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

Doris Haas [a], Bettina Pfeifer [a], Christoph Reiterich [b], Regina Partenheimer [b], Bernhard Reck [b], Walter Buzina [a,*]

[a] Institute of Hygiene, Microbiology and Environmental Medicine, Medical University Graz, Austria
[b] Food and Feed Analysis, R-Biopharm AG, An der neuen Bergstraße 17, D 64297 Darmstadt, Germany

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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 B_1, B_2, G_1, and G_2, fumonisins B_1, B_2, and B_3, 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%).

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

* Corresponding author at: Institute of Hygiene, Microbiology and Environmental Medicine, Universitaetsplatz 4, A 8010 Graz, Austria. Tel.: +43 316 380 7719; fax: +43 316 380 9648.
E-mail address: walter.buzina@medunigraz.at (W. Buzina).

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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 B_1, B_2, G_1 and G_2, fumonisins B_1 and B_2, 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 PureTM 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 B_1, B_2, G_1, and G_2 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 B_1 and B_2 (FB_1, FB_2) were measured quantitatively, FB_3 only qualitatively according to EN 14352:2004 "Foodstuffs — Determination of Fumonisin B_1 and B_2 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

Table 1
Quantification of fungi isolated from Pu-erh tea samples on different media. MEA, malt extract agar; DG18, dichloran glycerol agar; SGA, Sabourauds glucose agar; cfu, colony forming units.

Tea form	Range [cfu/g]			Mean [cfu/g] ± SD			Median [cfu/g]		
	MEA	DG18	SGA	MEA	DG18	SGA	MEA	DG18	SGA
Loose conventional (n = 16)	1.0×10^1–1.5×10^6	1.0×10^2–2.6×10^6	1.0×10^1–6.9×10^5	$1.3 \times 10^5 \pm 3.7 \times 10^5$	$1.2 \times 10^5 \pm 3.2 \times 10^5$	$8.1 \times 10^4 \pm 1.8 \times 10^5$	5.1×10^3	9.5×10^3	7.2×10^3
Loose organic (n = 9)	3.0×10^2–5.3×10^3	1.0×10^2–5.0×10^3	1.0×10^1–5.1×10^3	$1.9 \times 10^3 \pm 1.7 \times 10^3$	$1.7 \times 10^3 \pm 1.9 \times 10^3$	$1.6 \times 10^3 \pm 1.8 \times 10^3$	1.5×10^3	1.1×10^3	1.1×10^3
Compressed (n = 11)	1.0×10^1–2.0×10^2	1.0×10^1–1.0×10^2	1.0×10^1–1.0×10^2	$6.0 \times 10^1 \pm 6.4 \times 10^1$	$3.5 \times 10^1 \pm 4.2 \times 10^1$	$3.5 \times 10^1 \pm 4.2 \times 10^1$	1.0×10^1	1.0×10^1	1.0×10^1

of 11 compressed tea samples were comparatively low on all three culture media, ranging from 1.0×10^1 to 2.0×10^2 cfu/g (Table 1). However, because of the high variability of cfu values the differences between the three groups of Pu-erh teas were statistically not significant.

3.2. Identification of fungi from Pu-erh tea samples

The filamentous fungi isolated from 36 tea samples were identified microscopically and with molecular methods to genus and/or species level. A total of 39 different fungi (fungal taxa) comprising 19 genera and 31 species were identified (Fig. 1, Table 2).

In 16 conventionally produced loose teas, 13 genera and 25 species of fungi were identified, in 9 organic tea samples, 14 genera and 17 species and in 11 compressed tea samples, 4 genera and 10 species (Table 2).

In the majority of tea samples (29 from 36, 80.5%) black aspergilli were found. PCR of a part of the beta tubulin gene with primer pairs bt2a and bt2b resulted in an amplicon of 508–534 base pairs. Sequencing of these amplicons revealed three different species belonging to *Aspergillus* section *Nigri*. The most prevalent species was *Aspergillus acidus*, which occurred in all 29 black aspergilli-positive samples. The sequence data of *A. acidus* isolates were submitted to GenBank and assigned accession numbers KC433673–KC433701. Second in number was *Aspergillus tubingensis*, present in 6 samples (16.7%, GenBank accession numbers KC433702–KC433707), and *A. niger* was found in only one tea (2.8%, GenBank accession number KC433708). The latter two species occurred only together with *A. acidus*, never as sole *Aspergillus* section *Nigri* species. There was no significant difference in the occurrence of the species in the different forms of Pu-erh tea: *A. acidus* was present in 14 of 16 (87.5%) loose leaves, 7 of 11 (63.6%) compressed and 8 of 9 (88.9%) organic teas ($p = 0.75$), *A. tubingensis* was found in 4 of 16 (25.0%) loose teas but not in compressed tea leaves, and in 2 samples of 9 (22.2%) organic teas ($p = 0.26$). The one *A. niger* isolate was detected in a loose tea sample (Table 3).

The thermotolerant mold *Aspergillus fumigatus* was isolated quite frequently from Pu-erh tea samples (19; 52.8%). It was found more often in loose teas (11 of 16, 68.8%) than in organic teas (6 of 9, 66.7%) and in compressed leaves (2 of 11, 18.2%), but these differences were statistically not significant ($p = 0.17$). Other *Aspergillus* species isolated from Pu-erh tea samples were *Aspergillus reptans* (2; 5.6%), as well as *Aspergillus versicolor*, *Aspergillus ochraceus*, *Aspergillus penicillioides* and *Aspergillus tamarii* (all 1; 2.8%) (Table 3).

In the genus *Penicillium* 8 species were identified in the examined Pu-erh tea samples. The most predominant species were *Penicillium citrinum* and *Penicillium commune*.

Besides the different *Aspergillus* and *Penicillium* species filamentous fungi from 17 genera were identified (Fig. 1, Table 2).

3.3. Content of mycotoxins

None of the investigated tea samples showed an AFL content above the detection limit of 1.7 ppb (1.7 μg/kg), except for six samples (positive controls) spiked with 20 ppb AFL, which revealed recovery rates between 87% and 116% (Table 3).

The fumonisins B_1 and B_2 values were below the detection limit (10 μg/kg) in all samples investigated. FB_3 was also not detected in the samples investigated (Table 3).

OTA was positive in four samples (samples 6, 12, 16, 21) with values of 94.7, 14.8, 0.65 and 0.65 μg/kg, respectively. All other samples were below the detection limit of 0.5 μg/kg (Table 3).

4. Discussion

In this study the presence of fungi and mycotoxins in Pu-erh tea was investigated. After isolation and cultivation 19 fungal genera and 31 species were identified in 36 Pu-erh tea samples. The most frequently isolated fungi belonged to the genus *Aspergillus*. Similar results from

Pu-erh tea were reported by Zhao et al. (2010), who identified 41 species of 19 fungal genera including 13 species of *Aspergillus*, seven species of *Penicillium* and 21 species of other genera. Xu et al. (2005) confirmed that *Aspergillus* is the most prevalent genus found in the fermentation process of Pu-erh tea. *A. niger* was reported with high frequency in Pu-erh tea (Duh et al., 2004). Zhou et al. (2004) also reported *A. niger* as the most common species among all identified fungi. However, until recently all black aspergilli were identified as *A. niger* without further differentiation. Therefore publications reporting *A. niger* should be considered carefully. *Aspergillus* species which produce conidia in different shades of black (black aspergilli) were subsumed in the *A. niger* species group (Raper and Fennell, 1965). The section *Nigri* was introduced by Gams et al. (1985) and recently divided into five clades (Varga et al., 2011), it currently comprises 23 species. In our Pu-erh tea samples *A. acidus* was the most common fungal species, present in 29 samples (80.6%). *A. tubingensis* and *A. niger* (sensu stricto), two other species of *Aspergillus* section *Nigri*, were present in 6 (16.7%) and 1 (2.8%) samples, respectively. Interestingly, the latter two black aspergilli never occurred alone in a tea but always together with *A. acidus* (Table 3). Mogensen and coworkers isolated 47 black aspergilli from 10 tea samples (5 of them Pu-erh teas) and identified all of them as *A. acidus* (Mogensen et al., 2009). Identification of black aspergilli was done by sequencing part of the beta tubulin gene, which is an important tool for identification and differentiation of *Aspergillus* species. The three black aspergilli (*A. acidus*, *A. tubingensis*, *A. niger*) differed from each other in differences in the intron sequences (introns 3–5) of the beta tubulin gene, the exon sequences (exons 3–6) and hence the translated putative protein was identical in all three species (for details see accession numbers KC433673–KC433708 at GenBank http://www.ncbi.nlm.nih.gov/nucleotide).

Second most common fungal species in the investigated Pu-erh teas was *A. fumigatus* in 19 samples (52.8%). As this species is not described to have any beneficial effects on the fermentation of tea or other foodstuff, it is to be considered as contamination. *A. fumigatus* is a thermotolerant to thermophilic mold, found frequently in decomposing organic matter. It is also the most common mold causing invasive infections in immunosuppressed patients.

Table 2
Spectrum of fungal genera and species in Pu-erh tea. C, loose tea conventional (n = 16); O, loose tea organic (n = 9); P, compressed tea (n = 11).

Fungal genera and species	C	O	P	Fungal genera and species	C	O	P
Aspergillus				*Fusarium*			
Aspergillus acidus	14	8	7	*Fusarium* sp.		1	
Aspergillus fumigatus	11	6	2	*Geotrichum*			
Aspergillus niger	1			*Geotrichum* sp.		1	
Aspergillus ochraceus	1			*Geomyces*			
Aspergillus penicillioides	1			*Geomyces pannorum*		1	
Aspergillus reptans	2			*Mucor*			
Aspergillus tamarii	1			*Mucor hiemalis*	6	4	
Aspergillus tubingensis	4	2		*Mucor* sp.	3	1	
Aspergillus versicolor	1		1	*Mycelia sterilia*			
Byssochlamys				Mycelia sterilia	4	2	4
Byssochlamys spectabilis	1		1	*Paecilomyces*			
Chalara				*Paecilomyces* sp.	1		
Chalara microchona	1			*Penicillium*			
Cladosporium				*Penicillium citrinum*	2	3	3
Cladosporium cladosporioides	5	2		*Penicillium commune*	4	1	2
Emericella				*Penicillium crustosum*	1	1	1
Emericella nidulans	1			*Penicillium digitatum*			2
Engyodontium				*Penicillium glabrum*		1	
Engyodontium album		1		*Penicillium italicum*	2	1	
Eurotium				*Penicillium piceum*	1		
Eurotium amstelodami	4			*Penicillium polonicum*	1		1
Eurotium chevalieri	1			*Penicillium* sp.	5		3
Eurotium herbariorum	2	2		*Scedosporium*			
Eurotium sp.	2	3		*Scedosporium prolificans*		1	
Leptosphaerulina				*Rhizomucor*			
Leptosphaerulina chartarum		1		*Rhizomucor* sp.	7	4	
Lichtheimia				*Wallemia*			
Lichtheimia corymbifera	3	2		*Wallemia* sp.	1		

Beside these fungal species other taxa were also reported to be present quite often in Pu-erh and other fermented teas. In our study the genera *Rhizomucor*, *Mucor*, *Penicillium*, *Cladosporium* and *Eurotium* were found predominantly. The fermented Fuzhan brick tea studied by Xu et al. (2011) was dominated by the genera *Eurotium*, *Debaryomyces*, *Aspergillus* and *Beauveria*. These samples were collected at different fermentation stages in a single tea factory. Halt (1998) predominately

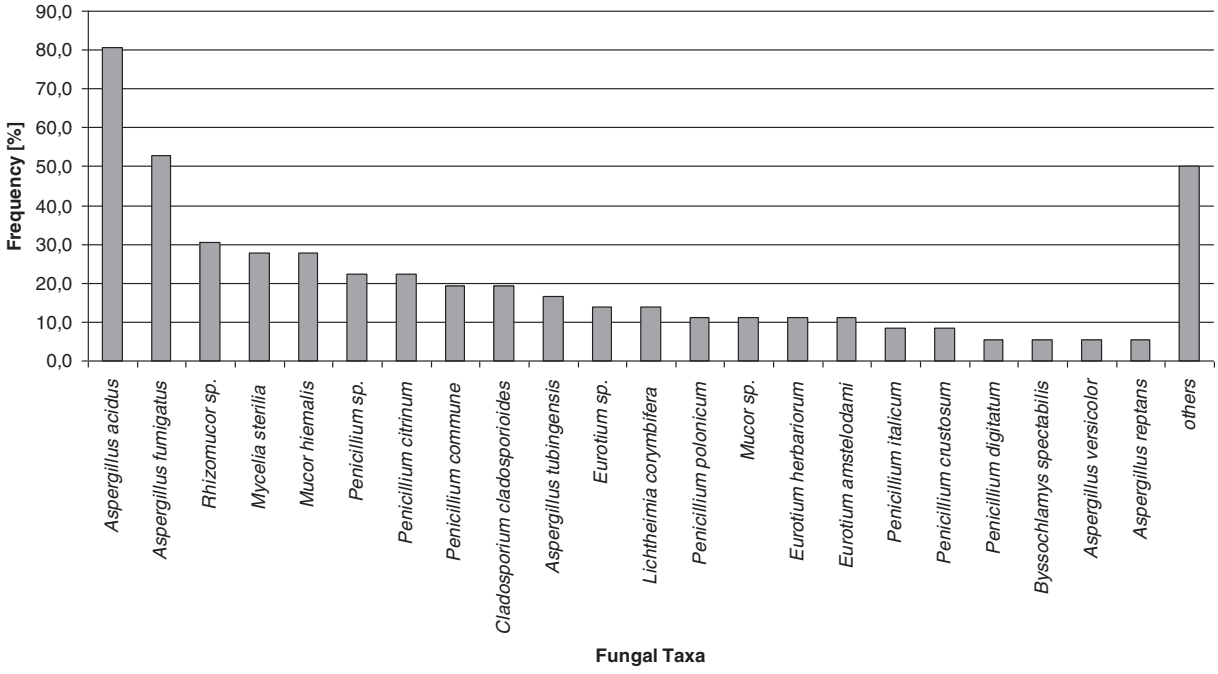

Fig. 1. Frequency of fungal taxa in Pu-erh tea.

Table 3
List of all Pu-erh tea samples investigated. C, loose tea conventional; O, loose tea organic; P, compressed tea; MEA, malt extract agar; DG18, dichlorane glycerol agar; SGA, Sabourauds glucose agar; cfu, colony forming units; atub, *Aspergillus tubingensis*; anig, *Aspergillus niger*; aaci, *Aspergillus acidus*; afum, *Aspergillus fumigatus*; aoch, *Aspergillus ochraceus*; peni, *Penicillium* sp., pcit, *Penicillium citrinum*; ×, fungi identified in the tea; AFL, aflatoxins B_1, B_2, G_1, and G_2 (detection limit 1.7 μg/kg); OTA, ochratoxin A (detection limit 0.5 μg/kg); FB, fumonisins B_1, B_2, (detection limit 10 μg/kg) and B_3 (qualitatively); <, below detection limit.

	MEA	DG-18	SGA	C	O	P	aaci	atub	anig	afum	aoch	peni	pcit	AFL	OTA	FB
#	cfu	cfu	cfu	16	9	11	29	6	1	19	1	23	8	[μg/kg]		
1	3.3×10^4	3.3×10^4	3.7×10^4	C			×			×				<	<	<
2	3.0×10^2	1.0×10^2	4.0×10^2		O		×			×				<	<	<
3	1.5×10^6	1.1×10^6	7.0×10^5	C			×			×				<	<	<
4	3.9×10^3	4.5×10^3	4.2×10^3		O		×					×		<	<	<
5	$<1.0 \times 10^2$	$<1.0 \times 10^2$	1.0×10^2			P	×							<	<	<
6	2.5×10^5	2.2×10^5	3.0×10^5	C			×			×				<	0.65	<
7	5.0×10^2	2.0×10^2	$<1.0 \times 10^2$		O					×				<	<	<
8	2.4×10^5	2.6×10^6	1.8×10^5	C			×	×		×				<	<	<
9	7.0×10^2	6.0×10^2	5.0×10^2		O		×							<	<	<
10	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$			P	×							<	<	<
11	5.3×10^3	5.0×10^3	5.1×10^3		O		×	×		×				<	<	<
12	4.2×10^4	4.4×10^4	3.3×10^4	C			×				×	×		<	0.65	<
13	1.0×10^3	3.4×10^3	3.3×10^3	C			×			×		×		<	<	<
14	1.6×10^4	1.6×10^4	1.1×10^4	C			×	×	×	×		×		<	<	<
15	2.0×10^2	$<1.0 \times 10^2$	1.0×10^2			P	×			×				<	<	<
16	4.0×10^2	4.0×10^2	7.0×10^2		O		×					×	×	<	14.8	<
17	2.2×10^3	1.1×10^3	1.2×10^3		O		×	×		×		×		<	<	<
18	1.0×10^2	1.0×10^2	1.0×10^2			P	×			×		×	×	<	<	<
19	2.3×10^3	2.6×10^3	1.6×10^3		O		×			×		×	×	<	<	<
20	8.0×10^3	1.3×10^4	1.1×10^4	C			×	×		×		×	×	<	<	<
21	1.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^2$			P						×	×	<	94.7	<
22	1.5×10^3	1.1×10^3	1.1×10^3		O		×			×		×	×	<	<	<
23	$<1.0 \times 10^2$	5.0×10^2	2.0×10^2	C			×			×		×	×	<	<	<
24	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$			P						×	×	<	<	<
25	5.0×10^2	6.0×10^3	1.1×10^3	C			×					×		<	<	<
26	1.0×10^2	$<1.0 \times 10^2$	$<1.0 \times 10^2$			P						×		<	<	<
27	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$	C			×							<	<	<
28	2.2×10^3	3.8×10^3	1.7×10^3	C			×			×				<	<	<
29	$<1.0 \times 10^2$	3.0×10^2	1.0×10^2	C								×		<	<	<
30	3.8×10^4	4.1×10^4	2.9×10^4	C			×	×		×		×		<	<	<
31	1.0×10^2	2.0×10^2	1.0×10^2	C								×		<	<	<
32	4.0×10^2	5.0×10^2	3.0×10^2	C			×			×		×		<	<	<
33	$<1.0 \times 10^2$	1.0×10^2	1.0×10^2			P						×		<	<	<
34	$<1.0 \times 10^2$	$<1.0 \times 10^2$	$<1.0 \times 10^2$			P	×					×		<	<	<
35	$<1.0 \times 10^2$	1.0×10^2	$<1.0 \times 10^2$			P	×					×		<	<	<
36	1.0×10^2	1.0×10^2	$<1.0 \times 10^2$			P	×					×		<	<	<

detected *Aspergillus*, *Penicillium*, *Mucor*, *Rhizopus*, *Absidia*, *Alternaria*, *Cladosporium* and *Trichoderma* in a variety of different samples of medicinal plants and herbal teas.

Above all *Penicillium* species are very common on various food products (Samson, 2010; Samson et al., 2004). In our study, high counts of *P. citrinum* and *P. commune* were found in different tea forms. *Penicillium* species may also have an effect on the biological transformation of the tea leaves during the Pu-erh tea fermentation process. *Penicillium* is believed to produce a variety of enzymes and organic acids during pile-fermentation process in tea. Also the metabolism effect of some species of *Penicillium* may inhibit and eliminate the growth of bacteria [http://www.tea-of-chinese.com/pu-erh-tea-details.php?cid=358] and may play a role in the quality of tea.

Fungi such as the zygomycetes *Rhizopus* and *Mucor*, and most notably *Cladosporium*, are almost everywhere in the environment and are able to grow on a variety of organic material (Haas et al., 2012). Their spores or conidia cover all surfaces, e.g. tea leaves, and therefore it is impossible to avoid contact with spores of fungi in the ripening process of tea.

Tea samples were all plated in three dilutions on three media: MEA, SGA and DG18. These media were chosen because of the wide spectrum of fungi able to grow on them, especially DG18 as medium for xerophilic fungi showed the widest spectrum of *Aspergillus* species. The quantity of fungi in cfu did not differ much (all within a decile) between the media. This study shows that the fungal concentrations may depend on the processing procedure and packing of the Pu-erh tea. In loose teas the fungal concentrations ranged from 10^1 to 10^6 cfu/g. Moreover, the highest concentrations of total fungal spores were found in tea forms containing *A. acidus*. Abe et al. (2008) reported that *A. niger* (sensu *largo*) and *Blastobotrys adeninivorans* play an important role in the fermentation process of Pu-erh tea leaves and the concentrations of fungi in dried tea leaves increased to 10^6 cfu/g. Hou et al. (2010) showed that the total microbial counts of *A. niger* decreased to the background level of 3.0×10^2 cfu/g after the drying process. Processing of Pu-erh tea was chemically investigated by Gong et al. (1993). This group discussed "three strains of basidiomycetes" as responsible for production of volatile organic compounds belonging to the group of methoxybenzene derivatives, but the authors did not report genera and species of these basidiomycetes. Basidiomycetes were not identified from our samples, however 10 strains of mycelia sterilia were cultured (Table 2), these could belong to basidiomycetes.

In the organic Pu-erh teas fungal concentrations were between 10^1 and 10^3 cfu/g and hence were lower than those of the conventionally produced loose Pu-erh teas. Two organic samples of Pu-erh tea in tea bags had higher fungal concentrations, but the variability of the fungi in tea bags was lower than those in other loose teas. One explanation might be the adherence of fungal spores to the filter material of the tea bags. Moreover, loose tea is more exposed to fungal contamination than packed tea. Herbal teas can be consistently contaminated by molds which may also cause tea spoilage. It is known that food products may be contaminated with fungi during processing, transport and

storage due to inappropriate conditions such as high temperature and humidity (Bouakline et al., 2000; Elshafie et al., 1999).

The compressed Pu-erh teas, compared to the investigated loose teas, showed the lowest fungal concentrations at an average of 2×10^2 cfu/g. Compressed tea forms are compact and have a small surface area which may limit the possibility of post-fermentation contamination. This may be the reason why Zygomycetes and *Cladosporium* spp. were also not isolated from compressed Pu-erh teas.

It cannot be expected that natural products such as teas are free from microorganisms which can be hazardous to human health. Besides in teas, *Aspergillus* species have been found in coffee beans, cereals, powdered milk, chocolate, soy sauce, and tofu, and have been linked to fungal infection in neutropenic patients (Bouakline et al., 2000; Manuel and Kibbler, 1998). Therefore it is advisable that tea leaves should not be handled by immunosuppressed persons, and that tea should be boiled or infused with hot (boiling) water to reduce the concentration of viable microorganisms. Above all the high number of Pu-erh teas (52.8%) containing the thermophilic and facultative pathogenic mold *A. fumigatus* may be a risk for immunosuppressed patients. The role of food and beverages which contain zygomycetes such as *Rhizomucor*, *Mucor* and *Lichtheimia* (former *Absidia*) species, which were found also in our tea samples, is discussed as health risk for neutropenic patients (Ribes et al., 2000; Willinger, 2013).

Three classes of mycotoxins were investigated in the tea samples: the aflatoxins B_1, B_2, G_1, and G_2 (AFL), the fumonisins B_1 and B_2 and ochratoxin A. Aflatoxins were below the detection limit of 1.7 ppb (1.7 μg/kg) in all samples. This correlates with the fact that the aflatoxin producing fungi *A. flavus* and *A. parasiticus* was also not present in our samples of fermented Pu-erh tea. Elshafie et al. showed that unfermented black tea is contaminated by fungi (i.e. *A. flavus*) that might constitute health hazards for humans, but aflatoxin was not found in contaminated tea samples (Elshafie et al., 1999). Only recently Mo et al. (2013) have shown that extracts of black and particularly Pu-erh tea show inhibitory effects on aflatoxin production by *A. flavus*.

One of the historical reasons for fermenting organic material intended for human consumption is to improve food safety through competitive inhibition of pathogenic or food spoiling microorganisms with the help of benign microbes (Bourdichon et al., 2012). In the case of Pu-erh tea these latter group of organisms are black aspergilli, particularly *A. acidus*. Mogensen et al. (2009) have shown that five Pu-erh teas investigated in their study did not contain OTA or FB_2. *A. niger* was shown to be able to produce fumonisins (Frisvad et al., 2011; Mogensen et al., 2009) and OTA (Abarca et al., 2001; Mogensen et al., 2009), but this species was only present in one sample without mycotoxin contamination. In our samples there were 2 teas (5.6%) with OTA contamination of 0.65 μg/kg, this is just above the detection limit (0.5 μg/kg), and 2 samples with 14.8 and 94.7 μg/kg respectively (Table 3). In none of these teas was *A. ochraceus*, a producer of OTA detected. In both teas with higher content of OTA *Penicillium* species were found, and in the tea with the highest OTA contamination (sample 21) *A. acidus* was not found, maybe showing that this tea was not properly fermented and thus enabling OTA producing molds to grow. *Penicillium* species are, after *Aspergillus* species, the main producers of OTA. Rodríguez et al. (2011) have shown, that a variety of species of these two fungal genera have the ability to produce OTA. In the genus *Aspergillus* most of the OTA producers are found in section *Nigri* and in section *Circumdati*; in the genus *Penicillium P. verrucosum* and *P. nordicum* are known to produce OTA (Cabañes et al., 2010). There are no limits in the European Union for OTA in tea; however, the limit for coffee beans (5 μg/kg) would be exceeded in the sample with the highest OTA contamination by almost 20 times. Most producers or distributers of Pu-erh tea recommend discarding the first brew; this may be advisable to remove part of the water soluble or suspendable contaminations. However, OTA has a very low solubility in water (<1 mg/mL). To which extent mycotoxins can be detected in the actual infusion, depending on water temperature and duration of brewing, may be a target for further studies.

Conflict of interest

D.H., B.P. and W.B. declare no conflicts of interest. C.R., R.P. and B.R. are employees of R-Biopharm (Darmstadt, Germany), which is the manufacturer of RIDASCREEN®FAST Aflatoxin used in this study.

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