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Biological System Responses to Zearalenone Mycotoxin Exposure by Integrated Metabolomic Studies

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S *Supporting Information*

ABSTRACT: This study aims to investigate the effect of zearalenone supplementation on rat metabolism. Rats received biweekly intragastric administration of zearalenone mycotoxin (3 mg/kg body weight) for 2 weeks. Urine and plasma samples after zearalenone administration were analyzed by NMR-based metabolomics. Zearalenone exposure significantly elevated the plasma levels of glucose, lactate, *N*-acetyl glycoprotein, *O*-acetyl glycoprotein, and propionate but reduced the plasma levels of tyrosine, branched-chain amino acids, and choline metabolites. Zearalenone supplementation decreased the urine levels of butyrate, lactate, and nicotinate. However, it increased the urine levels of allantoin, choline, and *N*-methylnicotinamide at 0−8 h after the last zearalenone administration and those of 1-methylhistidine, acetoacetate, acetone, and indoxyl sulfate at 8−24 h after the last zearalenone administration. These results suggest that zearalenone exposure can cause oxidative stress and change common systemic metabolic processes, including cell membrane metabolism, protein biosynthesis, glycolysis, and gut microbiota metabolism.

KEYWORDS: *zearalenone, metabolism, metabolomics, nuclear magnetic resonance*

INTRODUCTION

Mycotoxin contamination of agricultural commodities is a major problem worldwide in agricultural and livestock production. Consumption of contaminated products can cause acute and chronic teratogenic, carcinogenic, neurotoxic, estrogenic, and immunosuppressive effects in humans and animals.[1] Therefore, contamination of food, feed, and ingredients with mycotoxins presents a significant health risk for humans and animals. Zearalenone is an agriculturally important mycotoxin produced by various *Fusarium* species. These fungal species are present in cereals cultivated worldwide, such as wheat, corn, oats, barley, rice, maize, sorghum, and sesame seeds. The occurrence of this toxin is influenced by environmental conditions, such as moisture and temperature, oxygen level, physical damage, and the presence of the fungal spores.[2] Zearalenone is chemically stable and persists in storage, milling, food processing, and cooking. This toxin is easily absorbed from the alimentary canal and metabolized in the liver. Zearalenone is considered an endocrine disrupter because it can bind directly to estrogen receptors and interact with steroidogenic enzymes. In addition, zearalenone has numerous adverse effects on both males and females of different animal species.[3] For example, zearalenone can cause infertility, decrease serum testosterone level and sperm count, reduce fertility, and alter progesterone level.[4] Moreover, zearalenone exhibits hepatotoxicity by affecting several enzymatic parameters of hepatic functions in swine and rabbits and by inducing adverse liver lesions.[5] Furthermore, it displays hematotoxicity by changing hematological parameters and disrupting blood coagulation in rats. Zearalenone can also cause several alterations in immunological parameters.[6−8] Zearalenone shows cytotoxicity by inhibiting cell viability and macromolecule synthesis and by inducing stress response and apoptosis in different cultured cell lines.[9,10] Zearalenone exhibits genotoxicity by inducing micronuclear and chromosomal aberrations, DNA strand breaks, and DNA adduct formation.[6,7,11,12]

Traditional studies on zearalenone administration have been performed by measuring and comparing a single or several biochemical markers. However, these studies did not sufficiently reflect the overall metabolic status of animals or humans exposed to zearalenone. Molecular system biology has been proposed as a promising alternative for understanding and elucidating the metabolic mechanisms of zearalenone administration. The development of analytical techniques has improved the capability for global assessment of entire classes of biomolecules, such as genome, proteome, and metabolome. Metabolomics facilitates understanding global changes of metabolites in animals or humans in response to alterations in nutrition, genetics, environments, and gut microbiota.[13−15] Moreover, metabolomic approaches have theoretical advantages over genomic, transcriptomic, and proteomic ones because the metabolic network is downstream of gene expression and protein synthesis and because metabolic biomarkers are closely associated with real biological end

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points.[16] Metabolomics also provides a global system interpretation of biological effects. Thus, metabolomic investigations can reveal cellular activities at a functional level. Furthermore, metabolic profiles facilitate reliable data on metabolic alterations caused by certain genes and on toxicological effects and mechanisms of zearalenone. Current metabolic profiles can be more comprehensively characterized using high-throughput analytical tools, such as proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. NMR can facilitate simultaneous quantitative measurement of many metabolites. This method can be used to understand metabolic responses to diet and unrecognized mechanisms. However, only a few studies have focused on the response of animal or human biological systems to zearalenone supplementation.

In this study, we applied an ^{1}H NMR-based metabolomic strategy to examine the global metabolic response of rats to zearalenone administration. Metabolic profiling of rats exposed to zearalenone can be used to reveal the relationship between metabolites and zearalenone supplementation. This approach is also potentially useful to investigate zearalenone metabolism and search for further associations between zearalenone administration and health or disease risk. Furthermore, this study may help in the determination of biochemical mechanisms involved in carcinogenic, estrogenic, and immunosuppressive effects caused by zearalenone exposure. This study aims to investigate the effect of zearalenone administration on the urine and plasma compositions of rats using explorative metabolomic analysis through ^{1}H NMR spectroscopy and chemometrics.

MATERIALS AND METHODS

Animal Experiment and Sample Collection. The protocol of this study was approved by the Animal Care and Use Committee of the Animal Nutrition Institute, Sichuan Agricultural University, and was carried out according to the National Research Council's *Guide for the Care and Use of Laboratory Animals*. A total of 18 6-week-old female Sprague–Dawley rats weighing 130–150 g were placed in individual metabolic cages and allowed to acclimatize for a week. The rats received biweekly (Sunday and Wednesday) intragastric administration of either 3 mg/kg body weight zearalenone (Sigma Chemical Co., St. Louis, MO, USA) alcohol solution (treatment group) or 8% ethyl alcohol (control group) for 2 weeks. Nine rats were selected for each group. Urine samples were collected into ice-cooled vessels with the addition of 30 μL of sodium azide solution (1.0% w/v) at 0–8 and 8–24 h before the first and after the last zearalenone administration, respectively. Blood samples were also collected (9:00 a.m.) from the eye into Eppendorf tubes containing sodium heparin after anesthesia with ether at 24 h after the last zearalenone administration. Blood samples were centrifuged at 3500*g* for 10 min at 4 °C to obtain plasma. All urine and plasma samples were stored at −80 °C until NMR spectroscopic analysis. The dosage selected for this study was based on a prior experiment.[17,18] The rats were given free access to food and drinking water. The experimental conditions throughout the study were as follows: temperature, 22–25 °C; photoperiod, 12 h light/12 h dark; and humidity, 50–70%. Clinical observations were performed during the experimental period, and body weights were determined once a week.

Sample Preparation and NMR Spectroscopy. A 550 μL aliquot of each urine sample was mixed with 55 μL of phosphate buffer (1.5 M NaH_2PO_4/K_2HPO_4, pH 7.4, 100% v/v D_2O) containing 0.1% 3-(trimethylsilyl)propionic-(2,2,3,3-d_4) acid sodium salt (TSP) as chemical shift reference (δ 0.00). After centrifugation at 12000 rpm for 10 min, the supernatant was pipetted into 5 mm NMR tubes for NMR analysis. Plasma samples were prepared by mixing 200 μL of plasma with 400 μL of saline solution containing 50% D_2O (for field frequence lock purposes). Samples of 550 μL were then transferred into 5 mm NMR tubes after vortexing and centrifugation at 11180*g* for 10 min at 4 °C.

The proton NMR spectra of the urine and plasma samples were recorded at 300 K on a Bruker Avance III 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at a ^{1}H frequency of 600.13 MHz with broadband-inverse probe. For urine, a standard water-suppressed one-dimensional NMR spectrum was recorded using the first increment of nuclear Overhauser effect spectroscopy pulse sequence (recycle delay–90°–t_1–90°–t_m–90°–acquire data) with a recycle delay of 2 s, a t_1 of 3 μs, a mixing time (t_m) of 100 ms, and a 90° pulse length of 13.75 μs. A total of 64 transients were acquired into 32000 data points using a spectral width of 12019 Hz and an acquisition time of 1.36 s. A water-presaturated Carr–Purcell–Meiboom–Gill pulse sequence (recycle delay–90°–$(\tau–180°–\tau)_n$–acquisition) was used to attenuate NMR signals from macromolecules. A spin–spin relaxation delay ($2n\tau$) of 200 ms and a spin–echo delay τ of 400 μs were employed. Typically, 90° pulse was set to 10.6 μs, and 32 transients were collected into 32000 data points for each spectrum with a spectral width of 20 ppm. Other acquisition parameters were the same as described above. For assignment purposes, ^{1}H–^{1}H correlation spectroscopy and ^{1}H–^{1}H total correlation spectroscopy were acquired for selected samples.

NMR Spectroscopic Processes and Analysis. The free induction decays were multiplied by an exponential window function with a 1 Hz line-broadening factor prior to Fourier transformation. All NMR spectra were corrected for phase and baseline distortions. The plasma spectral region δ 0.5–9.5 was integrated into regions with an equal width of 0.002 ppm, and the urinary spectral region δ 0.5–10.0 was integrated into regions with an equal width of 0.005 ppm using the AMIX software package (V3.9.2, Bruker-Biospin, Germany). Plasma and urine chemical shifts were referenced to the peak of the methyl proton of L-lactate at δ 1.33 and the peak of TSP at δ 0.00, respectively. Signals from exogenous compounds, including ether, alcohol, and zearalenone metabolites, were carefully discarded together with regions containing urea and H_2O signals to obtain only the endogenous metabolite changes induced by zearalenone exposure. This treatment will avoid any contributions of zearalenone metabolites, alcohol, or ether to intergroup differentiations. In the plasma spectra, the discarded regions include δ 4.50–6.30 for H_2O and urea and δ 1.34–1.38 and δ 3.75–3.77 for ether. In the urine spectra, the discarded regions include δ 4.50–5.05 for H_2O, δ 5.44–6.48 for urea, δ 3.63–3.70 and δ 1.16–1.22 for alcohol, and δ 1.35–1.40, δ 1.50–1.80, δ 1.96–2.02, δ 2.09–2.14, δ 2.31–2.34, δ 2.37–2.40, δ 2.64–2.72, δ 2.81–2.85, δ 6.48–6.51, and δ 6.80–6.85 for zearalenone metabolites. Subsequently, each integral region was normalized to the total sum of all integral regions for each spectrum prior to pattern recognition analysis.

Multivariate data analysis was performed on the normalized NMR data sets with the software package SIMCA-P+ (version 11.0, Umetrics, Sweden). Principal component analysis (PCA) was performed on the data set to generate an overview. The projection to latent structure–discriminant analysis (PLS–DA) and the orthogonal projection to latent structure–discriminant analysis (OPLS–DA) were further carried out using the unit-variance scaled NMR data as *X*-matrix and class information as *Y*-matrix.[19] The quality of the model was assessed by the parameters R^2X and Q^2, which represent the total explained variations for *X* matrix and the model predictability, respectively. The models were certified using a 7-fold cross -validation method and a permutation test.[20,21] A model was considered significant if the Q^2 value was significant ($P < 0.05$) through permutation. The OPLS–DA models were interpreted by coefficient-coded loading plots. The loadings were backtransformed in Excel (Microsoft, Redmond, WA, USA) and plotted with color-coded absolute coefficient values ($|r|$) of the variables in MATLAB (The Mathworks Inc., Natwick, MA, USA; version 7.1).[20] Metabolites that contributed most to the prediction of the response (class) are shown in red, whereas those that had slight/no association with the response are shown in blue. In this study, appropriate correlation coefficients were used as the cutoff values (depending upon the number of animals used for each group) for the statistical significance based on the

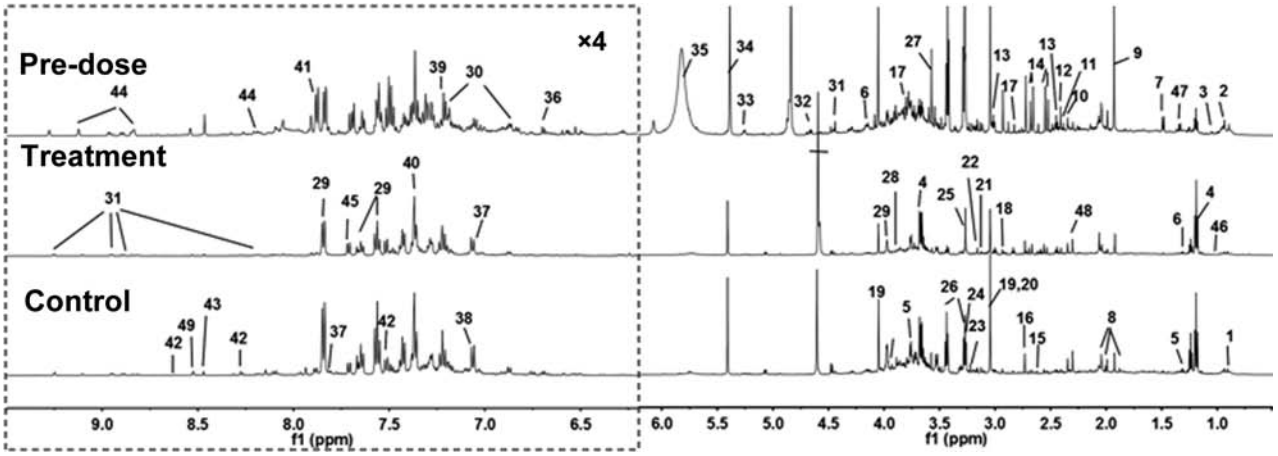

Figure 1. Representative one-dimensional ^{1}H NMR spectra of 0−8 h urine metabolites obtained from the control (alcohol), treatment (zearalenone and alcohol), and predose groups. The region of δ 6.2−9.5 was magnified 4 times compared with the corresponding region of δ 0.5−6.2 for the purpose of clarity. Metabolite keys are given in Table 1.

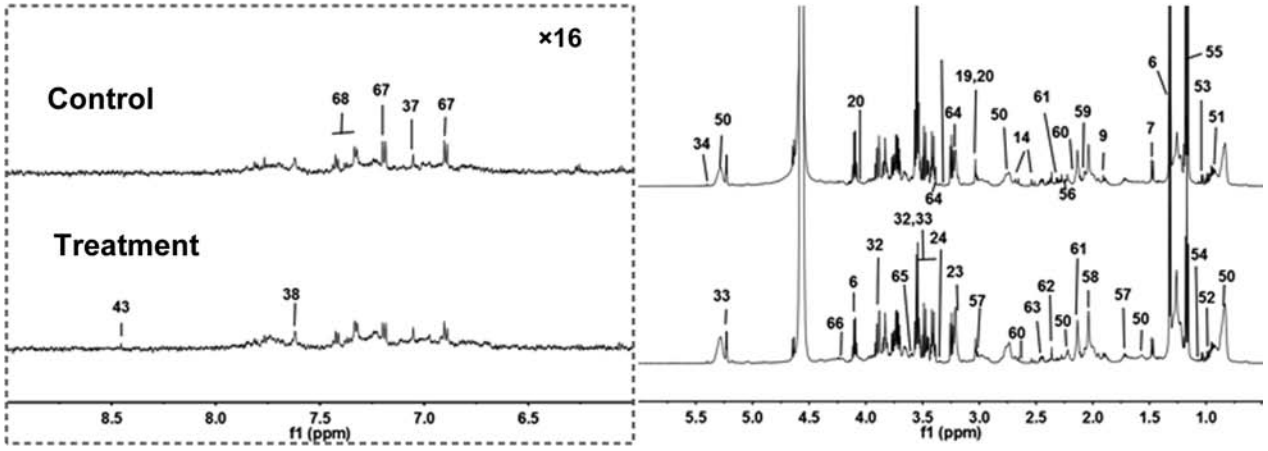

Figure 2. Typical 600 MHz ^{1}H NMR spectra of 24 h plasma metabolites obtained from the treatment (zearalenone and alcohol) and control groups (alcohol). The region of δ 6.0−9.0 was magnified 16 times compared with corresponding region of δ 0.5−6.0 for the purpose of clarity. Metabolite keys are given in Table 1.

discrimination significance ($P < 0.05$). These coefficients were determined using Pearson's product-moment correlation coefficient.[20]

RESULTS AND DISCUSSION

^{1}H NMR Spectra of Urine and Plasma Samples. Figure 1 shows the representative ^{1}H NMR spectra of 0−8 h urine samples taken from randomly selected rats of the treatment (zearalenone and alcohol), control (alcohol), and predose groups. Figure 2 shows typical spectra of rat plasma from the treatment (zearalenone and alcohol), control (alcohol). NMR signals were assigned to specific metabolites for ^{1}H resonances (Table 1). A total of 45 metabolites were unambiguously assigned for urine. The spectra of the urine samples contained resonances from several amino acids, glucose, organic acids, allantoin, and choline. Tricarboxylic acid cycle metabolites, such as succinate and citrate, were also detected in the urine samples. Moreover, it was found that plasma samples mainly contained glucose, lactate, lipids, and a series of amino acids.

Multivariate Data Analysis of NMR Data. PCA was initially performed on the plasma spectral data. Two principal components were calculated for the treatment groups, with 58.2 and 30.3% of the variables explained by PC1 and PC2, respectively. PCA results (Figure 3A) showed that separations in rats from the treatment and control groups were absent in their metabolic plasma profiles. Furthermore, the plasma metabolic changes in the rats from the treatment and control groups were analyzed using OPLS−DA. The corresponding coefficient analysis showed that zearalenone significantly increased the plasma levels of α-glucose, lactate, *N*-acetyl glycoprotein, *O*-acetyl glycoprotein, and propionate ($P < 0.05$). By contrast, zearalenone significantly decreased the plasma levels of glycerolphosphocholine, phosphocholine, isoleucine, leucine, valine, tyrosine, and lipid ($P < 0.05$, Table 3). PLS−DA was conducted on the urine spectra of the treatment, control, and predose groups at 0−8 h before the first and after the last zearalenone administration. The score plots (Figure 3B) clearly highlighted three clusters corresponding to the three groups. The metabolic profile of the treatment group was compared with that of the control group using OPLS−DA to further identify the important urine metabolic changes induced by zearalenone supplementation. Multivariate data analysis showed that the urine levels of allantoin, choline, and *N*-methylnicotinamide were higher in the treatment group than in the control group ($P < 0.05$). By contrast, the urine levels of butyrate, homogentisate, lactate, and nicotinate were lower in the treatment group than in the control group ($P < 0.05$, Figure

Table 1. ^{1}H NMR Data for Metabolites in Rat Urine and Plasma

key	metabolite	moieties	δ ^{1}H (ppm) and multiplicity	sample[a]
1	butyrate	CH_3	0.9 (t)	U
2	α-hydroxybutyrate	CH_3	0.94 (t)	U
3	isobutyrate	CH_3	1.14 (d)	U
4	ethanol	CH_3, CH_2	1.19 (t), 3.66 (q)	U
5	methylmalonate	CH_3, CH	1.26 (d), 3.76 (m)	U
6	lactate	αCH, βCH_3	4.13 (q), 1.33 (d)	U, P
7	alanine	αCH, βCH_3	3.77 (q), 1.48 (d)	U, P
8	*N*-acetylglutamate	αCH, βCH_2, γCH_2, CH_3	4.13 (m), 2.07 (m), 1.88 (m), 2.04 (s),	U
9	acetate	CH_3	1.92 (s)	U, P
10	acetone	CH_3	2.25 (s)	U
11	acetoacetate	CH_3	2.3 (s)	U
12	succinate	CH_2	2.41 (s)	U
13	α-ketoglutarate	βCH_2, γCH_2	2.45 (t), 3.01 (t)	U
14	citrate	CH_2	2.55 (d), 2.68 (d)	U, P
15	methylamine	CH_3	2.62 (s)	U
16	dimethylamine	CH_3	2.73 (s)	U
17	guanidoacetate	CH_2	3.8 (s)	U
18	dimethylglycine	CH_3	2.93 (s)	U
19	creatine	CH_3, CH_2	3.04 (s), 3.93 (s)	U, P
20	creatinine	CH_3, CH_2	3.04 (s), 4.05 (s)	U, P
21	ethanolamine	CH_2	3.13 (t)	U
22	malonate	CH_2	3.16 (s)	U
23	choline	OCH_2, NCH_2, $N(CH_3)_3$	4.07(t), 3.53(t), 3.20(s)	U, P
24	phosphocholine	$N(CH_3)_3$, OCH_2, NCH_2	3.22(s), 4.21(t), 3.61(t)	U, P
25	trimethylamine-*N*-oxide	CH_3	3.28 (s)	U
26	taurine	$-CH_2-S$, $-CH_2-NH_2$	3.26 (t), 3.43 (t)	U
27	glycine	CH_2	3.57 (s)	U
28	betaine	CH_3, CH_2	3.27 (s), 3.90 (s)	U
29	hippurate	CH_2, 3,5-CH, 4-CH, 2,6-CH	3.97 (d), 7.57 (t), 7.65 (t), 7.84 (d)	U
30	*p*-hydroxyphenylacetate	6-CH, 2-CH, 3,5-CH	3.6 (s), 6.87 (d), 7.15 (d)	U
31	*N*-methylnicotinamide	CH_3, 5-CH, 4-CH, 6-CH, CH_2	4.44 (s), 8.18 (d), 8.89 (d), 8.96 (d), 9.26 (s)	U
32	β-glucose	1-CH, 2-CH, 3-CH, 4-CH, 5-CH, 6-CH	4.65 (d), 3.25 (dd), 3.49 (t), 3.41 (dd), 3.46 (m), 3.73 (dd), 3.90 (dd)	U, P
33	α-glucose	1-CH, 2-CH, 3-CH, 4-CH, 5-CH, 6-CH	5.24 (d), 3.54 (dd), 3.71 (dd), 3.42 (dd), 3.84 (m), 3.78 (m)	U, P
34	allantoin	CH	5.40 (s)	U, P
35	urea	NH_2	5.82 (s)	U
36	homogentisate	6-CH, 5-CH	6.7 (d), 6.76 (d)	U
37	1-methylhistidine	4-CH, 2-CH	7.05 (s), 7.78 (s)	U, P
38	3-methylhistidine	4-CH, 2-CH	7.07 (s), 7.67 (s)	U
39	indoxyl sulfate	4-CH, 5-CH, 6-CH, 7-CH, CH3	7.51 (m), 7.22 (m), 7.28 (m), 7.71 (m), 7.37 (s)	U
40	phenylacetylglycine	2,6-CH, 3,5-CH, 7-CH, 10-CH	7.31 (t), 7.37 (m), 7.42 (m), 3.68 (s)	U
41	benzoate	2,6-CH, 3,5-CH, 4-CH	7.88 (d), 7.49 (dd), 7.56 (t)	U
42	nicotinate	2,6-CH, 4-CH, 5-CH	8.62 (d), 8.25 (d), 7.5 (dd)	U

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Table 1. continued

key	metabolite	moieties	δ ^{1}H (ppm) and multiplicity	sample[a]
43	formate	CH	8.46 (s)	U
44	trigonelline	2-CH, 4-CH, 6-CH, 5-CH, CH3	9.12 (s), 8.85 (m), 8.83 (dd), 8.19 (m), 4.44 (s)	U
45	4-aminohippurate	CH_2	7.71 (d)	U
46	unknown 1		1.05 (t)	U
47	unknown 2		1.24 (t)	U
48	unknown 3		2.35 (s)	U
49	unknown 4		8.54 (d)	U
50	lipid	$CH_3(CH_2)_n$, $CH_3CH_2CH_2C{=}$, $(CH_2)_n$, CH_2CH_2CO, $CH_2C{=}C$, CH_2CO, $C{=}CCH_2C{=}C$, $-CH{=}CH-$	0.84 (t), 0.89 (t), 1.28 (m), 1.58 (m), 2.01(m), 2.24 (m), 2.76 (m), 5.30 (m)	P
51	isoleucine	αCH, βCH, βCH_3, γCH_2, δCH_3	3.68 (d), 1.99 (m), 1.01 (d), 1.26 (m), 1.47 (m), 0.94 (t)	P
52	leucine	αCH, βCH_2, γCH, δCH_3	3.73 (t), 1.72 (m), 1.72 (m), 0.96 (d), 0.97 (d)	P
53	valine	αCH, βCH, γCH_3	3.62 (d), 2.28 (m), 0.99 (d), 1.04 (d)	P
54	propionate	CH_3, CH_2	1.08 (t), 2.18 (q)	P
55	ether	CH_3, CH_2	1.18 (t), 3.55 (q)	P
56	3-hydroxybutyrate	αCH_2, βCH, γCH_3	2.28 (dd), 2.42 (dd), 4.16 (m), 1.20 (d)	P
57	lysine	αCH, βCH_2, γCH_2, εCH_2	3.76 (t), 1.91 (m), 1.48 (m), 1.72 (m), 3.01 (t)	P
58	*N*-acetyl glycoprotein	CH_3	2.04 (s)	P
59	*O*-acetyl glycoprotein	CH_3	2.08 (s)	P
60	methionine	αCH, βCH_2, γCH_2, $S-CH_3$	3.87 (t), 2.16 (m), 2.65 (t), 2.14 (s)	P
61	glutamate	αCH, βCH_2, γCH_2	3.75 (m), 2.12 (m), 2.35 (m)	P
62	pyruvate	CH_3	2.37 (s)	P
63	glutamine	αCH, βCH_2, γCH_2	3.78 (m), 2.14 (m), 2.45 (m)	P
64	glycerolphosphocholine	CH_3, βCH_2, αCH_2	3.22 (s), 3.69 (t), 4.33 (t)	P
65	*myo*-inositol	1,3-CH, 2-CH, 5-CH, 4,6-CH	3.60 (dd), 4.06 (t), 3.30 (t), 3.63 (t)	P
66	threonine	αCH, βCH, γCH_3	3.58 (d), 4.24 (m), 1.32 (d)	P
67	tyrosine	2,6-CH, 3,5-CH	7.20 (dd), 6.91 (d)	P
68	phenylalanine	2,6-CH, 3,5-CH, 4-CH	7.32 (m), 7.42 (m), 7.37 (m)	P

[a]U, urine; P, plasma.

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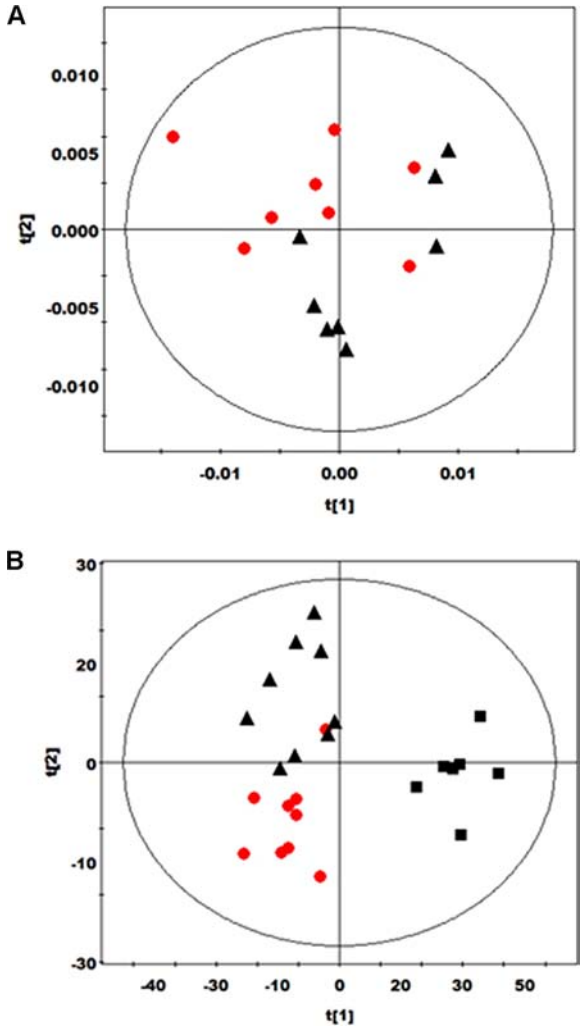

Figure 3. (A) PCA score plots (R^2X = 0.967, Q^2 = 0.923) based on the ^{1}H NMR spectra of 24 h plasma metabolites obtained from the treatment (zearalenone and alcohol) (red circles) and control groups (alcohol) (black triangles). One sample from treatment and control groups was excluded due to hemolysis, respectively. (B) PLS−DA score plots (R^2X = 0.548, R^2Y = 0.929, Q^2 = 0.73) based on the ^{1}H NMR spectra of the urine obtained from 0 to 8 h urine metabolites obtained from the treatment (zearalenone and alcohol) (red circles), control (alcohol) (black triangles), and predose groups (black squares). Two samples from predose groups were excluded due to contamination.

4 and Table 2). We also compared the metabolic profile of the treatment group with that of the predose group (0−8 h) using OPLS−DA to observe the effect of zearalenone and alcohol supplementation. The urine levels of 1-methylhistidine, 3-methylhistidine, acetoacetate, butyrate, choline, ethanolamine, hippurate, isobutyrate, lactate, methylmalonate, *N*-acetylglutamate, and unknown 2 were significantly higher in the treatment group than in the predose group ($P < 0.05$). By contrast, the urine levels of *α*-glucose, acetone, allantoin, benzoate, creatine, creatinine, dimethylamine, dimethylglycine, formate, guanidinoacetate, homogentisate, nicotinate, phenylacetylglycine, *p*-hydroxyphenylacetate, and trigonelline were lower in the treatment group than in the predose group ($P < 0.05$, Table 2).

OPLS−DA at the 8−24 h time point (last dose) was carried out to determine the degree of influence of zearalenone supplementation on metabolism. The urine levels of 1-methylhistidine, acetoacetate, acetone, indoxyl sulfate, *N*-acetylglutamate, and unknown 3 were significantly higher in the treatment group than in the control group ($P < 0.05$, Table 2). Moreover, most of the altered metabolites in the treatment group were similar to those in the predose group (8−24 h), with exceptional changes in the levels of 4-aminohippurate, *β*-glucose, *α*-ketoglutarate, acetate, dimethylamine, dimethylglycine, indoxyl sulfate, malonate, *N*-methylnicotinamide, succinate, and unknown 1 (Table 2).

Table 2. OPLS−DA Coefficients Derived from the NMR Data of 0−8 and 8−24 h Urine Metabolites Obtained from the (A) Control (Alcohol), (B) Treatment (Zearalenone and Alcohol), and (C) Predose Groups

	B (vs A)[a]		B (vs C)[a]	
metabolite	0−8 h after last dose	8−24 h after last dose	0−8 h after last dose	8−24 h after last dose
1-methylhistidine (37)	–	0.635	0.810	–
3-methylhistidine (38)	–	–	0.966	0.895
4-amimohippurate (45)	–	–	–	0.815
α-glucose (33)	–	–	−0.728	–
β-glucose (32)	–	–	–	−0.838
α-ketoglutarate (13)	–	–	–	0.876
acetate (9)	–	–	–	0.760
acetoacetate (11)	–	0.788	0.848	0.882
acetone (10)	–	0.706	−0.792	–
allantoin (34)	0.809	–	−0.743	–
benzoate (41)	–	–	−0.743	–
butyrate (1)	−0.673	–	0.809	0.938
choline (23)	0.677	–	0.878	–
creatine (19)	–	–	−0.726	–
creatinine (20)	–	–	−0.726	–
dimethylamine (16)	–	–	−0.937	0.847
dimethylglycine (18)	–	–	−0.761	0.714
ethanolamine (21)	–	–	0.840	0.828
formate (43)	–	–	−0.781	–
guanidoacetate (17)	–	–	−0.929	−0.886
hippurate (29)	–	–	0.803	0.751
homogentisate (36)	−0.635	–	−0.730	–
indoxyl sulfate (39)	–	0.700	–	−0.885
isobutyrate (3)	–	–	0.799	–
lactate (6)	−0.640	–	0.886	0.852
malonate (22)	–	–	–	0.763
methylmalonate (5)	–	–	0.959	0.913
N-acetylglutamate (8)	–	0.692	0.809	0.901
nicotinate (42)	−0.645	–	−0.914	−0.904
N-methylnicotinamide (31)	0.639	–	–	0.823
phenylacetylglycine (40)	–	–	−0.896	−0.871
p-hydroxyphenylacetate (30)	–	–	−0.959	−0.892
succinate (12)	–	–	–	−0.713
trigonelline (44)	–	–	−0.984	−0.973
unknown 1 (46)	–	–	–	0.842
unknown 2 (47)	–	–	0.895	0.921
unknown 3 (48)	–	0.720	–	–

[a]Correlation coefficients; positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of |r| > 0.707 (for B vs C) or 0.632 (for B vs A) was used as the cutoff value. "–" means the correlation coefficient |r| is <0.707 (for B vs C) or <0.632 (for B vs A).

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Table 3. OPLS−DA Coefficients Derived from the NMR Data of 24 h Plasma Metabolites Obtained from the (A) Control Groups (Alcohol) and (B) Treatment (Zearalenone and Alcohol)

metabolite	B (vs A)[a]
α-glucose (33)	0.784
glycerolphosphocholine (64)	−0.853
isoleucine (51)	−0.769
lactate (6)	0.675
leucine (52)	−0.843
N-acetyl glycoprotein (58)	0.711
O-acetyl glycoprotein (59)	0.721
phosphocholine (24)	−0.852
propionate (54)	0.703
tyrosine (67)	−0.790
valine (53)	−0.853
lipid (50)	−0.791

[a]Correlation coefficients; positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of $|r| > 0.666$ was used as the cutoff value.

Effects of Zearalenone Supplementation. Zearalenone exposure can improve lipid oxidation. We observed that the levels of urinary ketone bodies, such as acetone and acetoacetate, also increased in the treatment group. These ketone bodies are the products of β-oxidation of fatty acid in the mitochondria. The increase in the levels of these biochemicals suggested that zearalenone exposure promoted the β-oxidation of fatty acids. Furthermore, acetoacetate and 3-hydroxybutyrate are products of fatty acid oxidation in the liver, and their ratios are useful indicators of mitochondrial redox state. Zearalenone supplementation increased the urine level of acetoacetate but did not change that of 3-hydroxybutyrate. As a result, the acetoacetate/3-hydroxybutyrate ratio also increased. This result further suggests a more oxidized state of cells, which can be brought about by enhanced oxidation of fatty acids. This finding is in agreement with that of a previous study; that is, zearalenone can induce lipid peroxidation.[22] Lipid oxidation often leads to generation of hydrogen peroxide, thereby causing free radical oxidative damage. Oxidative stress followed by lipid oxidation has previously been reported in zearalenone toxicity; reactive oxygen species, such as H_2O_2 and O_2^-, participate in the main mechanism of zearalenone toxicity.[12] Thus, zearalenone supplementation can induce oxidative stress response in rats. The *O*-acetylated carbohydrate-bound protein resonance is found in rat blood plasma and can be considered as alternative "acute-phase" glycoproteins in animal models of human inflammatory conditions.[23] The concentrations of plasma "acute phase" *N*-acetyl glycoproteins are markedly elevated in a range of abnormal clinical conditions, including inflammatory disease, cancer, and certain liver diseases.[24] These "acute phase" acetyl glycoproteins are predominantly synthesized in liver parenchymal cells in response to cytokines.[25] In the current study, *O*-acetyl glycoproteins and *N*-acetyl glycoproteins were present with higher signal intensity in the spectra of plasma from the treatment group compared with the control group. Elevated levels of *N*-acetyl glycoproteins and *O*-acetyl glycoproteins in blood plasma of oxidatively stressed animals are consistent with previous investigations of the metabolic response to stress.[26]

Moreover, urinary allantoin levels were increased by zearalenone exposure. Allantoin is a product of purine metabolism in most mammals. The presence of allantoin in the urine can be created via nonenzymatic means through high levels of reactive oxygen species. Thus, allantoin can be used as a marker of oxidative stress. Furthermore, zearalenone supplementation increased the level of indoxyl sulfate at 8−24 h of the last dose. Indoxyl sulfate is a circulating uremic toxin stimulating glomerular sclerosis and interstitial fibrosis.

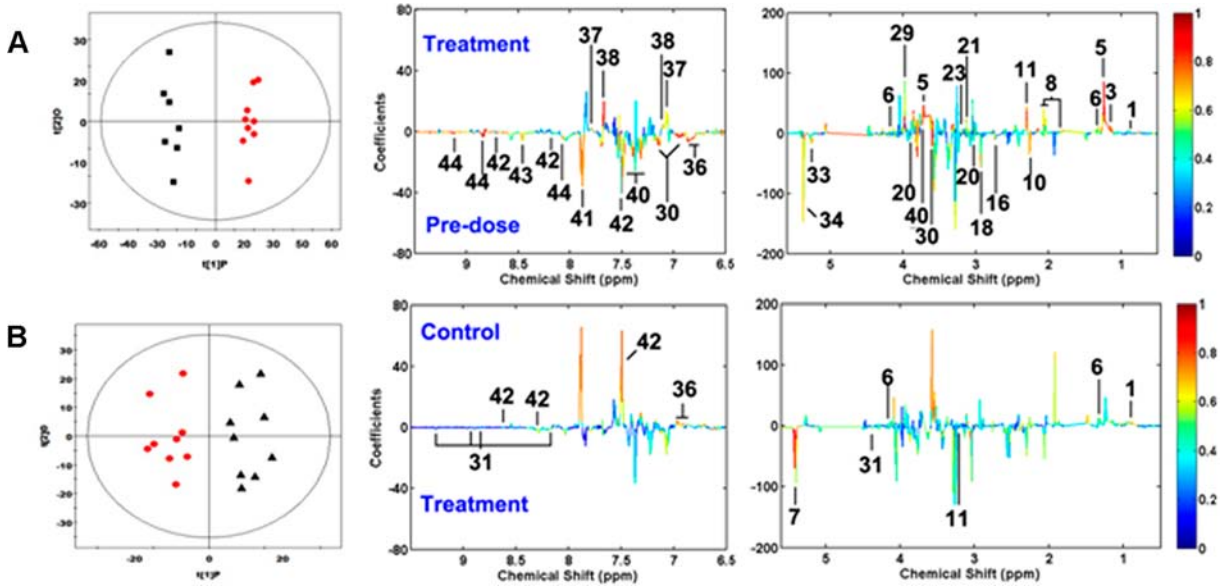

Figure 4. OPLS−DA scores plots (left panel) and the corresponding coefficient loading plots (right panel) derived from the ^{1}H NMR spectra of 0−8 h urine in the treatment (zearalenone and alcohol) (red circles), control (alcohol) (black triangles), and predose (black squares) groups (A, R^2X = 45.7%, Q^2 = 0.932; B, R^2X = 28.2%, Q^2 = 0.412). Two samples from predose groups were excluded due to contamination. The color map shows the significance of metabolite variations between the two classes. The peaks in the positive direction indicate the metabolites that are more abundant in the groups in the positive direction of the first principal component. The metabolites that are more abundant in the groups in the negative direction of the first primary component are presented as peaks in the negative direction.

Indoxyl sulfate is one of the well-known substances of a group of protein-bound uremic retention solutes. Some studies suggested that indoxyl sulfate is also involved in oxidative stress. Thus, the elevation of indoxyl sulfate level indicates that reactive oxygen species production may be increased in rats. Oxidative stress ultimately triggers an antioxidative response of an organism. In the present study, we observed elevated levels of *N*-methylnicotinamide and decreased levels of nicotinate in the treatment group. This result is consistent with the antioxidative responses activated by zearalenone. *N*-Methylnicotinamide is the methylated metabolite of nicotinamide, which can be generated during the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine in the biosynthesis of cysteine, an essential amino acid of glutathione synthesis.[27] This finding implies that rats can utilize an antioxidative vitamin B_3 cycle to decrease oxidative stress induced by zearalenone exposure. Taken together, zearalenone exposure can cause oxidative stress in rats.

Zearalenone exposure can promote glycogenolysis and glycolysis. In the present study, the treatment group exhibited significant elevation of plasma glucose compared with the control group. This finding is consistent with that of a previous study; that is, increased glucose utilization is a major metabolic effect of mycotoxin exposure.[28] Increased lactate concentration was also observed in the plasma of the treatment group. This result is in agreement with that of previous research; that is, zearalenone supplementation (100 μg/kg) increases serum lactate dehydrogenase in rabbits.[5] Lactate is associated with energy metabolism and is the end product of compounds involved in energy metabolism. Increased lactate level is related to increased anaerobic glycolysis. In addition, an increased plasma lactate level implies inhibited gluconeogenesis and altered carbohydrate and energy metabolism.

Zearalenone exposure can affect cell membrane metabolism. Choline, phosphorylcholine, and glycerolphosphocholine are elements essential for the structural integrity of the cell membrane. These elements also have important functions in cell metabolism and signaling processes.[29,30] In addition, choline, glycerolphosphocholine, and phosphorylcholine have crucial functions in lipid cholesterol transport and metabolism.[30] In the present study, choline was increased and phosphorylcholine and glycerolphosphocholine were decreased in the treatment group compared with the control group. The possible explanation is that the structural integrity of the cell membrane was decreased.

Zearalenone exposure can alter amino acid metabolism. In the present study, plasma tyrosine was decreased by zearalenone supplementation. This observation implies that zearalenone exposure can also inhibit protein synthesis because tyrosine is involved in protein synthesis. This result is in agreement with that of a previous study; that is, zearalenone can inhibit protein synthesis.[22] Moreover, 1-methylhistidine levels increased with zearalenone supplementation. This finding implies that muscle protein may be broken down by zearalenone exposure. Our results also showed that the levels of all branched-chain amino acids were decreased by zearalenone supplementation. The possible reason is that stress-induced increases in energy expenditure can also cause elevated consumption of amino acids, such as alanine, valine, and isoleucine, to provide energy.

Zearalenone exposure can change gut microbiota metabolism. Short-chain fatty acids (such as propionate and butyrate) produced by bacteria in the colon through fermentation of unabsorbed dietary fiber provide energy for metabolism in the colon. In this study, propionate was increased, whereas butyrate was decreased. The possible reason is that gut microbiota can either manufacture or utilize these products.

Effects of Zearalenone and Alcohol Supplementation. Dimethylglycine is produced in cells as an intermediate in the metabolism of choline to glycine. This compound also acts as a detoxifying agent and an antioxidant, protecting body cells from unwanted reactions caused by free radicals. Dimethylglycine can also serve as a stress reducer.[31] In the present study, dimethylglycine levels were decreased at 0−8 h but were increased at 8−24 h. Thus, the elevation of dimethylglycine can trigger antioxidative responses activated by zearalenone and alcohol supplementation. Moreover, 4-aminohippurate is an acyl glycine, a minor metabolite of fatty acids. Previous studies observed that the excretion of certain acyl glycines is increased in several inborn errors of metabolism. In certain cases, the measurement of these metabolites in body fluids can be used to diagnose disorders associated with mitochondrial fatty acid β-oxidation. In the present study, the 4-aminohippurate level was increased at 8−24 h of the last dose, indicating the development of disorders associated with mitochondrial fatty acid β-oxidation. Furthermore, the urinary lactate level was elevated. Increased lactate level is related to increased anaerobic glycolysis. Elevated urinary α-ketoglutarate and decreased urinary citrate in the treatment group suggest alterations in the tricarboxylic acid cycle. This study is the first to show decreased urinary excretion of formate and nitrogenous compounds (dimethylamine) and increased urinary excretion of short-chain fatty acids (acetate and isobutyrates) in rats exposed to zearalenone and alcohol supplementation. Notably, they are microbial metabolites of carbohydrates and amino acids,[32,33] which are possibly produced in the lumen of the small and large intestines. Intestinal microbes convert dietary nondigestible fibers into short-chain fatty acids and other nutrients that can be utilized by the mammalian host as energy sources and as precursors for fatty acid synthesis.[34] The introduction of zearalenone and alcohol supplementation into the mammalian system may displace baseline mammalian-to-microbial behavior, thereby causing a disruption in microbial populations and, hence, in metabolism. Changes in these metabolites can be attributed to reduced number and/or altered activity of intestinal microorganisms.[35] Results of the current study also indicated that zearalenone and alcohol increased the urinary excretion of hippurate produced through both renal and hepatic syntheses of glycine and benzoic acid. Hippurate is also the degradation product of flavonols acted upon by intestinal microorganisms.[36] Variations in urinary hippurate concentration have also been linked to changes in the distribution of intestinal microbial colonies.[37] The altered levels of gut microbial co-metabolites, including phenylacetylglycine and *p*-hydroxyphenylacetate, also verified the association of disturbance to gut microbiota with zearalenone and alcohol exposure. Phenylacetate was transformed from phenylalanine via the action of gut microbiota; phenylacetate was then conjugated with glycine to form phenylacetylglycine.[37] Previous studies suggested that elevated levels of urinary phenylacetylglycine are exhibited in abnormal accumulation of phospholipids in rat liver and that these levels can serve as a surrogate biomarker for associated changes in gut microbiota.[38] Acyl-CoA has a major function in glycine conjugation;[39] however, whether this enzyme is modified by zearalenone and alcohol exposure remains unclear. Moreover, *p*-hydroxypheny-

lacetate is a metabolite of tyrosine via enteric bacteria. Mammalian metabolism is significantly affected by its interaction with the complex gut microbial community.[40] Gut microbiota significantly affects the development and structure of the intestinal epithelium, the digestive and absorptive capabilities of the intestine, and the host immune system.[41] Possible disturbances of gut microbiota by zearalenone and alcohol administration can affect health status. Microbiological identification of specific changes in the microbiota community can be helpful in addressing the metabolic implications of zearalenone and alcohol supplementation.

In conclusion, zearalenone exposure affects the urine and plasma metabolome of rats. Zearalenone exposure can cause oxidative stress and some common systemic metabolic changes, including cell membrane metabolism, protein biosynthesis, glycolysis, and gut microbiota metabolism. To the best of our knowledge, this study is the first in vivo report on the response of animal biological systems to zearalenone supplementation. Future studies may be directed toward a mechanistic understanding of the effects of zearalenone on animal tissue intermediary metabolism.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Structure of zearalenone. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

PCA, principal component analysis; PLS−DA, projection to latent structure−discriminant analysis; OPLS−DA, orthogonal projection to latent structure−discriminant analysis; TSP, 3-(trimethylsilyl)propionic-(2,2,3,3-d_4) acid sodium salt

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