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^{1}H NMR and MVA metabolomic profiles of urines from piglets fed with boluses contaminated with a mixture of five mycotoxins

Sandra A. De Pascali[a], Lucia Gambacorta[b], Isabelle P. Oswald[c], Laura Del Coco[a], Michele Solfrizzo[b,*], Francesco Paolo Fanizzi[a,*]

[a] University of Salento, Di.S.Te.B.A., Campus Ecotekne, via Provle Lecce-Monteroni, 73100, Lecce, Italy
[b] Institute of Sciences of Food Production (ISPA), National Research Council of Italy (CNR), Via Amendola 122/O, 70126 Bari, Italy
[c] UMR 1331 Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, 180 Chemin de Tournefeuille, F-31027 Toulouse, Cedex, France

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ABSTRACT

Metabolic profile of urine from piglets administered with single boluses contaminated with mycotoxin mixture (deoxynivalenol, aflatoxin B_1, fumonisin B_1, zearalenone, and ochratoxin A) were studied by ^{1}H NMR spectroscopy and chemometrics (PCA, PLS-DA, and OPLS-DA). The mycotoxin levels were close to the established maximum and guidance levels for animal feed (2003/100/EC and 2006/576/EC). Urine samples were obtained from four groups of four piglets before (control, C) or within 24 h (treated, T) after receiving a contaminated boluses with increasing doses of mycotoxins (boluses 1–4). For the two highest dose groups, the urines were collected also after one week of wash out (W). For the two lowest doses groups no significant differences between the C and T samples were observed. By contrast, for the two highest doses groups the T urines separated from the controls for a higher relative content of creatinine, *p*-cresol glucuronide and phenyl acetyl glycine and lower concentration of betaine and TMAO. Interestingly, a similar profile was found for both W and T urines suggesting, at least for the highest doses used, serious alteration after a single bolus of mycotoxin mixture.

1. Introduction

Mycotoxins can be frequently found in the food chain because of fungal infection of crops to be either consumed directly by Humans or used as livestock feed. Consumption of contaminated foodstuffs can produce teratogenic, carcinogenic, neurotoxic, estrogenic, and immunosuppressive effects (both acute and chronic) in Humans and animals [1,2]. It is worth noting that the most frequently contaminated food crops are cereals and dried fruits. The production and accumulation of mycotoxins can take place at different levels in the food chain (pre-harvest, harvest and storage) and inadequate agricultural, harvesting, storage, packaging and transport practices could increase their content. The EU and several countries worldwide have in force specific regulations to restrain Human and animal exposure to the principal mycotoxins but also to avoid an impact on commerce worldwide.

The toxicokinetic of some mycotoxins such as ochratoxin A (OTA) and aflatoxin B_1 (AFB_1) could result in accumulation in different organs or tissues and excretion in milk. The mycotoxins can therefore enter the food chain also through meat, milk, or eggs obtained from livestock fed with contaminated feed, with a significant health risk for consumers. Recently, Streit and coworkers analyzed for contamination with several mycotoxins 17316 samples of feed and feed raw materials from all over the world during an 8-year period, reporting that overall, 72% of the samples tested positive for at least one mycotoxin and 38% were found to be co-contaminated [3].

The importance of the mycotoxin contamination of feed for farm animals has been recognized by the European Food Safety Authority [4–8]. Based on these EFSA opinions the Commission of the European Communities (CEC) established a maximum level for AFB_1 (20 μg/kg) in animal feed (Commission Directive 2003/100/EC) and the so-called guidance levels for deoxynivalenol (DON, 900 μg/kg), zearalenone (ZEN, 100–250 μg/kg) [9], OTA (50 μg/kg) and fumonisin $B_1 + B_2$ ($FB_1 + FB_2$, 5000 μg/kg) (Commission Recommendation 2006/576/EC). The purpose of these guidance levels is to protect farm animals from possible deleterious effects of contaminated feed and to ensure awareness of all involved economic parties and supervising authorities if the critical concentrations are exceeded [1]. A large number of fungal genera, belonging to various toxigenic fungi such as *Apergillus*, *Peni-*

* Corresponding authors.
E-mail addresses: michele.solfrizzo@ispa.cnr.it (M. Solfrizzo), fp.fanizzi@unisalento.it (F.P. Fanizzi).

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cillium, *Alternaria* and *Fusarium* spp. are able to produce several mycotoxins as secondary metabolites. The most relevant mycotoxins found in food and feed are produced by three fungal genera: aflatoxins, produced by *Aspergillus* species; OTA by both *Aspergillus* and *Penicillium*; trichothecenes (e.g. DON), ZEN and FB_1 produced mainly by *Fusarium* species [1]. Moreover, also specific environmental conditions, such as temperature, water activity, oxygen level, physical damage, insects, amount of fungal inoculum, are important factors that affect fungal growth and mycotoxin formation and accumulation both in the field and during storage of food crops [10]. DON, AFB_1, FB_1, ZEN and OTA are the main toxicologically relevant mycotoxins frequently occurring in cereals and cereal-based feed. These mycotoxins and their metabolites have been recently quantified in vivo in piglet urines in dose dependent manner [11].

In the present work the metabolic profile of 39 urine samples has been studied by proton nuclear magnetic resonance (^{1}H NMR) spectroscopy and multivariate analysis (MVA). These samples were already analyzed for their mycotoxin content [11]. This approach aims to assess potential differences in the overall metabolic response of piglets fed with single boluses contaminated, in most cases, within the guidance values for mycotoxins in feed. Metabolomic is a well-established methodology, based on ^{1}H NMR spectroscopy or HPLC-MS techniques assisted with multivariate statistical analysis [12], which provides an effective method for evaluating the metabolic responses of living organisms to physiological and pathological stresses. Due to its efficient and non-invasive characteristics, NMR spectroscopy, used in combination with multivariate data analyses, has been indicated as an effective analytical tool that can provide comprehensive information on the metabolic profiles in different fields. These include, among the others, drug toxicity evaluations [13,14], nutritional studies [15,16] and mammalian–parasite interactions [17,18].

The pig is an important model of human disease and nutrition. Moreover, urine is potentially the most useful metabolic diagnostic matrix in the pig to examine the relationship between metabolites and a specific pathophysiological status [19,20]. However, only few studies on animal or human biological system response to mycotoxin supplementation have been reported [21–23]. To the best of our knowledge, this work is the first in vivo study on an animal model, the pig, to a mycotoxin mixture administration considering the EC guidelines concentration limits.

2. Materials and methods

The urine samples of piglets analyzed in this study were previously obtained with an in vivo experiment aimed to validate urinary biomarkers of DON, AFB_1, FB_1, ZEN and OTA [11]. The design of the in vivo experiment used in the study [11] is reported below whereas the preparation of contaminated boluses and mycotoxin analysis of commercial diet are reported elsewhere [11].

2.1. Design of the in vivo experiments with piglets

Sixteen 4-week-old weaned piglets (Pietrain/Duroc/Large-white) and weighing 10.56 ± 1.88 kg at the beginning of the experiment were procured locally. Animals were acclimatized for 1 week in the animal facility of the INRA ToxAlim Unit (Toulouse, France) prior to being used in experimental protocols. During the acclimation and experimental periods, animals were given free access to water. Except during the urine collection periods, animals were fed a commercial diet ad libitum. Four groups of piglets (four piglets per group) were administered boluses contaminated with mixtures of DON, AFB_1, FB_1, ZEN and OTA at different levels (Bolus 1–4, Table 1). The feed was removed the evening before the experiment thus the animal had no access to feed overnight. Depending of the animal, the bolus was eaten within 1–2 h. After consumption of the bolus each piglet was housed in a metabolic cage to collect 24 h urine. Urine samples from each piglet were collected 3 times at regular intervals within 24 h (sample T) and their volumes were measured then the urines were pooled. Control urine samples (sample C) were collected from the same piglet the day before giving the contaminated bolus. Wash out urines were collected for 24 h for two groups (Bolus 3 and 4) after one week from the administration of contaminated bolus (sample W). Urine samples were frozen and stored at −20 °C until the NMR spectroscopy analysis. More details on the preparation of contaminated boluses, administration of boluses to piglets and urine collection are reported elsewhere [11].

2.2. NMR sample preparation

Thirty-nine samples of urine from sixteen piglets before and after administration of boluses contaminated with a mixture of 5 mycotoxins were obtained as described above and analyzed by ^{1}H NMR spectroscopy. Frozen samples were thawed at room temperature and shaken before use. Aliquots of each urine sample (630 µL) were added to 70 µL of potassium phosphate buffer (1.5 M K_2HPO_4 in D_2O, pH 7.4), to minimize variations in metabolite NMR chemical shifts arising from differences in urinary pH, also containing 0.1% sodium 3-(trimethylsilyl)-[2,2,3,3-d_4]propionate (TSP) and 2 mM sodium azide. Samples were centrifuged at 14,000*g* for 5 min at 4 °C to remove any solid debris. 600 µL of the supernatant were placed in a 5 mm outer diameter NMR tube.

2.3. NMR measurement

All measurements were performed on a Bruker Avance III 600 Ascend NMR spectrometer (Bruker, Karlsruhe, Germany) operating at 600.13 MHz for ^{1}H observation, equipped with a z axis gradient coil and automatic tuning-matching (ATM). A time delay of 5 min was set between sample injection and preacquisition calibrations to ensure complete temperature equilibration (300 K). For each sample a one-dimensional NOESY experiment (referred to as 1D ^{1}H-NOESY), including solvent signal saturation during relaxation and mixing time and a spoil gradient, was acquired using 64 free induction decays (FIDs) a spectral width of 12,019 Hz, an acquisition time of 2.7 s, a relaxation delay of 4 s, and a mixing time of 10 ms. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz before Fourier transformation phasing, and baseline correction. All spectra were referenced to the TSP signal (δ = 0.00 ppm). NMR data were processed using TopSpin 2.1 (Bruker). The metabolites were assigned on the basis of 2D NMR spectra analysis (2D ^{1}H *J*res, ^{1}H COSY, ^{1}H–^{13}C HSQC and HMBC) and comparison with published data [14,19,24,25].

2.4. Data analysis

NMR spectra were processed using Topspin 2.1 and visually inspected using Amix 3.9.13 (Bruker, Biospin, Italy). ^{1}H NMR spectra were segmented in rectangular buckets of fixed 0.04 ppm width and integrated. The spectral region between 6.1 and 4.5 ppm was discarded to avoid the effects of variable water suppression and to prevent cross-relaxation effects to the urea signal via proton exchange between urea and water. According to published procedures [26], the remaining 204 buckets in the range 10.00–0.30 ppm were then normalized to total area to minimize small differences due to total metabolites concentration and/or acquisition conditions among samples and subsequently mean-centered. The resulting data set was made of the ^{1}H NMR spectra bucket values (columns) measured for the above urine samples (rows). The description of statistical analyses refers to Pareto scaling data. The data table generated with all the spectra was submitted to multivariate data analysis.

Table 1
Mycotoxin intakes in four groups of piglets that received a bolus containing a mixture of deoxynivalenol (DON), aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), zearalenone (ZEN) and ochratoxin A (OTA). This table was already published in Gambacorta et al. [11].

Mycotoxin	Bolus 1 (n = 4)[a]		Bolus 2 (n = 4)[b]		Bolus 3 (n = 4)[c]		Bolus 4 (n = 4)[d]	
	µg/kg bw	µg/animal	µg/kg bw	µg/animal	µg/kg bw	µg/animal	µg/kg bw	µg/animal
DON	7.16	63.61	20.44	191.12	24.14	315.05	57.38	630.03
AFB_1	0.16	1.40	0.45	4.20	0.54	7.03	1.28	14.01
FB_1	3.71	32.96	10.60	99.14	63.19	824.60	150.19	1649.12
ZEN	0.68	6.05	1.94	18.15	2.38	31.08	5.66	62.15
OTA	0.16	1.45	0.46	4.35	0.56	7.25	1.32	14.50

[a] Mean body weight (bw) of 4 piglets ± standard deviation (SD): 8.88 ± 1.26 kg;
[b] Mean bw of 4 piglets ± SD: 9.35 ± 0.47 kg;
[c] Mean bw of 4 piglets ± SD: 13.05 ± 0.58 kg;
[d] Mean bw of 4 piglets ± SD: 10.98 ± 1.14 kg

2.5. Multivariate statistical analyses

1D ^{1}H-NOESY spectra were analyzed by multivariate statistical procedures. Specifically, an exploratory data analysis was performed using Principal component analysis (PCA), while Partial Least Squares Discriminant Analysis (PLS-DA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) methods were used to maximize the separation between samples. Multivariate statistical analysis and graphics were obtained using Simca-P version 14 (Umetrics, Sweden). PCA is a way of identifying patterns in data, expressing them in order to highlight their similarities and/or differences and was used to get an overview of the multivariate profiles. The PCA works by decomposing the X-matrix (buckets linked with the NMR signals) as the product of two smaller matrices (loading and score matrices) and was used to get an overview of the multivariate data. Values falling outside the Hotelling T2 95% confidence limit in score plots are considered outliers [27]. The PLS-DA is the regression extension of PCA, which gives the maximum covariance between the measured data (the X-matrix) and the response variable (Y variable, class membership). The confidence level for membership probability was considered to be 95% (observations at < 5% were considered outliers) [28]. In this work, the PLS-DA method was also performed in order to justify the number of t latent variables used in OPLS-DA model. The OPLS-DA analysis is a modification of the usual PLS-DA method which filters out variation that is not directly related to the response and produces models of clearer interpretation, as shown in several recent studies of metabolomics [29,30]. So, the further improvements made by the OPLS-DA resides in the ability to separate the portion of the variance useful for predictive purposes from the not predictive variance (which is made orthogonal) overcoming the problems of multicollinearity and autocorrelation of the variables. The quality of the models was described by R^2, Q^2 values and p[CV-ANOVA]. The R^2 value is a cross validation parameter defined as the proportion of variance in the data explained by the models and indicates goodness of the fit. The Q^2 parameter is defined as the proportion of variance in the data predictable by the model and indicates predictability. The models were validated using internal cross-validation default method (7-fold) and further evaluated with permutation test (400 permutations) of SIMCA 14 software, Umetrics, Umea, Sweden [28,31]. For each PLS-DA and OPLS-DA model built, a combination of the loading scores, variable influence on projection (VIP) parameters and p(corr) were examined to identify which metabolites most contributed to the data clustering. Loading scores describe the correlations between the original variables and the new component variables. VIP parameters are essentially a measure of the extent to which a particular variable explains the Y variance (class membership) and p(corr) represents the loadings scaled as a correlation coefficient (ranging from −1.0 to 1.0) between the model and original data [32].

3. Results

Urine metabolic profile of piglet fed with boluses contaminated with mixtures of DON, AFB_1, FB_1, ZEN and OTA at four increasing doses were studied by ^{1}H NMR spectroscopy and MVA. The values of mycotoxin intake in the four groups of piglets used in this study are reported in Table 1. These concentrations were chosen considering the maximum allowed and guidance levels reported in the Directive 2003/100/EC (EC, 2003) and the Recommendation 2006/576/EC (EC, 2006), respectively.

The piglets used in this work consumed about 350–400 g feed/day, therefore the values of mycotoxin intake were below the limits for all tested doses of OTA and FB_1. About AFB_1, DON, and ZEN, the levels were below the limits for three out of the four doses and about twice the limits for the highest dose. The urine samples were collected from the same piglets the day before administration (controls, C), within the 24 h post dose (treated, T) and after 1 week of wash out (wash out, W). All urines were analyzed by ^{1}H NMR spectroscopy and MVA.

Average one-dimensional (1D) ^{1}H NMR spectra from C, T, and W urine samples of piglets fed with Bolus 3 (characterized by the highest considered contamination values within the allowed limits) are reported in Fig. 1. Urine metabolites were identified in the ^{1}H NMR spectra and assigned on the basis of 2D NMR spectra analysis (2D ^{1}H *J*res, ^{1}H COSY, ^{1}H ^{13}C HSQC and HMBC) and by comparison with published data [14,19,25]. Relevant ^{1}H NMR data are reported in Table 2. Metabolic profiles of urine characterized by ^{1}H NMR spectroscopy were therefore studied by multivariate analyses (PCA, PLS-DA, OPLS-DA). Multivariate analysis was performed on bucket reduced ^{1}H NMR spectra (see Materials and Methods). The original dataset (204 buckets from the spectral region 10.00–0.30 ppm) was rearranged in a new multivariate coordinate space in which the dimensions are ordered by decreasing explained variance of the considered data. The principal components were displayed as a set of scores (t) and a set of loadings (p), which highlight clustering or outliers (score plot) and influence of input variables on t (loading plot), respectively. The first PCA model was built with the C and T urine samples (Fig. 2). In this model the first two components explained the 63% of total variance (PC1 = 36%, PC2 = 27%), with a Q^2 of 0.33. No relevant trends and some overlap among samples were observed in the PCA model applied on C and T urine samples of all groups, except for a marked separation of urine samples of piglets from Bolus 1 and 2 with respect to Bolus 3 and 4 before the treatments.

In order to improve the separation among samples based on maximizing covariance between the measured data (X) and the response variable (Y), PLS-DA and OPLS-DA models were applied. By these methods the identity of each group of samples is specified in the model such that the maximum variance of the groups can be attained in the hyperspace. Two performance indicators were used to assess the supervised model complexity and eventual overfit degree: the cross validation (CV) and the response permutation test (n = 400). The OPLS-

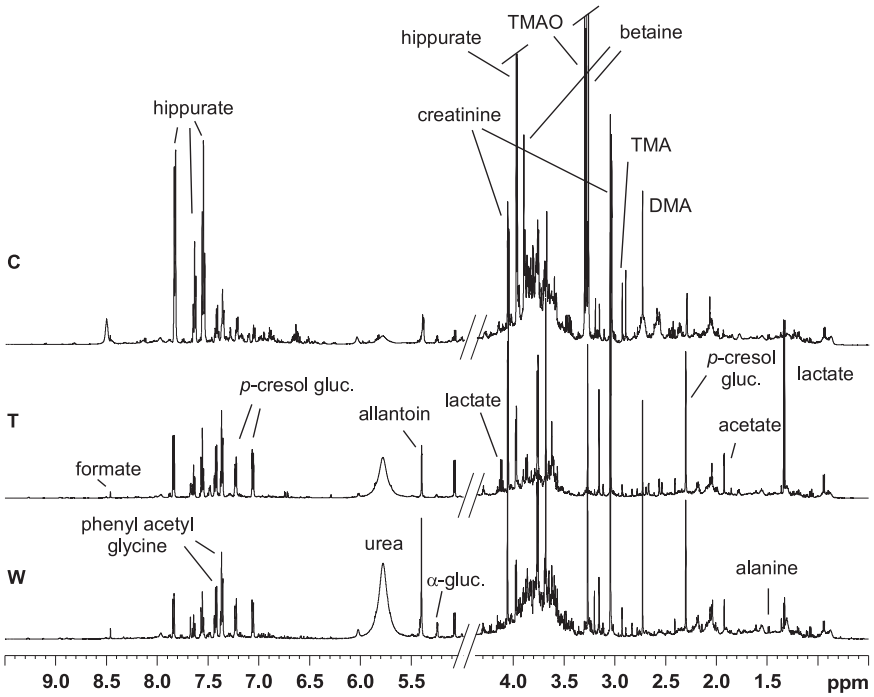

Fig. 1. 1D ^{1}H NMR average spectra from C, T, and W urine samples of piglets fed with Bolus 3 (characterized by the highest considered contamination values within the allowed limits). The most representative metabolites are indicated.

DA (1 predictive + 2 orthogonal components, R^2Y = 0.71, Q^2 = 0.47, p[CV-ANOVA] = 0.009) applied on all C and T samples (before/after treatment as responsible variable, Y) gave a model where the C samples moderately separated from T along the predictive component t[1] (Fig. 3).

Interestingly, along the orthogonal component to [1] the Bolus 3 and 4 well separated from Bolus 1 and 2, with the presence of a single outlier (Control sample from Bolus 2 piglets group). This clustering could be attributed to the original intrinsic differences of 1, 2 and 3, 4 piglets groups obtained from different suppliers. It is worth noting that the overall intra-class variability of the samples (dispersion along to [1] axis) was reduced after the mycotoxin treatment. Moreover, the Bolus 3 and 4 T samples were more separated than the Bolus 1 and 2 T samples from the corresponding controls. The variables responsible for the class separation observed in Fig. 3A could be determined by analysis of the p (corr)/VIP score plot (Fig. 3B) (see Experimental Section). Such variables indicate the chemical shift of the buckets responsible for the observed class separation in the bucket reduced urine spectra. Therefore, the metabolite differences between T and C could be assessed. The highest level of TMAO (bucket at 3.3 ppm), betaine (buckets at 3.26 and 3.9 ppm), TMA (2.90 ppm), DMA (2.74) and MA (2.58) resulted for C samples; whereas the T samples showed the highest content of *p*-cresol glucuronide (buckets at 2.30, 7.06, 7.22 ppm) and phenyl acetyl glycine (buckets at 7.42 and 7.38 ppm).

When the T samples, originated from the lower (Bolus 1 and 2) and higher doses (Bolus 3 and 4), were analyzed in comparison to the related controls (C), two models with a very different statistical significance were obtained. For Bolus 1 and 2 groups compared to the controls, unsupervised method (PCA) gave unclear results and the OPLS-DA model (Fig. 4) produced a very weak descriptive and predictive model (1 predictive and 1 orthogonal components, R^2 = 0.64 and Q^2 = 0.01).

On the other hand, for the Bolus 3 and 4, a good separation was already observed with PCA method without any *a priori* assumptions for the sample classification (Fig. 5). In this PCA model 2 PCs explained the 70% of variance (PC1 = 45%, PC2 = 25%), with a Q^2 of 0.55. In the t[2]/t[1] PCA score plot a good separation of the T from the C samples along the t[2] component was observed (Fig. 5A). By analysis of the related p[2]/p[1] loading plot (Fig. 5B) the T samples showed higher content of phenyl acetyl glycine and *p*-cresol glucuronide and lower level of TMAO and betaine with respect to the controls. The data were further analyzed by using OPLS-DA to maximize the separation between the classes and to further validate the model. The OPLS-DA (Fig. 5C) on the Bolus 3 and 4 produced a good descriptive and predictive model (1 predictive + 2 orthogonal components, R^2Y = 0.98 and Q^2 = 0.85, p[CV-ANOVA] = 0.003). By analysis of VIP/p(corr) score plot (Fig. 5D) the T samples exhibited, as well as a higher content of *p*-cresol glucuronide and phenyl acetyl glycine seen in the PCA model, also a higher concentration of creatinine (buckets at 3.06 and 4.06 ppm) with respect to the control. The latter showed again the highest level of TMAO, betaine, TMA, DMA and MA.

In order to investigate the prolonged effects of a single dose of high contaminated bolus, the urine samples were collected also after one week of wash out only for the animals fed with the highest doses (Bolus 3 and 4 groups). The PCA and OPLS-DA score and loadings plots on the Bolus 3 and 4 considering also the urine samples collected after one week of wash out (W) are reported in Fig. 6. In the t[2]/t[1] PCA score plot (Fig. 6A) (3 PCs, R^2X = 0.70, Q^2 = 0.44) the T and W urine samples separated from the control along t[2] axis (Fig. 6A). Moreover, the W samples resulted more distant from the controls. The OPLS-DA (supported by a preliminary meaningful PLS-DA analysis with 3 PLS, R^2Y = 0.85 and Q^2 = 0.75, p[CV-ANOVA] = $7.9*10^{-7}$) (Fig. S1), applied to maximize these differences, gave a good model (R^2Y = 0.85 and Q^2 = 0.69, p[CV-ANOVA] = $1.08*10^{-5}$). The OPLS-DA gave a good performance (described by 2 predictive + 1 orthogonal components, R^2Y = 0.85 and Q^2 = 0.69, p[CV-ANOVA] = $1.08*10^{-5}$) and it has been used as a descriptive model rather than for classification purposes, for the presence of more than two classes (control, C, treated, T, wash out, W) [29]. Interestingly, the urine samples collected after one week of wash out, when piglet had free access to diet free of mycotoxins, exhibited a marked difference from the controls along the predictive component t[1] and from treated samples along the orthogonal component t[2] (Fig. 6C). The metabolites responsible for these differences were again creatinine, *p*-cresol glucuronide and phenyl acetyl glycine.

Table 2
^{1}H peak assignments for identified metabolites.

Compound	δ_H
acetate	1.92 (s, CH_3)
alanine	1.49, (d, βCH$_3$), 3.80 (q, αCH)
β-alanine	3.19 (t, N-CH_2), 2.57 (t, CH_2COOH)
allantoin	5.40 (s, C4H)
betaine	3.26 (s, N-$(CH_3)_3$), 3.90 (s, CH_2)
choline	3.20 (s, N-$(CH_3)_3$)
trans-cinnamic acid	6.50 (d, =CH), 6.67 (d, =CH), 6.70 (d, =CH), 7.33 (d, =CH), 7.49 (d, =CH)
creatinine	3.04 (s, CH_2-N), 4.06 (s, CH_2-N)
p-cresol glucuronide	7.23 (d[a], C3H and C5H), 7.06 (d, C2H and C6H) 2.30 (s, CH_3), 5.07, 3.86, 3.61
dimethylamine (DMA)	2.72 (s, N-$(CH_3)_2$)
dimethylglycine	2.93 (s, N$(CH_3)_2$), 3.73 (s, CH_2)
formate	8.46 (s, HCOOH)
fructose	4.12, 3.89
fumarate	6.64 (s, CH=CH)
α-glucose	5.25 (d, C1H), 3.53 (dd, C2H), 3.71 (dd, C3H), 3.42 (dd, C4H), 3.84 (m, C5H), 3.78 (m, C6H)
β-glucose	4.65 (d, C1H), 3.89 (dd, C6H), 3.73 (dd, C5H), 3.49 (t, C3H), 3.25 (dd, C2H)
glycine	3.57 (s, CH_2)
hippurate	7.84 (d, C2H and C6H), 7.64 (t, C4H), 7.56 (t, C3H and C5H), 3.97 (d, CH_2)
lactate	1.33 (d, CH_3)
malonate	3.15 (s, CH_3)
methylamine (MA)	2.62 (s, N-(CH_3))
3-methylhistidine	7.00 (s, C4H), 7.67 (s, C2H)
methylmalonate	1.26 (d, CH_3), 3.76 (m, CH)
phenyl acetyl glycine	7.43 (m, C3H and C5H), 7.36 (m, C2H and C6H), 3.75 (d, CH_2), 3.68 (s, CH_2)
succinate	2.41 (s, CH_2)
taurine	3.28 (t, N-CH_2), 3.45 (s, S-CH_2)
trigonelline	9.10 (s, C1H), 8.82 (m, C3H and C5H), 8.07 (m, C4H), 4.43 (s, CH_3)
trimethylamine (TMA)	2.90 (s, N-$(CH_3)_3$)
trimethylamine-*N*-oxide (TMAO)	3.30 (s, N-$(CH_3)_3$)
urea	5.78 (broad signal, NH_2)

[a] Letters in parentheses indicate the peak multiplicities; s, singlet; d, doublet; t, triplet; dd, doublet of doublet; m, multiplet.

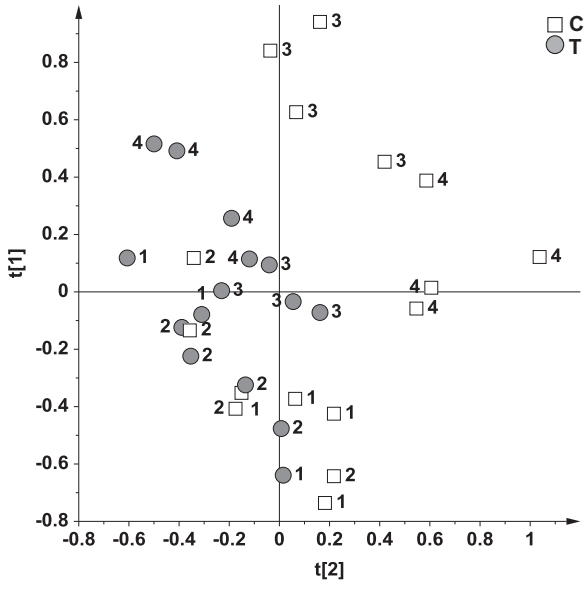

Fig. 2. PCA score plot on 32 samples using as responsible variable (Y) the two classes control (C, 16 samples) and treated (T, 16 samples). The numbers (1–4) indicate the Bolus group.

4. Discussion

In the present work the urine metabolome of piglets fed with contaminated boluses containing a mixture of mycotoxins (DON, AFB_1, FB_1, ZEN, and OTA) has been investigated and compared with own controls by ^{1}H NMR spectroscopy and chemometrics (PCA, PLS-DA and OPLS-DA). To the best of our knowledge, few in vivo studies have been focused on the metabolic response to mycotoxin ingestion [21–23]. Moreover, this is the first report on explorative metabolomic analysis of piglet urine after simultaneous oral administration of several mycotoxins at different concentrations and below the allowed guideline limits for most of the tested doses. Indeed, mycotoxigenic fungi are able to produce more than one mycotoxin simultaneously and food and feed crops can be contaminated by different fungi species at the same time. Thus, Humans and animals are generally exposed to several toxins at the same time as recently confirmed for Humans resident in three different countries [33–35]. The toxicity of mycotoxins combinations cannot always be easily predicted based on their individual toxicities [36]. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive or synergistic [37]. On the other hand, most of the studies concerning the toxicological effect of mycotoxins have been carried out taking into account only one mycotoxin. Therefore the information on the in vivo combined toxic effects of mycotoxin is scarce and the health risk derived from co-exposure of mycotoxins needs further studies [38].

We decided to conduct this study on piglet urines, previously collected during a mycotoxin biomarker validation study [11] for several reasons. Pigs are potentially exposed to high levels of mycotoxins due to their maize-rich diet and they are quite susceptible to mycotoxin toxicity [39–41]. On the other hand, typical lab animals, especially mice, are very resistant to most mycotoxins [41,42]. Finally, the many biological similarities of pigs and Humans, especially for the intestinal tract, make the pig a good model for this type of study.

Urine samples used in this work were previously collected from four groups of piglets (four animals per group) administered with boluses contaminated at four increasing mycotoxin levels (one level per group) [11]. The urine samples were collected from each piglet before and within the 24 h post dose administration. In order to investigate the effects of a single ingestion of mycotoxins on young animals, the urine samples were collected also after one week of wash out for the two higher doses groups (Bolus 3 and 4). At the lower dose groups (Bolus 1 and 2) no significant differences between the control and treated urine samples were detected. However, for the higher doses groups (Bolus 3 and 4) the treated samples were markedly separated from the controls due to highest content of creatinine, *p*-cresol glucuronide and phenyl acetyl glycine.

Creatinine is the metabolic end-product of creatine catabolism and the high concentration in 24 h post dose urines of piglets is a marker of kidney malfunction. On the other hand low creatinine concentration in urines could be an indicator of alterations in protein turnover and of metabolism in general [43]. The *p*-cresol glucuronide is a soluble glucuronide derivative of *p*-cresol. This is a uremic toxin which inhibits the proliferation of endothelial cells, prevents the repair of wounded endothelium [44], and hampers the response of endothelial cells to inflammatory cytokines [45,46]. The increased levels of urinary *p*-cresol glucuronide and phenyl acetyl glycine could be the result of the disturbance of gut microbiota by single exposure to the five mycotoxins mixture. Indeed, the aromatic amino acids, phenylalanine and tyrosine, are firstly converted into phenyl acetate and *p*-cresol through the action of the gut microbiota [47]. Then phenyl acetate and the *p*-cresol conjugate with glycine and glucuronide, respectively, to form phenyl acetyl glycine and *p*-cresol glucuronide in the liver and the gut mucosa [48–50]. Previous studies reported that elevated levels of urinary phenyl acetyl glycine are exhibited in rat abnormally accumulating phospholipids in liver and that these levels can be used as alternative biomarker suggesting changes in gut microbiota [51]. The development

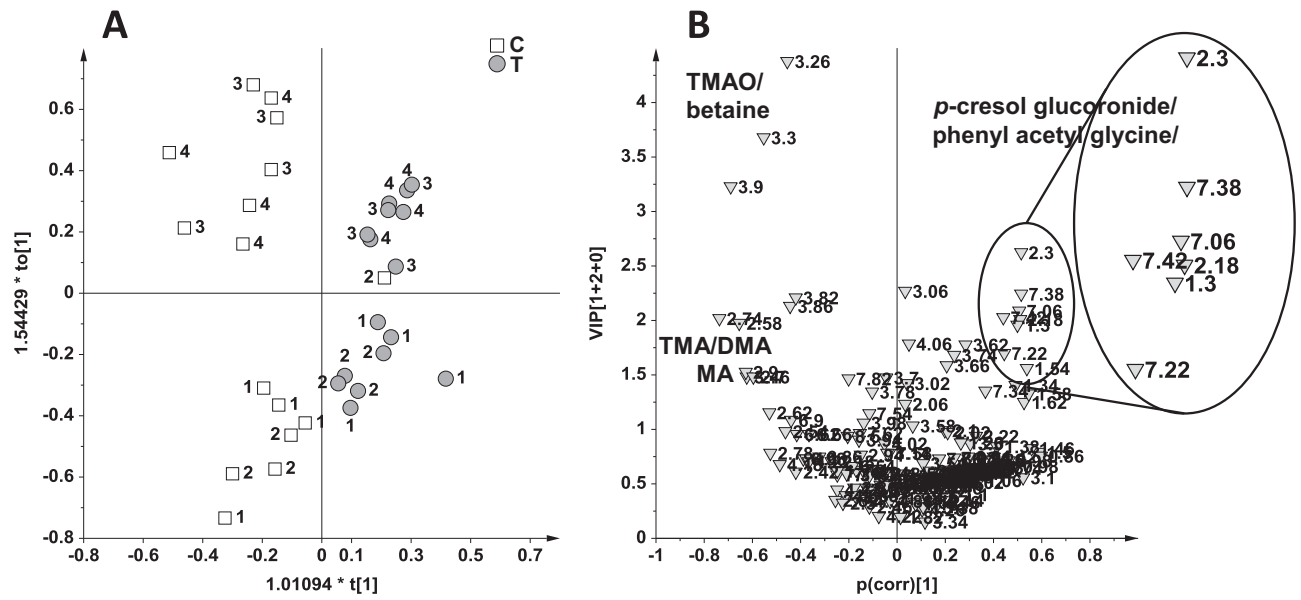

Fig. 3. A: OPLS-DA score plot on 32 samples using as responsible variable (Y) the two classes control (C, 16 samples) and treated (T, 16 samples). The numbers (1–4) indicate the Bolus group. B: p(corr)/VIP score plot. The numbers indicate the NMR spectra reduced buckets (ppm).

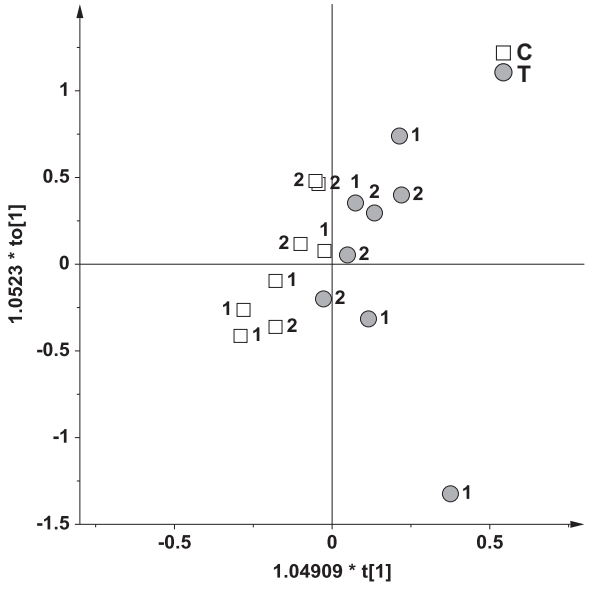

Fig. 4. OPLS-DA score plot on 16 samples from the Bolus 1 and 2 groups using as responsible variable (Y) the two classes: control (C, 8 samples) and treated (T, 8 samples).

and structure of the intestinal epithelium, the digestive and absorptive capabilities of the intestine, and the host immune system are considerably influenced by gut microbiota [52]. Possible disturbances of gut microbiota by mycotoxin administration can therefore affect the health status [53–55]. The two higher doses groups showed lower urinary concentration of betaine and TMAO with respect to control samples. Betaine is a well-known osmoregulatory biomolecule [56] and, therefore, its reduction in 24 h post dose urines could increase creatinine clearance. It has been reported that biomolecules with an electrophilic methyl group, e.g. as found in betaine, may represent an important control system for in vivo redox balance [57] by reducing oxidative stress in the liver. As oxidative stress has been reported to be inversely related to creatinine clearance, the higher urine creatinine excretion in treated animals could be consistent with the betaine reduction [58]. Interestingly, the three reactive methyl groups of betaine have a key role as a methyl donor in many biochemical pathways and take part in the methionine cycle [57]. A scarce dietary consumption of methyl groups rich molecules results into hypomethylation, which may correlate to several illnesses, such as coronary, cerebral, hepatic and vascular diseases [42]. Other physiological functions of betaine relate to a protective role against protein denaturation (as a 'chemical chaperone' [59]) and a cell volume preserving under osmotic stress [42]. Due to its physiological significance, the betaine decrease, after single contaminated bolus administration, must be considered extremely important. It has already been reported that trimethylammonium compounds such as choline and carnitine, which are ingested in the normal diet, are decomposed to TMA by several strains of gut bacteria, and further oxidized to TMAO in the liver and excreted with urine [60]. Therefore, the lower content of urinary concentration of TMAO after mycotoxin ingestion may be likely related to mycotoxin induced disruption of gut bacterial strains that degrade dietary choline to TMA in the intestinal tract. It has been reported a decline of urinary TMAO in rat treated with realgan [61]. TMAO reduction was related to realgar induced disruption of choline pathway degradation through antimicrobial activity to intestinal bacteria [61]. Our results suggest that the mixture of mycotoxins may have produced a similar anti microbial activity to intestinal bacteria with consequent disruption of choline degradation pathway. A decrease of DMA and TMAO in the urinary excretion when the gut microflora was reduced has been reported. Differently from our observation, such a decrease occurred with a concomitant increase of betaine [62].

Although our study did not address the gut microbial metabolism or activity, it has been reported that many different gut microbial species are impacted by xenobiotic toxicants, including AFB_1 that induced compositional changes in gut microbial communities of male F344 rats [55]. DON and FB_1 have also been demonstrated to impact the intestinal pig microbiota [53,54]. Thus microbiological identification of specific changes in the microbiota community can be helpful in addressing the metabolic implications of mycotoxin ingestion.

The aim of this study was also to investigate the effects of a single bolus contaminated with a mixture of five mycotoxins on a young organism such as piglets after one week from the exposure. For this purpose the urine samples were collected also after one week of wash out when the piglets had free access to a diet free of mycotoxins. Interestingly, both in PCA and PLS-DA models the post wash out urine samples showed a metabolic profile similar to the treated samples exhibiting the highest level of *p*-cresol glucuronide, phenyl acetyl glycine and creatinine. The results obtained in this study suggest that

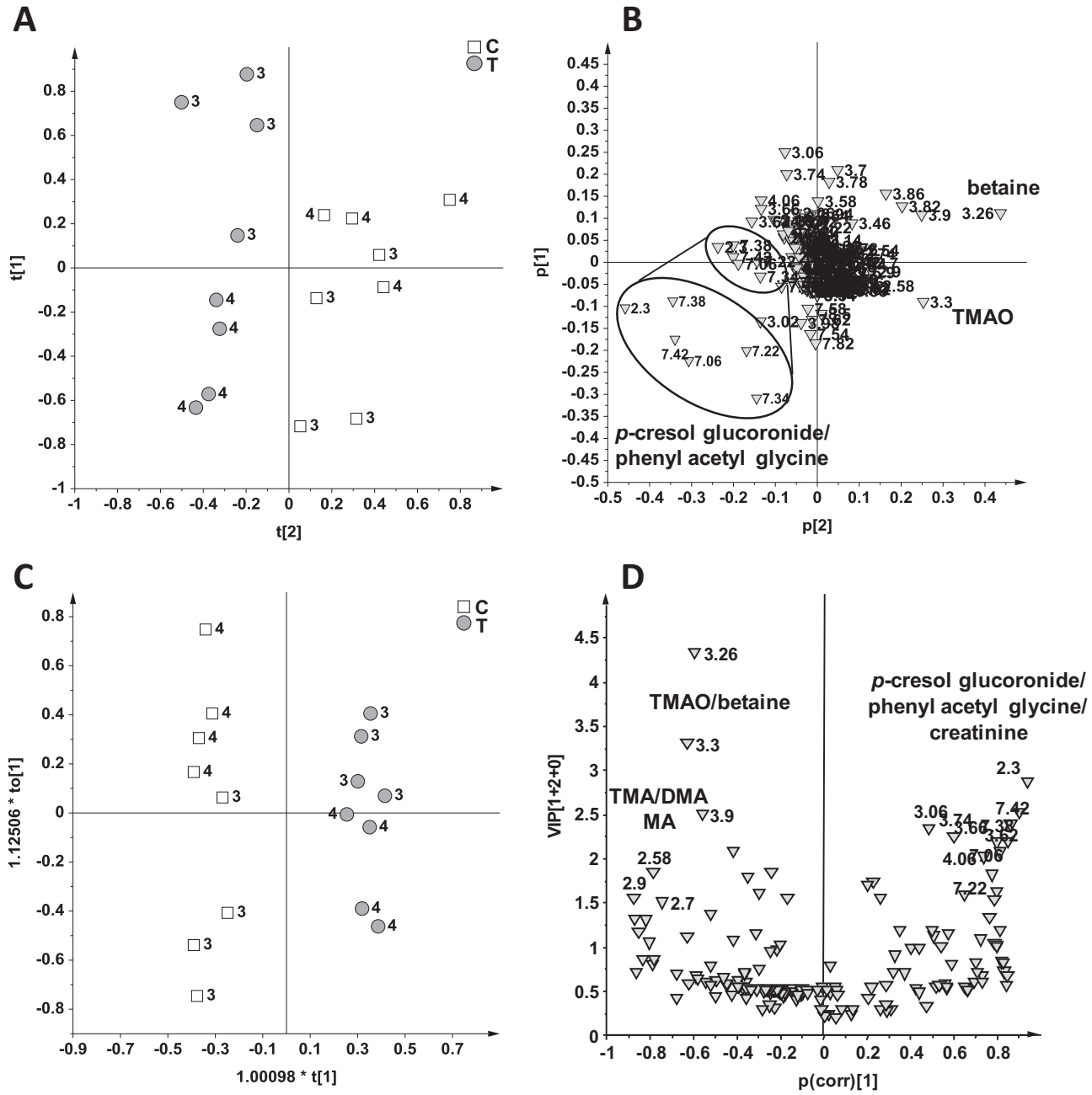

Fig. 5. PCA score plot (A) and relative loadings plot (B), OPLS-DA (C) and relative VIP/p(corr) (D) score plot on 16 samples from the Bolus 3 and 4 using as responsible variable (Y) the two classes: control (C, 8 samples) and treated (T, 8 samples).

a single dose of mycotoxin mixture, at least for the higher used doses, could produce serious alteration on urine metabolome of piglets for at least one week after exposure.

5. Conclusions

Urine metabolic profile of piglets, fed with boluses contaminated with mixtures of DON, AFB_1, FB_1, ZEN, and OTA at concentrations close to the maximum permitted levels in the EU showed higher urinary concentrations of creatinine, *p*-cresol glucuronide and phenyl acetyl glycine and lower concentrations of betaine and TMAO with respect to own controls. This suggest that the mycotoxin mixture impairs the urinary metabolome which is probably due to the alteration of the gut microbial ecosystem. Urines collected after a week of wash out showed a metabolic profile similar to that of urine collected in the 24 h post dose, exhibiting the highest level of *p*-cresol glucuronide, phenyl acetyl glycine and creatinine. These results also suggest that a single dose of mycotoxins could produce, serious impairments of gut microbiota of piglets. To the best of our knowledge, this work is the first in vivo metabolomic study on the response of animal, in particular pig, to a mycotoxin mixture supplementation close to the EC guidelines limits. Finally, it should be considered that in this work an explorative metabolomic analysis has been reported and a list of potential molecules which are statistically significant has been investigated. As well known, the metabolites differences observed in a metabolomic study may be statistically but not necessarily biochemically significant [63]. Some hypotheses related to the possible origin of the observed metabolic changes have been considered and are reported in this work. Nevertheless, further studies are required in order to assess the observed differences as specific biomarkers related to mycotoxins induced alterations (possibly on the basis of identified specific biochemical pathways disruption).

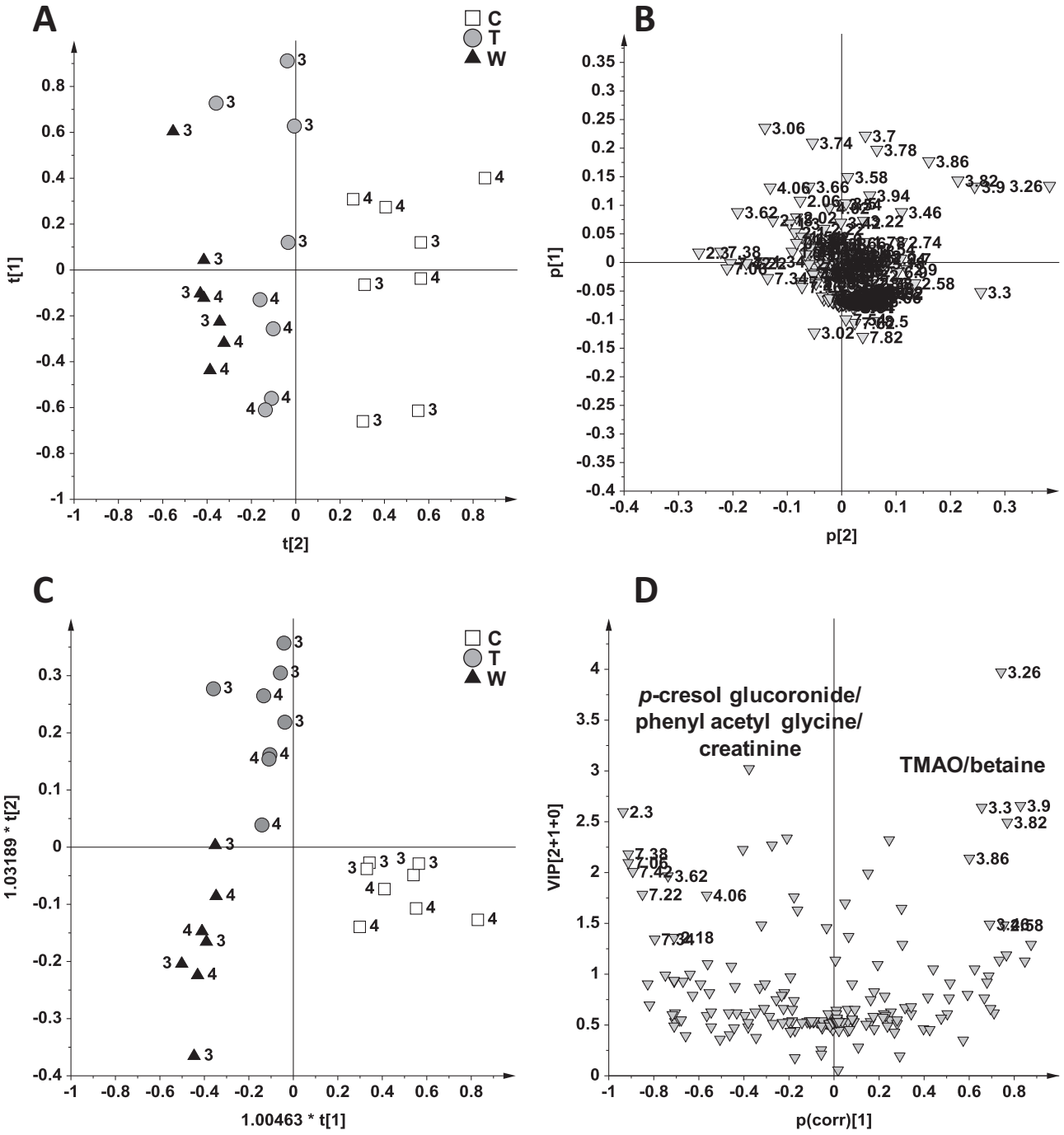

Fig. 6. PCA score plot (A) and relative loadings plot (B), OPLS-DA score plot (C) and relative loadings plot (D) on 23 samples from the Bolus 3 and 4 using as responsible variable (Y) the three classes: control (C, 8 samples), treated (T, 8 samples), wash out (W, 7 samples).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.05.004.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2017.05.004.

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