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Supplemental Material

Use of Shotgun Metagenomics and Metabolomics to Evaluate the Impact of Glyphosate or Roundup MON 52276 on the Gut Microbiota and Serum Metabolome of Sprague-Dawley Rats

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Figure S2. Correlations between the serum metabolome and the caecum metabolome. The Mantel.test function in the cultevo package of R was used to calculate the Mantel statistic for a Spearman's rank correlation coefficient between the log-transformed abundance values of the caecum metabolome and the serum metabolome datasets. The figure shows the density distribution of 1,000 permuted Spearman's rank correlation coefficients (Spearman's ρ, x-axis) from permutations of the samples. The blue arrow shows the Spearman's rank correlation coefficient for the unpermuted samples, with the empirical p-value of the Mantel test calculated as from a Monte-Carlo procedure described in North, et al. 2002, The American Journal of Human Genetics, 71 (2): 439—41.

Figure S3. Chromatograms following HPLC-MSMS for the detection of shikimic acid. Optimisation of the shikimic acid quantification assay were done in serum (top panel), water (middle panel) and in a 5% bovine serum albumin solution (lower panel). Y-axis: peak height (relative units).

Figure S4. Proteins interfere with the detection of shikimic acid in serum. Although a dose dependent increase in peak area (arbitrary units) was detected after spiking of samples with shikimic acid, the presence of proteins severely interfered with the detection of the signal and limited the reproducibility and sensitivity of this assay. BSA, bovine serum albumin.

Figure S5. The results of the analysis of 16S rRNA abundance correlates well with the results of the analysis of the complete shotgun metagenomics dataset. Statistical significance of the effects of the treatments was assessed with aldex2 using the complete shotgun metagenomics dataset (taxonomy inferred with the RefSeq database on the metagenomics RAST server) or a subset of 16S rRNA reads isolated from the shotgun metagenomics datasets using the metagenomics RAST server with a cut-off of 70% identity to ribosomal sequences from a reduced version of M5RNA with SortMeRNA. Statistical analysis was performed on a dataset corrected for asymmetry (uneven sequencing depths) using the inter-quartile log-ratio method, which identifies features with reproducible variance. We assessed statistical significance using a Kruskal–Wallis test. The p-values for statistical significance from these both analyses were transformed (- log10). The resulting -log10 p-values for each of the taxonomic group detected in both the complete shotgun metagenomics dataset and its 16S rRNA gene subset were correlated ($p = 0.003$) and plotted.

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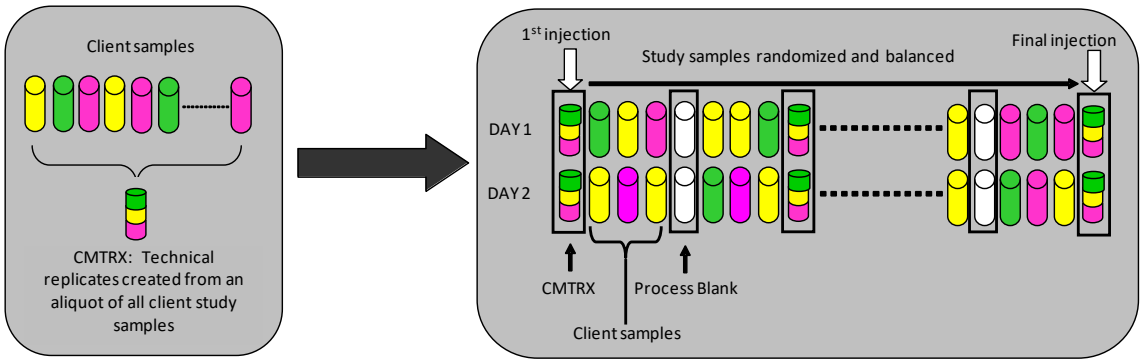
Additional File- Excel Document

Contaminant Screening.pdf File – Feed analysis to identify possible contaminants.



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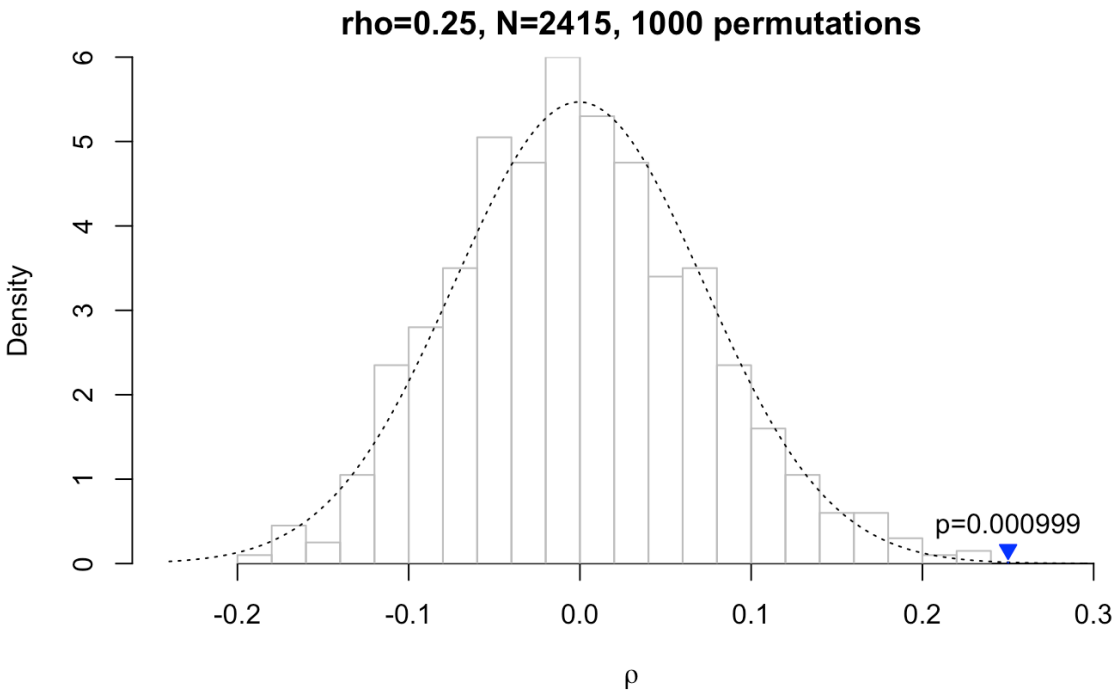

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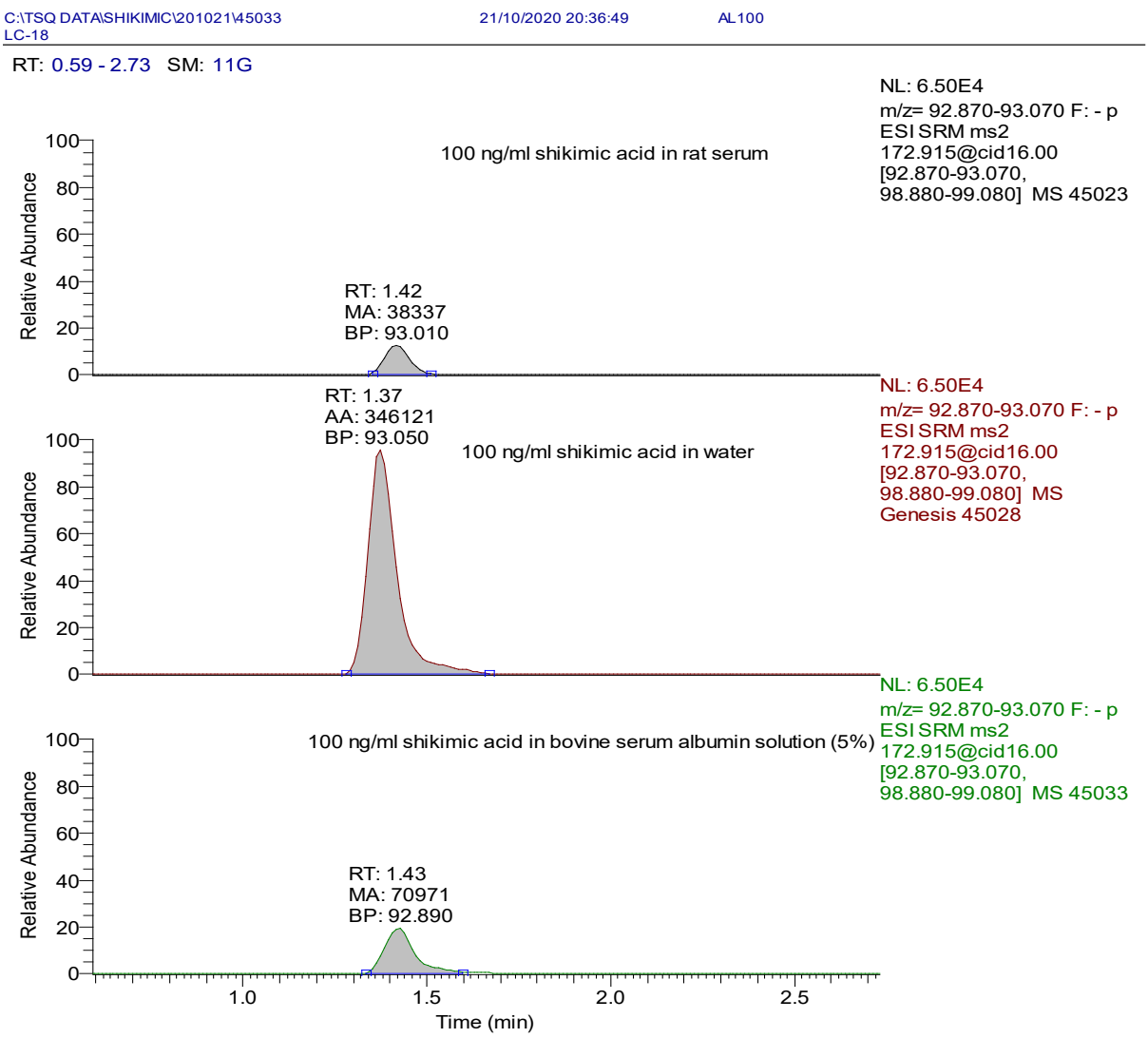

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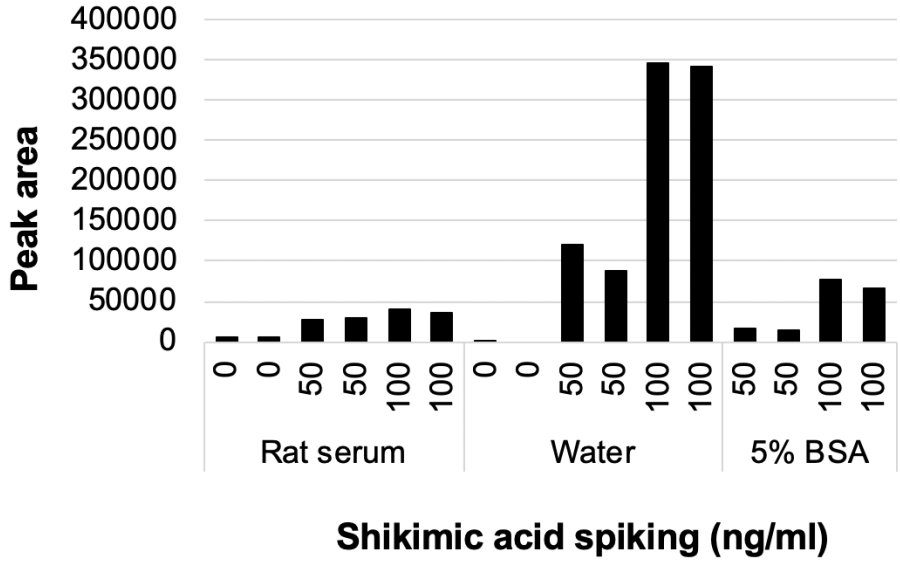

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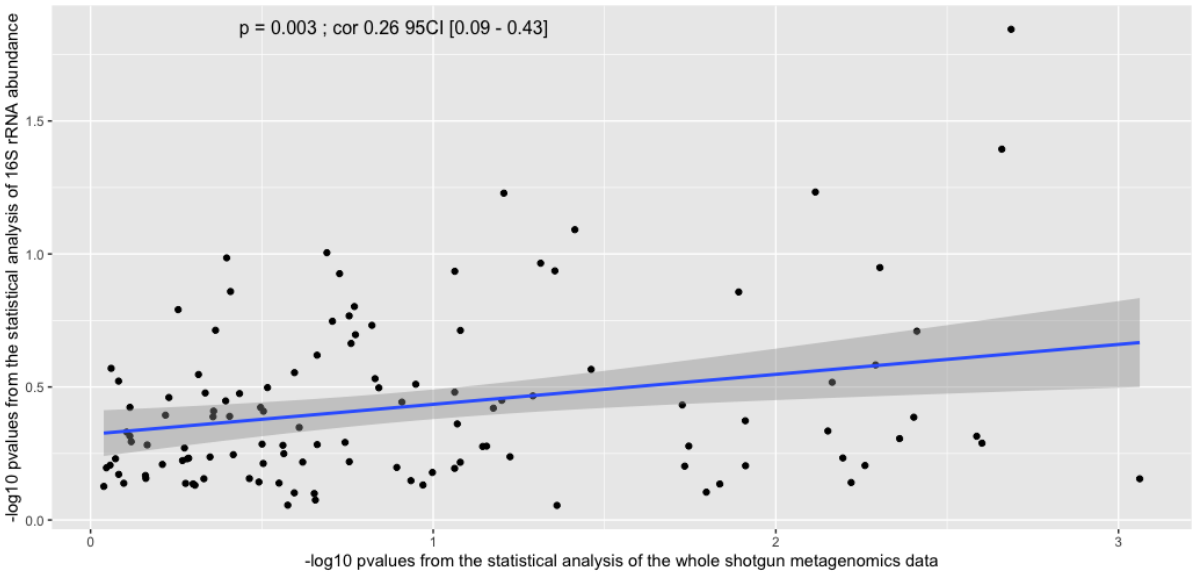

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Statistical significance of the effects of the treatments was assessed with aldex2 using the complete shotgun metagenomics dataset (taxonomy inferred with the RefSeq database on the metagenomics RAST server) or a subset of 16S rRNA reads isolated from the shotgun metagenomics datasets using the metagenomics RAST server with a cut-off of 70% identity to ribosomal sequences from a reduced version of M5RNA with SortMeRNA.

Statistical analysis was performed on a dataset corrected for asymmetry (uneven sequencing depths) using the inter-quartile log-ratio method, which identifies features with reproducible variance. We assessed statistical significance using a Kruskal–Wallis test. The p-values for statistical significance from these both analyses were transformed (- log10). The resulting -log10 p-values for each of the taxonomic group detected in both the complete shotgun metagenomics dataset and its 16S rRNA gene subset were correlated ($p = 0.003$) and plotted.

Table S1. Experimental design and sampling (N = number of animals measured)

Group	Treatment	Dose (mg/kg bw/ day glyphosate)	Body weights [e], food and water consumption [d]	caecum metabolomics [a,b]	serum metabolomics [a,c]	Metagenomics [a,b]
I	Control	0	yes (n =12)	yes (n =10)	yes (n =10)	yes (n=12)
II	Glyphosate	0.5	yes (n =12)	yes (n =10)	yes (n =10)	yes (n=12
III	Glyphosate	50	yes (n =12)	yes (n =10)	yes (n =10)	yes (n=12
IV	Glyphosate	175	yes (n =12)	yes (n =10)	yes (n =10)	no
V	MON 52276	0.5	yes (n =12)	yes (n =10)	yes (n =10)	yes (n=12
VI	MON 52276	50	yes (n =12)	yes (n =10)	yes (n =10)	yes (n=12
VII	MON 52276	175	yes (n =12)	yes (n =10)	yes (n =10)	no

[a.] The samples were collected at final sacrifice.

[b]. For each animal approximately 150 mg of caecum content and rectum faeces were collected at necropsy, put into labelled vials and stored at -70°C until metabolomic and microbiome analyses.

[c]. Blood samples were collected from all animals before sacrifice. Animals were anesthetized by inhalation using a mixture of CO_2/O_2 (70% and 30% respectively), and about 7.5 mL of blood were collected from the *vena cava*. The blood collected from each animal was centrifuged in order to obtain serum, which was aliquoted into labelled cryovials and stored at -70°C.

[d.] Water and food consumption: the daily water and food consumption per cage were measured before the start of the experiment, and weekly over the entire treatment period (13 weeks). Before sacrifice and after about 16 hours in a metabolic cage, water consumption, was recorded for each animal. The means of individual consumptions and related standard deviation were calculated for every group.

[e]. Body weight: body weight of experimental animals was measured before the start of the treatment, and then weekly for 13 weeks. All the experimental animals were weighed just before sacrifice. Average body weights and related standard deviations were calculated for each experimental group.

Table S2: Description of Metabolon quality control (QC) Samples

Description	Purpose
Large pool of human plasma maintained by Metabolon that has been characterized extensively.	Assure that all aspects of the Metabolon process are operating within specifications.
Pool created by taking a small aliquot from every customer sample.	Assess the effect of a non-plasma matrix on the Metabolon process and distinguish biological variability from process variability.
Aliquot of ultra-pure water	Process Blank used to assess the contribution to compound signals from the process.
Aliquot of solvents used in extraction.	Solvent Blank used to segregate contamination sources in the extraction.

Table S3: Metabolon QC Standards

Description	Purpose
Recovery Standard	Assess variability and verify performance of extraction and instrumentation.
Internal Standard	Assess variability and performance of instrument.

Table S4. Serum and caecum metabolomics quality control

QC Sample	Measurement	Median RSD	
		Cecal Contents	Serum
Internal Standards	Instrument Variability	3 %	4 %
Endogenous Biochemicals	Total Process Variability	10 %	9 %

Note: Instrument variability was determined by calculating the median relative standard deviation (RSD) for the internal standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the samples, which are technical replicates of pooled client samples. Values for instrument and process variability as shown in the table above meet Metabolon's acceptance criteria.

Table S5. Determination of Shikimic Acid in serum by LCMSMS. Version 201023

INSTRUMENTATION:

Thermo Accela Pump and CTC Autosampler coupled to a Thermo TSQ Quantum Access mass spectrometer

LIQUID CHROMATOGRAPHY (LC):

LC column: Thermo Accucore 100x2.1mm 2.6µ (LC18)
LC column temperature: 40°C
Injection tray temperature: Cool, not able to set temperature
Injection Volume: 10 µL
Flow rate: 200 µL
Run Time: 10 min

LC gradient:

Time, min	Solvent A: 0.01% Formic Acid in H_2O, %	Solvent B: Acetonitrile, %
0.0	100	0
0.2	100	0
2.0	80	20
2.2	80	20
3.5	0	100
5.0	0	100
5.5	100	0
10.0	100	0

LC Solvent Preparation:

Solvent A:
0.1 mL of Formic Acid dilute up to 1L with Fisher HPLC HPLC grade Water

Solvent B:
1L Fisher HPLC HPLC grade Acetonitrile

Retention Times:

Shikimic Acid: 1.5 min;
Diclofenac: 5.9 min;

MASS SPECTROMETRY (MS):

Tune File: shikimic-F02-200921
Method Name: Shikimic-200928grad-C18-A
Ionisation: Electrospray (ESI)

Polarity:	negative
Spray Voltage:	4000 V
Capillary Temperature:	350°C
Auxiliary Gas Flow:	0 Arb.
Sheath Gas Flow:	50 Arb.
Scan type:	SRM
Scan Width:	0.2 m/z
Scan Time:	0.150 sec
Numbers of Microscans:	1
Data type:	Profile
Divert Valve:	into Waste: 0 - 0. 5 m, into MS: 0.5 – 10 m

SRM Transitions:

Substance	Transitions m/z	Collision energy, Arb	Tube Lens Offset, V
Shikimic Acid	172.915 → 92.87 172.915 → 98.98	16	81
Diclofenac	293.74 → 249.65 293.74 → 213.720	10	76

SRM transitions were determined from Infusion data acquired in central mode data type and peak width 0.4 m/z.

STOCK SOLUTIONS:

1 mg/mL Shikimic Acid (ShA) stock solution:

Dissolve 1 mg Shikimic Acid in 1 mL HPLC grade MeOH. Keep in -20ºC for 12 months.

1 mg/mL Diclofenac (D) stock solution (IS):

Dissolve 1 mg Diclofenac.Na in 1 mL HPLC grade MeOH (approximate concentration of 1 mg/ml). Keep in -20ºC for 12 months.

WORKING SOLUTIONS:

10 µg/mL ShA solution:

Dilute 10 µL of 1 mg/mL ShA up to 1 mL with HPLC grade H2O. Keep in -20ºC for 1 month.

100 ng/mL ShA solution:

Dilute 10 µL of 10 µg/mL ShA up to 1 mL with blank serum. Prepare fresh before use.

Internal Standard (IS)solution:

10 µg/mL D:

Dilute 10 µL of 1 mg/mL D up to 1 mL with HPLC grade MeOH. Keep in -20ºC for 6 months.

50 ng/mL D (IS-50n):
Dilute 5 µL of 10 µg/mL D up to 1 mL with HPLC grade H2O. Prepare fresh before use.

CALIBRATION SAMPLES PREPARATION:

Sample	ShA concentration, ng/ml	100 ng/mL ShA solution, µL	Serum, µL
C0	0.0	X	1000
C1	10	100	900
C2	25	250	750
C3	50	500	500
C4	100	neat	X

SAMPLE PROCESSING:

C0-C4, test serum samples:

1. Pipette 100 µL of C0-C4 and test samples into individual 1.7 ml Eppendorf vials, add 10 µL of **IS-50n** solution, and 500 µL of acetonitrile, vortex.
2. Keep at -20°C for 1 hour.
3. Centrifuge 5 min at 14,000 rpm,4°C.
4. Transfer clear supernatants into 1.7 ml Eppendorf vials, evaporate to dryness in rotavap at 30°C.
5. Reconstitute in 80 µL Acetonitrile:H_2O (1:1), vortex.
6. Centrifuge 5 min at 14,000 rpm,4°C.
7. Transfer to injection vials.

Table S6. Composition of the de Man, Rogosa et Sharpe (MRS) broth used in the *in vitro* study of bacterial growth

Compounds	Quantity (g)	Providers (Cat. Number)
Tween80	1.08	Fisher scientific (T164500)
Yeast Extract	5.00	Biokar Diagnostics (A1202)
Glucose	20.00	Sigma (D9434)
Potassium phosphate dibasic	2.00	Sigma (60353)
Sodium acetate	5.00	Sigma (71183)
Ammonium citrate dibasic	2.00	Sigma (247561)
Magnesium sulfate	0.20	Sigma (M7506)
Manganese sulfate monohydrate	0.05	Sigma (M7634)