from 10 beans Indonesia 1A	1B	1C	1D	1E	**Total Population (Indonesia)**	Frequency of isolation from 10 beans North Queensland 2A	2B	2C	2D	2E	2F	2G	2H	2I	**Total population (Queensland)**
						Aspergillus & teleomorphs										
A. flavus	5	0	6	0	0	**11**	0	0	1	3	3	0	0	0	0	**7**
A. parasiticus	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
A. niger	2	1	3	0	0	**7**	0	0	3	2	3	1	0	0	0	**9**
A. carbonarius	1	0	0	0	0	**1**	0	0	0	0	0	0	0	0	0	**0**
A. clavatus	2	3	0	1	0	**6**	1	0	1	1	1	0	2	0	0	**6**
A. wentii	4	0	2	0	0	**7**	1	1	0	1	0	0	0	0	0	**3**
A. ochraceus	1	0	0	0	0	**1**	0	0	3	0	0	0	0	0	0	**3**
A. versicolor	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Eurotium chevalieri	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Total Aspergillus & teleeomorphs	**15**	**4**	**11**	**1**	**0**	**33**	**2**	**1**	**8**	**7**	**7**	**1**	**2**	**0**	**0**	**28**
						Penicillium										
P. spinolosum	0	0	2	0	0	**2**	1	1	3	1	1	0	1	0	0	**8**
P. citrinum	0	0	2	0	0	**2**	1	0	1	2	3	1	1	0	0	**9**
P. corylophilum	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Eupenicillium cinnamopurporeum	0	0	1	1	1	**3**	0	0	0	0	0	0	0	0	0	**0**
Total Penicillium	**0**	**0**	**5**	**1**	**1**	**7**	**2**	**1**	**4**	**3**	**4**	**1**	**2**	**0**	**0**	**17**
						Others										
Mucor pyriformis	0	0	3	0	0	**3**	0	0	0	0	0	0	0	0	0	**0**
Stemphylium	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	0	**0**
Cladosporium	0	0	0	0	0	**0**	0	0	1	0	0	0	0	0	0	**1**
Chaetomium globosum	0	0	0	0	0	**0**	0	0	0	0	0	0	1	0	0	**1**
Epicoccum nigrum	0	0	1	0	0	**1**	0	0	0	0	0	0	0	0	0	**0**
Phoma sp.	0	0	0	0	0	**0**	0	0	0	0	0	0	0	0	1	**1**
Fusarium spp.	0	0	1	0	0	**1**	0	0	0	0	0	0	0	0	0	**0**
Total others	**0**	**0**	**5**	**0**	**0**	**5**	**0**	**0**	**1**	**0**	**0**	**0**	**1**	**0**	**1**	**3**

1A = naturally fermented, sun dried (Indonesian cocoa beans, Penajam)

1B = naturally fermented, sun dried (Indonesian cocoa beans, Malinau)

1C = naturally fermented, sun dried (Indonesian cocoa beans, Samarinda)
1D = naturally fermented, sun dried (Indonesian cocoa beans, Sulawesi)
1E = naturally fermented, sun dried (Indonesian cocoa beans, Irian Jaya)
2A = naturally fermented, sun dried (Queensland cocoa beans)
2B = naturally fermented, oven dried (Queensland cocoa beans)
2C = naturally fermented, sun and oven dried (Queensland cocoa beans)
2D = box fermented using mixed yeast cocktail, sun dried (Queensland cocoa beans)
2E = box fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2F = barrel fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2G = heap fermented using mixed yeast cocktail, sun dried (Queensland cocoa beans)
2H = washed, fermented using mixed yeast cocktail, oven dried (Queensland cocoa beans)
2I = box with long fermentation using mixed yeast cocktail, oven dried (Queensland cocoa beans)

Table 4 Fungal populations associated with Indonesian and Queensland, Australia as determined by plate culture on DRBC and DG-18 agar

Sample origin	DRBC (CFU.g^{-1})	DG-18 (CFU.g^{-1})	Fungal species isolated
Penajam, East Kalimantan (naturally fermented, sun dried)	2,100,000	7,200,000	*Aspergillus flavus, A. niger, A wentii, Cladosporium sp, Penicillium citrinum, yeasts*
Malinau, East Kalimantan (naturally fermented, sun dried)	38,000	23,000	*Aspergillus niger, A. flavus, A. carbonarius, Fusarium sp., Penicillium citrinum*
Samarinda, East Kalimantan (naturally fermented, sun dried)	200,000	200,000	*Aspergillus wentii, A. flavus, A. niger, Penicillium citrinum, Fusarium sp., Geotricum candidum, yeasts*
Sulawesi, Indonesia (naturally fermented, sun dried)	< 100	220	*Aspergillus niger,A. flavus, Mucor pyriformis, Stemphylium sp., Penicillium citrinum*
Irian Jaya, Indonesia (naturally fermented, sun dried)	< 100	< 100	*Aspergillus niger, A. flavus, A. wentii, Penicillium citrinum, P. spinolosum*
North Queensland (naturally fermented, sun dried)	< 100	250	*Aspergillus niger, Penicillium citrinum*
North Queensland (naturally fermented, oven dried)	< 100	< 100	*Aspergillus niger, A. fumigatus, A. carbonarius, Cladosporium sp, Penicillium citrinum, P. spinolosum*
North Queensland (naturally fermented, sun & oven dried)	< 100	< 100	*Aspergillus carbonarius, yeasts*
North Queensland (box fermented using mixed yeast cocktail, sun dried)	< 100	< 100	*Aspergillus carbonarius, A. niger, A. clavatus, Penicillium citrinum*
North Queensland (box fermented using mixed yeast cocktail, oven dried)	< 100	< 100	*Penicillium spinolosum*
North Queensland (barrel fermented using mixed yeast cocktail, oven dried)	< 100	< 100	*Aspergillus niger, Stemphylium sp., Penicillium citrinum*
North Queensland (heap fermented using mixed yeast cocktail, sun dried)	< 100	< 100	*Aspergillus niger, A. clavatus, A. carbonarius, Penicillium spinolosum*
North Queensland (washed, fermented using mixed yeast cocktail, oven dried)	< 100	< 100	*Aspergillius niger, Penicillium spinolosum*
North Queensland (box with long fermentation using mixed yeast cocktail, oven dried)	< 100	130	*Aspergillus niger, A. flavus, A. fumigatus, Penicillium citrinum*

Table 5 Populations of total plate count, lactic acid bacteria and Bacillus species on non-chlorinated Indonesia and Queensland, Australia.

Samples	TPC[a] ($CFU.g^{-1}$)	LAB[b] ($CFU.g^{-1}$)	*Bacillus* ($CFU.g^{-1}$)
Malinau, East Kalimantan (naturally fermented, sun dried)	2.6×10^8	n/d	3.4×10^8
Penajam, East Kalimantan (naturally fermented, sun dried)	1.6×10^9	n/d	2.8×10^8
Samarinda, East Kalimantan (naturally fermented, sun dried)	1.8×10^9	n/d	6.5×10^8
Sulawesi, Indonesia (naturally fermented, sun dried)	2.7×10^9	n/d	3.4×10^8
Irian Jaya, Indonesia (naturally fermented, sun dried)	9.1×10^9	n/d	3.1×10^8
North Queensland (naturally fermented, sun dried)	2.2×10^9	9.1×10^7	5.6×10^8
North Queensland (naturally fermented, oven dried)	7.3×10^8	5.4×10^8	8.3×10^8
North Queensland (naturally fermented, sun & oven dried)	7.1×10^7	3.5×10^8	5.8×10^7
North Queensland (box fermented using mixed yeast cocktail, sun dried)	1.6×10^9	6.8×10^8	4.0×10^8
North Queensland (box fermented using mixed yeast cocktail, oven dried)	3.3×10^8	6.0×10^6	2.0×10^8
North Queensland (barrel fermented using mixed yeast cocktail, oven dried)	1.4×10^9	6.2×10^7	4.0×10^8
North Queensland (heap fermented using mixed yeast cocktail, sun dried)	1.0×10^9	9.1×10^7	2.0×10^8
North Queensland (washed, fermented using mixed yeast cocktail, oven dried)	5.1×10^8	5.4×10^7	5.0×10^7
North Queensland (box with long fermentation using mixed yeast cocktail, oven dried)	4.6×10^9	5.9×10^8	8.0×10^8

[a] total plate count, [b] lactic acid bacteria, n/d = not detected result (< 100 $CFU.g^{-1}$)

Table 6 Reduction in frequency of occurrences of *Aspergillus* and *Penicillium* populations from Indonesia and Queensland cocoa bean samples after 0.4% chlorine treatment compared with untreated samples

Total moulds	Indonesia	Queensland
	Aspergillus	
Untreated	90 isolates	47 isolates
Chlorine treated	33 isolates	28 isolates
Reduction	63 %	40 %
	Penicillium	
Untreated	11 isolates	29 isolates
Chlorine treated	7 isolates	17 isolates
Reduction	36 %	41 %