

## Electronic Supplementary Information

for

### GUT MICROBIOME MODULATES THE TOXICITY OF HYDRAZINE: A METABONOMIC STUDY

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## **Rat model and husbandry**

Male Sprague-Dawley conventional rats ( $n = 6$ ) and germ-free (OFA) Sprague-Dawley rats ( $n = 6$ ) were purchased from Charles River and housed in individual metabolism cages. Three animals from each group were treated as controls and were given an oral dose, by gavage, of physiological saline, whilst the remaining animals were administered a 60 mg/kg oral gavage of hydrazine hydrochloride dissolved in physiological saline.

Animals were maintained in metabolism cages after dosing in a standard laboratory environment at room temperature, with a 12/12-light/dark cycle. Food (Rat & Mouse No.1 Modified Irradiated Diet, Special Diet Services) and water were provided throughout the study *ad libitum*.

## **Collection and preparation of rat biofluids and faeces**

Urine samples were collected pre dose, and for the periods 0-8, 8-24, 24-48, 48-72 and 72-96 hours after dosing. Faeces were collected 24 hours post dose. Blood (0.5 mL) was taken at 8 and 24 hours after dosing into heparinized sample tubes and plasma prepared by centrifugation (1500 g for 10 minutes). Animals were sacrificed by halothane inhalation 96 hours post dose with the exception of animals B and C whom were sacrificed 24 hours post dose. All samples were immediately frozen on dry ice and were stored at  $-80^{\circ}\text{C}$  prior to analysis by  $^1\text{H-NMR}$  spectroscopy.

For  $^1\text{H-NMR}$  spectroscopic analysis, an aliquot of urine (480  $\mu\text{L}$ ) from each sample was placed in an eppendorf to which phosphate buffer (240  $\mu\text{L}$ ) at pH 7.4 containing 10%  $\text{D}_2\text{O}$  and 0.05% sodium 3-(trimethylsilyl) propionate-2,2,3,3- $\text{d}_4$  (TSP, an internal standard, chemical shift  $\delta$  0.0) was added. The sample was centrifuged to remove particulate matter (3000 g for 4 minutes) and transferred to a 5 mm outer diameter NMR tube. Plasma samples (240  $\mu\text{L}$ ) were combined with 480  $\mu\text{L}$  of saline containing 10%  $\text{D}_2\text{O}$ , centrifuged and then placed in a 5 mm NMR tube. Chemical shifts in the plasma spectra were referenced to the anomeric proton of  $\beta$ -glucose at  $\delta$  5.223.

## **Histopathological assessment**

At necropsy, liver and kidneys were excised quickly and immersed into 10% neutral buffered formalin and fixed for 24-48 hours followed by conventional histological tissue processing and the generation of tissue sections stained with haematoxylin and eosin. These sections were examined using light microscopy.

## **One-dimensional $^1\text{H}$ NMR spectroscopy of urine and plasma samples.**

All samples were analysed on a Bruker DRX-600 NMR Spectrometer (Bruker Analytische GmbH, Rheinstetten, Germany) at 300 K operating at 600.13 MHz for  $^1\text{H}$  observation. For the urine samples, a standard one-dimensional NMR spectrum was acquired with water peak suppression using a standard pulse sequence (recycle delay- $90^\circ$ - $t_1$ - $90^\circ$ - $t_m$ - $90^\circ$ -acquisition).<sup>(21)</sup> The recycle delay was set at 2 s and the mixing time ( $t_m$ ) 100 ms. For each urine sample, 8 dummy scans were followed by 64 scans and collected into 32K data points. A spectral width of 20 ppm and an acquisition time per scan of 3.41 s were used.

One-dimensional  $^1\text{H}$  NMR spectra of plasma were acquired with 128 transients collected into 32 K data points using the same standard pre-saturation pulse sequence as for urine. Water suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin-echo spectra were also acquired for the plasma samples for observing changes in the low molecular weight metabolite profile by suppression of the contribution of the larger metabolites such as lipids and proteins. These were acquired with 8 dummy scans followed by 128 scans collected in 32K data points, with a spectral width of 20 KHZ and an acquisition time per scan of 1.36 s.

## **Data reduction and analysis**

In-house software was used to phase the spectra and to correct for baseline distortions. The  $^1\text{H}$  NMR spectra ( $\delta$  0.2-10.0) were digitized into consecutive integrated spectral regions of equal width (0.04 ppm). Although it is becoming increasingly common to use full resolution spectra, given the small group sizes employed in the current study, it

was deemed expedient to use a 'binning' approach to minimize peak positional shift due to pH variation. The area for each segmented region was expressed as an integral value. The region  $\delta$  4.50-5.98 was removed from the analysis in order to exclude the effects of variation in the suppression of water resonance and, in the case of urine, variation in the urea signal caused by exchanging protons. Each spectrum was then normalized to unit area.

SIMCA-P10.5 software (Umetrics, Umea, Sweden) was utilized to perform Principal Components Analysis (PCA). PCA is an unsupervised, multiparametric statistical method helpful in data reduction from data sets containing many more measurement variables than samples and for visualizing patterns within the data and within groups and has been widely used in metabonomic toxicology studies. There is no inclusion of class information in the model and therefore, the data cannot be overfitted. Here PCA is used for the sole purpose of visualization of metabolic patterns within the data.

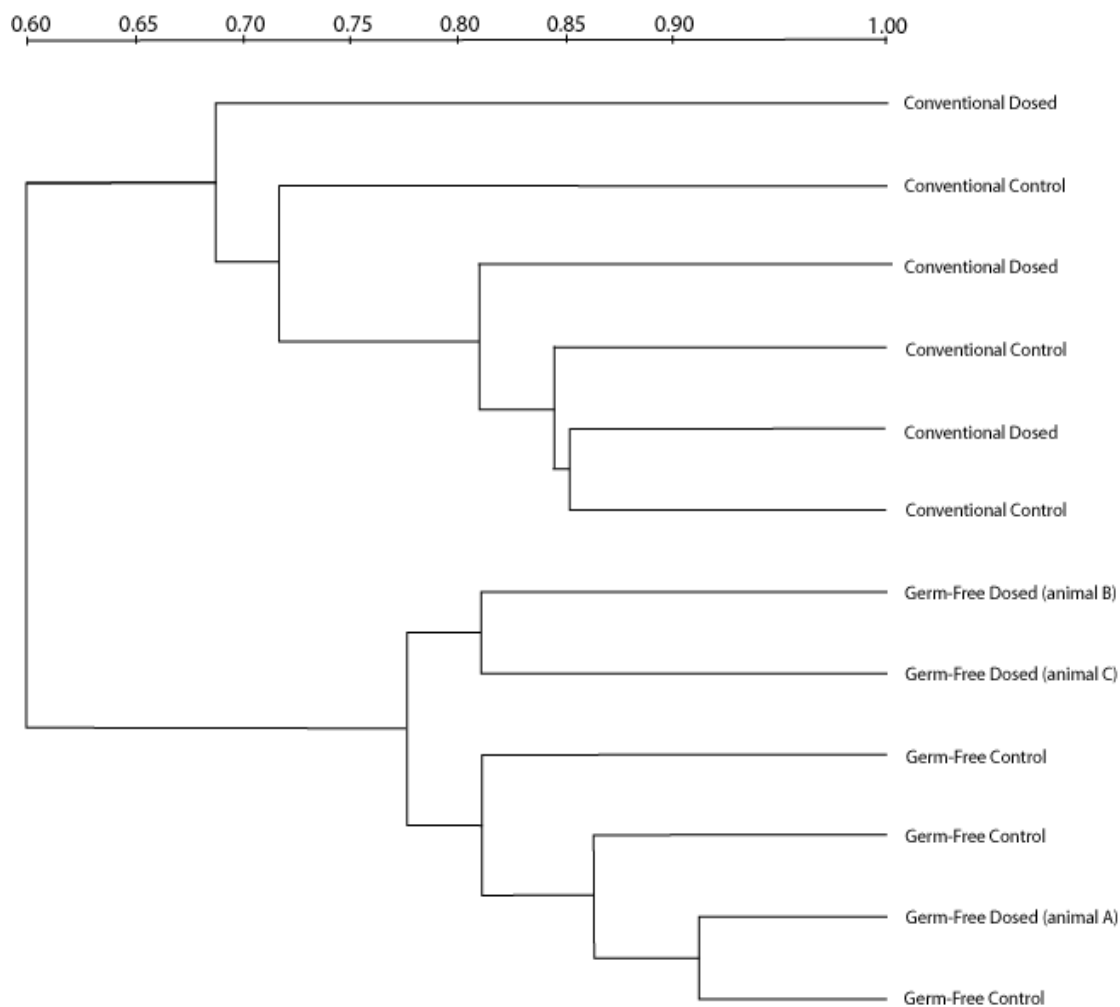
#### **DNA extraction and PCR amplification of the 16S rRNA gene**

Frozen faecal samples of each animal were thawed on ice before DNA extraction. DNA was isolated using FastDNA Spin Kit for Soil (Qbiogene, Cambridge, UK) according to manufacturer's instruction. DNA was used as a target for amplification of approximately 200 bp of the variable V3 region of the 16S ribosomal RNA (rRNA) gene corresponding to positions 341-534 using the primers P2 (5'-ATTACCGCGCTGCTGGCTGG-3') and P3 (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3')<sup>1</sup>. The programme used for the amplification was a touch down PCR: an initial denaturation at 95 °C for 5 min, followed by 20 cycles at 95 °C for 1 minute, 65 °C for 1 minute and 72 °C for 1 min, going down after every two cycles 1 degree in the annealing temperature (from 65 °C to 56 °C) and 8 cycles at 95 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 min, with a final extension period at 72 °C for 3 min. The resulting amplicons were visualized on a 1.5% (w/v) TAE buffer (National diagnostic, UK) agarose gel to check for PCR products within the predicted size range (200–250 bp).

## **Denaturing Gradient Gel Electrophoresis (DGGE)**

PCR fragments were separated by denaturing gradient gel electrophoresis (DGGE) consisting of 8% polyacrylamide was performed using the BDH system from VWR International Ltd (UK), following the manufacturer's guidelines. PCR products (6  $\mu$ l) were loaded onto 8% (w/v) TAE polyacrylamide gels (40 mmol/l of Tris base, 20 mmol/l of acetic acid and 1 mmol/l of EDTA, pH 8.3), which contained a 40–60% denaturant gradient (100% denaturant, 7 mol/l of urea and 40% (v/v) deionized formamide). Electrophoresis was performed at a constant voltage of 100 V and at a constant temperature of 60°C for 16 h. Gels were then stained with AgNO<sub>3</sub> as described previously<sup>2</sup> and the gel image scanned using Canon Scanning software (Canon Scanner Lide 50, Surrey, UK). Scanned DGGE images were analyzed by Quantity One software (Version 4.5.2, Bio-Rad Laboratories, Herts, UK) to generate a dendrogram of similarity between DGGE profiles.

Dendrogram constructed from the DGGE data showing the microbial diversity of faeces collected 24 hours post dose from all study animals.



## References

1. Muyzer, G.; de Waal, E.C.; Uitterlinden, A.G.; *Appl. Environ. Microbiol.* **1993**, 59, 695-700.
2. Sanguinetti, C.J.; Dias Neto, E.; Simpson, A.J.; *Biotechniques* **1994**, 15, 914-921.