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Masked Mycotoxins Are Efficiently Hydrolyzed by Human Colonic Microbiota Releasing Their Aglycones

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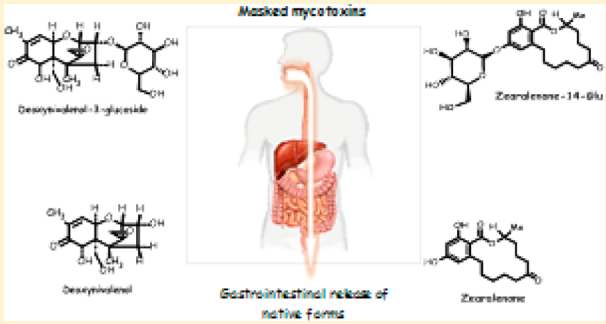
ABSTRACT: *Fusarium* mycotoxins are secondary metabolites produced by *Fusarium* spp. in cereals. Among them, deoxynivalenol (DON) and zearalenone (ZEN) are widespread worldwide contaminants of cereal commodities and are ranked as the most important chronic dietary risk factors. Their conjugates, known as masked mycotoxins, have been described but are still not accounted for in risk assessment studies. This study demonstrates for the first time that DON and ZEN are effectively deconjugated by the human colonic microbiota, releasing their toxic aglycones and generating yet unidentified catabolites. For this reason, masked mycotoxins should be considered when evaluating population exposure.



INTRODUCTION

Fusarium mycotoxins are secondary metabolites produced by *Fusarium* spp. in grains, such as wheat and maize. Among them, deoxynivalenol (DON) and zearalenone (ZEN) are widespread contaminants of cereal commodities worldwide. Mycotoxins were ranked as the most important chronic dietary risk factors, higher than synthetic contaminants, plant toxins, and pesticide residues.[1,2] In particular, ZEN toxicological effects in humans and mammals are due mainly to its strong estrogenic activity,[1] while pathophysiologic effects associated with DON include altered neuroendocrine signaling, proinflammatory gene induction, disruption of the growth hormone axis, and altered gut integrity.[2]

In the past decade, several studies involving *Fusarium* mycotoxins have been designed to elucidate the formation and role of their masked forms in food. Masked mycotoxins are plant-derived phase II metabolites formed by conjugation of parent mycotoxins to polar groups.[3] Generally, these forms are not considered in risk assessment studies due to their as yet unknown biological effects. However, concerns about their potential effects on human health are growing, as the conjugates consistently occur alongside their parent compounds in food and feed.[3] Specifically, deoxynivalenol-3-glucoside (D3G), zearalenone-14-glucoside (Z14G), and zearalenone-14-sulfate (Z14S) are the most representative masked mycotoxins occurring in food commodities.[3] These compounds could have direct toxicological properties and/or might be totally or partially hydrolyzed to release the parent aglycone after ingestion, thus leading to an increased exposure compared to the estimates made with conventional analytical approaches, which do not consider conjugates. Although this assumption is not yet supported by *in vivo* data, a recent study showed that specific intestinal bacteria are capable *in vitro* of partially converting D3G into DON.[4]

The role of the gut microbiota is becoming a key point for the investigation of xenobiotic bioactivity in humans and animals. The vast amount of different bacterial strains and associated enzymes in the human colon makes the gastrointestinal tract a remarkable bioreactor able to chemically transform most of the compounds ingested by humans that reach the large intestine. The overall toxic effect of mycotoxins is a function of their bioactivity per se but also of their bioavailability and catabolism. As an example, rumen bacteria are often supposed to degradate mycotoxins to less toxic compounds.[5]

Inspite of the increasing number of reports about the occurrence of masked mycotoxins in food, toxicological aspects related to these compounds have still to be clarified.[4,6,7] There are several reports dealing with *in vitro* studies using single bacterial strains[4] and trials with Caco-2 cell lines.[6] There is, however, only one report about the *in vivo* metabolic fate of masked mycotoxins in rats,[7] while nothing is known about the catabolic fate of these compounds in humans. Mycotoxin conjugates, indeed, may represent a detoxification product, in the case where the aglycone is resistant to release in the gastrointestinal tract. However, when partial or total cleavage occurs in the digestive system, masked forms may exert the same toxic effects reported for their parent compound.[4] Finally, their own toxicity should also be considered, although very little is currently known about their potential bioactivity. For all these reasons, classification of these conjugates from a

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Table 1. Constituents and Concentrations of the Synthetic Juices Used for the *in Vitro* Gastrointestinal Digestion

	saliva	gastric juice	duodenal juice	bile juice
inorganic solution	5 mL KCl 89.6 g/L	7.85 mL NaCl 175.3 g/L	20 mL NaCl 175.3 g/L	15 mL NaCl 175.3
	5 mL KSCN 20 g/L	1.5 mL NaH_2PO_4 88.8 g/L	20 mL $NaHCO_3$ 84.7 g/L	34.15 mL $NaHCO_3$ 84.7 g/L
	5 mL NaH_2PO_4 88.8 g/L	4.6 mL KCl 89.6 g/L	5 mL KH_2PO_4 8 g/L	2.1 mL KCl 89.6 g/L
	5 mL Na_2SO_4 57 g/L	9 mL $CaCl_2$ 16.65 g/L	3.15 mL KCl 89.6 g/L	75 μL HCl 37% g/g
	850 μL NaCl 175.3 g/L	5 mL NH_4Cl 30.6 g/L	5 mL $MgCl_2$ 5 g/L	
	10 mL $NaHCO_3$ 84.7 g/L	3.25 mL HCl 37% g/g	90 μL HCl 37% g/g	
organic solution	4 mL urea 25 g/L	5 mL glucose 65 g/L	2 mL urea 25 g/L	5 mL urea 25 g/L
		5 mL glucuronic acid 2 g/L		
		1.7 mL urea 25 g/L		
		5 mL glucosamine hydrochloride 33 g/L		
other constituents	290 mg/L alpha-amylase	1 g/L BSA	9 mL/L $CaCl_2$ 16.65 g/L	10 mL/L $CaCl_2$ 16.6 g/L
	15 mg/L uric acid	2.5 g/L pepsin	1 g/L BSA	1.8 g/L BSA
	25 mg/L mucin	3 g/L mucin	9 g/L pancreatin	30 g/L bile
			1.5 g/L lipase	
pH	6.8 ± 0.2	1.30 ± 0.02	8.1 ± 0.2	8.2 ± 0.2

toxicological perspective is very cumbersome and requires further investigation, leading to the necessity of including the occurrence of masked mycotoxins in risk assessment studies in order to avoid possible underestimation of total exposure.

This article is thus aimed to demonstrate whether the human microbiome is able to modify masked mycotoxins and to release parent compounds in the intestinal tract. For this purpose, the D3G, Z14G, and Z14S stability under digestive conditions and microbial colonic fermentation has been investigated.

MATERIALS AND METHODS

Chemicals. Mycotoxin standard zearalenone, deoxynivalenol, and deoxynivalenol-3-glucoside were purchased from RomerLabs (Tulln, Austria). Zearalenone-14-β-D-glucopyranoside (Z14G) was synthesized in our laboratory as reported below. Zearalenone-14-sulfate was kindly provided by Professor Franz Berthiller, IFA-Tulln (BoKu University, Vienna, Austria).

All solvents (HPLC grade) were from Carlo Erba (Milan, Italy); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore, Marlborough, MA, USA). Salts were from Fluka (Chemika-Biochemika, Basil, Switzerland). 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosylbromide and tetrabutylammonium bromide were purchased from Sigma (Stuttgard, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (urea 98%, D-(+)-glucose 99.5%, D-glucuronic acid, D-(+)-glucosamine hydrochloride 99%, type III mucin from porcine stomach, uric acid, type VIII A R-amylase from barley malt, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancrease, and bovine and ovine bile were purchased from Sigma (Stuttgart, Germany).

For fecal fermentation, L-cysteine hydrochloride monohydrate and Fe(II)-sulfate heptahydrate were supplied by AppliChem (Darmstadt, Germany). Bile salts, calcium chloride, (+)-arabinogalactan, tryptone, yeast extract, inulin, buffered peptone water, Dulbecco's phosphate buffer saline, casein sodium salt from bovine milk, pectin from citrus fruits, mucin from porcine stomach-type III, xylan from Birchwood, sodium hydrogen carbonate, potassium hydrogen phosphate, magnesium sulfate monohydrate, guar gum, Tween 80, and resazurin redox indicator were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Zearalenone-14-glucoside Preparation and Characterization. Zearalenone-14-glucoside (Z14G) was synthesized according Zill et al., with slight modifications.[8] Zearalenone (25 mg) was dissolved in chloroform and added to acetobromoglucose (2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosylbromide, 1 g) and tetrabutylammonium bromide (252 mg) as transfer phase catalyst (molar ratio 1:30:10), both dissolved in 50 mM Cs_2CO_3. The mixture was magnetically stirred for 24 h at 30 °C. Afterward, the organic layer was collected; the aqueous layer was washed with chloroform, and the organic layers were pooled and reduced to dryness *in vacuo*. The residue was redissolved in 0.1 N NaOH and stirred for 5 h to allow Z14G deprotection. The reaction was checked by TLC (buthanol-ethanol−water, v/v, 5:4:1). After neutralization with CH_3COOH, the final product was evaporated to dryness and the residue dissolved in methanol and stored at −8 °C. The reaction yield was calculated as 88%.

The final product was characterized by ^{1}H- and ^{13}C-NMR after dissolving 1 mg in CD_3OD (1 mL). The NMR spectra were consistent with those already reported by Kamimura et al.[9] and by Berthiller et al.,[10] allowing for the univocal identification of the product as zearalenone-14-β-D-glucopyranoside.

***In Vitro* Digestion Assay.** The *in vitro* digestion experiments were performed using a procedure based on the protocol of Versantvoort et al.[11] with some modifications. Table 1 resumes constituents and their respective concentrations used for the preparation of the synthetic juices. The inorganic and the organic solutions must be prepared separately and augmented to 250 mL with bidistilled water. After mixing the inorganic and organic solutions, enzymes and other constituents are added to a selected volume and dissolved by heating to 37 °C while stirring. If necessary, the pH of each juice is adjusted to the appropriate value using either 1 M HCl or 1 M NaOH.

Before each experiment, all digestive juices were heated at 37 ± 2 °C. The digestion was started by adding 3 mL of saliva to 2 g of ground sample, followed by an incubation step of 5 min. Then, 6 mL of gastric juice was added and the mixture incubated for 2 h. Finally, 6 mL of duodenal juice, 3 mL of bile, and 1 mL of bicarbonate solution (1 M) were added simultaneously to the mixture, and a final incubation step of 2 h was performed. During the *in vitro* digestion, the mixture was stirred with a magnetic stirrer (250 rpm) to obtain a gentle but systematic mixing of the matrix with the digestive juices. The pH of the chyme varied in the range 6.5−7. At the end of the experiment, the digestion tubes were centrifuged for 15 min at 3500 rpm (Alc Centrifuge pk110, DJB Labcare Ltd., Newport Pagnell, Buckinghamshire, UK), yielding the chyme (the supernatant) and the digested matrix (the pellet). At the end of the experiment, the volume was adjusted to 2 mL with bidistilled water before LC-MS/MS analysis.

Each experimental set (D3G, Z14G, and Z14S) was run in triplicate; a control sample was prepared for each set: the target compound was added to the inorganic and organic media without enzymes and reacted as described above. A blank sample was also prepared by mixing all the digestive juices (inorganic and organic media and enzymes) in the absence of the target compound.

***In Vitro* Fecal Fermentation Assay.** The fecal fermentation was performed according to Dall'Asta et al.,[12] with slight modifications. The composition for 1 L of growth medium was 5 g of soluble starch,

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5 g of peptone, 5 g of tryptone, 4.5 g of yeast extract, 4.5 g of NaCl, 4.5 g of KCl, 2 g of pectin, 4 g of mucin, 3 g of casein, 2 g of arabinogalactan, 1.5 g of $NaHCO_3$, 0.69 g of $MgSO_4 \cdot H_2O$, 1 g of guar, 0.8 g of L-cysteine $HCl \cdot H_2O$, 0.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.4 g of bile salt, 0.08 g of $CaCl_2$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 1 mL of Tween 80, and 4 mL of resazurin solution (0.025%, w/v) as an anaerobic indicator. The growth medium was sterilized at 121 °C for 15 min in glass vessels (50 mL) before sample preparation.

For the fecal slurry preparation, fresh fecal samples were collected from three healthy donors with no previous intestinal disease and who had not been treated with antibiotics for 3 months prior to fecal collection. Samples were immediately stored in an anaerobic jar and then diluted with Dulbecco's phosphate buffer saline at 10% (v/v) and homogenized to obtain a 20% (w/w) slurry to be used as the fermentation starter.

The final fermentation volume was 4 mL: 50% growth medium and 50% fecal slurry. Toxin amount was added in order to get a final concentration of 0.5 mg/L. The fermentation starter was introduced in the vessel containing sterilized growth medium, sealed with a rubber seal, and flushed through a double needle with nitrogen to create anaerobic conditions. Samples were then introduced into the vessel through the needle and incubated for 24 h at 37 °C at 200 strokes/min in a Dubnoff bath (ISCO, Milan, Italy). After 30 min and 24 h of incubation, the fermentation samples were centrifuged, and 0.5 mL was filtered through a 0.2-mm nylon filter and stored at −80 °C. All samples were fermented independently, and all experiments were carried out in triplicate.

Control samples were prepared by adding 10% acetonitrile to the fecal slurry before toxin addition, in order to inhibit enzymatic activity, and left to react for t = 0 min, t = 30 min, and t = 24 h. Negative control samples were prepared by adding target toxin to the growth media without the addition of fecal slurry and were allowed to react for 30 min and 24 h. A blank sample was also prepared by mixing growth media and fecal slurry in the right proportions without the addition of any toxins.

UPLC-ESI-MS Analysis. The UPLC/MS analyses were carried out with an Acquity UPLC separation system (Waters Co., Milford, MA, USA) equipped with an Acquity Single Quadrupole MS detector with an electrospray source. Chromatographic conditions were the following: column, Acquity UPLC BEH C18 (1.7 μm, 2.1 × 50 mm); flow rate, 0.35 mL/min; column temperature, 30 °C; injection volume, 5 μL; and gradient elution was performed using 0.1 mM sodium acetate solution in water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid. Initial conditions were set at 2% B for 1 min, then eluent B was increased to 20% in 1 min. After an isocratic step (6 min), eluent B was increased to 90% in 9 min. After a 3 min isocratic step (90% B), the system was reequilibrated to initial conditions for 2 min. The total analysis time was 22 min. The ESI source was operated in positive ionization mode, with the exception of Z14S, which was monitored under ESI- conditions. MS parameters were as follows: capillary voltage, 2.50 kV; cone, 30 V; source block temperature, 120 °C; desolvation temperature, 350 °C; cone gas, 50 L h^{-1}; desolvation gas, 850 L h^{-1}.

A good linearity was obtained for all the considered mycotoxins (r^2 > 0.99). For all the target compounds, limit of quantification (LOQ) and limit of detection (LOD) were lower than 30 μg/L and than 10 μg/L, respectively. Recovery experiments were performed by spiking a blank chime or a blank fecal slurry at the target concentration level (0.5 mg/L). Samples were analyzed using both single ion monitoring (SIR) and scan mode (m/z 100−600), as reported in Table 2. Matrix-matched calibration experiments were performed in the range 0.05−1.00 mg/L for the target analytes. Recoveries, LOD, and LOQ were calculated for each mycotoxin in both chyme and fecal slurry, as reported in Table 3.

Compound Confirmation by ITMS Analysis. Target compound confirmation was performed by a MS^n experiment using Accela UHPLC 1250 equipped with a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc., San Jose, CA, USA). Experiments were performed in both positive and negative ionization modes. Analyses were carried out using full scan, data-dependent MS^2 and MS^3 in the conditions reported in Table 4. Collected spectra were compared to those obtained for reference standards as well as with those reported in the literature.

Table 2. Monitored Ions and Ionization Parameters Applied

	ionization mode	monitored ions (m/z)	capillary voltage (kV)	cone voltage (V)
D3G	ESI+	481.3, 459.3	2.5	30
DON	ESI+	319.3, 297.3	2.5	30
Z14G	ESI+	503.4, 341.4, 319.4	2.5	30
Z14S	ESI−	397.4, 317.4	2.5	30
ZEN	ESI+	341.4, 319.4	2.5	30

Table 3. Quality Parameters of the UPLC/MS Method

		D3G	DON	Z14G	Z14S	ZEN
chyme	recovery (%)	89%	95%	92%	88%	96%
	LOQ[b] (μg/L)	80	50	80	100	80
	LOD[a] (μg/L)	30	10	25	50	25
fecal slurry	recovery (%)	87%	93%	91%	85%	93%
	LOQ[b] (μg/L)	150	150	150	200	150
	LOD[a] (μg/L)	50	50	50	100	50

[a]LOD was determined as signal-to-noise ratio >3. [b]LOQ was determined as signal-to-noise ratio >10.

Table 4. Linear Ion Trap (ITMS) Data-Dependent Parameters Applied to Confirmatory Analysis

	ionization mode	MS (m/z)	CID	MS^2	CID	full scan (m/z)
D3G	ESI−	503 $[M+HCOO]^-$	11.5	457 $[M-H]^-$	15.0	125−600
DON	ESI−	341 $[M+HCOO]^-$	12.0	295 $[M-H]^-$	12.0	80−600
Z14G	ESI−	524 $[M+HCOO]^-$	22.0			75−600
Z14S	ESI−	397 $[M-H]^-$	22.0			90−600
ZEN	ESI−	317 $[M-H]^-$	22.0			85−350

RESULTS

A first set of experiments was set up to simulate the gastrointestinal digestive process, according to a comprehensive protocol already in use in our laboratory.[13,14] In brief, the salivary step (5 min), the gastric step (120 min), and the duodenal step (120 min) were reproduced *in vitro* by sequential enzymatic treatments, each associated with adequate buffering and ionic strength. The digested samples were then collected and analyzed by LC-ESI-MS/MS. Control experiments proved the stability of the precursor mycotoxins, DON and ZEN, during the entire digestive simulation. However, the parent mycotoxins aglycones were undetectable under the applied conditions and the conjugates D3G, Z14G, and Z14S were fully recovered after digestion (99.5%, 97.3%, and 98.6%, respectively), excluding any other possible degradation (see Scheme 1). Results are in agreement with data recently reported for D3G[4,6] and fumonisin conjugation products.[14]

The effect of human microbiota fermentation on D3G, Z14G and Z14S was then evaluated *in vitro*, according to the protocol developed by Dall'Asta et al.[12] Samples were collected after 30

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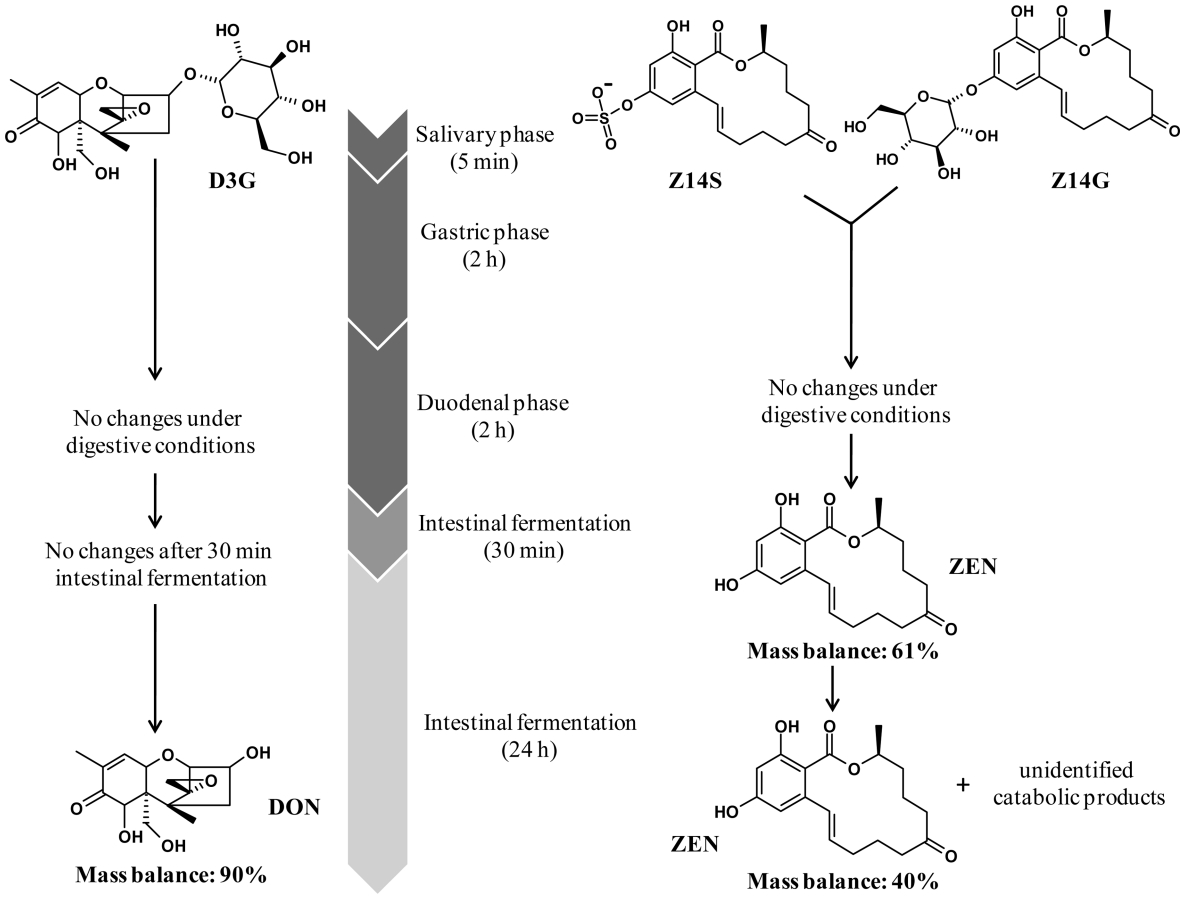
Scheme 1. Masked Mycotoxin Degradation under Human Gastrointestinal Conditions[a]



[a]Degradation steps observed along all the digestive phases considered in this study are reported for deoxynivalenol (on the left) and zearalenone (on the right) derivatives, according to the results.

min and 24 h of fermentation and compared with control samples (t = 0 min). Negative control samples were also obtained by incubation of D3G, Z14G, and Z14S in the growth media in order to check possible chemical reactions co-occurring during the incubation period. In all the experiments, masked mycotoxins were stable in 24 h incubations in the absence of microbiota. Moreover, masked mycotoxins were fully recovered after 30 min and 24 h in control samples prepared by adding 10% acetonitrile to the fecal slurry before the mycotoxin addition.

The analysis was performed by LC-ESI-MS/MS using a full scan mode to check the possible presence of unknown metabolites, and MS^n analysis was performed for target compound confirmation. The results obtained for Z14G and D3G are illustrated in Figures 1 and 2, respectively.

To minimize the interindividual variability, each set of experiments was repeated 3 times over a 6 month period, using fecal starters derived from three different healthy volunteers on each occasion. The ZEN derivatives Z14G and Z14S were completely cleaved at t = 30 min; however, ZEN was only partially recovered in the fecal slurry. The ZEN degradation rate at 30 min was 39%, while after 24 h only 40% of ZEN was present. The ZEN confirmation in the fecal slurry was obtained by ITMS analysis, as reported in Figure 3. Data-dependent MS^2 spectra (ESI−) obtained for standard solutions of Z14G and ZEN were compared to that obtained for the major compound found in the fecal slurry (t = 30 min), which was univocally identified as ZEN. The same experiment was performed also for Z14S, as reported in Figure 4. Also in this case, ZEN was univocally identified in the fecal slurry after 30 min.

The identification and structural elucidation of at least 10 major catabolites is currently ongoing. As far as D3G is concerned, after 30 min deglucosylation was not significant, while the complete degradation of D3G was obtained at t = 24 h. In this case, the main product found in the fecal slurry was DON (90%). The DON and D3G confirmation in fecal slurry was obtained by ITMS analysis, as reported in Figure 5. Data-dependent MS^3 spectra (ESI−) obtained for standard solutions of D3G and DON were compared to that obtained for the major compound found in the fecal slurry at t = 30 min and at t = 24 h, which were univocally identified as D3G and DON, respectively. Only traces of the de-epoxydation product DOM-1 were detected, although this compound has been reported as the major metabolite produced by gut microbes in rats.[7] Further experiments are ongoing taking into consideration intermediate sampling points to investigate the kinetic rate of D3G cleavage as well as the formation of ZEN catabolic products.

DISCUSSION

This is the first article describing the role played by intestinal microbiota in the chemical modification of masked mycotoxins in humans. The role of our commensal bacteria is becoming paramount in an increasing number of health related situations,

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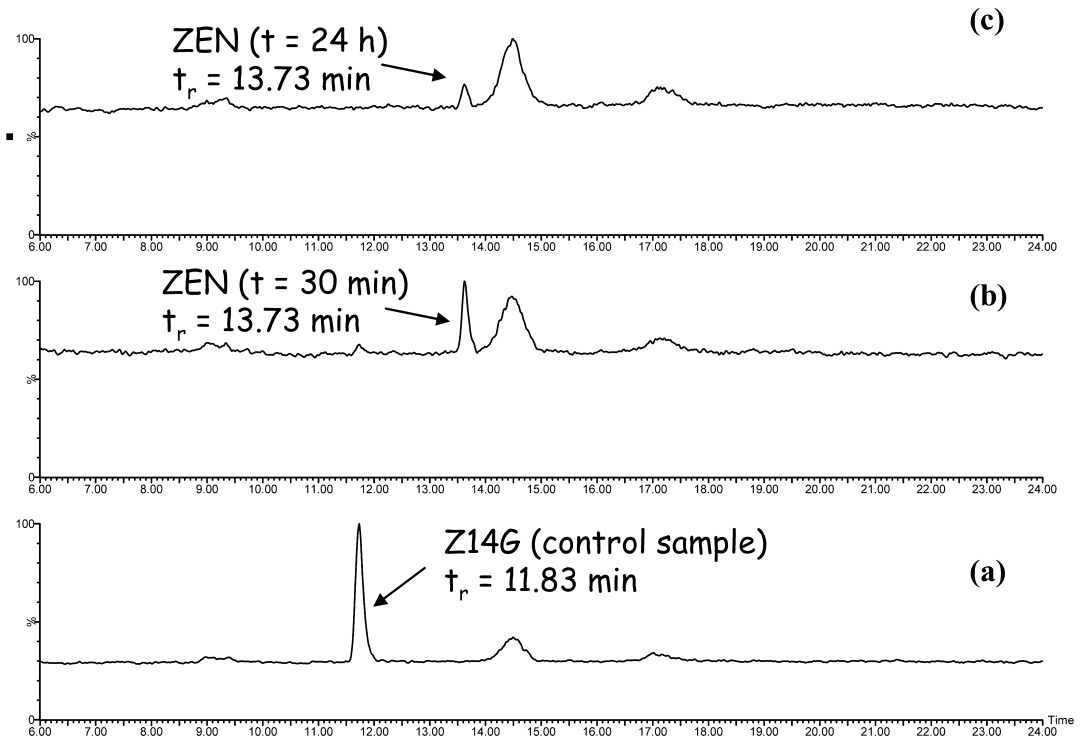

Figure 1. Chromatograms obtained for the fecal fermentation of Z14G compared to that of the control sample: control sample containing Z14G (a), ZEN released from Z14G degradation after 30 min of fermentation (b), and ZEN released from Z14G degradation after 24 h of fermentation (c).

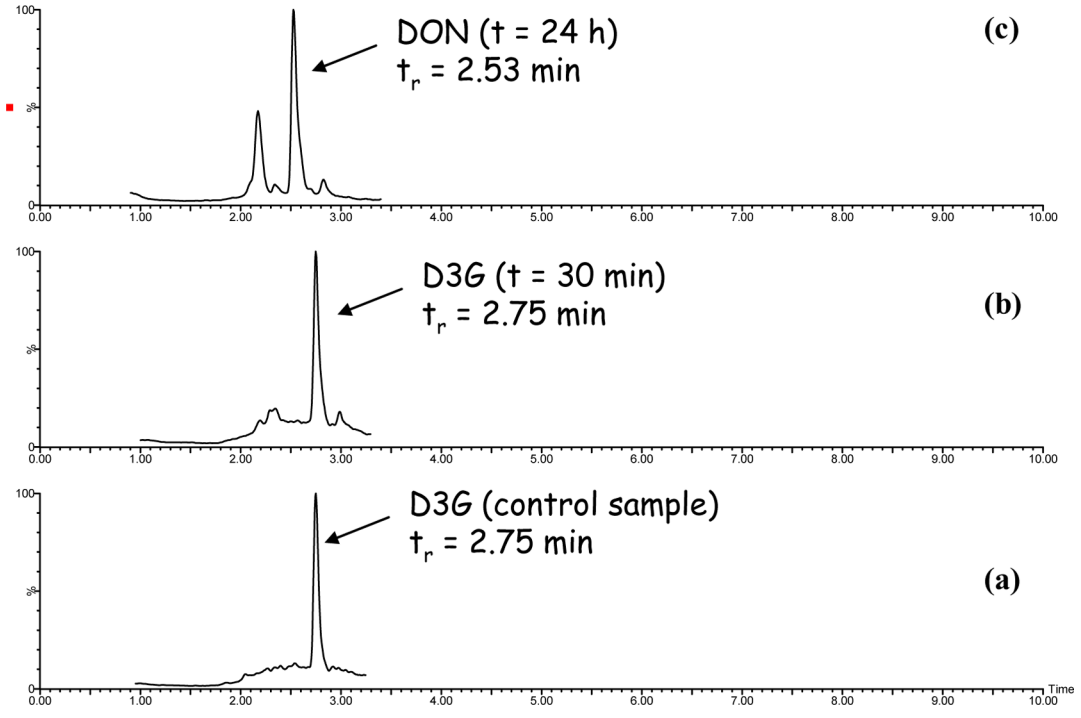

Figure 2. Chromatograms obtained for the fecal fermentation of D3G compared to that of the control sample: control sample containing D3G (a), D3G still present after 30 min of fermentation (b), and DON released from D3G degradation after 24 h of fermentation (c).

including excessive weight and obesity, diabetes, and other pathologies associated with the systemic inflammatory burden.[15] More recently, the action of the colonic microbiota has been strongly associated with the bioavailability of phenolic substances,[16] but nothing so far has been hypothesized about its possible role in the chemical modification and consequent local and systemic toxicity of potentially dangerous food contaminants. Very few data are so far available about masked mycotoxin toxicity in mammals,[7,17] and practically nothing has been reported for humans. Although several authors hypothesized about the cleavage of the glucoside moiety upon human digestion, this was never previously demonstrated directly. Gareis et al.[17] showed that after the ingestion of Z14G, ZEN was recovered in swine urine and feces and proposed that

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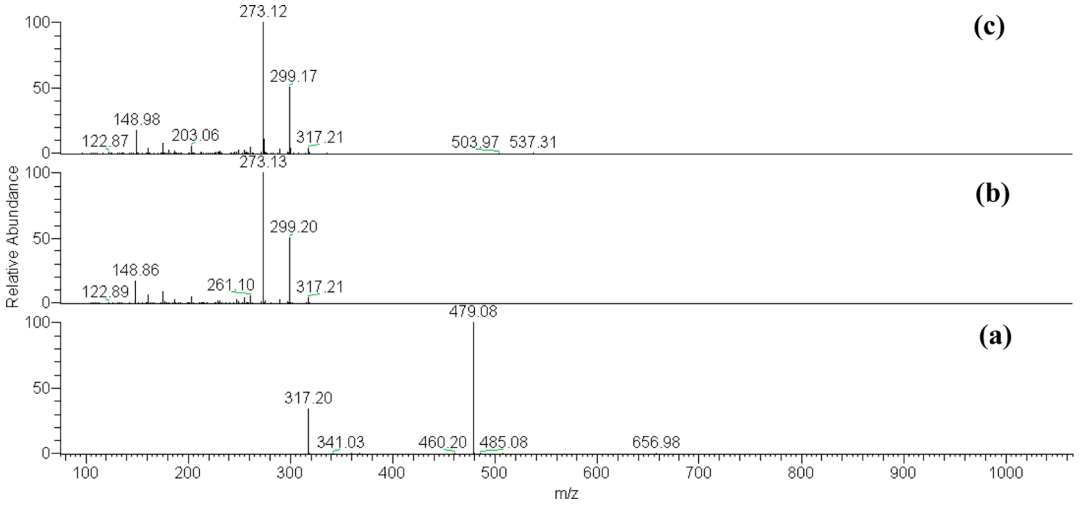

Figure 3. Comparison of mass fragmentation spectra (MS^2, ESI−) obtained for Z14G standard (a), ZEN standard (b), and ZEN in fecal slurry obtained from Z14G fermentation at t = 30 min (c).

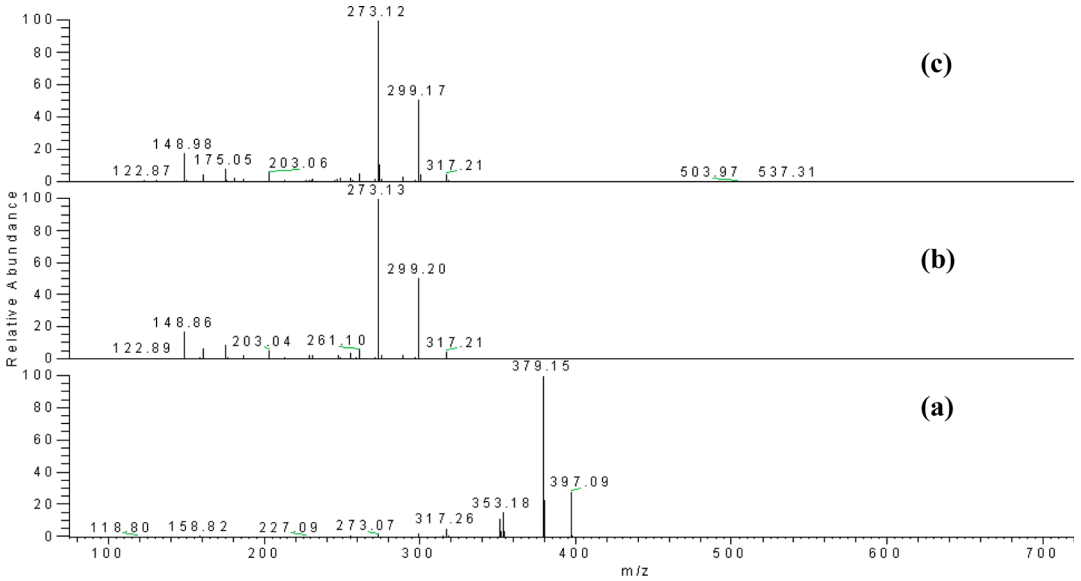

Figure 4. Comparison of mass fragmentation spectra (MS^2, ESI−) obtained for Z14S standard (a), ZEN standard (b), and ZEN in fecal slurry obtained from Z14S fermentation at t = 30 min (c).

animal or microbial enzymes were able to catalyze the reaction. The partial cleavage of D3G upon bacterial fermentation was also reported,[4] but since single strain experiments were performed, the effect of the whole human microbiota was not considered.

Toxicokinetics of DON and ZEN in animal species are different: metabolism by pig gastrointestinal and rumen microflora plays a major role in their detoxification. However, it might be speculated that humans are more sensitive to these compounds than pigs, although human gut microflora is strongly influenced by the diet and the general health condition.

The results reported in this study suggest that Z14G and Z14S are easily and rapidly deconjugated by the colonic microbiota, which opens up the possibility that the released aglycone might exert toxic effects on local epithelial cells. Moreover, since the aglycone is much less polar than Z14G and Z14S, an increased absorption may take place. It should also be considered that intestinal mucosal cells may convert ZEN to their more estrogenic phase I metabolites α- and β-ZOL.[2]

Concerning D3G, we demonstrated that this masked form is efficiently deconjugated by colonic microbiota. Although it is well known that DON absorption takes place mainly in the duodenum and in the small intestine, the release of DON from D3G in the colon cannot be ignored when neuroendocrine effects in humans are considered. As recently reviewed by Pestka,[1] DON impairment of gut motility and appetite appears to involve altered neuroendocrine signaling at both the enteric and central levels. More specifically, it has been suggested that an elevation of serotonin levels in the gut could be related to peripheral serotonergic effects of DON. Serotonin from the gut might also enter into the circulation, resulting in central effects.[1,18]

Several experiments reported that decreased feed intake and weight gain occur in pigs fed with naturally contaminated feed but not in pigs fed a diet spiked with equivalent DON amounts.[19] This evidence, which is related to the neuro-

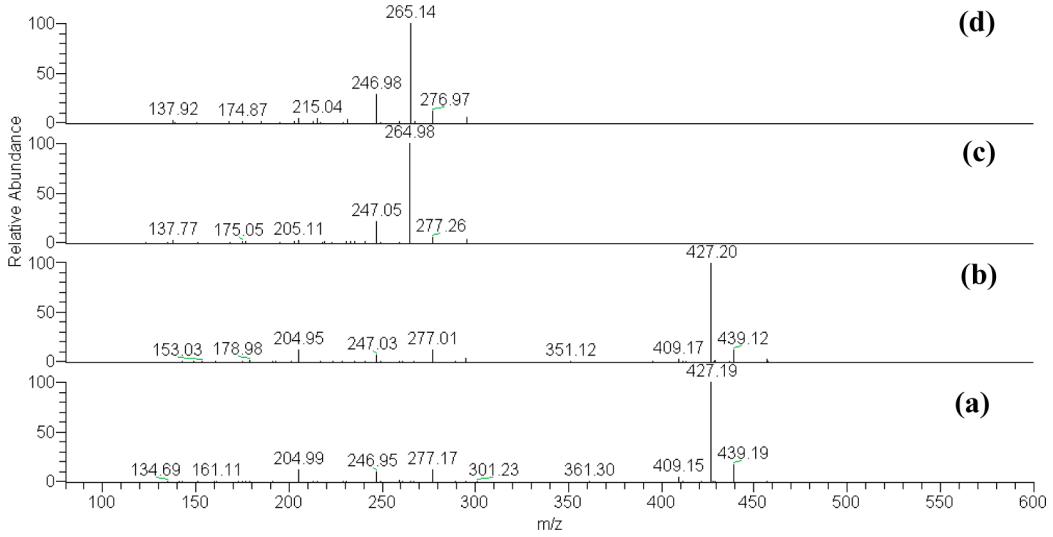

Figure 5. Comparison of mass fragmentation spectra obtained for the D3G standard (a), D3G in fecal slurry at t = 30 min (b), DON standard (c), and DON in fecal slurry obtained from D3G fermentation at t = 24 h (d).

endocrine disruptive activity of DON, may be a consequence of additional amounts of D3G and other masked forms being cleaved to DON by gut microflora.

In our study, DOM-1, which has been previously described as the main DON detoxification product in mammal feces, was only a minor component after a 24 h fecal fermentation. Similar results have been reported by Sundstol-Eriksen and Petterson.[20] These investigators demonstrated that 3-acetyldeoxynivalenol was metabolized to DON during the fecal incubation and that no de-epoxidation metabolites were detected in the incubates. This implies that humans may lack the microflora for a key DON detoxification step; further studies are needed in order to clarify the role of the diet in human gut microflora modulation and DON detoxification.

In our opinion, according to the release of parent compound due to the effect of human gut microflora and on account of occurrence data,[3] a reconsideration of legal limits for *Fusarium* mycotoxins is required. Since data on exposure and bioactivity are still lacking, it is currently impossible to perform a dose–response assessment (e.g., NOAEL) as well as a proper risk assessment for masked mycotoxins in food. In addition, although an increasing number of masked forms of *Fusarium* mycotoxins have been identified, information on their occurrence in foods is lacking. All these forms clearly contribute to the toxicity of a given food and should be taken into consideration in setting future regulations. As an example, D3G usually occurs in cereals at levels up to 30% of its respective aglycone. On the basis of what we report here, commodities which are immediately below the legal limit could well exceed it when the masked forms are considered. A possible approach can be the definition of proper maximum levels based on the sum of free mycotoxin, and masked mycotoxins.

In conclusion, our results demonstrate for the first time that the cleavage of the masked mycotoxin glucosyl- and sulfate moieties is complete in a period of time fully compatible with the presence of food remnants in the large intestine. This cleavage takes place through the action of microbial enzymes, whereas masked mycotoxins are untouched by human enzymes in the digestive tract. On the basis of these findings, masked mycotoxins should be included in risk assessment studies since they are likely to contribute to the overall toxicity as they are potentially transformed into their dangerous parent compounds. In the past few years, an increasing number of reports about D3G occurrence data have been published; however, information about its stability and transformation along the manufacturing food chains is still lacking. Nonetheless, recently the Joint FAO/WHO Expert Committee on Food Additives (JECFA) considered D3G, 3ADON, and 15ADON as additional contributing factors for dietary exposure to DON,[21] suggesting a reconsideration of the admitted maximum levels in food. ZEN, because of its estrogenic activity and widespread occurrence in cereal-based products, was recently recommended by the European Food Safety Authority (EFSA) for further risk assessment studies in children as a potentially overexposed category,[22] and the new scenario should be carefully considered. The results obtained in this study clarify the catabolic fate of masked mycotoxins in humans, confirming the importance of their inclusion in risk assessment studies.

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Notes

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ABBREVIATIONS

ZEN, zearalenone; DON, deoxynivalenol; D3G, deoxynivalenol-3-glucoside; Z14G, zearalenone-14-glucoside; Z14S, zearalenone-14-sulfate; DOM-1, deepoxy-deoxynivalenol

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