



Identification and quantification of fungi and mycotoxins from Pu-erh tea



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ABSTRACT

Pu-erh tea originates from the province of Yunnan in south-western China. As this tea is produced by so called *Aspergillus* post-fermentation the question arises which molds and mycotoxins may be found in this tea. In total 36 samples of Pu-erh tea were investigated for their content of filamentous fungi and the mycotoxins aflatoxins B₁, B₂, G₁, and G₂, fumonisins B₁, B₂, and B₃, and ochratoxin A. Fungi were isolated from all samples in a concentration of 1.0×10^1 to 2.6×10^6 colony forming units (cfu)/g tea, all together 19 fungal genera and 31 species were identified. The most prevalent species were *Aspergillus acidus* and *Aspergillus fumigatus*, followed by *Zygomycetes* and *Penicillium* species. Aflatoxins and fumonisins were not found in the samples investigated, ochratoxin A was detected in 4 of 36 teas (11.1%).

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1. Introduction

Tea is – after water – the most common beverage in the world. It is produced by pouring hot (boiling) water over processed leaves of the tea plant, *Camellia sinensis*. Tea can be classified according to the processing of the tea leaves: non-fermented (green tea), slightly fermented (white and yellow tea), half-fermented (cyan tea), completely fermented (black or red tea), and post-fermented (Pu-erh tea) (Jidong, 2009). The latter is produced in the province of Yunnan in south-western China and has been consumed in China for centuries. Recently it has become popular in other countries too as a functional beverage. Pu-erh tea is said to have cholesterol- and lipid-lowering effects (Chiang et al., 2005; Yang and Koo, 1997), as well as antioxidative properties (Duh et al., 2004).

In the past, large batches of Pu-erh tea were transported for months on caravans to Tibet and other remote destinations. The tea matured continuously along the way, factors such as exposure to the sun, wind, rain and humidity affected the raw tea leaves (Jidong, 2009). Today, most Pu-erh tea is produced through artificial pile fermentation for 48 days, also called “*Aspergillus* post-fermentation”. For this process the leaves are picked from tea bushes, followed by airing (withering), heating, rubbing and twisting, and sun drying. The sun-baked crude

tea leaves are combined with water and piled into a windrow shape in fermentation rooms and covered with straw mats, bringing the tea leaves in contact with microorganisms inhabiting straw mats and fermentation rooms (Abe et al., 2008). Until recently, *Aspergillus niger* was discussed as the main fermenting mold in Pu-erh tea production (Abe et al., 2008; Sano et al., 1986; Xu et al., 2005).

The fungal genus *Aspergillus* is one of the most important genera for the man, for its industrial use, its ability to spoil food and not least its medical impact as cause of a variety of diseases (Buzina, 2013). *Aspergillus* is subdivided into 8 subgenera and 22 sections. *Aspergillus* section *Nigri* was introduced by Gams et al. (1985) and recently divided into five clades (Varga et al., 2011). Currently it comprises 23 species. Some of them are used widely for production of enzymes and organic acids (Pariza and Johnson, 2001; Raper and Fennell, 1965). *A. niger* fermentation is “generally recognized as safe” (GRAS) by the United States Food and Drug Administration (FDA) under the Federal Food, Drug, and Cosmetic Act (Schuster et al., 2002). However many investigations did not or do not distinguish between different species in the section *Nigri*, and it has been shown that only some (or none) of black aspergilli in tea fermentation are *A. niger sensu stricto* (Mogensen et al., 2009; Zhao et al., 2010).

Previous studies have shown a variety of fungi to be isolated from Pu-erh tea (Halt, 1998; Mogensen et al., 2009; Xu et al., 2011; Zhao et al., 2010). Whether all these fungi were involved in the fermentation process or rather are contaminants is not yet fully understood, but some of them are potentially able to produce mycotoxins. *A. niger* was shown to be able to produce fumonisins (Frisvad et al., 2011; Mogensen et al., 2009) and ochratoxin A (OTA) (Mogensen et al., 2009). Aflatoxins are

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mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, molds commonly occurring in products which are stored under humid and warm conditions.

The aim of our study was to investigate the fungal community in different Pu-erh teas with particular attention to the black aspergilli (*A. niger* sensu lato, *Aspergillus* section *Nigri*), and the content of the mycotoxins aflatoxins B₁, B₂, G₁ and G₂, fumonisins B₁ and B₂, and ochratoxin A.

2. Material and methods

2.1. Pu-erh tea samples

For this investigation 36 different Pu-erh tea samples were purchased from tea shops. Samples were available in compressed form as cakes and bowls, in loose form and sealed in tea bags. As well as conventionally produced Pu-erh teas, samples from organic farming were investigated also. Fungi were isolated from tea samples by suspension and dilution series, followed by cultivation on mycological culture media to determine the qualitative and quantitative outcome.

2.2. Isolation and characterization of fungi in the Pu-erh tea

Compressed tea samples were loosened carefully with the help of a mortar and pestle. For fungal spore cultivation 10 g Pu-erh tea sample was suspended in 90 mL 0.1% buffered peptone water by using a shaker at 150 rpm for 15 min. The suspension was poured through a sieve (1 mm mesh) and serially diluted (1:10, 1:100, 1:1000) in sterile distilled water. From these dilutions 100 µL each were plated onto agar plates of malt extract (MEA), Sabourauds glucose (SGA) and dichloran 18% glycerol (DG18) according to the methods of Mogensen et al. (2009). These media were chosen because of the wide spectrum of fungi that may occur in Pu-erh tea, including many xerophilic species which will grow on DG18. Agar plates were incubated at 25 °C for 7–14 days. After cultivation fungal colonies were counted on each agar plate. Calculation of colonies was reported as colony forming units (cfu)/g tea. Pure cultures of each colony were prepared for fungal differentiation and identification.

2.3. Identification of fungi

Filamentous fungi were identified to genus and species level, respectively, by examining the culture morphology and by microscopy according to the Atlas of Clinical Fungi (De Hoog et al., 2000) and other identification keys (Klich, 1988; Pitt, 1985; Samson, 2010). Fungi which were not identified unambiguously by morphology were examined with molecular methods. Therefore DNA was extracted from mycelia using the Master Pure™ Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). PCR and sequencing of the Internal Transcribed Spacer (ITS) region of the DNA coding for ribosomal RNA (rDNA) using primers ITS1 and ITS4 were carried out as described previously (White et al., 1990). To distinguish between species of *Aspergillus* section *Nigri* part of the β -tubulin gene was amplified using primers bt2a and bt2b (Glass and Donaldson, 1995) and sequenced thereafter. Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready reaction Kit (Applied Biosystems, Carlsbad, CA, USA) and sequence data were analyzed on the 3130 Genetic Analyzer (Applied Biosystems). The DNA sequences were edited with the Sequencing Analysis 5.2 computer program (Applied Biosystems). Alignments of sequence data were compared with Clustal Omega software (<http://www.ebi.ac.uk/Tools/msa/clustalo/>).

2.4. Analytical methods for aflatoxins, fumonisins and ochratoxin A

2.4.1. Aflatoxins (AFL)

The aflatoxins B₁, B₂, G₁, and G₂ were determined with the commercial kit RIDASCREEN®FAST Aflatoxin (R5202) (R-Biopharm, Darmstadt,

Germany) as described by the manufacturer. Briefly, 5 g tea samples were extracted with 25 mL methanol 70% by shaking at 300 rpm for 10 min. Then the extract was centrifuged at 3000 g for 10 min and neutralized with 2 mL 10 × PBS (pH 7.3). After supplement of 250 µL Tween 20 and mixing for 5 min the extract was poured into an EASI-EXTRACT® AFLATOXIN column (R-Biopharm), rinsed with 20 mL PBS 20 mM, pH 7.4 and demineralized water and eluted with 1.5 mL methanol and 1.5 mL demineralized water. The detection limit for AFL was 1.7 ppb. Six samples were spiked with 20 ppb AFL for internal control. These spiked samples were analyzed both with ELISA and HPLC for control. The recovery rate ranged between 87% and 116%.

The HPLC analysis was performed essentially according to the instruction of R-Biopharm for EASI-Extract® Aflatoxin immunoaffinity column application. The aflatoxins were separated over Waters Spherisorb ODS-2 (5 µm; 4.6 × 250 mm) with water:methanol (60/40 v/v) as mobile phase medium and analyzed with fluorescence detection at λ_{ex} = 362 nm, and λ_{em} = 440 nm after post-column derivatization by means of a Kobra cell.

2.4.2. Fumonisins

Fumonisins B₁ and B₂ (FB₁, FB₂) were measured quantitatively, FB₃ only qualitatively according to EN 14352:2004 “Foodstuffs – Determination of Fumonisin B₁ and B₂ in Maize Based Foods – HPLC Method with Immunoaffinity Column Clean Up” by an accredited laboratory (Wessling, Bremen, Germany). Briefly, 20 g tea samples were extracted with 50 mL elution solution (12.5 mL acetonitrile, 12.5 mL methanol, 25 mL deionized water) by shaking for 20 min and centrifugation for 10 min at 2500 g. The supernatant was filtered and the pellet extracted again as described above. Both extracts were merged and 10 mL thereof mixed with 40 mL PBS buffer (pH 7.0) and poured in an immunoaffinity column. After washing the column with PBS the fumonisins were eluted slowly (1–2 drops per sec) with 1.5 mL methanol and determined by liquid chromatography–mass spectrometry (LC/MS).

2.4.3. Ochratoxin A (OTA)

Determination of OTA was carried out commercially as described by Thellmann and Weber (1997) by an accredited laboratory (Wessling). Briefly, tea samples were homogenized and 10 g each were extracted with 40 mL acetonitrile:water (80:20 v/v). After clean-up of extracts using immunoaffinity columns (IAC), OTA was determined by high-performance liquid chromatography (HPLC) using RP18 column and fluorometric detection at 333 nm excitation and 460 nm emission.

2.5. Statistical analysis

The statistical evaluations were carried out by using the Chi square (χ^2) test from the statistical tools of Microsoft Excel 2010 for Windows XP. All p-values below 0.01 were considered statistically significant.

3. Results

3.1. Quantification of fungi from Pu-erh tea samples

In total 36 different Pu-erh tea samples were investigated. Of them 25 (69%) were loose and 11 (31%) were compressed tea samples. Within the loose samples 9 (25%) were organic as indicated on the packaging, the other 16 loose samples (44%) were considered conventionally produced. Fungal cultures were grown from all 36 Pu-erh tea samples; and more than 250 fungal isolates were cultured.

The fungal concentrations of 16 conventionally produced (non-organic) loose Pu-erh teas ranged from 1.0×10^1 to 2.6×10^6 cfu/g (Table 1). The fungal concentrations of 9 organic loose teas ranged from 1.0×10^1 to 5.3×10^3 cfu/g (Table 1). The highest concentrations in organic loose teas were 1–3 logs lower than in conventionally produced loose teas. In organic tea samples, the highest concentrations were found in two teas sealed in tea bags. The fungal concentrations

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