Supplementary Information

Dynamic Metabonomic and Microbiological Response of Rats to Lincomycin Exposure: An Integrated Microbiology and Metabonomics Analysis

Manna Lin,[#ab] Zhiyong Xie, [#b] Yuting Zhou,[b] Yemeng Li,[b] Jian Ren,[c] Xuan-xian Peng,[c] Meicun Yao,[b] Zhongzhou Yang[b] and Qiongfeng Liao*[a]

[a]*School of Chinese Materia Medica, Guangzhou University of Chinese Medicine, Guangzhou, 510006, P. R. China*

[b]*School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, 510006, P. R. China*

[c]*School of Life Sciences, Sun Yat-sen University, Guangzhou, 510006, P. R. China*

*Corresponding author: Qiongfeng Liao

Tel. +86 20 3935 8081

Fax +86 20 3935 8050

E-mail address: liaoqf2075@yahoo.com

[#] These authors contributed equally to this work.

Supplementary information text:

Materials and methods

Chemicals

Lincomycin (LM) was purchased from Dalian Meilun Biotech Co., LTD (Shandong, China) and authorized for experimental use by School of Pharmaceutical Sciences, Sun Yat-sen University (Guangzhou, China). D_2O (with 0.05% sodium 3-trimethylsilyl-(2, 2, 3, 3-d_4)-1-propionate, TSP) was obtained from Qingdao Teng Long Technology Co., LTD (Qingdao, China). Phosphate buffer was prepared with $K_2HPO_4 \cdot 3H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ essentially refer to the previously reported.[23] TIANamp tool DNA Kit and *Escherichia coli* DH5*α* were obtained from Tiangen Biotech Co., LTD (Beijing, China). All the primers and Taq PCR Master Mix (2×, blue dye) were purchased from Sangon Biotech Co., LTD (Shanghai, China). p GEM-T Easy Vector was purchased from Promega Biotech Co., LTD (Beijing, China).

Sample preparation and NMR spectroscopy

A previously optimized method was adopted for preparation of urine samples.[23] Briefly, samples were removed from –80 ˚C storage and thawed at room temperature. Urine samples were prepared by blending 550 μL of urine with 55 μL of a phosphate buffer (K_2HPO_4/NaH_2PO_4, 1.5 M, pH 7.4, 100% D_2O) containing 0.05% TSP for chemical shift reference (δ 0.00). Faecal samples were extracted as previously optimized[24] with slight modifications. In brief, samples (100 ± 2 mg) were sonicated twice with 800 μL phosphate buffer (0.1 M, K_2HPO_4/NaH_2PO_4, pH 7.4)

containing 0.005% TSP, 10% D_2O and 0.01% NaN_3. After 10 min of centrifugation (4 ˚C, 16000 g), 500 μL samples of supernatant was pipetted into 5 mm NMR capillary tubes for NMR analysis.

Microbiological analysis of faecal samples

Briefly, the selected bands were excised from the DGGE gels and their fragments were reamplified with primers 357f and 518r. Following verification by agarose gel electrophoresis, the PCR products were ligated with p GEM-T Easy Vector and then transformed into *Escherichia coli* DH5*α*. The inserted DNA of positive clones was amplified using the nest approach. The primers for the first PCR were T7 and SP6, and for the second, they were 357f_GC and 518r. The resulting PCR products, which confirmed to the same position as the original band in DGGE gels were sequenced (Sangon Biotech, Shanghai, China).

Supplementary figures and tables:

Figure S1 Overview of experimental workflow. The experimental workflow combined urinary and faecal metabolic profiling and 16S rRNA gene PCR-DGGE profiling to investigate the impact of lincomycin exposure on the metabolites and gut microbiome.

Figure S2 Changes of body weight and body weight ratio of rats in lincomycin group (LIN) compared with control group (CTR). A, body weight of rats in the whole experimental period; B, increased body weight ratio on days 5, 14 and 21. The data are expressed as the means ± SD (* $p < 0.05$, ** $p < 0.005$).

Figure S3 Trajectorises of different datasets (A) and (B) are score plots of PCA derived from urinary (A) and faecal (B) metabonomic data for the control group (CTR, red) and lincomycin group (LIN, blue) across the time course of pre-dose (day -1), lincomycin dose (day 5, day 14) and recovery period (day 21). Each dot in the plots represents mean values of the scores from the first and second principal components at a time point. PCA scores of urinary (C) and faecal (D) metabolic profiles show similar time-dependent changes of the lincomycin at different time points.

Figure S4 Permutation test plots (200 permutations) for urine at day 5 (A1), day 14 (A2) and day 21 (A3).

Figure S5 Permutation test plots (200 permutations) for faece at day 5 (A1), day 14 (A2) and day 21 (A3).

Figure S6 OPLS-DA score plots (left panel) and PCR-DGGE profiles (right panel) derived from the 16S rRNA gene *Bacteroides* spp. (A) and 16S rRNA gene *Clostridium leptum* (B), indicating the discrimination between control group (CTR, black) and lincomycin group (LIN, red) rats at day 14.

Table S1 The primers of V3 region, *Bacteroides* spp. and *Clostridium leptum*.

Table S2 PCR amplification system of V3 region, *Bacteroides* spp. and *Clostridium leptum.*

Table S3 PCR program and DGGE analysis condition of V3 region, *Bacteroides* spp. and *Clostridium leptum.*

Table S4 ^{1}H NMR data for metabolites in rat urine and faeces.

Table S5 Significant changes in urine metabolites of the LIN at day 5, day 14 and day 21.

Table S6 Significant changes in faecal metabolites of the LIN at day 5, day 14 and day 21.

Table S7 Correlation coefficients between the bacterial species from bands and relative concentration of fecal metabolites obtained from samples collected at day 14 post-treatment.

76

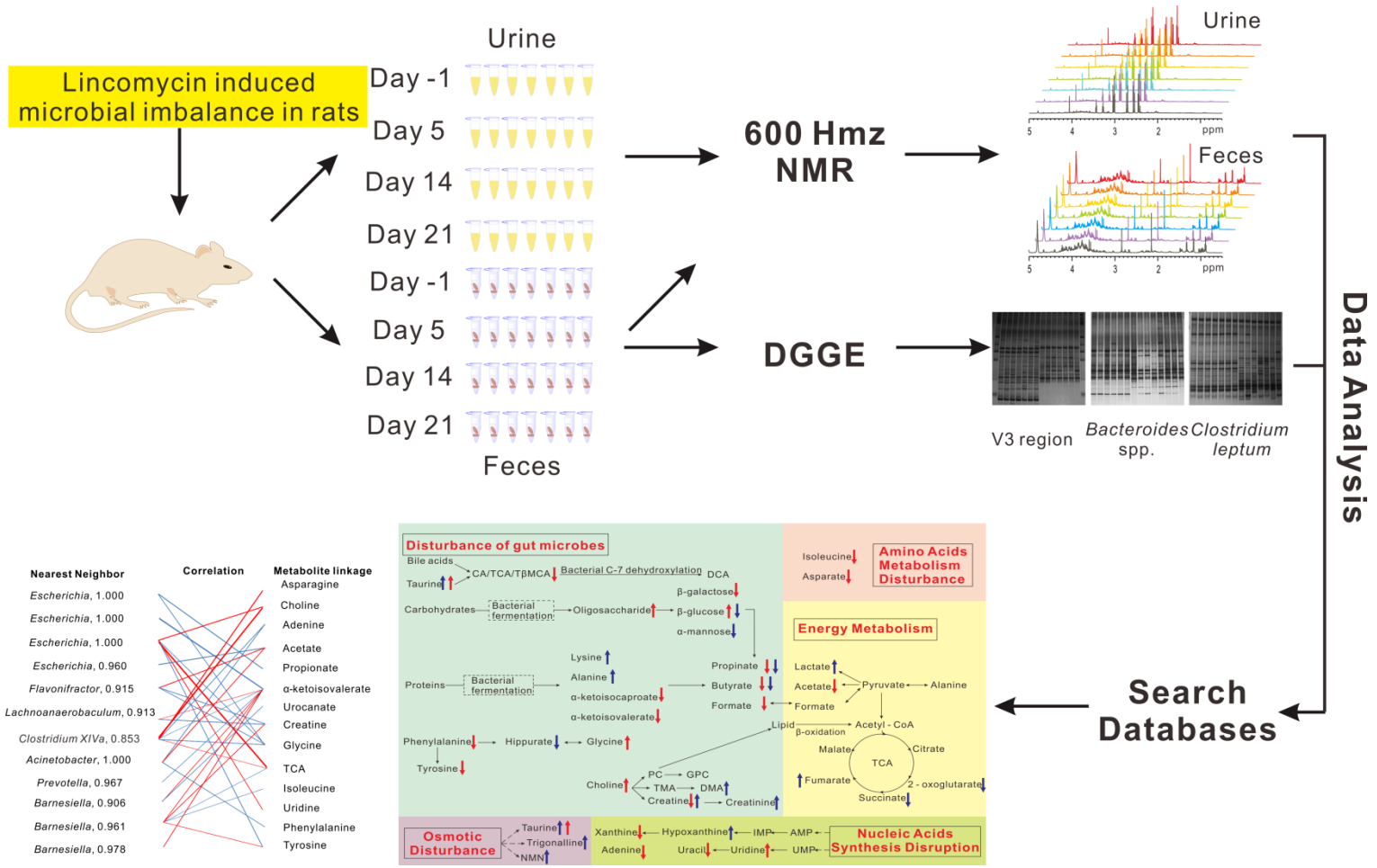

Figure S1 Overview of experimental workflow. The experimental workflow combined urinary and faecal metabolic profiling and 16S rRNA gene PCR-DGGE profiling to investigate the impact of lincomycin exposure on the metabolites and gut microbiome.

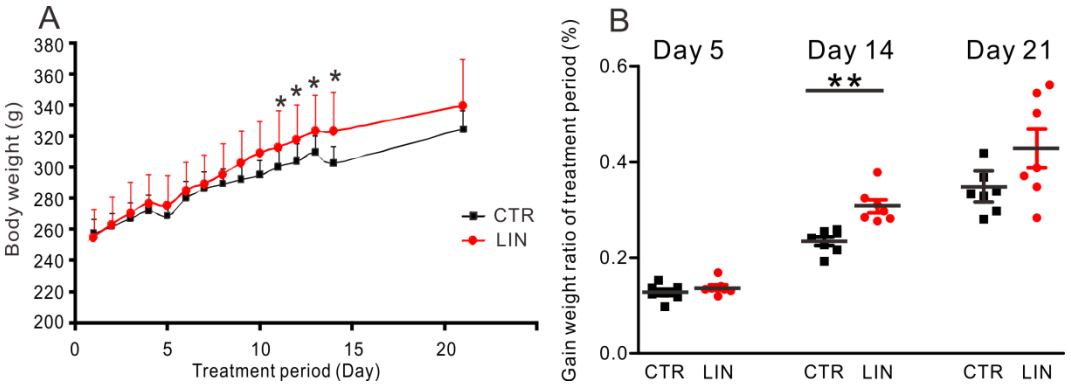

Figure S2 Changes of body weight and body weight ratio of rats in lincomycin group (LIN) compared with control group (CTR). A, body weight of rats in the whole experimental period; B, increased body weight ratio on days 5, 14 and 21. The data are expressed as the means ± SD (* $p <$ 0.05, ** $p < 0.005$).

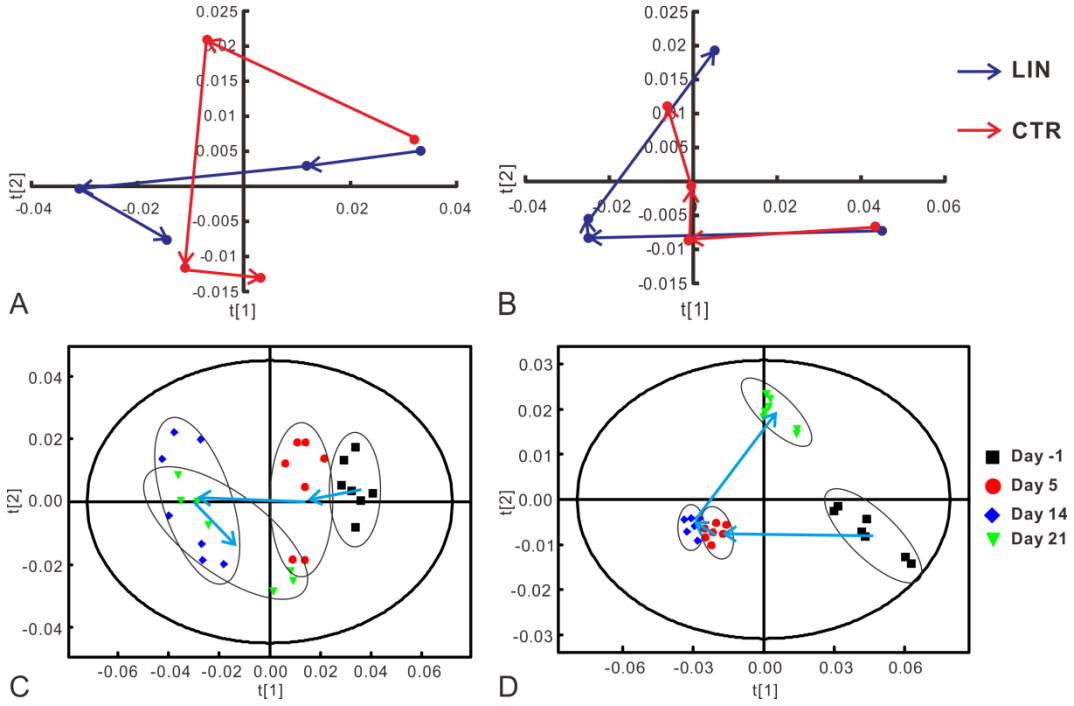

Figure S3 Trajectorises of different datasets (A) and (B) are score plots of PCA derived from urinary (A) and faecal (B) metabonomic data for the control group (CTR, red) and lincomycin group (LIN, blue) across the time course of pre-dose (day -1), lincomycin dose (day 5, day 14) and recovery period (day 21). Each dot in the plots represents mean values of the scores from the first

and second principal components at a time point. PCA scores of urinary (C) and faecal (D) metabolic profiles show similar time-dependent changes of the lincomycin at different time points.

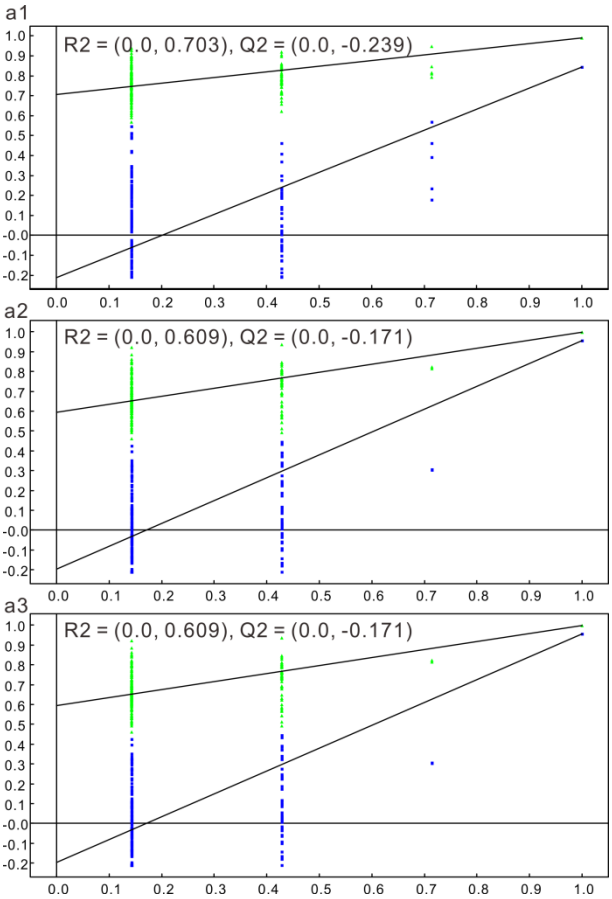

Figure S4 Permutation test plots (200 permutations) for urine at day 5 (A1), day 14 (A2) and day 21 (A3).

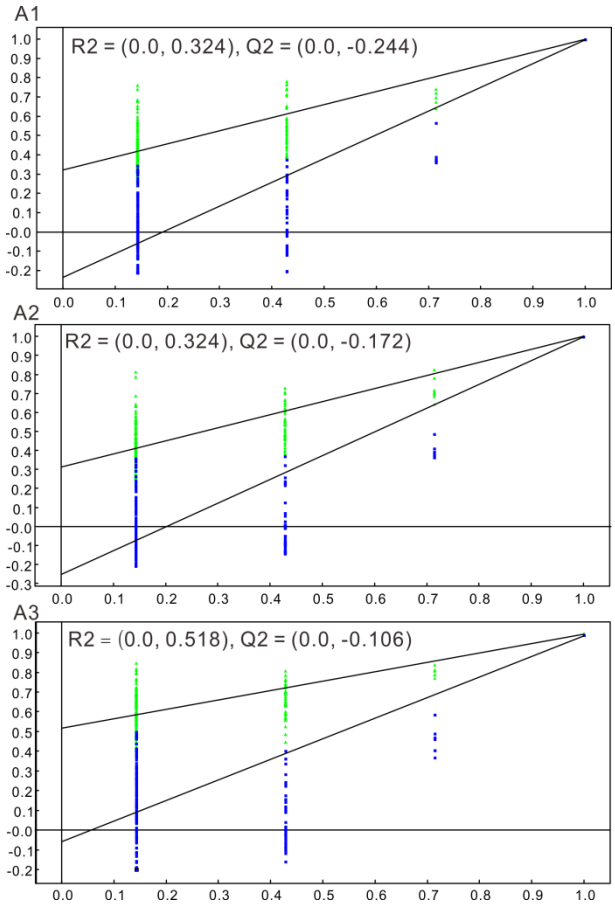

Figure S5 Permutation test plots (200 permutations) for faece at day 5 (A1), day 14 (A2) and day 21 (A3).

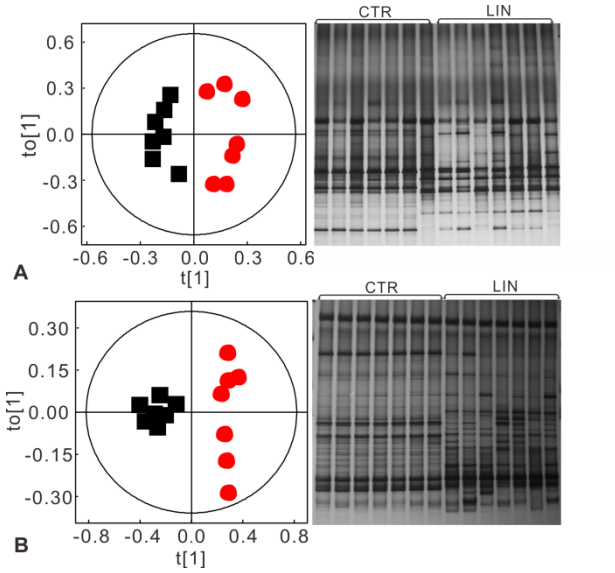

Figure S6 OPLS-DA score plots (left panel) and PCR-DGGE profiles (right panel) derived from the 16S rRNA gene *Bacteroides* spp. (A) and 16S rRNA gene *Clostridium leptum* (B), indicating

the discrimination between control group (CTR, black) and lincomycin group (LIN, red) rats at day 14.

Table S1 The primers of V3 region, *Bacteroides* spp. and *Clostridium leptum*.

	Primers	Sequences
V3 region	357f_GC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCA-3'
	518r	5'-ATTACCGCGGCTGCTGG-3'
Bacteroides spp.	Bfr-R	5'-CCGCAAACTTTCACAACTGACTTA-3'
	Bfr-F	5'-CTGAACCAGCCAAGTAGCG-3'
Clostridium leptum	Clept-F	5'-GCACAAGCAGTGGAGT-3'
	Clept-R	5'-CTTCCTCCGTTTTGTCAA-3'

Table S2 PCR amplification system of V3 region, *Bacteroides* spp. and *Clostridium leptum.*

Reagents	357f_GC (μL)	518r (μL)	Bfr-R (μL)	Bfr-F (μL)	Clept-F (μL)	Clept-R (μL)	Taq PCR Master Mix (μL)	Templates (ng)	dd H_2O
V3 region	2	2	0	0	0	0	25	20	Add water to total volume of 50 μL
Bacteroides spp.	0	0	2	2	0	0	25	20	
Clostridium leptum	0	0	0	0	2	2	25	20	

Table S3 PCR program and DGGE analysis condition of V3 region, *Bacteroides* spp. and *Clostridium leptum.*

	V3 region of 16S rRNA gene		*Bacteroides* spp.		*Clostridium leptum*	
	Temperature (°C)	Time	Temperature (°C)	Time	Temperature (°C)	Time
Pre-Duration	95	5 min	95	4 min	94	5 min

Duration	95	1 min	95	30 s	94	30 s
Anneal	55	1 min	52	30 s	55	30 s
Extend	72	1 min	72	1 min	72	30 s
Post-Extend	72	10min	72	5 min	72	10min
DGGE	38%-58%		28%-50%		35%-50%	
Voltage	70 V		70 V		70 V	
DGGE Time	12 h		13 h		13 h	

Table S4 ^{1}H NMR data for metabolites in rat urine and faeces.

keys[a]	Metabolites	δ ^{1}H (ppm) [b] and multiplicity	δ^{13}C	Samples[c]
1	Acetone	2.227(s)	28.2	U
2	Leucine	3.74(t),0.96(d),0.98(d),1.72(m)	24.7	U,F
3	Valine	0.95(d),1.05(d),2.28(m),3.614(d)	19.6, 20.5	U,F
4	Inosine	8.355(s),8.25(s)	143.0, 149.5	U
5	Guanidinoacetate	3.80(s)	47.5	U
6	Carnitine	3.23(s)	#	U
7	Xanthosine	7.88(s),5.86(d),4.73(m),4.41(m)	141.0, 90.2	U
8	3-Hydroxyisovalerate	1.26(s),2.35(s)	30.2	U
9	Lactate	1.33(d),4.12(q)	23.0, 71.3	U,F
10	α-Hydroxyisobutyrate	1.35s	27.5	F
11	Alanine	1.492(d),3.78(q)	53.0, 19.2	U,F
12	Hypoxanthine	8.22(s),8.20(s)	145.2, 147.5	U
13	Cytidine	7.85(d),6.07(d)	26.4	U
14	*N*-Acetylglycine	2.04(s)	24.2	U
15	*O*-Acetylcholine	2.13(s)	23.8	U
16	NADP	9.35(s),9.16(s),8.84(d),8.19(m)	#	U
17	Acetate	1.92(s)	26.1	U,F
18	Pyruvate	2.375(s)	29.5	U,F
19	Succinate	2.407(s)	37.3	U,F

20	Citrate	2.56(d),2.71(d)	48.7, 48.7	U
21	Methylamine	2.615(s)	27.0	U
22	Dimethylamine	2.729(s)	36.8	U
23	Trimethylamine	2.887(s)	47.4	U,F
24	3-Hydroxybutyrate	2.32(dd),4.16(d t),1.203(d)	49.6, 24.5	U
25	*N*,*N*-Dimethylglycine	2.91(s),3.71(s)	46	U,F
26	Creatinine	3.043(s),4.05(s)	33.1, 59.8	U
27	Creatine	3.045(s),3.93(s)	38.2, 56.2	U,F
28	Hydroquinone	6.81(s)	118.0	U
29	2-Oxoglutarate	2.454(t),3.02(t)	33.2, 39.0	U
30	cis-Aconitate	3.133(d),5.72(t)	127, 46.7	U
31	Malate	2.38(dd),2.67(dd),4.295(d)	45.5, 98.0	U,F
32	*α*-Mannose	5.195(s)	97.5	U
33	Isovalerylglycine	0.94(m),2.17(d),3.76(d)	24, 47.6, 45.5	U
34	Methylmalonate	1.25(d),3.18(q)	18.0	U
35	Choline	4.075(t),3.515(t),3.20(s)	58.2, 56.9	U,F
36	Taurine	3.275(t),3.437(t)	49.5, 37.5	U
37	Acetoacetate	2.282(s)	32.3	U
38	Betaine	3.27(s),3.91(s)	57.0, 68.0	U
39	Formate	8.46(s)	#	U,F
40	*N*-Acetylglutamate	2.228(t),1.99(s)	#	U
41	Benzoate	7.88(d),7.49(t),7.55(t)	131.2, 130.0, 134.0	U
42	3-Methyl-2-Oxovalerate	1.10(d),0.88(t)	13.2, 16.4	U
43	Pantothenic acid	0.84(s)	21.8	U
44	Hippurate	3.975(d),7.55(t),7.64(t),7.835(t)	46.1, 131.5, 134.9, 129.9	U
45	Glycine	3.57(s)	44.5	U,F
46	4-Cresol sulfate	2.35(s),7.22(d),7.29(d)	#	U
47	NMN	4.48(s),8.90(d),8.97(d),9.29(s)	#	U
48	CDP	6.13(d),7.97(d),5.98(d)	#	U

49	CMP	6.14(d),8.10(d),6.01(d)	99.2, 91.5	U
50	ITP	8.51(s),8.24(s)	149.0	U
51	*N*-Methyl nicotinate	4.45(s),8.83(d),9.13(s)	#	U
52	Dihydroxyacetone	4.457(s)	67.6	U
53	Ttrigonelline	4.44(s),8.09(m),8.85(m),9.125(s)	51.3	U
54	trans-Aconitate	3.50(s),6.57(m)	39.2	U
55	Allantoin	5.39(s)	#	U
56	Fumarate	6.53(s)	137.5	U,F
57	*p*-Cresol	7.14(d),6.835(d),2.255(s)	117.9, 22.1	U
58	Histidine	7.09(s),7.907(s)	119, 139.1	U,F
59	Butyrate	0.90(t),1.56(m),2.15(t)	42.3, 16	U,F
60	α-Ketoisocaproate	0.92(d),2.06(m),2.61(d)	#	F
61	Isoleucine	0.94(t),1.01(d),1.25(m),1.48(m), 1.98(m),3.67(d)	62.4, 17.2, 14.7	F
62	Propionate	1.06(t),2.19(q)	13.5, 19.2	U,F
63	1,3-Dihydroxyacetone	4.42(s),3.57(s)	67.5	F
64	α-Ketoisovalerate	1.13(d),3.02(m)	18.8	U,F
65	Threonine	1.32(d),3.58(d)	23.5, 63.2	U,F
66	Lysine	3.03(t),3.76(t)	41.0	U,F
67	Citrulline	1.57(m),1.87(m),3.15(t),3.75(t)	42.0, 57.9	F
68	Glutamate	2.10(m),2.09(m),2.36(m),3.77(m)	#	F
69	Methionine	2.14(s),2.16(m),2.65(t),3.86(t)	16.0, 31.0, 56.7	F
70	TMAO	3.275(s)	61.5	U,F
71	Aspartate	2.68(dd),2.82(dd),3.91(m)	39.3	F
72	Asparagine	2.86(dd),2.96(dd),4.00(m)	#	F
73	β-Xylose	3.24(dd),4.57(d)	99.7	U,F
74	*N*-Acety-Glycoprotein	2.03(s)	24.5	F
75	Uracil	5.81(d),7.54(d)	104.2, 146.2	F
76	Tyrosine	6.91(d),7.20(d)	133.7, 117.0	U,F
77	Tryptophan	3.31(dd),3.49(dd),4.06(dd),7.21(t),	#	F

		7.29(t),7.33(s),7.55(d),.774(d),		
78	Phenylalanine	3.13(dd),3.29(dd),3.98(dd),7.33(m), 7.38(m),7.43(m)	38.9, 58.0	F
79	Urocanate	6.40(d),7.31(d),7.43(s),7.89(s),	144.4, 124.0	F
80	Sucrose	5.40(d),4.21(d),4.04(t),3.82(m), 3.67(s),3.55(m)	94.5, 78.9, 76.2	F
81	5-Aminovalerate	1.62(m),1.65(m),2.24(t),3.02(t)	38.9, 41.0	F
82	*α*-Ketoglutarate	2.45(t),3.01(t)	33.4, 38.3	F
83	*α*-Arabinose	5.21(d),5.30(d),4.51(d),4.10(m), 3.89(dd)3.66(m)	93.8	F
84	*α*-Glucose	5.243(d),3.72(dd)3.53(dd)	94, 73.8	U,F
85	Adenine	8.19(s),8.21(s)	144.2, 156.2	F
86	*α*-Galactose	5.26(d),4.58(d),4.08(m),3.64(dd)	94.0, 99.2	F
87	*β*-Arabinose	3.53(dd),4.52(d),	#	U,F
88	*β*-Galactose	3.48(dd),3.65(dd),3.93(m),3.71(m), 4.59(d),3.74(m)	94.0	F
89	*α*-Xylose	5.25(d),3.52(dd)	93.8	U,F
90	Uridine	3.81(d),3.92(d),4.14(q),4.24(t),4.36 (t),5.90(d),5.91(d),7.87(d)	143.9, 92.0,104.8	F
91	*β*-Glucose	4.657(d),3.235(d),3.733(dd)	98.9, 75.9,77.5	U,F
92	TCA	0.67(s)	12.5	F
93	T*β*MCA	0.69(s)	#	F
94	DCA	0.72(s)	16.1	F
95	Xanthine	7.91(s)	144.2	U,F
96	Urea	5.78(s)	#	U,F

[a]Keys: NADP, nicotinamide adenine dinucleotide phosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; ITP, inosine triphosphate; TCA, taurocholic acid; T*β*MCA, tauro-*β*-muricholic acid; DCA, deoxycholic acid; TMAO, trimethylamine oxide; NMN, *N*-methylnicotinamide. [b]s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets. [c]F, faeces; U, urine; #, not determined.

Table S5 Significant changes in urine metabolites of the LIN at day 5, day 14 and day 21.

Urine metabolites	Correlation coefficients (r[a]) LIN vs. CTR		
	day 5	day 14	day 21
	R^2X=0.667	R^2X=0.487	R^2X=0.724
	Q^2=0.908	Q^2=0.960	Q^2=0.866
Fumarate	-	0.79	-
Taurine	-	0.83	-
Dimethylamine	-	0.79	-
Lactate	0.79	0.79	-
Hippurate	-0.90	-0.9	-0.82
Butyrate	-0.78	-0.83	-0.81
3-Methyl-2-oxovalerate	-0.85	-0.89	-0.77
α-ketoisovalerate	-0.86	-0.84	-
Propionate	-0.86	-0.90	-
Acetate	-0.79	-	-
α-mannose	-	-0.76	-
β-glucose	-	-0.81	-
2-Oxoglutarate	-	-0.78	-
Trigonelline	0.87	0.90	-
Allantoin	0.76	0.87	0.78
Creatine	0.88	0.76	0.79
Creatinine	0.89	0.76	-
Lysine	0.76	0.77	-
Glycine	-	0.77	-
N-methyl nicotinamide (NMN)	0.81	0.77	-
Alanine	-	-0.82	-
Hypoxanthine	-	0.78	-

[a] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. P = 0.05, [r] = 0.755 was used as the corresponding cutoff value of correlation coefficient for the statistical significance based on the discrimination significance, respectively. ‘‘-’’ means the correlation coefficient [r] is less than the cutoff value.

Table S6 Significant changes in faecal metabolites of the LIN at day 5, day 14 and day 21.

Urine metabolites	Correlation coefficients (r[a]) LIN vs. CTR		
	day 5	day 14	day 21
	R^2X=0.964	R^2X=0.943	R^2X=0.746
	Q^2=0.999	Q^2=0.999	Q^2=0.987
β-Glucose	0.78	0.79	0.77
Uridine	0.78	0.82	0.77
Threonine	0.76	0.78	0.79
Taurine	0.77	0.80	0.76
Choline	0.77	0.79	0.82
Glycine	0.79	0.85	-
Formate	-0.77	-0.77	-0.78

Adenine	-0.78	-0.81	-0.77
Uracil	-0.78	-0.80	-0.77
Urocanate	-0.77	-0.79	-0.78
Tyrosine	-0.79	-0.84	-0.78
β-Galactose	-0.78	-0.79	-
Creatine	-0.80	-0.86	-
Aspartate	-0.78	-0.86	-0.77
taurocholic acid (TCA)	-0.78	-0.82	-0.77
α-Ketoisocaproate	-0.77	-0.81	-0.78
Acetate	-0.86	-0.92	-0.8
Propionate	-0.78	-0.84	-0.77
Butyrate	-0.78	-0.82	-0.80
Isoleucine	-0.77	-0.84	-0.78
α-Ketoisovalerate	-0.78	-0.87	-
Phenylalanine	-0.79	-0.77	-0.78
Xanthine	-	-0.82	-0.79

[a] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. $P = 0.05$, [r] = 0.755 was used as the corresponding cutoff value of correlation coefficient for the statistical significance based on the discrimination significance, respectively. ‘‘-’’ means the correlation coefficient [r] is less than the cutoff value.

Table S7 Correlation coefficients between the bacterial species from bands and relative concentration of fecal metabolites obtained from samples collected at day 14 post-treatment.

Bands	correlation coefficient (r[a])													
	Choline	Glycine	Tyrosine	TCA	Uridine	α-ketoisovalerate	Adenine	Asparagine	Acetate	Creatine	Phenylalanine	Propionate	Urocanate	Isoleucine
Band 1	0.85	-0.80	-0.90	0.85	0.77	-	-	-	-	-	-	-	-	-
Band 2	-0.80	-0.88	-	0.76	-	-0.77	-0.96	0.90	-	-	-	-	-	-
Band 3	-	-0.88	-	-	-	-	-	-	0.77	-0.80	-	-	-	-
Band 4	-	-	-	-	-	0.81	-	-	-	-0.76	-	-	-	-
Band 5	-	-	-	-	-	-	-0.89	-	0.80	-	-0.81	-	-	-
Band 6	-	-	-	-	-	-0.90	-	-	-	-	-	-	-	-
Band 7	-	-0.78	-	-	-	-	-	-	-	-	-	-0.89	-	-
Band 8	-	-	-	-	-	-	-	-	-0.77	-	-	-	-	-
Band 9	-	-	-	-	-	-0.84	-	-	-	-	-	-	-	-
Band 10	-	-	-	-	-	0.90	-	-	-	-	-	-	-	-
Band 11	-	-	-	-	-	0.86	-	-	-	-	-	-	-0.91	-
Band 12	-	-	0.77	0.84	-	0.94	-	-	-	0.84	-	-	-	-0.90

[a]Correlation coefficients, post-treatment.positive and negative signs indicate positive and negative correlation in the concentrations, respectively. P = 0.05, [r] = 0.755 was used as the corresponding cutoff value of correlation coefficient for the statistical significance based on the discrimination significance, respectively. ‘‘-’’ means the correlation coefficient [r] is less than the cutoff value.