World Mycotoxin Journal, 2018; 11 (3): 471-481
SPECIAL ISSUE: *Mycotoxins in Africa*

Diversity and fate of fungal metabolites during the preparation of *oshikundu*, a Namibian traditional fermented beverage

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Received: 3 May 2018 / Accepted: 27 June 2018

RESEARCH ARTICLE

Abstract

Sorghum and pearl millet, ingredients for the popular Namibian traditional fermented beverage *oshikundu*, are prone to fungal infection, raising concerns for consumer health from mycotoxin exposure. This study aimed at determining the diversity of fungal metabolites in street-vended ingredients and their transfer rates into *oshikundu*. A total of 105 samples (40 sorghum malt, 40 pearl millet, 25 *oshikundu*) were analysed for 700 fungal, bacterial and plant metabolites, using liquid chromatography-tandem mass spectrometry. Of 98 quantified metabolites, 84 were fungal, some being mycotoxins. *Aspergillus* metabolites were most prevalent (50%, n=42), including aflatoxins, aflatoxin precursors, cyclopiazonic acid and 3-nitropropionic acid from *Aspergillus flavus*; helvolic acid, gliotoxin and fumiquinazolines from *Aspergillus fumigatus* and cytochalasin E, patulin and tryptoquivalines from *Aspergillus clavatus*. High levels of up to 2,280 µg/kg for cyclopiazonic acid and 11,900 µg/kg for 3-nitropropionic acid were quantified in sorghum malts. Other metabolites included fumonisins, curvularin, alternariol and dihydroergosine produced by *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps* genera, respectively. European Union legislated mycotoxins occurred in cereals at a prevalence range of 3-75%, while none were quantifiable in *oshikundu*. Aflatoxin B_1 was quantified in pearl millet meals (13%) and sorghum malts (50%), with 15% sorghum malts having levels above the European Union regulatory limit of 5 µg/kg. Fumonisin B_1 was quantified in pearl millet meals (50%) and sorghum malts (75%) at maximum levels of 3,060 µg/kg and 123 µg/kg respectively, and levels in 5% pearl millet meals were above the European Union regulatory limit of 2,000 µg/kg. Zearalenone and ochratoxin A were quantified in the cereals at levels below European Union regulatory limits. For most metabolites quantifiable in *oshikundu*, transfer rates from cereals to *oshikundu* were above 50%, necessitating the use of good quality ingredients for preparing *oshikundu* and assessment of consumer exposure to mycotoxins.

Keywords: *Aspergillus*, sorghum, pearl millet, beverage, mycotoxin

1. Introduction

Production and consumption of cereal-based traditional fermented beverages is of socio-economic, nutritional and cultural significance in most African countries (Aka *et al.*, 2014; Amadou *et al.*, 2011). Meals and malts of the cereals, maize (*Zea mays*), pearl millet (*Pennisetum glaucum*), finger millet (*Eleusine coracana*) and sorghum (*Sorghum bicolor*) are common substrates used in the preparation of a variety of traditional fermented beverages (Aka *et al.*, 2014; Ezekiel *et al.*, 2015, 2018; Lee and Ryu, 2017; Misihairabgwi and Cheikhyoussef, 2017). *Oshikundu* is a widely consumed opaque, non-alcoholic fermented beverage in the North central and Kavango regions of Namibia, which are suitable for pearl millet and sorghum cultivation (Mu Ashekele *et al.*, 2012; Shapi *et al.*, 2012). The beverage is a very important

daily beverage in northern Namibia, being brewed both for household consumption and for income generation. Culturally, the serving of *oshikundu* to visitors is considered a gesture of welcome and hospitality and the beverage is produced as part of the traditional initiation of young girls into womanhood (Misihairabgwi and Cheikhyoussef, 2017; Mu Ashekele *et al.*, 2012; Shapi *et al.*, 2012). *Oshikundu* is served at weddings and other important ceremonies as well as at daily social interactions (Embashu *et al.*, 2013). The main ingredients for *oshikundu* production are pearl millet meal (locally known as *mahangu*), sorghum malt and water (Embashu *et al.*, 2013; Mu Ashekele *et al.*, 2012). Pearl millet bran may be added optionally. Fermentation occurs by chance inoculation, under uncontrolled environmental conditions, and the peculiar nutritional and sensory properties of *oshikundu* derive mainly from the ingredients, with variable quality beverages being produced between households. In some cases, back slopping is conducted by the local producers to enhance fermentation and product quality.

Fungal, bacterial and plant metabolites play an important role in determining the quality and safety of processed beverages prepared using cereal ingredients (Mastanjević *et al.*, 2018). The cereal ingredients for *oshikundu* production are prone to infection, mainly by fungi of the mycotoxin producing genera *Aspergillus*, *Fusarium* and *Penicillium* (Ezekiel *et al.*, 2018; Kaushik, 2015; Lee and Ryu, 2017). Traditional malting conditions, which include germination of wet cereal grains under hot, humid environments, are ideal for fungal growth and mycotoxin production (Milani and Maleki, 2014). Malting reportedly has the potential to elevate mycotoxin contents up to three fold during processing (Ezekiel *et al.*, 2015; Matumba *et al.*, 2011). Dominant fungal species which have been reported to develop during the sorghum malting process include *Aspergillus clavatus*, *Aspergillus flavus* and *Fusarium* spp. (Rabie and Lubben, 1984). Aflatoxins (AFs), sterigmatocystin (STE), ochratoxins, fumonisins, patulin (PAT), gliotoxin (GLI) and cyclopiazonic acid (CPA) are among the main mycotoxins produced by *Aspergillus* species, which have serious health impacts in humans and animals (Varga *et al.*, 2015). There is great possibility of mycotoxins and other fungal metabolites in the cereal raw materials being carried over into processed beverages, and of continued fungal growth and metabolite production during fermentation, posing health threats to consumers (Ezekiel *et al.*, 2018; Milani and Maleki, 2014).

AFs, fumonisins, ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) commonly contaminate cereals, although AFs and fumonisins are more prevalent in Southern Africa (Darwish *et al.*, 2014; Lee and Ryu, 2017; Misihairabgwi *et al.*, in press). These mycotoxins and several others are regulated in many developed countries of the world after thorough risk assessment, considering their acute and chronic toxic effects, occurrence, consumption data and other factors (Gruber-Dorninger *et al.*, 2016; Kovalsky *et al.*, 2016). Emerging mycotoxins, which are currently unregulated and commonly include aflatoxin precursors, ergot alkaloids, enniatins (ENN), beauvericin (BEA), alternariol (AOH), alternariolmethylether (AME) and moniliformin (MON), have been shown to occur frequently in agricultural products (Fraeyman *et al.*, 2017; Gruber-Dorninger *et al.*, 2016; Kovalsky *et al.*, 2016).

Transfer of food contaminants from agricultural raw ingredients to processed food is a major consideration in determining the safety of the final product (Mastanjević *et al.*, 2018). Despite the numerous reports on the spectrum and quantities of fungal metabolites, including mycotoxins, in crops used in traditional beverage processing, few studies have analysed the transfer rate of mycotoxins and other fungal metabolites from ingredients to beverages, their concentrations in the beverages, and exposure rates for consumers (Ezekiel *et al.*, 2018). No regulations targeted at mycotoxin levels in African traditional fermented beverages exist, despite the high mycotoxin levels in their raw materials and the high consumption levels of the beverages (Ezekiel *et al.*, 2018). Fungal metabolite detection, quantification and determination of fate during processing is therefore imperative in the cereals and traditionally fermented beverages. No research on mycotoxin diversity and fate has been carried out on any food commodities in Namibia and no strict process control is imposed on traditional brewing, fermentation or storage of raw cereal grains. The aim of this study, therefore, was to investigate the diversity of fungal metabolites in *oshikundu* beverage and the raw materials used in its production, as well as to determine the fate of metabolites from cereal ingredients to processed product.

2. Materials and methods

Chemicals and reagents

Methanol (Merck, Darmstadt, Germany) and acetonitrile (ACN) (VWR, Leuven, Belgium) were of LC gradient grade and ammonium acetate (Sigma Aldrich, Vienna, Austria) was of MS grade. Water was purified successively to 18.2 MU by reverse osmosis using an Elga Purelab ultra analytic system (Veolia Water; Bucks, UK). Fungal metabolite standards, obtained from commercial sources, were predominantly constituted in ACN to yield a multi-analyte working solution, which was subsequently used to prepare different working solutions for calibrations. All standard solutions were stored in amber vials in the dark at 20 °C; before use they were kept at room temperature for 15 min. For the metabolites for which quantitative standards were not available, relative quantification was carried out based on the peak areas. Extraction (ACN:water:glacial acetic acid, 79:20:1, v/v/v) and dilution (ACN:water:acetic

acid, 20:79:1, v/v/v) solvents were freshly prepared and kept at room temperature before use. Two eluting solvents (eluent A and eluent B) that each contained 5 mM ammonium acetate were prepared using MeOH:water:glacial acetic acid (10:89:1, v/v/v) (eluent A) and (97:2:1, v/v/v) (eluent B).

Sampling

A total of 105 samples were analysed in this study. In May 2016, forty samples of milled pearl millet were randomly purchased at open-markets, twenty samples from Oshakati open market in the Oshana region of Namibia and twenty samples from Rundu open market in the Kavango East region of Namibia. Forty samples of milled sorghum malt were purchased from Oshakati and Omuthiya open-markets, both in the Oshana region of Namibia. Approximately 500 g of the milled samples each were collected following the sampling procedure described in the European Commission regulation EC No. 1881/2006 (EC, 2006). Samples were placed in khaki paper bags and stored at 4 °C until analysis. Following metabolite analysis of the milled pearl millet and sorghum malt samples, 25 samples of the traditional non-alcoholic beverage *oshikundu* were prepared using 25 combinations of pearl millet meal and sorghum malt samples purchased from Oshakati market in the Oshana region of Namibia. Twenty beverages were prepared from random combinations of the ingredient samples while 5 beverages were prepared using a selection of 5 of the most contaminated pearl millet and 5 of the most contaminated sorghum malt samples (considering legislated mycotoxins). *Oshikundu* samples were kept frozen at -20 °C before analysis.

Preparation of *oshikundu* beverage

Oshikundu was prepared according to previously described methods (Misihairabgwi and Cheikhyoussef, 2017). Boiled water (10 ml) was added to pearl millet meal (5 g) and the mixture was stirred to make a thick porridge. The porridge was left to cool to room temperature after which sorghum malt (1.25 g) was added to the porridge and thoroughly stirred. Cold water (25 ml) was added to the porridge-sorghum malt mixture. The mixture was left to ferment for 24 h, at ambient temperature.

Metabolite analysis

Extraction

All pearl millet meal, sorghum malt and *oshikundu* beverage samples were analysed for the presence of 760 fungal, bacterial and plant metabolites, including the regulated and emerging mycotoxins. Mycotoxins were extracted using ACN:water:acetic acid (79:20:1, v/v/v) as extraction solvent. For the pearl millet and sorghum malt samples, 5 g of each ground sample was extracted with 20 ml of solvent in a 50 ml polypropylene tube (Sarstedt, Nümbrecht, Germany) for 90 min and 180 rpm shaking speed using a GFL 3017 rotary shaker (GFL 3017, Burgwedel, Germany). For the extraction of metabolites in *oshikundu* beverage samples, 5 ml of each sample was extracted using 15 ml of acidified ACN (ACN:acetic acid 1.5%, v/v) at 180 rpm for 15 min on a rotary shaker.

LC-MS/MS analysis

Metabolite analysis was carried out using a 1290 Series HPLC System (Agilent, Waldbronn, Germany) coupled to a QTrap 5500 LC-MS/MS System (Applied Biosystems SCIEX, Foster City, CA, USA) equipped with Turbo Ion Spray electrospray ionization source as described earlier (Malachova *et al.*, 2014). Chromatographic separation was performed at 25 °C on a Gemini® C_{18}-column, 150×4.6 mm i.d., 5 µm particle size, equipped with a C_{18} 4×3mm i.d. security guard cartridge (Phenomenex, Torrance, CA, USA). Confirmation of positive metabolite identification was carried out by the acquisition of two time scheduled multiple reaction monitoring (MRMs) which yielded 4.0 identification points according to the European Commission decision 2002/657 (EC, 2002).

For the pearl millet and sorghum malt samples, 500 µl of each extract was transferred into a 1.5 ml glass vial containing an equal volume of the dilution solvent and vortexed for 30 s. After appropriate mixing, 5 µl of the diluted extract was injected into the LC-MS/MS system. For the *oshikundu* samples, 625 µl of extract was diluted in 375 µl of dilution solvent, vortexed for 30 s and 5 µl was obtained and directly injected into the LC-MS/MS system for analysis. Quantification was performed using external calibration based on serial dilution of a multi-analyte stock solution. Results were corrected using apparent recoveries that were determined for each of the investigated matrices by spiking experiments. The accuracy of the method is verified on a continuous basis by participation in a proficiency testing scheme organised by BIPEA (Gennevilliers, France) with a current success rate (z score between -2 and 2) of >94% of the >850 results submitted.

For the determination of transfer rates to the beverage, the expected metabolite concentration was calculated based on the related concentration in the cereal ingredients and the dilution factor deriving from the beverage preparation procedure. This was compared to the actual metabolite levels determined in *oshikundu*.

Data analysis

Descriptive statistical analysis was carried out for all data using SPSS version 24 (SPSS, Chicago, IL, USA).

3. Results and discussion

Fungal metabolites produced by *Aspergillus* in pearl-millet, sorghum malt and their fate in *oshikundu* preparation

A total of 98 fungal, bacterial and possibly plant metabolites were quantified in the food commodities analysed, with 84 (86%) being fungal metabolites. Forty (50%) of the fungal metabolites quantified in the food commodities are produced by fungi from the genus *Aspergillus*, which falls among the most common mycotoxin producing genera (Varga *et al.*, 2015). Table 1 shows the fungal metabolites produced by *Aspergillus* species in open market vended pearl millet and sorghum malt samples, as well as in the beverage *oshikundu*.

While no regulated mycotoxins were quantified in the *oshikundu* beverage, regulated mycotoxins of public health concern produced by *Aspergillus flavus* were quantified in the pearl millet meal and sorghum malt samples, including aflatoxin B_1 (AFB_1) and B_2 (AFB_2) (Table 1). AFB_1 was quantified in 13% of the pearl millet meal samples (mean: 0.9±0.7 µg/kg, range: 0.1-2.0 µg/kg) and in 50% of the sorghum malt samples (mean: 4.5±5.5 µg/kg, range: 0.2-25.4 µg/kg). Several reports exist of AF occurrence in sorghum malt samples intended for beverage production in sub-Saharan Africa (Bationo *et al.*, 2015; Ezekiel *et al.*, 2015; Matumba *et al.*, 2011). In a study carried out in Burkina Faso, Bationo and co-workers (2015) reported a lower incidence of AFB_1 in sorghum malt samples compared to our study (25%, n=20), although the mean level (97.6±88.2 µg/kg) was higher than the level reported in this study. Similarly, no aflatoxins were detected in the beverage *dolo* prepared from sorghum malt samples. However, a study in Malawi (Matumba *et al.,* 2011) detected AFs in 100% of the sorghum malt samples used for the production of the traditional opaque beverage *tobwa* (n=6) and in 100% of samples used for producing the traditional sorghum opaque beer (n=21), as well as in 3 samples of *tobwa* (mean: 4.50± 1.45 µg/kg, range: 2.1-7.1 µg/kg) and in 5 samples of sorghum opaque beer (mean: 22.32± 4.93 µg/kg; range: 8.8-34.5 µg/kg). In our study, AFB_1 levels were significantly higher in sorghum malt compared to pearl millet samples, whereas in Nigeria, Apeh and co-workers (2016) reported that millet was contaminated with AFB_1 at slightly higher incidence but lower levels (≤10 µg/kg) than sorghum (Apeh *et al.*, 2016).

OTA, a regulated mycotoxin produced by *Aspergillus niger*, was quantified in 3% of the pearl millet meal samples (mean: 0.5±0.0 µg/kg) and in 5% of the sorghum malt samples (mean: 0.7±0.1 µg/kg, range: 0.7-0.8 µg/kg). The incidence and levels of OTA in our samples are lower than those reported for the occurrence of OTA in sorghum and pearl millet samples from Nigeria by Makun and co-workers (2013). They reported the occurrence of OTA in all 18 millet samples (mean: 24.74 µg/kg; range: 10.2-46.57 µg/kg) at levels above the EU regulation limit of 5 µg/kg limit for OTA in foods.

Although CPA was not quantifiable in *oshikundu*, the *A. flavus* metabolite was quantifiable in all the sorghum malt samples at the high concentration range of 99.9-2,280 ug/kg. This poses a health concern as high levels of CPA in foods have been associated with immunosuppression in humans (Hymery *et al.*, 2014).

AF precursors produced by *A. flavus*, which were quantifiable in the food commodities included averantin, averufin, norsolorinic acid, STE, O-methylsterigmatocystin (O-methylSTE) and versicolorin A. High incidence rates of 98% and 100%, and maximum levels of 31.90 and 51.20 µg/kg were recorded for averantin and averufin in sorghum malt samples, respectively. Averufin was the only aflatoxin precursor quantified in *oshikundu* at a mean level of 0.2±0.2 µg/kg and maximum of 1.0 µg/kg. Incidence rates of 65% and 38% and maximum concentrations of 10.4 and 2.7 µg/kg were recorded for STE and O-methylSTE, respectively, in sorghum malt samples. Averantin, averufin and norsolorinic acid were detected in the pearl millet samples at maximum levels of 0.8 µg/kg, 1.1 µg/kg and 1.4 µg/kg, respectively. Due to the limited occurrence and exposure data, health risk assessment has not been possible for most of the aflatoxin precursors.

3-Nitropropionic acid was quantified in all sorghum malt and *oshikundu* beverage samples and in 63% of the pearl millet samples at very high concentrations of up to 11,900, 346 and 89.4 µg/kg, respectively, thereby posing a health risk for reduced motor performance (Roitberg *et al.*, 2002) and defective oxidative energy production (Scarlet *et al.*, 2003) among beverage consumers. The transfer rate of 3-nitropropionic acid from sorghum malt and pearl millet to *oshikundu* beverage was 66.8±11.4% (Table 2). Kojic acid, another *A. flavus* metabolite that was transferred from the raw ingredients to *oshikundu*, with a recorded transfer rate of 132.3±37.3%, has been reported to augment the effects of AFB_1 (Dowd, 1988).

The sorghum malt samples exhibited a distinct metabolite pattern indicating the presence of *Aspergillus fumigatus*, evidenced by the presence of the metabolites GLI, bis-(methylthio)gliotoxin, helvolic acid, trypacidin, fumagillin, fumiquinazoline A, fumiquinazoline D), as well as *A. clavatus*, evidenced by the presence of the metabolites cytochalasin E and PAT. Some metabolites, such as pseurotin A and the tryptoquivaline derivatives are produced by both species. The related concentrations exceeded 100 µg/kg in the most heavily contaminated samples for most of these metabolites, which have not been found at such high levels in any of the other surveys

Table 1. Fungal metabolites produced by the genus *Aspergillus* in open-market vended pearl millet and sorghum malt samples and in *oshikundu*.

Metabolite	Pearl millet (n=40)			Sorghum malt (n=40)			*Oshikundu* (n=25)		
	+ve (%)[1]	Mean (µg/kg)	Range (µg/kg)	+ve (%)[1]	Mean (µg/kg) or Peak area[2]	Range (µg/kg) or Peak area[2]	+ve (%)[1]	Mean (µg/kg) or Peak area[2]	Range (µg/kg) or Peak area[2]
Aspergillus flavus									
Aflatoxin B_1	13	0.9±0.7	0.1-2.0	50	4.5±5.5	0.2-25.4	0	n/a	n/a
Aflatoxin B_2	3	0.2±0.0	0.2-0.2	8	0.3±0.1	0.1-0.4	0	n/a	n/a
Aflatrem	0	n/a	n/a	10	125,000±34,700*	103,000-177,000*	0	n/a	n/a
Asperfuran	0	n/a	n/a	88	520±651	12.8-3180	0	n/a	n/a
Averantin	13	0.3±0.3	0.1-0.8	98	6.4±5.7	0.1-31.9	0	n/a	n/a
Averufin	63	0.4±0.2	0.2-1.1	100	8.2±10.2	0.3-51.2	96	0.2±0.2	0.0-1.0
Cyclopiazonic acid	0	n/a	n/a	100	550±1,460	99.9-2,280	0	n/a	n/a
Kojic acid[3]	10	2,920±3,950	379-8,750	100	51,200±43,600	1,130-161,000	100	3,010±3,110	121-10,100
Nidurufin	0	n/a	n/a	18	104,000±78,500*	285,00-237,000*	0	n/a	n/a
3-Nitropropionic acid	63	18.0±17.7	6.5-89.4	100	4,560±3,010	131-11,900	100	139±91.0	7.8-346
Norsolorinic acid	5	1.4±0.1	1.4-1.5	78	2.1±0.9	0.8-4.9	0	n/a	n/a
O-methylsterigmatocystin	0	n/a	n/a	38	0.4±0.7	0.1-2.7	0	n/a	n/a
Paspalin	0	n/a	n/a	5	45,300±5,540*	41,400-49,200*	0	n/a	n/a
Sterigmatocystin	0	n/a	n/a	65	2.6±2.1	0.5-10.4	0	n/a	n/a
Versicolorin A	0	n/a	n/a	35	1.7±1.8	0.4-7.4	0	n/a	n/a
Aspergillus niger									
Aurasperon C	0	n/a	n/a	75	670,000±1,560,000*	23,900-8,400,000*	0	n/a	n/a
Flavipucin	0	n/a	n/a	10	5.8±6.3	0.4-12.5	0	n/a	n/a
Fonsecin	0	n/a	n/a	30	609,000±842,000*	63,800-3,120,000*	0	n/a	n/a
Malformin C	0	n/a	n/a	50	4.1±6.1	0.0-23.8	0	n/a	n/a
Ochratoxin A[4]	3	0.5±0.0	0.5-0.5	5	0.7±0.1	0.7-0.8	0	n/a	n/a
Aspergillus fumigatus									
Bis(methylthio)gliotoxin	0	n/a	n/a	93	30.4±28.8	2.1-114	0	n/a	n/a
Deoxynortryptoquivaline	0	n/a	n/a	95	136±192	3-695	48	11.3±7.6	2.7-24.1
Deoxytryptoquivaline A	0	n/a	n/a	95	60.7±89.7	1.4-354	40	8.9±4.4	3.2-16.1
Fumagillin	3	8.8±0.0	8.8-8.8	55	42±33.6	2.9-153	0	n/a	n/a
Fumagillol	0	n/a	n/a	83	47.3±32.4	9-130	0	n/a	n/a
Fumiquinazolin A	0	n/a	n/a	98	178±208	1.1-795	52	18.5±14.9	1.0-41.8
Fumiquinazolin D	3	1.7±0.0	1.7-1.7	100	687±878	3.3-3,550	76	23.1±26.6	1.9-70.8
Fumiquinazolin F	0	n/a	n/a	78	618,000±585,000*	37,200-214,0000*	0	n/a	n/a
Fumitremorgin C	0	n/a	n/a	73	47.1±43.7	2.2-158	0	n/a	n/a
Gliotoxin	0	n/a	n/a	58	25.6±18.0	2.8-70.7	0	n/a	n/a
Helvolic acid	0	n/a	n/a	93	192±192	4.3-784	36	26.7±14.9	8.9-47.3
Methylsulochrin	5	0.2±0.1	0.1-0.2	55	16.0±43.5	0.0-202	0	n/a	n/a
Pseurotin A	0	n/a	n/a	88	200±202	10.3-840	28	21.0±18.2	1.6-47.1
Pyripyropene A	0	n/a	n/a	8	12.8±15.5	2.4-30.6	0	n/a	n/a
Quinadoline A	0	n/a	n/a	70	11.0±11.9	0.4-42.2	36	2.4±1.3	0.3-4.7
Trypacidin	0	n/a	n/a	48	3.5±9.4	0.1-40.6	0	n/a	n/a
Tryprostatin A	0	n/a	n/a	70	170±187	2.2-632	0	n/a	n/a
Tryptoquialanine derivative	0	n/a	n/a	60	8.9±8.5	0.9-31.0	0	n/a	n/a
Tryptoquivaline A	3	n/a	n/a	85	38.8±58.5	0.1-257	0	n/a	n/a
Tryptoquivaline F	0	n/a	n/a	80	111±91.4	26.8-357	0	n/a	n/a
Aspergillus clavatus									
Cytochalasin E	0	n/a	n/a	93	90.8±117	3.1-362	32	10.0±2.9	5.0-14.2
Patulin	0	n/a	n/a	10	68.6±46.1	43.2-138	0	n/a	n/a

[1] +ve = positive (percentage of samples); n/a = not applicable. [2] Values indicated by * are based on relative quantification of chromatography peak areas.
[3] Produced by other *Aspergillus* species. [4] Also produced by *Penicillium* spp.

Table 2. Percentage transfer of selected fungal metabolites from open-market vended pearl millet and sorghum malt samples to *oshikundu* (mean ± standard deviation from 25 beverages).

Metabolite	Transfer (%)[1]
3-Nitropropionic acid	66.8±11.4
Alternariol	123±43.1
Alternariolmethylether	11.2±4.4
Agroclavin	62.7±27.9
Curvularin	87.8±21.2
Cytochalasin E	75.0±14.6
Deoxynortryptoquivalin	80.5±6.5
Deoxytryptoquivaline A	96.5±8.6
Festuclavin	72.5±28.6
Fumigaclavin	51.4±23.3
Fumiquinazolin A	173±38.2
Fumiquinazolin D	72.5±9.4
Helvolic acid	175±33.5
Kojic acid	132±37.3
Monocerin	133±46.0
Pseurotin A	109±13.9
Quinadoline A	203±28.9
Tryptoquivaline A	750±1,850*

[1] Transfer rate indicated by * based on relative quantification of chromatography peak areas.

performed in our laboratory so far (Abia *et al*., 2013; Chala *et al.,* 2014). As a consequence, part of these analytes were quantifiable in *oshikundu* despite the dilution factor deriving from the fermentation process with the highest concentration being found for helvolic acid (8.9-47.3 µg/kg) and transfer rates ranging from 72.5±9.4% to 750±1,850% (Table 2). *Aspergillus* metabolites that were quantified in *oshikundu* at transfer rates higher than 100% included fumiquinazolin A, helvolic acid, kojic acid, pseurotin A, quinadoline A and tryptoquivaline A. The higher levels of these metabolites in *oshikundu* than what is expected from the transfer from raw materials suggests that there could be unknown derivatives in the cereal ingredients (not included in the LC-MS/MS method list) which were hydrolysed or degraded to form the target compounds during beverage processing.

In a number of studies, fermentation has been reported to reduce the level of mycotoxins in contaminated raw materials (Batiano *et al.*, 2015; Ezekiel *et al.*, 2015; Karlovsky *et al.*, 2016; Okeke *et al.*, 2015) and to cause conversion of ZEA to its less oestrogenic form, β-zearalenol (Ezekiel *et al.*, 2015; Mizutani *et al.*, 2011;). No AFB_2 and OTA were expected in *oshikundu* as the concentrations in the cereal ingredients were very low.

Fungal metabolites produced by *Fusarium*, *Alternaria*, *Penicillium* and *Claviceps* and their fate in *oshikundu* preparation

Of the 42 other fungal metabolites, excluding *Aspergillus* metabolites, 17, 9, 6 and 4 of the fungal metabolites quantified in the food commodities are produced by fungi from the genera *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*, respectively. Fumonisins B_1, B_2, B_3 and B_4 (FB_1, FB_2, FB_3 and FB_4), as well as ZEA, were among the regulated mycotoxins produced by the genus *Fusarium* in the ingredients used for *oshikundu* preparation, while none were quantifiable in the beverage. FB_1 was detected in 50% of the pearl millet samples (mean: 984.1±1,200, range 0.1-3,060 µg/kg) (Table 3), with 5% being above the European Union regulatory limit of 2,000 µg/kg (EC, 2006). In the sorghum malt samples, FB_1 was quantified in 75% of the samples at levels below the European Union regulatory limits (mean: 28.2±33.3, range 0.8-123 µg/kg). Although only 5% of the pearl millet samples had higher levels of FB_1 than the set regulatory limits, chronic exposure to low amounts of various mycotoxins has detrimental health effects. FB_2, FB_3 and FB_4 were also quantified in pearl millet meal and sorghum malt samples at significantly lower concentrations than FB_1 (P<0.05). The fumonisins were significantly more prevalent in pearl millet compared to sorghum malt (P<0.05). Incidence of fumonisin B classes suggests susceptibility of the pearl millet and sorghum crops to the fumonisin B producing fungal species, *Fusarium verticillioides* and *Fusarium proliferatum* (Chilaka *et al.*, 2016). Although Chilaka *et al* (2016) reported a low incidence of FB_1 in pearl millet samples (10.3%, n=87), the levels were notably higher (mean: 2,333 µg/kg, maximum: 18172 µg/kg) compared to our findings. Although no fumonisins were detected in *oshikundu*, studies from Nigeria reported the occurrence of fumonisin B classes in two fermented traditional cereal-based beverages (*kunu-zaki* and *pito*) (Ezekiel *et al.*, 2015), while Roger (2011) also reported the occurrence of FB_1 in 78.5% (n=70) of the traditional sorghum beer, *Bil-bil* from Cameroon (mean: 150±24 µg/kg, range: 0.0-230 µg/kg).

ZEA was quantified in 40% of the pearl millet meal samples (mean: 2.3±1.8 µg/kg, range: 0.1-7.9 µg/kg) and in 53% of the sorghum malt samples (mean: 12.3±12.6 µg/kg, range: 0.2-48.8 µg/kg) (Table 3). Quantified levels of ZEA were all below the EU regulatory limits of 100 µg/kg. Levels of ZEA detected in sorghum malt samples are higher than levels reported by Ezekiel *et al.* (2015) who reported a mean of 3.85 µg/kg for sorghum malt samples from Nigeria. There was no quantifiable ZEA in *oshikundu* while Ezekiel *et al.* (2015) reported a mean level 0.2 µg/kg in the sorghum processed beverage, *pito*. An incidence of 14% (n=87) was reported for ZEA occurrence in pearl millet samples from Nigeria, with a mean of 419 µg/kg and a maximum level

Table 3. *Fusarium*, *Alternaria*, *Penicillium* and *Claviceps* metabolites in open-market vended pearl millet and sorghum malt samples and in *oshikundu*.

	Pearl millet (n=40)			Sorghum malt (n=40)			*Oshikundu* (n=25)		
Metabolite	**+ve (%)[1]**	**Mean (µg/kg)**	**Range (µg/kg)**	**+ve (%) [1]**	**Mean (µg/kg) or Peak area[2]**	**Range (µg/kg) or Peak area[2]**	**+ve (%) [1]**	**Mean (µg/kg) or Peak area[2]**	**Range (µg/kg) or Peak aea[2]**
***Fusarium* metabolites**									
Antibiotic Y	3	62.7±0.0	62.7-62.7	0	n/a	n/a	0	n/a	n/a
Aurofusarin	33	31.0±18.0	4.3-66.5	100	447±463	34.5-2,550	0	n/a	n/a
Beauvericin	100	3.5±6.2	0.1-29.0	100	3.3±3.4	0.0-15.0	96	0.3±0.3	0.0-1.2
Chrysogin	25	2.1±2.0	0.0-6.0	5	1.3±1.4		0	n/a	n/a
Enniatin A	0	n/a	n/a	15	0.1±0.1	0.0-0.2	0	n/a	n/a
Enniatin A_1	0	n/a	n/a	5	0.4±0.5	0.0-0.8	0	n/a	n/a
Enniatin B	5	0.2±0.1	0.2-0.3	3	0.5±0.0	0.5-0.5	0	n/a	n/a
Enniatin B_1	0	n/a	n/a	3	1.6±0.0	1.6-1.6	0	n/a	n/a
Equisetin	100	8.1±13.2	0.2-55.3	100	27.6±25.2	0.7-205	0	n/a	n/a
Fusapyron	3	1.4±0.0	1.4-1.4	5	1.9±0.4	1.6-2.2	0	n/a	n/a
Fusaric acid	3	35.2±0.0	35.2-35.2	100	587±839	36.2-5,060	0	n/a	n/a
Fumonisin A_1 precursor	25	36.4±19.9	1.6-66.3	8	3.1±1.2	1.9-4.3	0	n/a	n/a
Fumonisin B_1	50	984±1,200	0.1-3,060	75	28.2±33.3	0.8-123	0	n/a	n/a
Fumonisin B_2	40	292±29.11	0.1-760	50	7.7±7.3	0.2-26.7	0	n/a	n/a
Fumonisin B_3	28	387±239	9.2-695	20	8.9±6.0	0.9-19.0	0	n/a	n/a
Fumonisin B_4	25	127±63.1	5.5-205	13	4.6±2.9	2.2-8.1	0	n/a	n/a
Siccanol	0	n/a	n/a	83	745,000±574,000*	106,000-2,930,000*	16	50,800±10,400*	44,792-66,424*
Zearalenone	40	2.3±1.8	0.1-7.9	53	12.3±12.6	0.2-48.8	0	n/a	n/a
***Alternaria* metabolites**									
Alternariol	13	1.6±2.0	0.1-4.7	93	7.8±7.4	0.2-32.0	80	0.8±1.0	0.1-4.3
Alternariol methyl ether	98	6.6±10.1	1.0-51.4	100	58.3±53.4	2.4-294	92	0.7±0.7	0.1-3.2
Altersetin	3	7.5±0.0	7.5-7.5	78	23.8±28.5	0.4-153	0	n/a	n/a
Altersolanol	0	n/a	n/a	68	920±1,030	41.5-4,180	0	n/a	n/a
Macrosporin	98	9.5±11.5	0.0-43.4	100	16.6±11.7	0.0-53.3	100	2.3±2.1	0.1-8.4
Tentoxin	95	1.6±2.5	0.0-9.9	83	0.4±0.3	0.1-1.3	0	n/a	n/a
***Penicillium* metabolites**									
Andrastin A	3	3.8±0.0	3.8-3.8	58	47.9±59.0	1.8-204	16	1.8±0.4	1.4-2.4
Andrastin C	0	n/a	n/a	38	10.9±10.0	0.7-35.0	0	n/a	n/a
Curvularin	90	11.2±16.7	0.5-77.8	100	1,480±1,484	14.2-6,660	100	76.5±80.3	1.5-275.0
Dehydrocurvularin	0	n/a	n/a	65	95.1±81.1	10.0-412	36	14.6±8.8	1.4-30.4
Griseofulvin	8	1.4±1.2	0.3-2.7	23	1.1±0.7	0.2-2.4	0	n/a	n/a
Hydroxycurvularin	0	n/a	n/a	88	53.7±50.5	5.7-248	0	n/a	n/a
Methylfunicone	0	n/a	n/a	70	1.9±2.7	0.0-9.1	0	n/a	n/a
Skyrin	15	1.9±1.8	0.5-5.0	93	2.6±2.1	0.9-10.5	0	n/a	n/a
***Claviceps africana* metabolites**									
Chanoclavine	3	1.9±0.0	1.9-1.9	50	39.8±31.2	0.1-131	52	0.6±0.7	0.1-1.9
Dihydroergosine	3	0.1±0.0	0.1-0.1	63	11.0±13.4	0.1-44.8	28	1.4±1.3	0.1-3.0
Dihydrolysergol	0	n/a	n/a	73	55.9±48.3	4.8-165	0	n/a	n/a
Elymoclavine	0	n/a	n/a	68	11.2±12.8	1.8-54.2	0	n/a	n/a

[1] +ve = positive (percentage of samples); n/a = not applicable.

[2] Values indicated by * are based on relative quantification of chromatography peak areas.

of 1,399 µg/kg (Chilaka *et al.*, 2016), which is much higher than the mean levels obtained in this study for pearl millet.

Emerging mycotoxins quantified in the food commodities, included the *Fusarium* metabolites BEA, ENN A, ENN A_1, ENN B and ENN B_1, and the *Alternaria* metabolites AOH and AME. AOH, AME and BEA were found in pearl millet, sorghum malt and *oshikundu* at quantifiable levels (Table 3). Incidence rates of 80 and 92% and maximum concentrations of 4.3 and 3.2 µg/kg were recorded for AOH and AME in *oshikundu* samples, respectively. Recorded transfer rates of AOH and AME from the ingredients to *oshikundu* were 123±43.1 and 11.2±4.4%, respectively. ENNs were not quantifiable in *oshikundu*. BEA was quantifiable in all pearl millet meal samples (mean: 3.5±6.2 µg/kg, range: 0.1-29.0 µg/kg) and sorghum malt samples (mean: 4.5±5.5 µg/kg, range: 0.2-25.4 µg/kg), as well as in 96% of the *oshikundu* samples (mean: 3.3±3.4 µg/kg, range: 0.0-1.2 µg/kg). BEA levels quantified in this study were lower than the levels quantified by Chala and co-workers (2013), who first reported the occurrence of BEA in sorghum at a mean of 17.01 µg/kg and a maximum level of 289.94 µg/kg. ENNs were detected at trace levels in the sorghum malt samples in this study. BEA and ENNs were completely lost during beverage production (Table 2). The results are consistent with the results of Ezekiel *et al.* (2015), who reported complete loss of ENNs and 64.3% loss of BEA during processing to produce the traditional sorghum beverage *pito*. Other *Fusarium* metabolites quantified in the food commodities include equisetin, which was detected in all pearl millet and sorghum malt samples at maximum concentrations of 55.3 µg/kg and 205 µg/kg, respectively.

In a scientific opinion from 2014, the European Food Safety Authority (EFSA, 2014) concluded that acute exposure to BEA and ENNs is not a concern to human health, but no conclusion could be drawn with respect to chronic exposure due to the lack of relevant *in vivo* toxicity data (Gruber-Dorninger *et al.*, 2016). While *in vitro* studies suggest genotoxic effects of ENN A, A_1 and B_1, BEA, MON, AOH and AME, immunomodulating effects of most emerging toxins and a reproductive health hazard of AOH, BEA and ENN B, there are still knowledge gaps regarding occurrence, toxicity and toxicokinetic data for a proper health risk assessment of these mycotoxins (Fraeyman *et al.*, 2017; Gruber-Dorninger *et al.*, 2016).

Penicillium metabolites detected in *oshikundu* ingredients and transferred to *oshikundu* included andrastin A, curvularin and dehydrocurvularin (Table 3). The transfer rate of curvularin from ingredients to *oshikundu* was 87.8±21.1%. Metabolites produced by *Claviceps africana*, i.e. chanoclavine, dihydroergosine, dihydrolysergol and elymoclavine, were mainly detected in more than 50% of the sorghum malt samples. The main contaminating ergot alkaloid was dihydrosergol, which was quantifiable in 73% of the sorghum malt samples at a range of 4.8-165 µg/kg, though not quantifiable in *oshikundu*. Shimsoni and co-workers (2017) reported the presence of dihydroergosine and dihydrolysergol as the major ergot alkaloids in sorghum silages from Israel.

Other fungal metabolites, bacterial metabolites and unspecific metabolites in pearl-millet, sorghum malt and their fate in *oshikundu* preparation

Other fungal metabolites, which were quantifiable in all pearl millet, sorghum malt and *oshikundu* samples, include brevianamide F, emodin (EMO), monocerin and rugulusovin (Table 4), with brevianamide F having the highest range of 4.3±64.5 µg/kg in *oshikundu*. The bacterial metabolites chloramphenicol, monactin and nonactin were quantified only in the sorghum malt samples at low concentrations ranging from 0.3-1.3 µg/kg. Of the unspecific metabolites which could be either of fungal, bacterial or plant origin, tryptophol was quantifiable at the highest concentration range in *oshikundu*, ranging from 12.8-93.2 µg/kg (Table 4).

4. Conclusions

This study has, for the first time, reported research data on the diversity of fungal and bacterial metabolites in food commodities from Namibia, focusing on the spectrum of multi-mycotoxins contaminating the popular non-alcoholic beverage *oshikundu* and its raw materials. A number of regulated mycotoxins of public health concern were quantifiable in the raw materials used for *oshikundu* production, although none were quantifiable in the processed beverage due to the dilution factor deriving from the processing procedure. Aflatoxin B_1, which was quantifiable in some of the sorghum malt samples at levels above the European Union regulatory limit of 5 µg/kg is of health concern. Despite the absence of regulated mycotoxins in *oshikundu*, a degree of caution among consumers is advised, as risk assessment studies still need to be carried out for emerging mycotoxins and other fungal metabolites quantified in *oshikundu*, especially those produced by *A. fumigatus* and *A. clavatus*, based on beverage consumption data. A considerable number of *Aspergillus* metabolites, fumiquinazoline A, fumiquinazoline D, helvolic acid, kojic acid, pseurotin A, quinadoline A and tryptoquivaline A were detected in *oshikundu* at higher concentrations than the raw materials, suggesting hydrolysis or breakdown of unknown metabolites to produce the increased fungal metabolites during beverage preparation. Given that carryover of some mycotoxins occurs from the cereal ingredients into the beverage, and that continued processing may increase metabolite concentrations in the beverage, it is imperative to educate the producers and consumers on the health risk of mycotoxins and possible ways to reduce or avoid contamination and on the necessity of hygienic conditions

Table 4. Other fungal, bacterial and unspecific metabolites in open-market vended pearl millet, sorghum malt and in *oshikundu*.

Metabolite	Pearl millet (n=40)			Sorghum malt (n=40)			*Oshikundu* (n=25)		
	+ve (%)[1]	Mean (µg/kg)	Range (µg/kg)	+ve (%) [1]	Mean (µg/kg)	Range (µg/kg)	+ve (%) [1]	Mean (µg/kg)	Range (µg/kg)
Other fungal metabolites									
Bassianolide	10	1.3±2.2	0.1-4.6	48	1.4±3.2	0.1-13.2	8	0.3±0.4	0.1-0.6
Brevianamide F	100	34.3±26.3	9.1-139	100	22.1±14.3	5.6-54.5	100	20.2±15.5	4.3-64.5
Calphostin	5	3.1±1.3	2.2-4.0	25	3.3±1.4	1.5-5.6	0	n/a	n/a
Flavoglaucin	68	7.0±11.0	0.4-48.9	80	8.3±21.3	0.3-118	60	90.4±110	2.1-289.8
Heptelidic acid	3	0.8±0.0	0.8-0.8	83	20.2±19.5	1.8-72.4	0	n/a	n/a
Monocerin	100	10.4±14.1	0.8-66.7	100	62.5±50.4	1.2-245	0	n/a	n/a
Unspecific metabolites									
Chrysophanol	0	n/a	n/a	85	122±114	20.8-446	0	n/a	n/a
Citreorosein	45	2.1±1.1	0.5-5.1	100	26.6±26.1	1.2-110	64	8.0±4.4	1.2-16.6
Cyclo(L-Pro-L-Tyr)	100	145±83.9	49.9-390	100	78.2±34.2	29.1-156	100	37.2±22.8	8.8-90.0
Cyclo(L-Pro-L-Val)	100	38.3±83.9	12.5-130	100	29.6±14.4	7.6-66.0	100	20.0±12.3	5.6-62.4
Emodin	100	4.3±2.6	1.2-15.2	100	54.8±51.7	1.5-212	100	6.0±5.2	0.2-16.7
Iso-Rhodoptilometrin	18	0.3±0.2	0.1-0.5	100	1.6±1.4	0.1-7.1	28	0.1±0.1	0.1-0.3
N-Benzoyl-Phenylalanine	8	2.0±2.4	1.5-2.2	3	2.2±0.0	2.2-2.2	0	n/a	n/a
Neoechinulin A	43	8.9±9.6	1.3-38.6	40	16.1±34.7	1.6-145	0	n/a	n/a
Rugulusovin	100	16.6±11.6	3.3-49.5	100	15.8±7.4	4.5-39.4	100	6.3±3.5	1.7-16.6
Tryptophol	90	86.2±93.0	12.1-523	98	293±416	46.8-2,090	96	47.5±22.2	12.8-93.2
Bacterial metabolites									
Chloramphenicol	53	0.2±0.1	0.1-0.3	65	0.3±0.2	0.1-0.7	0	n/a	n/a
Nonactin	0	n/a	n/a	40	0.1±0.1	0.1-0.3	0	n/a	n/a
Monactin	0	n/a	n/a	63	0.3±0.3	0.1-1.3	0	n/a	n/a

[1] +ve = positive (percentage of samples); n/a = not applicable.

during fermentation to avoid *A. fumigatus* and *A. clavatus* proliferation and metabolite production.

Although none of the legislated mycotoxins were quantifiable in *oshikundu*, it is important to note that reduction of mycotoxins during food processing is dependent on the initial concentrations of mycotoxins in the raw ingredients, therefore good quality cereal grains should be selected for *oshikundu* production. Occurrence of multiple mycotoxins in the cereals (especially in malt) and *oshikundu* is of concern because of the lack of knowledge of the effects of such complex metabolite mixtures in humans. Although the levels of other mycotoxins quantified in the samples were low, it cannot be ruled out that their occurrence may present health risks considering that *oshikundu* is consumed almost on daily basis. Data from the present study serves as a starting point for more detailed investigation of fungal metabolite contamination rates of food and the influence of processing on a nationwide scale. Further research on the fate of mycotoxins during beverage preparation is necessary considering the possible formation of masked/bound mycotoxins which may not have been quantifiable in the present study. *In vivo* toxicity determinations are also necessary to determine the effect of chronic exposure and high consumption levels of mycotoxins. Surveillance of the occurrence and the level of mycotoxin contamination in other food commodities, determination of exposure and awareness creation of mycotoxins and their health effects among producers and consumers of sorghum and millet in Namibia is necessary.

Acknowledgements

Financial support for this study was availed through an OeAD Ernst Mach Follow up grant awarded by the Austrian Academic Exchange Department. The authors thank Borjan Sarkanj for the technical assistance provided.

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