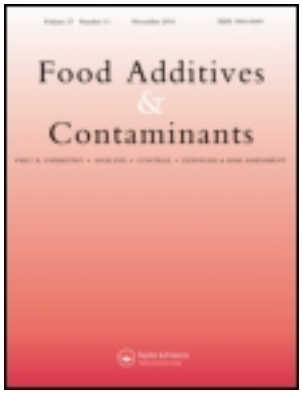

Food Additives & Contaminants: Part A
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tfac20

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Accepted author version posted online: 01 May 2012. Version of record first published: 22 Jun 2012

To cite this article: C.N. Ezekiel, R. Bandyopadhyay, M. Sulyok, B. Warth & R. Krska (2012): Fungal and bacterial metabolites in commercial poultry feed from Nigeria, Food Additives & Contaminants: Part A, 29:8, 1288-1299

To link to this article: http://dx.doi.org/10.1080/19440049.2012.688878

Food Additives and Contaminants
Vol. 29, No. 8, August 2012, 1288–1299

Taylor & Francis
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Fungal and bacterial metabolites in commercial poultry feed from Nigeria

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(*Received 23 October 2011; final version received 23 April 2012*)

Metabolites of toxigenic fungi and bacteria occur as natural contaminants (e.g. mycotoxins) in feedstuffs making them unsafe to animals. The multi-toxin profiles in 58 commercial poultry feed samples collected from 19 districts in 17 states of Nigeria were determined by LC/ESI–MS/MS with a single extraction step and no clean-up. Sixty-three (56 fungal and seven bacterial) metabolites were detected with concentrations ranging up to 10,200 $\mu g\,kg^{-1}$ in the case of aurofusarin. *Fusarium* toxins were the most prevalent group of fungal metabolites, whereas valinomycin occurred in more than 50% of the samples. Twelve non-regulatory fungal and seven bacterial metabolites detected and quantified in this study have never been reported previously in naturally contaminated stored grains or finished feed. Among the regulatory toxins in poultry feed, aflatoxin concentrations in 62% of samples were above 20 $\mu g\,kg^{-1}$, demonstrating high prevalence of unsafe levels of aflatoxins in Nigeria. Deoxynivalenol concentrations exceeded 1000 $\mu g\,kg^{-1}$ in 10.3% of samples. Actions are required to reduce the consequences from regulatory mycotoxins and understand the risks of the single or co-occurrence of non-regulatory metabolites for the benefit of the poultry industry.

Keywords: chromatography, LC/MS; mycotoxins; veterinary drug residues, antibiotics; animal feed

Introduction

The poultry industry in Nigeria is an important, rapidly growing (2.17% annual growth rate) sub-sector of the agricultural sector and it provides food, employment and other economic resources for the country. Categories of birds raised in Nigeria as poultry include chickens, turkeys, ducks, guinea fowls, pigeons and ostrich (Killebrew and Plotnick 2010). Chickens broadly fall into two categories: broilers for meat and layers for eggs. In 2008, Nigeria with a population of 151,212 million produced 1.61 kg per capita of chicken meat and 3.66 kg per capita of eggs, a slight increase from 2000 to 2007 (Killebrew and Plotnick 2010). In addition, about 90% of broilers are sold as frozen, fried or roasted meat to consumers via fast food companies, commercial distributors, supermarkets and hotels or other hospitality industry operators (Adene and Oguntade 2006). The commercial poultry sector now ranges from the small scale, peri-urban or rural operations to very large farms with vertically integrated facilities. Since the meat and eggs sourced from the birds are major protein sources for many Nigerians (USDA-FAS 2010), there is a need for appropriate monitoring and legislation of all aspects of quality issues in this sector.

The feed ingredients for chicken in Nigeria are composed of energy and protein sources, such as processed cereals and nuts (maize, soft wheat, soybean meal and groundnut cake), additives (iron tabs, salt and premixes) and mould inhibitors or toxin binders (organic acids and their salts). There are five major classes of chicken feeds in Nigeria: chick mash, the first feed given to chicks of the layer type from day-old to 4 weeks; broiler starter, the first feed for broiler type from chick stage to 3 weeks; grower mash, the next feed given to layer type from 5 to 14 weeks; broiler finisher, the last feed fed to broiler type from 4 weeks till time of consumption; and layer mash, the feed given to the layer type to initiate egg production in matured birds. Varying proportions of the previously stated ingredients constitute these feed types. Some of the feed ingredients and compounded feed may be contaminated with microbial metabolites arising from fungal and bacterial infestation of grains before, during and after feed production leading to several kinds of toxicities and high bird mortality.

Poultry birds are very sensitive to mycotoxicoses, a leading cause of low productivity and death (Huff et al. 1988). Several mycotoxins occur in poultry feed and their ingredients. These include aflatoxins,

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ISSN 1944–0049 print/ISSN 1944–0057 online

http://dx.doi.org/10.1080/19440049.2012.688878
http://www.tandfonline.com

ochratoxins, fumonisins, cyclopiazonic acid, deoxynivalenol (DON), zearalenone (ZEN), nivalenol and ergot alkaloids (Dhavan and Choudary 1995; Cespedes and Diaz 1997; Zollner et al. 1999; Dalcero et al. 2002; Biselli and Hummert 2005; Oliviera et al. 2008; Spanjer et al. 2008). Among these, maximum concentration of aflatoxins, DON, ZEN and ochratoxin A (OTA) are regulated in poultry feed in various countries albeit at different levels (FAO 2004). Although Nigeria does not have regulations on mycotoxins in poultry feed, the risk of mycotoxins exist in the Nigerian poultry sector since the common feed ingredients, such as maize and groundnut cake, are known to contain high levels of mycotoxins (Kpodo and Bankole 2008). However, there is no report of the occurrence of a wide array of non-regulatory and potentially toxic fungal metabolites in poultry feed in Nigeria. Consequently, ingestion of contaminated feed by poultry may place the birds and, by extension, human consumers at risk. This may further create a situation of stress for the agricultural sector or the economy at large.

Due to the need for effective detection of mycotoxins in food and feed and legislation directives recommended by the European Commission (2002), analytical detection techniques for mycotoxins have graduated over the years from simple thin layer chromatography (TLC), through the enzyme-linked immunosorbent assays (ELISA), gas chromatography with mass spectrometry (GC–MS) to the very reliable, highly sensitive and selectively qualitative and quantitative liquid chromatography (LC) coupled with tandem mass spectrometry (LC–MS/MS) with single extraction and no clean-up (Biselli and Hummert 2005; Oliviera et al. 2008; Shephard 2008). In this study, we adopted an LC–MS/MS protocol that was previously developed and validated for food samples (Sulyok et al. 2006, 2007) in an accredited laboratory specialising in analytical determinations of multi-toxins in food, feed and other substances, including indoor scrapings (Vishwanath et al. 2009).

There is a need to understand the level of occurrence, distribution and concentrations of regulatory and non-regulatory toxic metabolites in finished feed products to enable the feed industry and policy-makers to determine the potential risk of these toxicants. This research was carried out to survey 17 states for commercial poultry feed types and assessing each collected sample for the simultaneously contaminating toxic fungal metabolites. Some bacterial metabolites were also analysed. This work provides reliable data of mycotoxin risk in poultry birds in Nigeria for rapid intervention and legislation purposes. It will also contribute to the creation of a long-awaited mycotoxin map for Nigeria. Therefore, we have not extensively reported on the efficiency of extraction method or matrix effects in sample types as this is beyond the scope of the study.

Materials and methods

Chemicals and reagents

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany), acetonitrile (LC gradient grade) from VWR (Leuven, Belgium) and ammonium acetate (MS grade) was obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and an Elga Purelab ultra analytic system from Veolia Water (Bucks, UK). Standards of fungal and bacterial metabolites were obtained either as gifts from various research groups or from commercial sources. Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water (1 : 1, v/v), methanol, methanol/water (1 : 1, v/v) or water. Thirty combined working solutions were prepared prior to the spiking experiments by mixing the stock solutions of the corresponding analytes, followed by a further dilution in neat solvent. All solutions were stored at −20°C and were brought to room temperature before use.

Approximately one third of the fungal metabolite reference standards (including all mycotoxins addressed by regulations) were obtained as certified reference solution from Romer Labs (Tulln, Austria). Other sources were Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausen, Switzerland) and LGC Promochem GmbH (Wesel, Germany). The purity of the solid substances was 95% or higher.

Sampling

Fifty-eight commercial poultry feed formula in vendor's store were collected from three points of the bulk feed bag (50 kg) into clean zip-lock bags. One bag was sampled for every 10 bags in the store. The subsamples (1 kg each from top, middle and bottom of the feed bag) were taken from each bag and mixed thoroughly to form each sample lot. Samples were transported to the Mycotoxin Laboratory of International Institute of Tropical Agriculture (IITA, Ibadan) and 90–100-g representative sub-samples were obtained from each lot by the quartering method. Representative samples were comminuted and stored at 4°C to prevent further metabolite liberation by fungi and bacteria within samples prior to multi-toxin analysis.

Samples

The samples were collected during October and November 2009 from retail or wholesale stores of feed vendors located in 19 districts from 17 states (Table 1). The sampled locations and number of sample types collected from each district were uneven

Table 1. Number of commercial poultry feed samples and the districts from where they were collected in Nigeria.

Feed type	Total no. samples per feed type	Districts
Chick mash	7	Ijebu-Ode, Ikorodu, Kaduna, Minna, Mushin and Ogere
Grower mash	14	Aba, Benin, Birnin Kebbi, Damaturu, Kano, Ogere, Onitsha, Owerri, Port-Harcourt and Warri
Layer mash	13	Aba, Abakpa, Benin, Birnin Kebbi, Jalingo, Kano, Ogere, Owerri, Port-Harcourt, Warri and Yenegoa
Layer premixed	1	Ogere
Broiler starter	11	Abakpa, Benin, Birnin Kebbi, Kano, Makurdi, Owerri, Port-Harcourt, Warri and Yenegoa
Broiler finisher	12	Abakpa, Benin, Kano, Makurdi, Minna, Owerri, Port-Harcourt, Warri and Yenegoa
Total	58	19

and were based on availability of each feed type and cooperation from the marketers. The identity of vendors and commercial manufacturers of feed are not disclosed due to the confidential nature of this information. Only samples stored for 1–2 months were collected from the vendors. Based on their uses, the samples were classified into six types of uneven sample sizes, viz. chick mash, grower mash, layer mash, layer premixed mash, broiler starter and broiler finisher. Nevertheless, we categorised the samples into five (combining the layer premixed mash and the layer mash) since the layer premixed type had only one sample and is a new formulation meant to induce prompt egg formation during the early days of maturity of the layer bird. The feed types were composed of the following cereal and nut ingredients in varying quantities depending on the requirements of each poultry class: maize, wheat offal, groundnut cake and soybean meal.

A 50-g portion of each sample was dispatched from IITA by courier to the Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln, Tulln, Austria) where the multi-toxin analysis was conducted. Following a 5-day transit period, the samples were stored at −20°C in IFA-Tulln until analysed.

Sample preparation and estimation of matrix effects

The representative, homogenised samples were weighed into 50-ml polypropylene tubes (Sarstedt, Nümbrecht, Germany) and covered with extraction solvent (acetonitrile/water/acetic acid, 79:20:1, v/v) in a ratio of 4 ml solvent per g sample. For spiking experiments, 0.25 g (and for all other experiments 5 g) of the sample were applied for extraction. Samples were extracted for 90 min on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany), diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid, 20:79:1, v/v) and injected as described in detail by Sulyok et al. (2007). Centrifugation was not necessary due to sufficient sedimentation by gravity alone.

For the estimation of matrix effects, three different individual samples expected not to be, or only to minor extent, contaminated by mycotoxins were spiked using a multi-analyte standard at one concentration level. The spiked samples were stored overnight at room temperature to allow evaporation of the solvent and to establish equilibrium between the analytes and the sample. Extraction, dilution and analysis were performed as described above. The corresponding peak areas of the spiked samples not containing the target toxin were used for estimation of the apparent recovery by comparison to a standard, prepared and diluted in neat solvent. Results were corrected by the calculated correction factor.

LC–MS/MS parameters

LC–MS/MS screening of target fungal and bacterial metabolites was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionisation (ESI) source and an 1100 Series HPLC system (Agilent, Waldbronn, Germany). 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 × 3 mm I.D. security guard cartridge (all from Phenomenex, Torrance, CA, USA). The chromatographic method, as well as chromatographic and mass spectrometric parameters for 186 of the investigated analytes, are as described by Vishwanath et al. (2009).

ESI–MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time, ±30 and ±60 s in the positive and the

negative modes, respectively. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that exhibit only one fragment ion), which yielded 4.0 identification points according to commission decision 2002/657/EC. In addition, the LC retention time and the intensity ratio of the two MRM transition agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively.

Results and discussion

Analytical method quality assurance

The performance characteristics for the analytical method used as established from spiked blank samples are reported in Table 2. The limits of detection (LOD) ranged between 0.007 and 640 $\mu g\,kg^{-1}$ for enniatin B3 (ENN-B3) and kojic acid (KA), respectively. With respect to recoveries calculated from samples spiked at one concentration level, the largest deviations from 100% were observed for fumonisins, aflatoxins, ergot alkaloids and bacterial metabolites. This agrees well with the results from previous studies (Sulyok et al. 2006, 2007), where incomplete extraction was found to be critical for fumonisins, whereas aflatoxins and ergot alkaloids were particularly affected by matrix effects and epimerisation phenomena, respectively. The relative standard deviations of the apparent recoveries exceeded 10% for approximately one third of the analytes, which are acceptable since three individual samples from a complex matrix (with potentially significant differences in the compositions) were used in spiking experiments.

Multi-toxin surveillance data

Fifty-six fungal and seven bacterial metabolites in the feed samples were detected and quantified in varying concentrations ranging from $<$LOD to 10,300 $\mu g\,kg^{-1}$ (Table 3). The results are reported as corrected data using a correction factor equivalent to the reciprocal of the value for the apparent recovery for each analyte. The occurrence, distribution (Figures 1–3) and level of contamination (Table 3) of the 63 metabolites in the different feed samples indicate that the toxins liberated by *Fusarium* were more frequent than those of *Aspergillus, Penicillium* or bacteria. This corresponds with the reports of D'Mello et al. (1999) who stated that *Fusarium* toxins are more abundant in animal feeds than toxins of other fungi.

Of the 63 toxic metabolites reported in this study, 12 fungal and seven bacterial non-regulatory toxins have to our knowledge never been reported previously in naturally contaminated stored grains (feed ingredients) and finished feed. These are fungal toxins: macrosporin A (MAC-A), *O*-methylsterigmatocystin (O-STER), cyclosporin H (CYC-H), cytochalasin H (CYT-H), cytochalasin J (CYT-J), apicidin (APIC), equisetin (EQUS), chlamydosporol (CLAM), cycloaspeptide A (CA-A), curvularin (CURV), secalonic acid D (S-A-D) and rugulosin (RUG); and bacterial metabolites: chloramphenicol (CHLR), geldanamycin (GELD), monactin (MONC), nigericin (NIG), nonactin (NONC), radicicol (RAD) and valinomycin (VAL). However, 15 of these were found in various indoor matrices analysed by Vishwanath et al. (2009) using the same HPLC–MS/MS approach. ENN-B3, festuclavine (FEST), CA-A and alternariol methylether (AME) occurred only in one sample (1.7% contamination) each at concentrations of 0.007, 3.2, 14 and 106 $\mu g\,kg^{-1}$, respectively (Figures 2 and 3), while all the 58 samples were contaminated with beauvericin (BEAU) and EQUS (Figure 2).

Regulated mycotoxins

Various countries have legislations for maximum regulatory limits for aflatoxins, DON, OTA and ZEN in animal feeds. Among these regulated toxins, 62% of all samples had total aflatoxin concentrations exceeding 20 $\mu g\,kg^{-1}$, the regulatory level in most countries where regulations exist (FAO 2004). All the chick mash samples exceeded the total aflatoxin limit whereas the percentage of samples over the limit ranged 43–73% for other feed types (Table 4). DON concentration exceeded 1000 $\mu g\,kg^{-1}$ in 10.3% samples but was not high enough to be a problem by itself but could be a problem when other metabolites (e.g. fusaric acid) co-occur. The concentrations of OTA and ZEN in none of the samples were above the set limits of 1000 $\mu g\,kg^{-1}$. For aflatoxins, B1 was most prevalent (44 of 58 samples) followed by aflatoxin G1 which occurred in 33 of 58 samples (Figure 1). When considering the distribution of various aflatoxins in feed types, B-aflatoxins contaminated all chick mash samples while the layer mash, grower mash, finisher feed and starter feed had contamination of 46.4, 53.6, 62.5 and 72.7%, respectively. Therefore, the significant proportion of samples with aflatoxin concentration $>20\,\mu g\,kg^{-1}$ demonstrates the high prevalence of unsafe levels of aflatoxins in poultry feed in Nigeria.

Non-regulated metabolites

The 12 fungal metabolites being reported for the first time contaminated all feed types except chick mash. CYT-H and CYT-J, although not previously reported to naturally contaminate food, were sought for but not detected in naturally contaminated mouldy foods analysed by Sulyok et al. (2010). This makes our report the first natural occurrence of these two

Table 2. Method performance characteristics for 63 metabolites in poultry feed samples.

Metabolite category	Analyte	LOD (μg kg^{-1})[a]	Spiking concentration (μg kg^{-1})	Recovery (%)[b]
Aflatoxins				
	Aflatoxin B1 (AFB1)	4.0	34.2	71.6 ± 6.2
	Aflatoxin B2 (AFB2)	10.0	34.2	80.0 ± 12.8
	Aflatoxin G1 (AFG1)	6.0	34.6	60.6 ± 6.2
	Aflatoxin G2 (AFG2)	10.0	34.6	59.5 ± 7.5
	Aflatoxin M1 (AFM1)	10.0	82.5	81.0 ± 3.1
Alternaria toxins				
	Alternariol (AOH)	20.0	90.2	77.8 ± 2.3
	Alternariolmethylether (AME)	45.0	90.2	83.0 ± 5.7
	Macrosporin A (MAC-A)	20.0	309.4	91.7[c]
	Tentoxin (TNTX)	8.0	35.8	112.0 ± 13.3
Aspergillus toxins				
	Kojic acid (KA)	640.0	2034	78.0 ± 2.9
	3-Nitropropionic acid (3-NPA)	20.0	565.1	69.7 ± 8.8
	Sterigmatocystin (STER)	3.0	90.2	97.0 ± 8.7
Bacterial metabolites				
	Chloramphenicol (CHLR)	3.0	103.1	120.0 ± 8.2
	Geldanamycin (GELD)	3.0	29.7	54.8 ± 7.7
	Monactin (MONC)	0.2	13	58.0 ± 8.3
	Nigericin (NIG)	0.4	31.6	45.0 ± 7.6
	Nonactin (NONC)	0.5	13	58.0 ± 7.0
	Radicicol (RAD)	32.0	53.4	37.0 ± 15.0
	Valinomycin (VAL)	1.0	25.5	39.0 ± 7.4
Chaetomium toxins				
	O-Methlysterigmatocystin (O-STER)	3.0	68.8	94.7 ± 1.4
Cyclosporins				
	Cyclosporin H (CYC-H)	30.0	412.5	63.4 ± 2.6
Cytochalasins				
	Cytochalasin H (CYT-H)	140.0	257.8	136.0 ± 1.6
	Cytochalasin J (CYT-J)	32.0	257.8	66.0[c]
Depsipeptides				
	Enniatin A (ENN-A)	0.3	0.3	93.0 ± 9.4
	Enniatin A1 (ENN-A1)	0.8	2.0	111.0 ± 4.7
	Enniatin B (ENN-B)	0.1	2.1	100.0 ± 9.4
	Enniatin B1 (ENN-B1)	0.6	5.6	92.4 ± 6.9
	Enniatin B2 (ENN-B2)	1.2	2.6	90.8 ± 10.7
	Enniatin B3 (ENN-B3)	0.007	5.2	89.1 ± 11
	Beauvericin (BEAU)	0.3	5.2	76.0 ± 19.0
Ergot alkaloids				
	Agroclavin (AGV)	8.0	180.5	96.0 ± 3.8
	Chanoclavin (CNV)	8.0	7.2	43.3 ± 2.2
	Elymoclavin (ECV)	3.0	6.1	66.6 ± 7.4
	Ergocorninine (ERCN)	4.0	7.3	67.6 ± 11.8
	Ergocristine (ERT)	4.0	10.3	50.8 ± 14.2
	Ergocristinine (ERTN)	2.0	7.3	45.0 ± 14.7
	Ergocryptinine (ERCT)	2.0	7.3	97.0 ± 15.6
	Ergometrine (ERM)	1.0	20.6	90.7 ± 5.9
	Ergometrinine (ERMN)	2.0	4.6	129.0 ± 26.0
	Festuclavin (FEST)	3.0	180.5	95.4 ± 2.7
	Lysergol (LYS)	6.0	18.8	95.0 ± 7.6
Fumonisins				
	Fumonisin B1 (FB1)	40.0	679.4	51.4 ± 5.4
	Fumonisin B2 (FB2)	40.0	694.1	51.6 ± 4.8
	Fumonisin B3 (FB3)	40.0	87.6	52.1 ± 4.5
	Fumonisin B4 (FB4)	40.0	–[d]	–[d]
	Hydrolysed Fumonisin B1 (HFB1)	2.0	257.1	114.0 ± 6.0
Other *Fusarium* metabolites				
	Apicidin (APIC)	0.3	18.8	99.0 ± 3.6
	Aurofusarin (ARF)	40.0	12.1	31.0[c]
	Chlamydosporol (CLAM)	12.0	68.8	102.0 ± 8.2
	Equisetin (EQUS)	16.0	128.9	175.0 ± 24.0

(*continued*)

Table 2. Continued.

Metabolite category	Analyte	LOD (μg kg^{-1})[a]	Spiking concentration (μg kg^{-1})	Recovery (%)[b]
	Moniliformin (MON)	60.0	451.2	78.7 ± 9.5
Ochratoxins				
	Ochratoxin A (OTA)	4.0	146.9	90.5 ± 5.0
	Ochratoxin B (OTB)	4.0	29.4	96.0 ± 8.0
Penicillium toxins				
	Curvularin (CURV)	40.0	103.1	58.0[c]
	Cycloaspeptide A (CA-A)	14.0	103.1	71.2 ± 7.0
	Emodin (EMOD)	12.0	90.24	104.0[c]
	Rugulosin (RUG)	18.0	68.75	97.2 ± 18.0
	Secalonic acid D (S-A-D)	32.0	1062	97.0 ± 8.7
Trichothecenes				
	Deoxynivalenol (DON)	55.0	180.5	91.4 ± 1.9
	DON-Glucoside (DON-G)	20.0	121.2	38.0 ± 1.1
	Nivalenol (NIV)	15.0	180.5	73.5 ± 5.0
Zearalenone-derivatives				
	Zearalenone (ZEN)	19.0	182.3	78.0 ± 3.0
	Zearalenone-Sulfate (ZEN-S)	3.0	3.7	90.7 ± 7.9

Notes: [a]LOD, Limit of Detection [$S/N = 3{:}1$] expressed as μg kg^{-1} sample.
[b]Average ± standard deviation calculated from spiking experiments of three different samples.
[c]Calculation was restricted to one spiked sample since the others exhibited background concentrations of the related metabolite.
[d]No standard available; estimation of concentration based on response and recovery of fumonisin B2.

non-regulated metabolites in feed materials. The cytochalasins occurred at concentration level of up to 656 μg kg^{-1}. They are known to be cytotoxic and act by binding actin filaments (Brown and Spudich 1979; Cooper 1987), preventing their polymerisation and, thus, inhibiting cell division amongst many other processes. The anthraquinoid and xanthone dimers, RUG and S-A-D, respectively coupled with CA-A and CURV are liberated by some *Penicillium* species, inducing toxic effects such as mutagenicity, teratogenicity, nephrotoxicity, cytotoxicity and hepatocarcinogenicity (Ueno et al. 1980; Rout et al. 1989; Vesely et al. 1992; Hanumegowda et al. 2002; Schmeda-Hirschmann et al. 2008). These non-regulated metabolites occurred in almost all feed types in quantities below 100 μg kg^{-1} except CURV which occurred at concentrations up to 277 μg kg^{-1}. CA-A contaminated only one sample (layer mash) while RUG and S-A-D were not detected in chick mash samples (Table 3).

Very low concentration of the suspect mutagenic agent CYC-H (< 50 μg kg^{-1}) and MAC-A (< 300 μg kg^{-1}) occurred in chicken feed in contrast to high levels (up to 7000 μg kg^{-1} for both metabolites) in indoor matrices reported by Vishwanath et al. (2009). The occurrence of APIC, EQUS and CLAM are suggestive of feed contamination by a wide array of *Fusarium* species which can produce these toxic substances in the field or in culture (Bosch and Mirocha 1992; Samson et al. 2000). APIC and CLAM (a pyranopyrone) have been reported to be cytotoxic (Park et al. 1999; Uhlig et al. 2006), whereas there is no known zootoxic effect of EQUS. CLAM was detected in rice cultures of some *Fusarium* species from stored sugar beet at concentrations of 68–4708 μg kg^{-1} and the isolates obtained from rice have been documented to also occur in sorghum (Savard et al. 1990). In addition, this toxin has been detected in four naturally infected apples with apple core rot in Slovenia (Sorensen et al. 2009) but we are not aware of any report of its natural occurrence in grains or finished feed. APIC is highly toxic to rats and brine shrimps but has a weak toxicity potential to human and mouse tumour cell lines (Park et al. 1999). This may, therefore, imply that the low concentrations of APIC in the analysed samples (0.3–3.9 μg kg^{-1}) may not necessarily be problematic. However, the low to moderately high concentrations of the other non-regulatory toxins being reported for the first time here may constitute hazards of unknown potential to the poultry industry.

VAL was the most prevalent bacterial metabolite contaminating various feed types at levels up to 85% and these were lower than the frequencies reported to occur to indoor matrices (Vishwanath et al. 2009). The other bacterial metabolites, which are mostly byproducts from *Streptomyces* were present in all feed types except chick mash. RAD was detected at its highest concentration of 392 μg kg^{-1} in the feed samples. Although it may appear that these bacterial metabolites are beneficial since they are antibiotics, their occurrence does not reflect quality of the affected feeds considering the zero tolerance limits established in Europe for CHLR in foods in 1994. An unintended exposure to very low levels of antibiotics may even be

Table 3. Overview of the occurrence and concentration of 63 metabolites in poultry feed types in Nigeria.

		Concentration (μg kg^{-1})				Number of contaminated feed types				
Metabolite[a]	Incidence (%)	Mean	SD	Min	Max	Chick mash ($n^b = 7$)	Broiler finisher ($n = 12$)	Grower mash ($n = 14$)	Layer mash ($n = 14$)	Broiler starter ($n = 11$)
AFB1	76	198	246	6	1067	7	8	12	10	8
AFB2	50	34	23	10	114	7	7	4	3	8
AFG1	60	45	46	8	235	7	7	8	5	8
AFG2	10	13	4	10	20	3	2	0	0	1
AFM1	26	15	5	10	29	6	4	2	0	3
AGV	17	107	106	10	350	1	3	3	3	0
AME	2	106	0	106	106	0	1	0	0	0
AOH	10	33	14	23	61	0	3	2	0	1
APIC	34	2	1	0.3	4	0	5	6	4	5
ARF	86	1148	2482	40	10,252	4	11	14	11	10
BEAU	100	15	9	3	39	7	12	14	14	11
CA-A	2	14	0	14	14	0	0	0	1	0
CHLR	3	3	0	3	4	2	0	0	0	0
CLAM	72	22	8	12	44	6	10	6	10	10
CNV	7	9	3	6	13	0	0	1	3	0
CURV	40	85	52	44	277	1	5	7	6	4
CYC-H	5	39	5	33	43	2	0	0	0	1
CYT-H	7	200	42	142	231	0	0	1	1	2
CYT-J	81	245	154	33	656	6	11	7	13	10
DON	36	651	786	80	2336	1	5	5	5	5
DON-G	29	170	179	22	482	0	2	6	6	3
ECV	26	38	45	3	147	1	5	4	4	1
EMOD	60	36	21	10	96	3	7	11	7	7
ENN-A	74	2	3	0.3	15	3	9	12	9	10
ENN-A1	79	12	17	0.5	101	3	11	12	10	10
ENN-B	91	15	23	0.1	141	5	11	14	12	11
ENN-B1	81	22	31	1	182	3	11	13	10	10
ENN-B2	22	3	2	1	8	0	3	4	3	3
ENN-B3	2	0.007	0	0.007	0.007	0	0	0	0	1
EQUS	100	176	115	20	570	7	12	14	14	11
ERCN	5	8	6	5	15	0	1	2	0	0
ERCT	10	5	1	2	6	0	2	3	1	0
ERM	26	2	1	1	5	1	4	4	2	4
ERMN	24	3	1	2	6	1	3	5	2	3
ERT	22	15	12	5	43	0	3	5	2	3
ERTN	26	8	6	3	25	1	4	4	3	3
FB1	83	964	621	31	2733	4	10	13	12	9
FB2	81	359	217	51	1130	4	10	13	11	9
FB3	76	161	86	37	369	4	9	11	11	9
FB4	67	51	22	18	115	4	9	9	8	9
FEST	2	3	0	3	3	0	0	1	0	0
GELD	16	15	16	3	44	1	4	3	0	1
HFB1	16	5	3	2	11	1	2	2	2	2
KA	67	3196	2794	649	10,072	7	7	10	9	6
LYS	7	11	4	6	17	0	0	2	2	0
MAC-A	41	134	68	40	299	1	5	8	5	5
MON	53	115	42	61	217	1	6	9	9	6
MONC	21	0.2	0	0.2	1	1	2	4	2	3
NIG	21	1	1	0.4	3	1	4	4	2	1
NIV	38	31	15	15	72	0	6	7	5	4
NONC	21	1	0	0.5	2	1	2	4	2	3
3-NPA	81	123	155	21	947	7	7	12	12	9
O-STER	48	5	3	3	19	7	7	4	3	7
OTA	34	10	6	4	26	5	4	4	2	5
OTB	28	10	6	4	26	4	3	1	2	6
RAD	14	116	124	32	392	1	1	2	1	3
RUG	22	43	24	20	92	0	2	7	3	1
S-A-D	19	46	17	32	88	0	3	3	2	3

(*continued*)

Table 3. Continued.

Metabolite[a]	Incidence (%)	Concentration (μg kg^{-1}) Mean	SD	Min	Max	Number of contaminated feed types: Chick mash (n[b] = 7)	Broiler finisher (n = 12)	Grower mash (n = 14)	Layer mash (n = 14)	Broiler starter (n = 11)
STER	24	5	4	3	16	2	6	4	1	1
TNTX	19	13	5	8	23	1	2	3	3	2
VAL	62	1	1	1	3	6	6	8	7	9
ZEN	22	45	32	21	134	1	4	3	2	3
ZEN-S	14	11	11	4	36	0	2	3	1	2

Notes: [a]See Table 2 for the complete names of the metabolites.
[b]n = number of analysed samples per feed type.

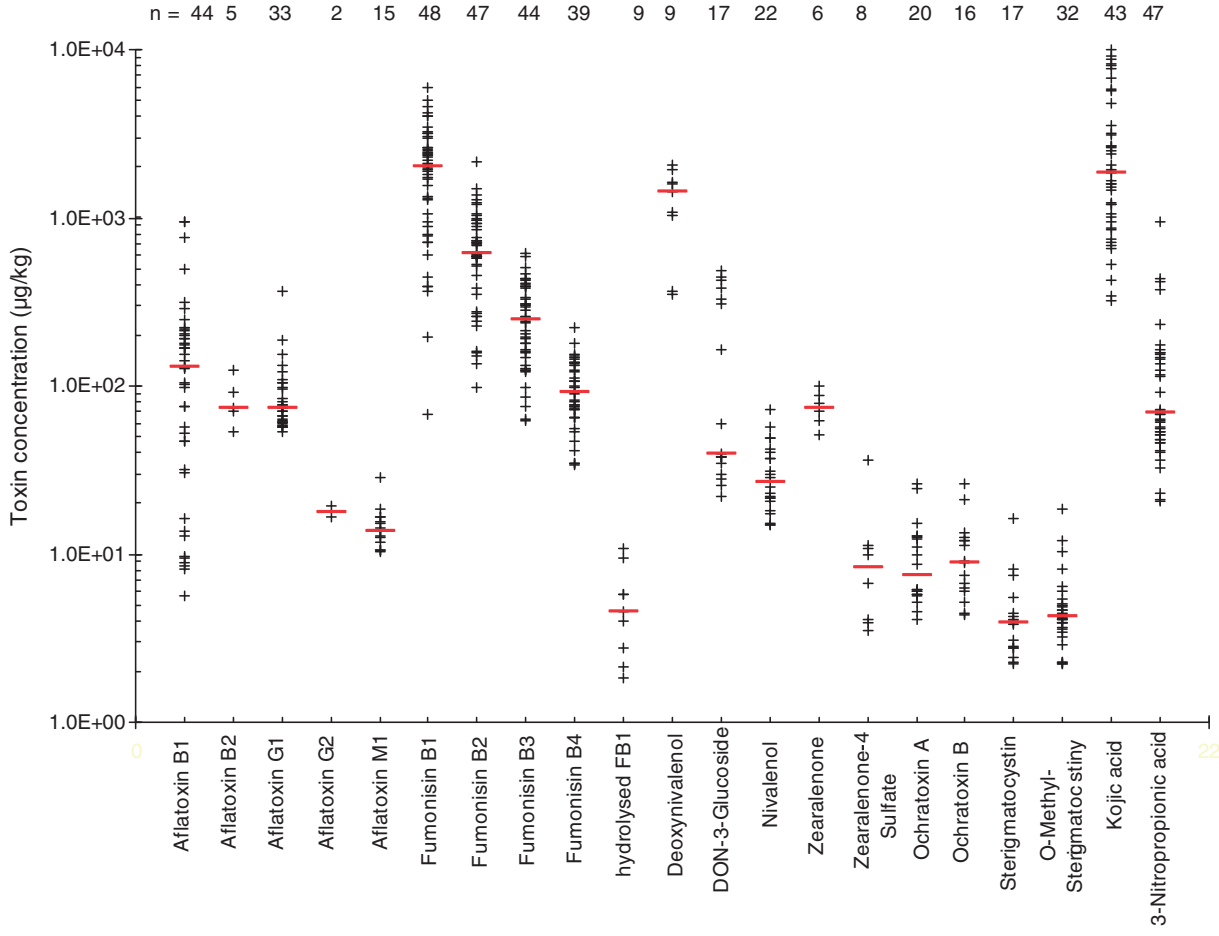

Figure 1. Range (with median as the horizontal line) of concentrations of regulated toxins and related derivatives in feed samples (n = number of positive samples of 58).

responsible for the increase in bacteria that are resistant to antibiotics.

Among other non-regulated metabolites that have been previously implicated in natural food contamination; aurofusarin (ARF), 3-nitropropionic acid (3-NPA), enniatins, some ergot alkaloids and KA appear to be important. Reasons include their wide distribution in the feed samples, previously reported toxic effects and the moderate-to-high concentration of their presence. ARF (naphthoquinone dimer), first documented as a metabolite of *F. culmorum* (Ashley et al. 1937), is produced by at least 10 other species (Medentsev and Akimenko 1998; Samson et al. 2000). Although Kotyk and Trufanova (1998) found ARF to occur in 11 samples of wheat in Ukraine, we report the first occurrence of this toxic metabolite in

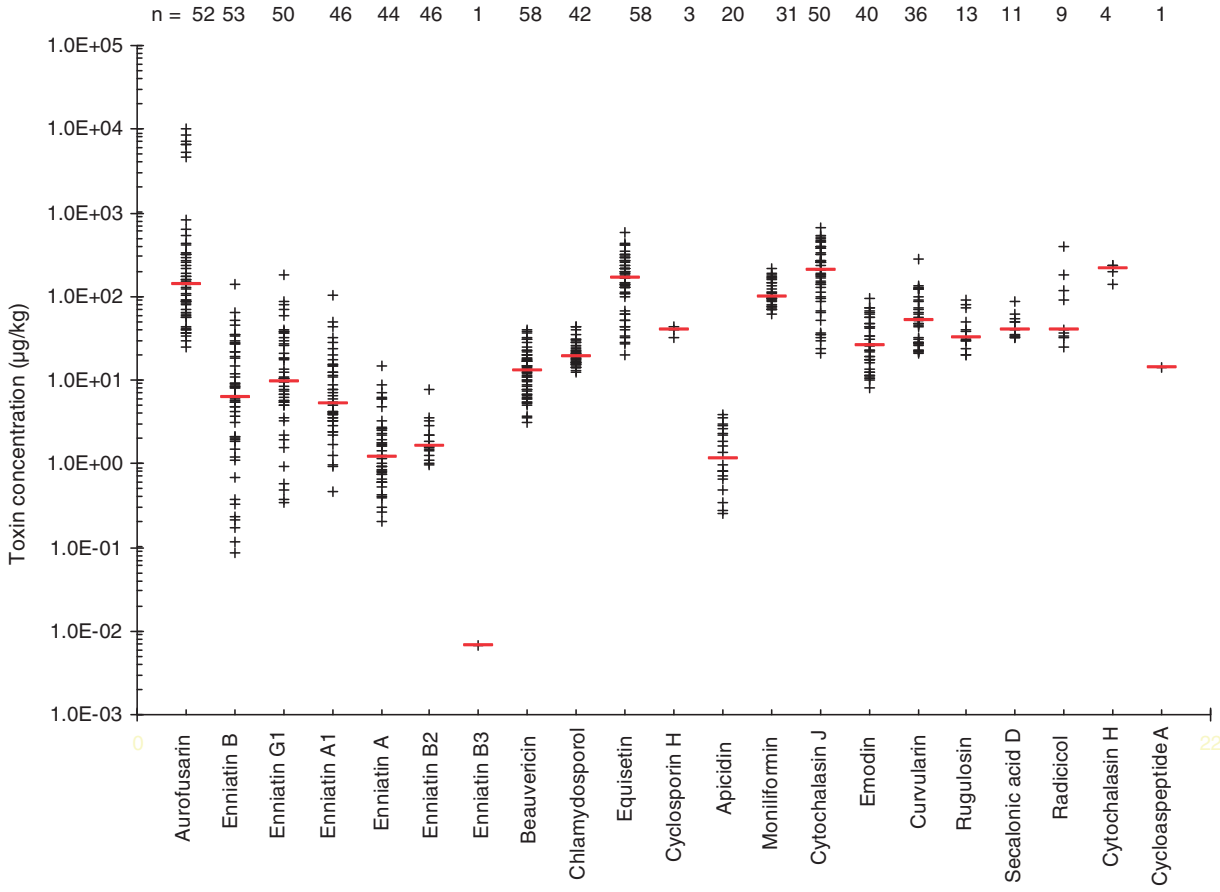

Figure 2. Range (with median as the horizontal line) of concentrations of *Fusarium* and other fungal metabolites in feed samples (n = number of positive samples of 58).

finished and stored feed. In this study, ARF had the highest concentration of 10,300 µg kg^{-1} in layer mash sample. The concentrations of ARF in chick mash samples was quite low (<100 µg kg^{-1}) but its high concentrations (>800 µg kg^{-1} mean concentration) in other feed types, especially layer mash, may be a concern for the layer industry. ARF is transferrable from feed to egg yolk and impairs egg quality by changing the colour of egg yolk from orange to dark brown (Kotyk et al. 1995). Its consumption by birds may also affect chicken meat quality by reducing protein (alanine, leucine, valine, glycine, glutamic acid and lysine) and fat contents significantly in red muscles (Dvorska 2000). ARF can also significantly decrease the concentration of essential vitamins A and E, total carotenoids, lutein and zeaxanthin even at concentrations below 30 µg kg^{-1}, thereby increasing the susceptibility of the egg yolk to lipid peroxidation, a mechanism of action similar to that of aflatoxins (Dvorska et al. 2001).

Other *Fusarium* metabolites encountered in this study were the enniatins, that occurred in moderate quantities (up to 140.8 µg kg^{-1}) virtually in all feed types, with ENN-B2 and ENN-B3 having very low concentrations (0.007 to 7.6 µg kg^{-1}), but was not present in the chick mash samples. Ivanova et al. (2006) recorded that enniatins, including ENN-B2 and ENN-B3, which occur frequently in grains from Northern Europe due to grain contamination by *Fusarium avenaceum,* have an underestimated cytotoxic potential even at very low concentrations. The depsipeptide, BEAU, occurred in all feed types at concentrations up to 37.5 µg kg^{-1}. Although the concentrations of BEAU in our samples may be low compared to the reports of Sulyok et al. (2006) (up to 800 µg kg^{-1} in maize samples), there is a risk posed to the birds and workers in feed mills and poultry farms who handle these feeds due the potential toxicity of this peptide to brine shrimps and genotoxicity to human lymphocytes (Moretti et al. 2007; Celik et al. 2010).

The presence of ergot alkaloids, which indicates contamination of feed by *Claviceps,* is worrisome because these metabolites were found at concentrations up to 350 µg kg^{-1}, with agroclavin (AGV) having the highest concentration although elymoclavin (ECV) was the most prevalent. Regardless of the low-to-moderate levels of ergot alkaloids in our samples, there is an impending danger in the continuous consumption of this potent group of non-regulatory toxins (Sulyok et al. 2010). 3-NPA, a metabolite produced by

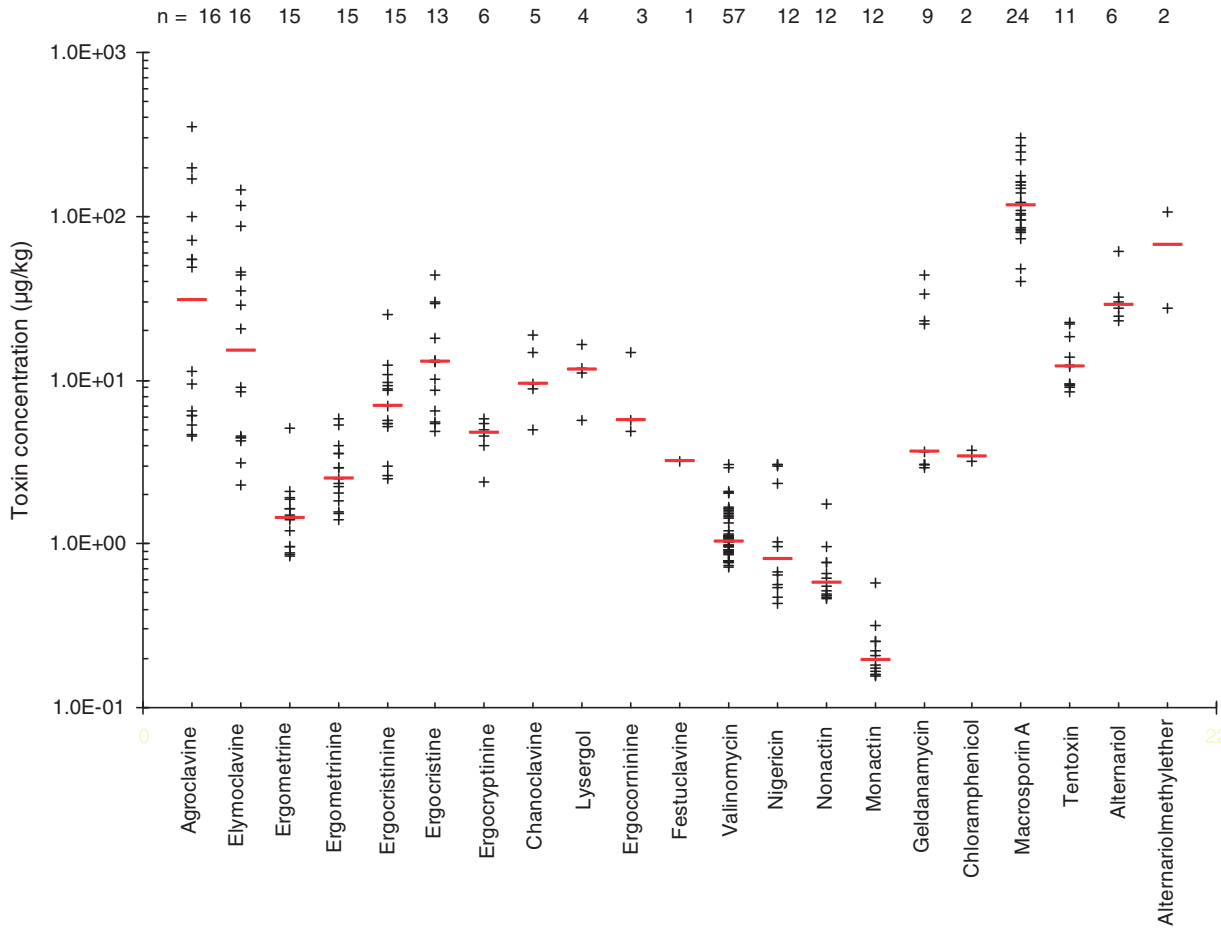

Figure 3. Range (with median as the horizontal line) of concentrations of ergot alkaloids, bacterial metabolites and *Alternaria* metabolites in feed samples (n = number of positive samples of 58).

Table 4. Poultry feed samples (%) of various types contaminated with different levels of aflatoxin in Nigeria.

	Mean aflatoxin concentration (µg kg^{-1})[a]			
Feed type and number of samples	<20	>20	>100	>500
Chick mash (n[b] = 7)	0.0	100.0	100.0	57.1
Broiler finisher (n = 12)	41.7	58.3	58.3	0.0
Grower mash (n = 14)	42.9	57.1	28.6	0.0
Layer mash (n = 14)	57.1	42.9	21.4	0.0
Broiler starter (n = 11)	27.3	72.7	63.6	0.0
All samples (n = 58)	37.9	62.1	48.3	6.9

Notes: [a]Total aflatoxin (AFB1, AFB2, AFG1, AFG2).
[b]Number of analysed samples per feed type.

Aspergillus, occurred in all feed types and is known to contaminate mouldy sugar cane and garlics in very high concentrations (Ming 1995; Sulyok et al. 2010). KA is another *Aspergillus* metabolite that occurred in all feed types at very high quantities ranging from 649 to 10,100 µg kg^{-1}. The high quantities of KA indicate fungal deterioration of cereal components of the feed since KA is synthesised as a metabolic by-product during *Aspergillus* colonisation of cereals.

Our study demonstrates the usefulness of the LC–MS/MS analytical technique to the feed industry as a broad variety of metabolites from different fungi and bacteria were shown to occur at various concentrations. We have also highlighted the high mycotoxin risks in the Nigerian poultry industry due to the occurrence of a wide array of toxic metabolites, including those reported to occur naturally in food for the first time in this study. Of particular concern is

the high prevalence of aflatoxins in poultry feed sold in the Nigerian market. Given the importance of the poultry industry to the Nigerian economy, and the harmful effects of aflatoxins and some other mycotoxins to the birds, the agriculture and poultry sectors must increase their efforts for mitigation of aflatoxins and other mycotoxins in grains, which are the raw materials of poultry feed. We also recommend establishment of regulatory limits for aflatoxins in animal feed where they do not exist and mechanisms developed to enforce it in a phased manner in partnership with all stakeholders in the poultry value chain for increasing the productivity and profitability of the poultry industry. This paper is a first ''snapshot'' towards achieving a mycotoxin map in Nigeria for poultry feed. Analysis of more samples is needed for an overview of the mycotoxin problem in the Nigerian poultry sector.

Acknowledgements

The authors thank EU MycoRed project for supporting this research and the senior author by granting a Short-Term Visit (STV) Fellowship under the WP 8.2.7 IFA/BOKU section. The support of the graduate school program Applied Bioscience Technology of the Vienna University of Technology and the University of Natural Resources and Life Sciences Vienna is acknowledged. The laboratories from which we received standards as gifts are thanked.

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