Electronic Supplementary Information

Multi-omics and temporal dynamics profiling reveal disruption of central metabolism in *Helicobacter pylori* on bismuth treatment

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Supplementary Methods and Figures

Reagents and Instrumentation. All chemicals used were of analytical grade. Columbia blood agar base was purchased from Thermo Scientific. Brucella broth was purchased from BD (Difico). Deuteroxide (NMR quality) was purchased from Sigma. All solutions and glasswares were sterilized by autoclaving at 121 °C for 20 mins, at 15 psi. ^{1}H and 2D NMR spectra were recorded on a Bruker AV-600 spectrometer equipped with a 5 mm CPTCI probe (Bruker, BioSpin, Germany). An Agilent 7890A / 5975C GC-MS system was used for metabolite profiling.

Culture of *H. pylori*. *Helicobacter pylori* 26695 was obtained from American Type Culture Collection (ATCC 700392). Bacterial glycerol stock was recovered from -80 °C and cultured on Columbia agar (Oxoid) plates supplemented with 7% laked horse blood and selective antibiotics (Oxoid) at 37 °C for 3 days under microaerobic conditions (5% CO_2, 4% O_2 and 91% N_2) generated by CampyGen (Oxoid). Bacterial cells were then transferred into freshly prepared Brucella Broth medium supplemented with 0.2% β-cyclodextrin (Sigma) and cultured at 37 °C in a bacterial culture shaker with speed setting to 105 rpm. *H. pylori* grown to early log phase were treated by CBS (colloidal bismuth subcitrate, Livzon Pharmaceutical Ltd) and harvested at appointed time points by centrifugation at 4,000 g for 15 mins at 4 °C for further experiments.

Transcriptomics Assay and Data Processing. Total RNA was isolated from *H. pylori* treated with different concentrations of CBS for 12 h using total RNA isolation system (Promega). RNA concentration was measured using Qubit RNA assay kit in Qubit 2.0 flurometer (Life Technologies, CA, USA). The RNA degradation and contamination was monitored by 1% agarose gel electrophoresis.

For gene expression arrays, quality control and quantification of gene expression levels were performed with HTSeq v0.6.1 (1). Briefly, the initial preprocessing of the raw intensity data were firstly processed through in-house perl scripts to remove reads with low quality, and then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. Differential expression analysis between two groups (three biological replicates per condition) was performed using the DESeq R package (1.18.0). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting *p*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted *p*-value < 0.05 found by DESeq were assigned as differentially expressed. Subsequent

enrichment analysis including Gene Ontology (GO) and KEGG pathways enrichment analysis were implemented by the GOseq R package and KEGG database resource.

NMR Acquisition and Data Processing. Sample preparation procedure for one-dimensional ^{1}H NMR was as previously described with minor modification (2). Bacteria in mid-log phase were collected with cold PBS washing for three times, then extracted twice with 600 μL of ice-cold methanol / H_2O (2 : 1 v/v) extraction buffer using a tissue-lyser. After centrifugation at 16,000 g for 10 min at 4 °C, the combined supernatants were lyophilized after removal of methanol under vacuum. The extracts were then reconstituted in 600 μL of phosphate buffer (1.5 M, pH 7.4, TSP 0.05%, K_2HPO_4 / NaH_2PO_4 = 4:1) for NMR analyses.

^{1}H NMR spectra of bacteria were acquired at 298 K with a Bruker Avance III 600 MHz NMR spectrometer. A total of 256 transients spanning a spectral width of 8992.8 H_Z were collected with a relaxation delay of 2.0 s, an acquisition time of 1.82 s, and a mixing time of 100 ms, while water suppression was achieved by using a standard presaturation pulse sequence. For assignment purposes, a range of two-dimensional NMR spectra, including *J*-resolved, total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), homo- and heteronuclear correlation spectroscopy were also acquired for selected bacterial samples.

The ^{1}H NMR spectra (δ 10 - 0.2) were manually adjusted for phase and baseline distortions using TOPSPIN (version 2.1, Bruker, Biospin) and referenced to the TSP signal (δ = 0.0). The spectral region δ 0.2 - 9.8 was automatically integrated into regions with equal widths of 0.002 ppm (1.2 Hz) using AMIX software package (version 3.8, Bruker-Biospin, Germany). To remove the effects of variation in the suppression of the water resonance, the region from δ 5.0 through 4.7 was set to zero integral (3). The data were then normalized to total integrated intensity and par scaling was applied before pattern recognition analyses. Principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) were carried out using the SIMCA-P software package (version 13.0, Umetrics, Sweden). The validity of all models was additionally ensured with CV-ANOVA ($p < 0.05$) and permutation tests (200 permutations). Finally, the data were visualized by the color-coded correlation coefficient plots generated in MATLAB (R2015b, The Mathworks Inc., Natwick, USA). A correlation coefficient of $|r| > 0.602$ was considered to be statistically significant ($p < 0.05$).

GC-MS Acquisition and Data Processing. The extraction procedure was used as a previous protocol with modification (4). The bacteria in mid-log phase of control group and dosing groups were adjusted to the same amount according to the OD_{600}. Then cold methanol was added to stop the metabolism of bacteria. Cell pellets were thawed on ice and then resuspended

in 1 mL of methanol by pipetting. The solution was lysed by ultrasonication for 3 min (5 s pulses, 10 s stops) at a 15 % power setting. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was transferred to new tubes. Then the supernatant containing 5 μg ribitol as an internal standard was dried using a rotary vacuum centrifuge device.

Samples were derivatized according to the literature (5). Briefly, 80 μL of 20 mg/mL methoxylamine (Sigma Aldrich) in pyridine were added to the dried sample and incubated for 90 min at 37 °C, followed by the addition of 80 μL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Sigma Aldrich) and incubated at 37 °C for another 30 min. The 140 μL of derivated samples were transferred to the GC-MS autosampler vial. Analysis was carried out on an Agilent Technologies 7890 gas chromatograph coupled to mass spectrometry 5975C which was equipped with a HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, Agilent J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a constant flow of 1 mL/min. Electron impact ionization (EI) mode was selected, while the ionization energy was 70 eV and the mass range scanned was 33 - 550 m/z at a rate of 2 scan/s. 1 μL of derivatized sample was injected to the injector at 280 °C, and the initial column temperature of 85 °C was held for 3 min and then ramped at 20 °C/min to 310 °C and held for 7 min. Two technical replicates were prepared for each sample.

R statistical package, MSD chemstation with NIST MS search 2.0 program and internal standards were used to perform the spectral deconvolution and calibration. Deconvolution and alignment with quantification and identification of metabolites from the GC-MS spectra were conducted through eRah and National Institute of Standards and Technology (NIST 11) Mass Spectra Library (6). The resulting data matrix was normalized to the integrated peak area of ribitol and the total spectral intensity of each sample. Multivariate statistical analysis including principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to reduce the high dimension of the data set. One-way ANOVA followed by Tukey's multiple comparisons test in SPSS statistics (version 24) was used to test the significance of metabolites. GraphPad Prism (version 5.01) was used to draw the histogram and the scatter plot. MetaboAnalyst 3.0 was employed to analyze the statistically enriched pathways over the identified metabolites (7).

Quantitative real-time PCR. The total RNA was extracted from *H. pylori* treated with different concentrations of CBS using the total RNA extraction kit as described above in transcriptomics assay. The RNA concentration and purity were estimated with NanoDrop 2000

(Thermo Scientific, Waltham, MA) by measuring absorbance at 260 and 280 nm, respectively. Then the extracted RNA was used to synthesize cDNAs using the GoScript reverse transcriptase system (Promega) according to the manufacturer's instructions. Real-time PCR was performed in 20 μL reaction volume using an ABI step one plus thermal cycler (Applied Biosystems, Foster City, CA). The thermal cycling conditions were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Melt curve analysis and agarose gel electrophoresis were then conducted to monitor the purity of the PCR products. 16S rRNA of *H. pylori* was used as an endogenous control. The expression levels of the target genes (Table S4) were calculated according to the amount of cycles in three replications.

Evaluation of the activities of key enzymes in TCA cycle. Activities of citrate synthase, aconitase, isocitrate dehydrogenase, fumarase and malate dehydrogenase were evaluated by the corresponding enzymatic activity colorimetric assay kits (Sigma-Aldrich). Citrate synthase activity was measured by monitoring the production of CoA during the synthesis of citrate from oxaloacetate. Aconitase was measured by monitoring the production of isocitrate during the isomerization of citrate to isocitrate. Isocitrate dehydrogenase activity was measured by monitoring the production of NADPH during the conversion of isocitrate to α-ketoglutarate using isocitrate and NADP as substrates. Fumarase activity was measured by monitoring the production of NADH during the hydration of fumarate to malate and a further transition step for NADH production. Malate dehydrogenase activity was measured by monitoring the production of NADH during the oxidation of malate to oxaloacetate. In brief, equal amounts of cells were collected by centrifugation, washed, and then resuspended in lysate buffer (from the assay kits). Following centrifugation, 50 μL of supernatant was transferred to 96-well plate, after the addition of substrates and developer, activities of different enzymes in *H. pylori* TCA cycle were determined by colorimetric assay. The standard curves were prepared according to kit instructions.

Measurement of ATP. Cellular ATP concentrations of *H. pylori* cells exposed to CBS were measured with BacTiter-Glo Microbial Cell Viability Assay (Promega) according to the manufacturer's instruction. Generally, cells were harvested, washed with PBS and diluted to 1 $\times 10^8$ cells/mL based on OD_{600}. To an aliquot of 100 μL culture, 100 μL of CellTiter-Glo reagent were added. The mixture was incubated at room temperature for 15 min and then the luminescence was measured on Biotek cytation 3. A standard curve was simultaneously constructed using 10-fold serial dilutions of ATP in the range of 1 μM to 10 pM.

Urease activity assay. *H. pylori* strains were cultured in Brucella Broth medium containing 0.2% β-Cyclodextrin at 37 °C in the presence of 80 μg/ml CBS. *H. pylori* cells were harvested at different time points and washed twice with 1 mL 50 mM Hepes (pH 7.5) and re-suspended in 800 μL of the same buffer. Cells were lysed, and 10 μL supernatants of the lysates were diluted in 250 μL 50 mM Hepes buffer (pH 7.5) containing 25 mM urea and incubated in 37 °C for 20 min to allow ammonia to be released. The phenol-hypochlorite assay was used to quantify the amount of ammonia. Meanwhile, the concentration of total proteins was measured by BCA protein quantification kit (23225, Thermo Scientific). The urease activity was defined as nmol ammonia produced per min per mg of total proteins (nmol · min^{-1} · μg^{-1}) in three biological replicates.

ROS detection. *H. pylori* was grown to log-phase and diluted to an OD_{600} of 0.3 in fresh media. For ROS analysis, cells with or without CBS treatment were taken at the indicated time points, and cells were harvested at equal amounts, then washed twice with PBS. After centrifugation at 4,000 g for 10 min, cell pellets were incubated with CM-H_2DCFDA (10 μM) for 30 min at 37 °C in dark. Cells were then harvested and re-suspended in PBS and 200 μL suspension was transferred to 96-well plate. The level of ROS was measured by multifunctional microplate reader (Cytation™ 3, Biotek) with 491 nm and 518 nm as excitation wavelength (Ex) and emission wavelength (Em), respectively.

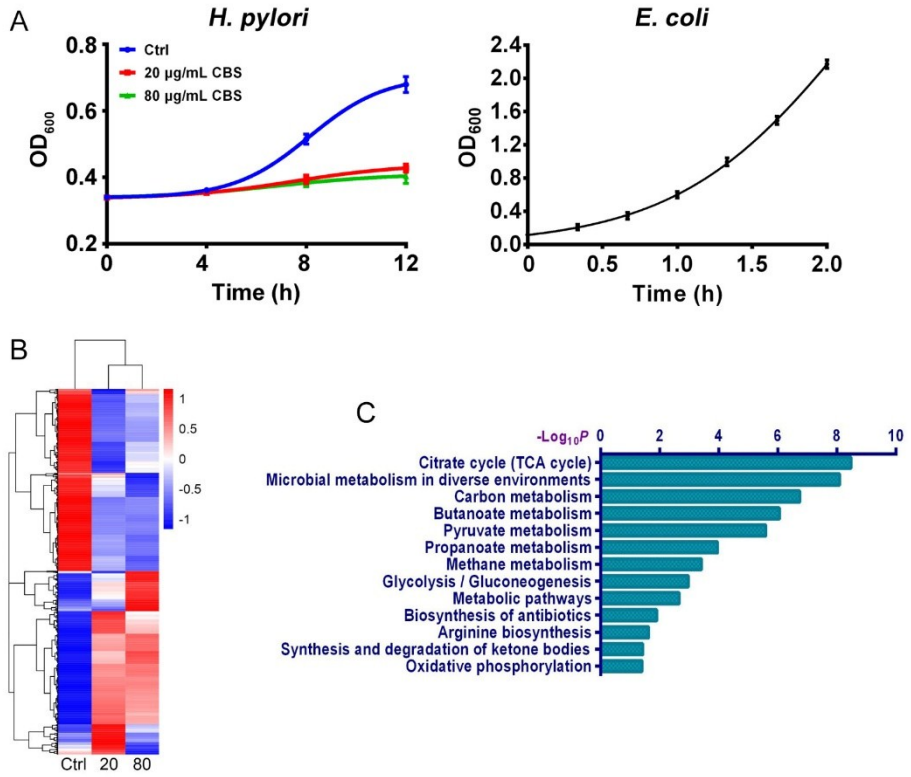

Fig. S1 (A) Growth curves of *H. pylori* (in Brucella Broth + 0.2% β-cyclodextrin) and *E. coli* (in LB Broth). (B) Cluster analysis of differentially expressed genes in control and CBS treated groups. The cluster map was created based on the normalized $\log_{10}$ (FPKM+1) values. (C) Enriched pathways of significantly altered common genes of CBS treated groups at a concentration of 20 and 80 μg/mL.

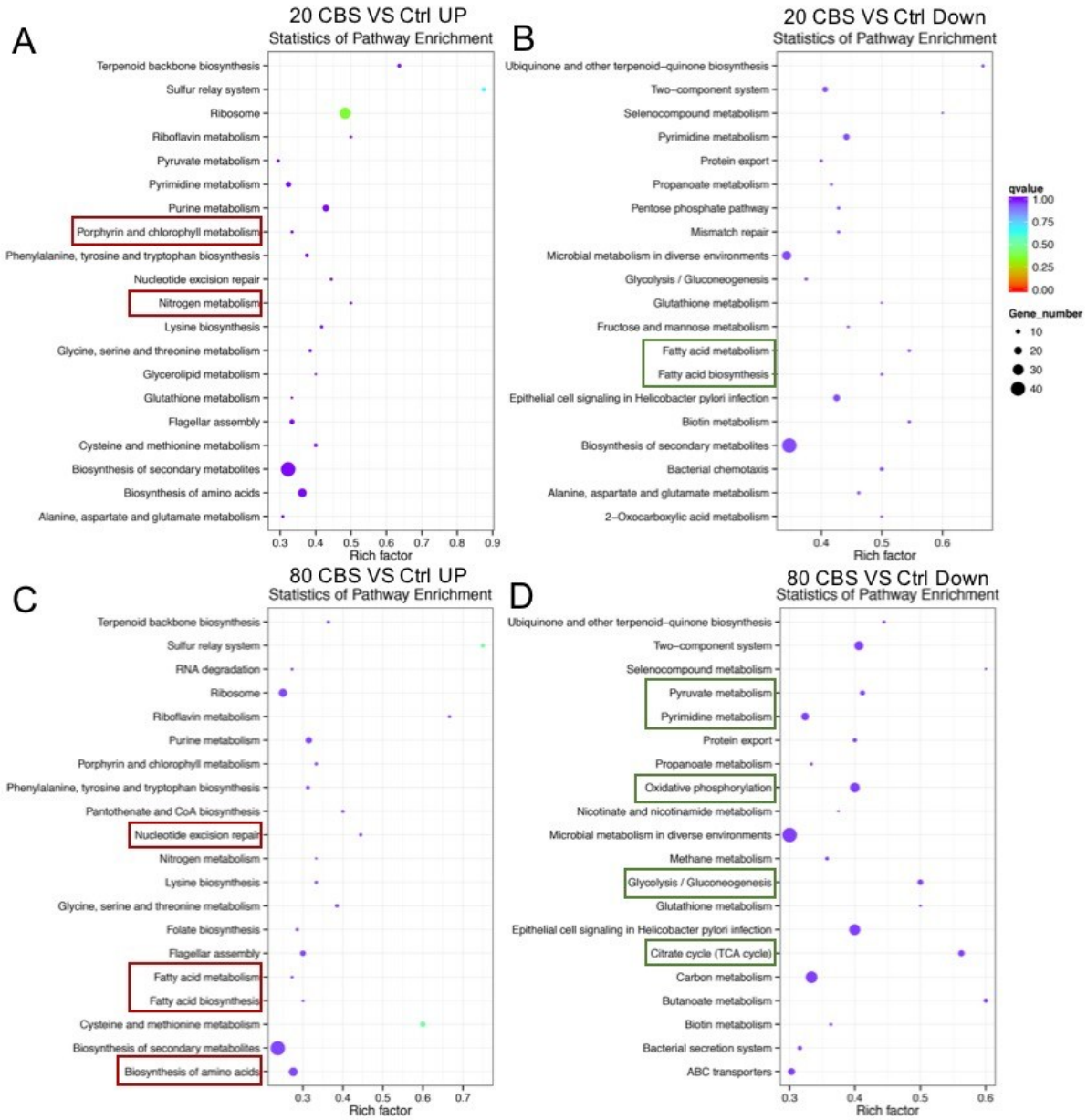

Fig. S2 Statistical enrichment KEGG pathway scatter plot of differentially expressed genes (DEGs). (A) Up-regulated gene sets of 20 μg/mL CBS treated group. (B) Down-regulated gene sets of 20 μg/mL CBS treated group. (C) Up-regulated gene sets of 80 μg/mL CBS treated group. (D) Down-regulated gene sets of 80 μg/mL CBS treated group.

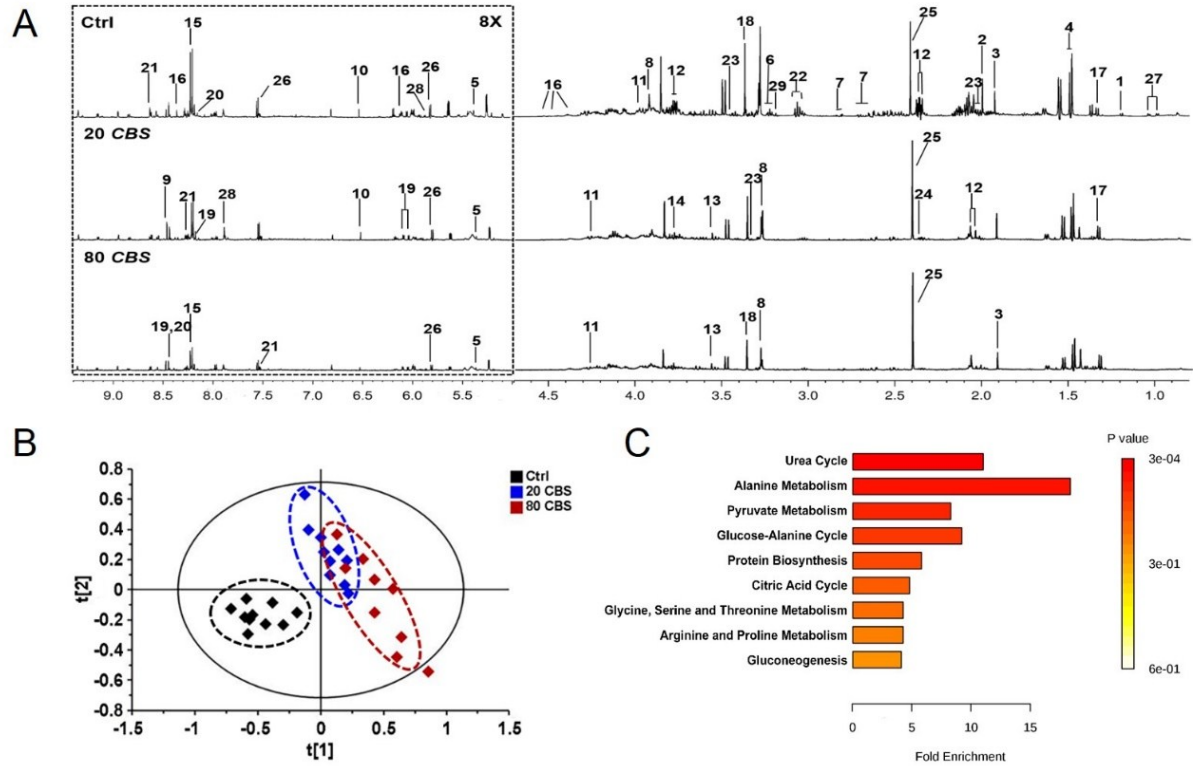

Fig. S3 NMR-based metabolomics study. (A) Representative 600 MHz ^{1}H NMR spectra of aqueous extracts from untreated and CBS-treated *H. pylori* cells. Key: 1. 3-Hydroxybutyrate; 2. Acetamide; 3. Acetate; 4. Alanine (Ala); 5. Allantoin; 6. Arginine (Arg); 7. Aspartate (Asp); 8. Betaine; 9. Formate; 10. Fumarate; 11. Galactarate 12. Glutamate (Glu); 13. Glycine (Gly); 14. Guanidoacetate; 15. Hypoxanthine; 16. Inosine; 17. Lactate; 18. Methanol; 19. Nicotinamide adenine dinucleotide (NAD^{+}); 20. Nicotinamide adenine dinucleotide phosphate ($NADP^{+}$); 21. Nicotinate; 22. Ornithine; 23. Proline (Pro); 24. Pyruvate; 25. Succinate; 26. Uracil; 27. Valine (Val); 28. Xanthosine; 29. β-Alanine. (B) PCA analysis of NMR profiles obtained from control and CBS-treated *H. pylori* metabolite extracts. Each square represents metabolites profiling data from one individual sample. (C) Overview of the enrichment pathway analysis based on metabolites alterations.

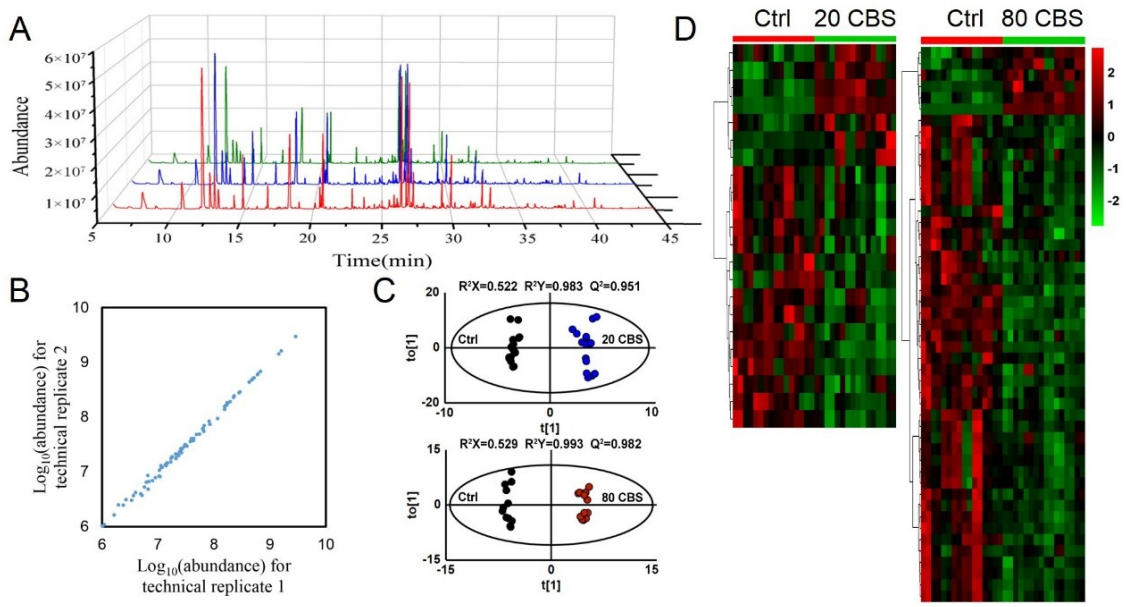

Fig. S4 GC-MS-based metabolomics study. (A) Representative total ion current (TIC) chromatograms from the control and CBS treatment groups. (B) Reproducibility of the metabolic profiling platform used in the discovery phase. Metabolite abundances quantified in cell samples over two technical replicates are shown. The correlation coefficient between the two technical replicates varies between 0.996 and 0.999. (C) OPLS-DA score plots showing the differences between untreated control group and CBS treated groups. (D) Heat map of the significantly changed metabolites. Red and green indicate increased and decreased metabolite levels relative to the median metabolite level, respectively (see the color scale).

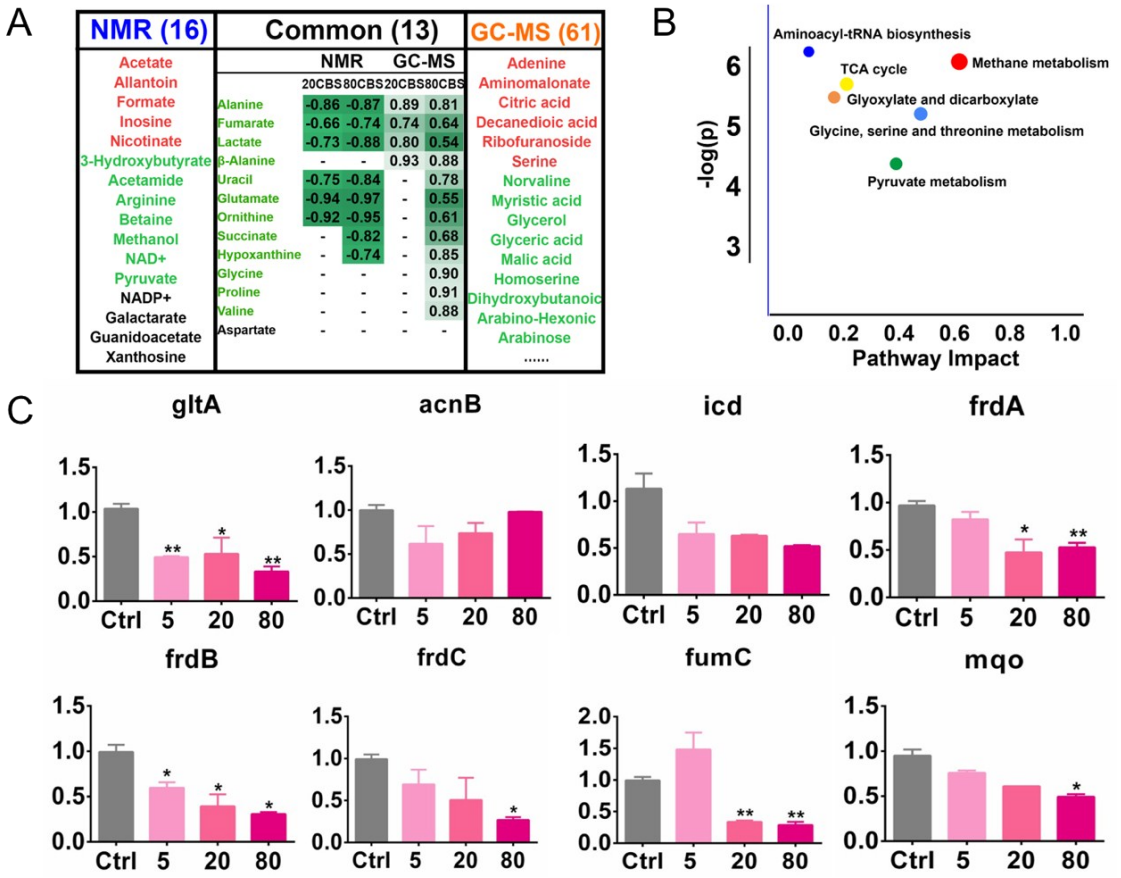

Fig. S5 (A) The altered metabolites of *H. pylori* treated by CBS versus control as revealed by NMR and GC-MS analyses. Data of the differential metabolites in common are indicated by values of correlation coefficients and relative fold changes of metabolites from NMR and GC-MS analysis, respectively. (B) Pathway mapping based on changed metabolites by integrating NMR and GC-MS data. Colored dots represent enriched KEGG pathways. (C) Transcriptional level of genes in TCA cycle in *H. pylori* treated with CBS for 12 h was determined by qRT-PCR and normalized against 16s rRNA and untreated control (n = 3). Two-tailed t-test was used for all comparisons between two groups. Data are presented as mean ± SEM. *, $0.01 < p < 0.05$ and **, $0.001 < p < 0.01$.

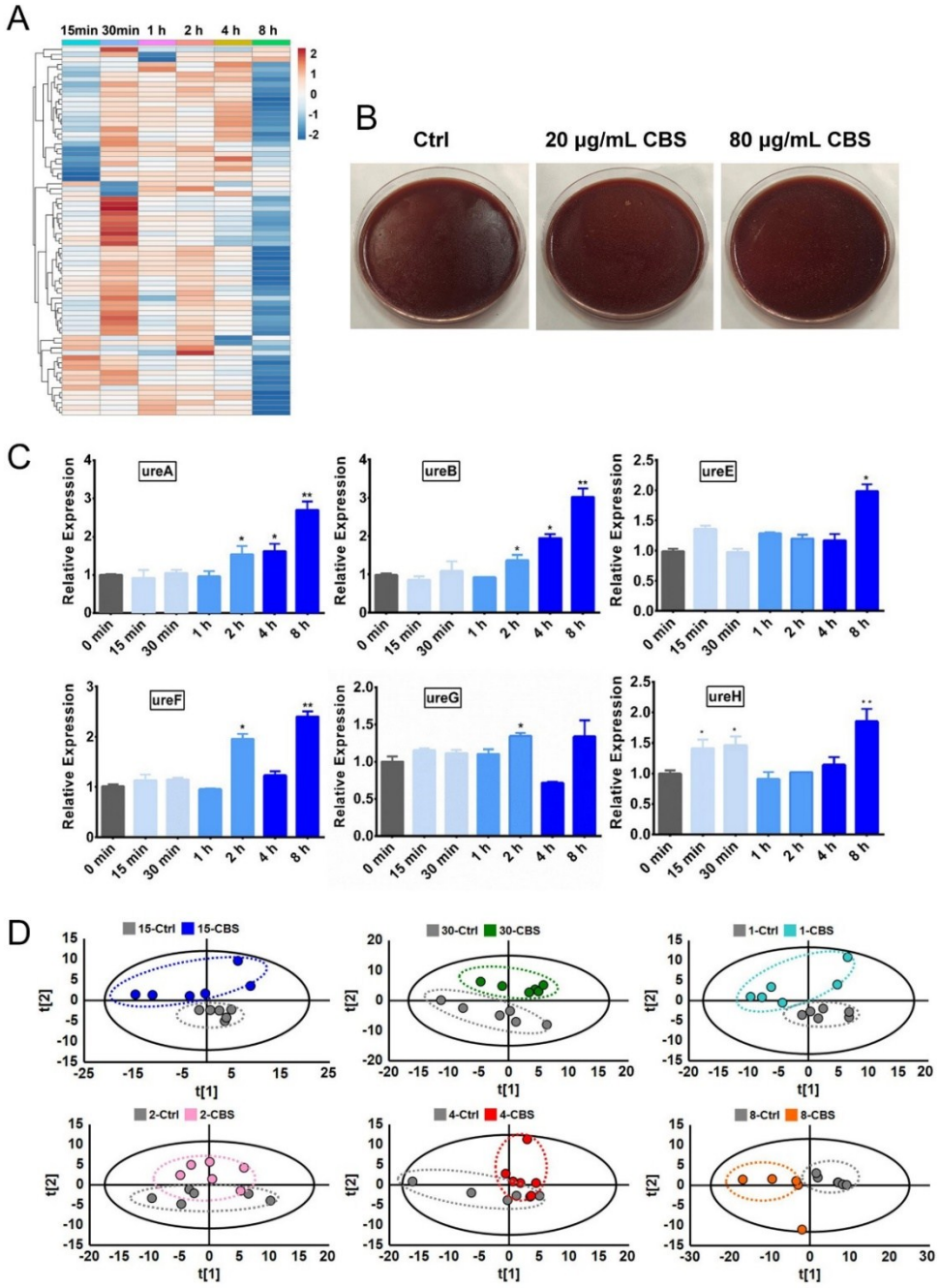

Fig. S6 (A) Heat map of all metabolites in *H. pylori* at different time points according to the values of $\log_2$ relative fold changes (80 μg/mL CBS treatment versus control group). Abundance of increased metabolites in CBS treated groups is shown in red, while the decreased metabolites are in blue. (B) Growth of *H. pylori* on agar plates after incubation with CBS for 8 h. Growth of *H. pylori* on agar plates could still be observed after 12 h CBS treatment but with smaller colonies. (C) Relative expressions of urease-related genes *ureA, ureB, ureE, ureF, ureG* and *ureH* in *H. pylori* cells treated with 80 μg/mL CBS at different time points. Gene expressions were normalized against 16s rRNA and the untreated control. Data are presented as mean ± SEM of triplicate samples from three biological replicates. Two-tailed t-test was used for comparisons between two groups. (*, $0.01 < p < 0.05$ and **, $0.001 < p < 0.01$) (D) PCA score plots of metabolites in *H. pylori* at different time points (15 min, 30 min, 1 h, 2 h, 4 h and 8 h) to show separation between control groups and 80 μg/mL CBS-treated groups.

Table S1 Significantly regulated genes in *H. pylori* upon CBS treatment for 12 h.*

Gene ID	Gene Description	Log2FC (20 CBS/Ctrl)	*p*-adj (20 CBS/Ctrl)	Log2FC (80 CBS/Ctrl)	*p*-adj (80 CBS/Ctrl)
HP0011	co-chaperone (groES)	1.01	1.50E-35	1.22	4.08E-09
HP0048	carbamoyltransferase (hypF)	-	-	0.35	1.99E-02
HP0067	urease accessory protein (ureH)	0.53	1.54E-12	0.40	1.04E-03
HP0068	urease accessory protein (ureG)	0.35	7.52E-06	-	-
HP0069	urease accessory protein (ureF)	0.50	8.33E-07	0.68	1.24E-07
HP0070	urease accessory protein (ureE)	-	-	0.42	9.84E-04
HP0144	cytochrome c oxidase, heme b and copper-binding subunit, membrane-bound (fixN)	-0.31	1.13E-03	-0.46	4.48E-05
HP0145	cytochrome c oxidase, monoheme subunit, membrane-bound (fixO)	-	-	-0.31	1.31E-02
HP0146	cbb3-type cytochrome c oxidase subunit Q (CcoQ)	-	-	-0.96	5.27E-05
HP0147	cytochrome c oxidase, diheme subunit, membrane-bound (fixP)	-	-	-0.30	1.32E-02
HP0153	recombinase (recA)	-0.37	1.64E-06	-	-
HP0191	fumarate reductase, iron-sulfur subunit (frdB)	-0.21	3.97E-03	-0.47	3.41E-05
HP0192	fumarate reductase, flavoprotein subunit (frdA)	-	-	-0.34	2.63E-03
HP0193	fumarate reductase, cytochrome b subunit (frdC)	-0.52	4.87E-09	-0.52	3.64E-05
HP0194	triosephosphate isomerase (tpi)	-0.42	1.49E-05	-0.43	9.34E-04
HP0198	nucleoside diphosphate kinase (ndk)	-0.36	6.36E-06	-0.40	7.42E-04
HP0349	CTP synthetase (pyrG)	-0.41	1.49-07	-0.35	3.41E-03

HP0390	adhesin-thiol peroxidase (tagD)	-0.27	4.69E-02	-	-
HP0557	acetyl-coenzyme A carboxylase (accA)	-	-	0.34	2.66E-02
HP0572	adenine phosphoribosyltransferase (apt)	-0.84	3.54E-17	-0.88	2.53E-13
HP0621	DNA mismatch repair protein (MutS)	-0.47	2.41E-02	-0.46	2.60E-02
HP0653	nonheme iron-containing ferritin (pfr)	2.40	8.83E-34	2.56	1.33E-33
HP0687	iron(II) transport protein (feoB)	1.11	5.09E-46	1.01	1.92E-17
HP0807	iron(III) dicitrate transport protein (fecA)	1.19	5.82E-58	1.84	2.57E-17
HP0824	thioredoxin (trxA)	-1.01	4.42E-55	-1.07	4.20E-22
HP0825	thioredoxin reductase (trxB)	-0.16	1.88E-02	-0.38	1.03E-03
HP0828	ATP synthase F0, subunit a (atpB)	-0.47	1.48E-09	-0.55	4.68E-06
HP0869	hydrogenase expression/formation protein (hypA)	-0.92	8.64E-08	-0.43	1.56E-02
HP0889	iron(III) dicitrate ABC transporter, permease protein (fecD)	0.58	2.66E-08	0.57	4.58E-05
HP0900	hydrogenase expression/formation protein (hypB)	0.61	2.83E-21	0.70	2.72E-06
HP0929	geranyltranstransferase (ispA)	-0.35	4.33E-03	-	-
HP0950	acetyl-CoA carboxylase beta subunit (accD)	0.33	6.24E-06	0.50	4.39E-02
HP1027	ferric uptake regulation protein (fur)	0.22	2.37E-02	0.69	1.48E-08
HP1045	acetyl-CoA synthetase (acoE)	-0.19	3.72E-03	-0.24	4.11E-02
HP1077	nickel transport protein (nixA)	-0.66	2.77E-19	-0.57	6.85E-07
HP1084	aspartate transcarbamoylase (pyrB)	-0.63	1.83E-07	-0.44	3.57E-03
HP1101	glucose-6-phosphate dehydrogenase (g6pD)	-0.72	2.54E-18	-0.59	1.23E-06
HP1102	glucose-6-phosphate 1-dehydrogenase (devB)	-0.56	3.80E-07	-0.64	2.85E-06
HP1103	glucokinase (glk)	-0.58	4.98E-14	-0.61	2.42E-07

HP1108	pyruvate ferredoxin oxidoreductase, gamma subunit	-0.20	1.53E-02	-0.27	2.71E-02
HP1109	pyruvate ferredoxin oxidoreductase, delta subunit	-	-	-0.37	1.88E-03
HP1110	pyruvate ferredoxin oxidoreductase, alpha subunit	-	-	-0.25	3.46E-02
HP1123	peptidyl-prolyl cis-trans isomerase, FKBP-type rotamase (slyD)	0.64	2.15E-16	0.61	7.73E-07
HP1131	ATP synthase F1, subunit epsilon (atpC)	0.61	9.82E-04	-	-
HP1132	ATP synthase F1, subunit beta (atpD)	0.29	3.83E-05	-	-
HP1133	ATP synthase F1, subunit gamma (atpG)	-	-	-0.34	6.46E-03
HP1134	ATP synthase F1, subunit alpha (atpA)	-	-	-0.29	1.62E-02
HP1137	ATP synthase F0, subunit b' (atpF')	-0.56	2.95E-09	-0.47	3.22E-04
HP1341	siderophore-mediated iron transport protein (tonB)	0.46	1.31E-08	0.56	2.72E-06
HP1360	4-hydroxybenzoate octaprenyltransferase (ubiA)	-0.62	4.18E-05	-	-
HP1386	D-ribulose-5-phosphate 3 epimerase (rpe)	-1.19	2.31E-15	-0.77	1.38E-08
HP1432	histidine and glutamine-rich protein (hpnl)	0.90	4.55E-08	2.11	6.03E-84
HP1483	Ubiquinone/menaquinone biosynthesis C-methyltransferase (ubiE)	-0.31	8.36E-03	-0.31	8.36E-03
HP1540	ubiquinol cytochrome c oxidoreductase, Rieske 2Fe-2S subunit (fbcF)	-0.40	7.47E-07	-0.36	3.27E-03

* Only significantly regulated genes mentioned in the manuscript are listed. Adjusted p value < 0.05 was used as the cutoff value for statistical significance. "-" means the gene was not significantly regulated with adjusted p values > 0.05.

Table S2 Metabolites identified by NMR (12 h CBS treatment).

Metabolites	**Assignment**	**δ ^{1}H (ppm) (multiplicity*)**	**δ ^{13}C (ppm)**	**Correlation coefficients (r)†**	
				20 CBS *vs* Ctrl	**80 CBS *vs* Ctrl**
				$R^2X = 0.656$ $Q^2 = 0.962$	$R^2X = 0.737$ $Q^2 = 0.960$
3-Hydroxybutyrate	α-CH_2 β-CH γ-CH_3 COOH	2.30(dd) 4.16(m) 1.19(d)	49.7 69.1 24.6 183.3	-0.84	-0.69
Acetamide	CH_3 CO	1.99(s)	24.0 176.3	-0.88	-0.92
Acetate	CH_3 COOH	1.92(s)	26.6 185.3	0.78	-
Alanine	α-CH β-CH_3 COOH	3.79(q) 1.48(d)	53.5 20.0 178.2	-0.86	-0.87
Allantoin	CH	5.36(s)	66.3	-	0.78
Arginine	α-CH β-CH_2 γ-CH_2 δ-CH_2 C=NH COOH	3.76(m) 1.90(m) 1.70(m) 3.21(t)	57.0 30.5 26.5 43.4 159.5 177.2	-0.74	-0.77

Aspartate	β-CH_2 β-CH_2' α-COOH β-COOH	2.68(dd) 2.83(dd)	39.3 39.9 176.4 180.2	-	-
Betaine	CH_3 CH_2 COO^-	3.27(s) 3.91(s)	56.2 69.8 172.0	-0.85	-0.9
Formate	CH	8.46(s)	173.4	0.73	0.76
Fumarate	CH COOH	6.52(s)	139.6 177.5	-0.66	-0.74
Galactarate	C1H C2H	3.94(s) 4.25(s)	69.9 73.8	-	-
Glutamate	α-CH β-CH_2 γ-CH_2 α-COOH	3.76(m) 2.05(m) 2.35(dd)	57.2 29.0 35.4 177.2	-0.94	-0.97
Glycine	CH_2 COOH	3.56(s)	43.8 175.3	-	-
Guanidoacetate	CH_2 C=NH COOH	3.78(s)	47.0 159.6 177.9	-	-
Hypoxanthine	C1H C2H	8.20(s) 8.21(s)	147.8 144.5	-	-0.74

Inosine	CH_2 CH_2' 5-H' 4-H' 3-H' 2-H' 8-H' 2-H	3.84(dd) 3.92(dd) 4.29(dd) 4.44(dd) 4.78(t) 6.10(d) 8.24(s) 8.35(s)	64.2 63.8 87.1 73.7 74.6 91.3 150.4 144.5	0.64	0.80
Lactate	α-CH β-CH_3 α-COOH	4.11(q) 1.33(d)	69.3 21.5 184.3	-0.73	-0.88
Methanol	CH_3	3.36(s)	51.3	-0.80	-0.83
NAD^+	2'-CH 2'''-CH 5-CH 2''-CH 6-CH 4-CH 2-CH	6.04(d) 6.09(d) 8.17(dd) 8.43(s) 8.83(d) 9.14(d) 9.34(s)	86.5 102.6 130.1 143.3 150.1 145.5 143.0	-0.76	-0.89
$NADP^+$	2'''-CH 7''-CH 2''-CH 6-CH 4-CH 2-CH	6.10(d) 8.14(dd) 8.42(s) 8.82(d) 9.10(d) 9.30(s)	102.6 132.3 142.9 145.0 146.0 142.1	-	-
Nicotinate	C1H C2H C3H C4H	8.25(m) 8.62(dd) 8.94(d) 7.52(dd)	141.2 153.4 152.0 127.3	0.96	-

Ornithine	α-CH β-CH_2 γ-CH_2 δ-CH_2	3.8(t) 1.84(m) 1.94(m) 3.05(t)	54.5 27.6 30.2 40.5	-0.92	-0.95
Proline	α-CH γ-CH_2 δ-CH_2 δ-CH_2' COOH	4.12(dd) 2.01(m) 3.34(m) 3.42(m)	64.0 26.6 49.0 49.0 176.1	-	-
Pyruvate	CH_3 C=O COOH	2.37(s)	29.2 173.4 207.9	-0.91	-0.95
Succinate	CH_2 COOH	2.4(s)	36.9 182.5	-	-0.82
Uracil	CH CH' C=O	5.81(d) 7.54(d)	102.5 145.0 155.9	-0.75	-0.84
Valine	α-CH β-CH γ-CH_3 γ'-CH_3 COOH	3.61(d) 2.28(m) 0.98(d) 1.03(d)	62.3 31.2 19.4 20.7 177.3	-	-
Xanthosine	CH_2 C1H C2H C3H C4H C5H	3.82(m) 5.85(d) 4.69(t) 4.38(dd) 4.25(q) 7.87(s)	62.2 92.1 73.4 73.1 86.0 145.5	-	-

β-Alanine	α-CH_2	2.54(t)	35.10	-	-
	β-CH_2	3.17(t)	39.09		
	COOH		176.23		

* Multiplicity: singlet (s), doublet (d), triplet (t), quartet (q), doublet of doublets (dd), multiplet (m).

† Positive and negative values of correlation coefficient values indicate positive and negative correlations in metabolite concentrations respectively, by comparing the CBS treatment group with the control group. $p < 0.05$ and $|r| > 0.602$ was used as the cutoff values for statistical significance. "-" means the correlation coefficient is less than the cutoff value.

Table S3 Metabolites identified by GC-MS and their regulations by CBS at different time points.

Identified Compound	Match Factor	PubChem CID	KEGG Entry	*p*-values (Ctrl vs CBS treatment)*						
				15 min	30 min	1 h	2 h	4 h	8 h	12 h
Lactic acid (2TMS)	94.15	107689	C00186	0.707	0.171	**0.015**	**1.03E-05**	0.127	0.473	**0.000**
Norvaline (1TMS)	98.96	439575	C01799	0.414	0.316	0.643	0.943	0.744	0.404	**0.002**
Alanine (3TMS)	99.84	5950	C00041	**0.021**	0.271	0.355	0.501	0.622	0.161	**0.017**
Oxalic acid (2TMS)	81.30	971	C00209	**0.005**	0.652	0.489	0.067	**0.001**	**0.043**	0.505
Leucine (1TMS)	99.81	6106	C00123	0.190	0.198	0.630	0.854	0.463	0.307	**0.017**
Isoleucine (1TMS)	99.21	6306	C00407	0.445	**0.015**	**0.046**	0.333	0.762	0.067	**0.031**
Valine (2TMS)	99.76	6287	C00183	0.426	0.624	0.409	0.110	0.251	**0.006**	**0.001**
Serine (2TMS)	98.34	5951	C00065	0.215	0.555	0.902	0.387	0.350	0.248	**0.000**
Norleucine (2TMS)	99.32	21236	C01933	**0.035**	**0.040**	0.603	0.481	0.723	0.069	0.236
Phosphoric acid (3TMS)	95.61	1004	C00009	0.626	**0.015**	0.516	0.250	0.694	0.087	**0.041**
Glycerol (3TMS)	97.27	753	C00116	0.147	**0.004**	0.843	0.535	0.337	0.069	**0.002**
2-Piperidinecarboxylic acid (2TMS)	96.61	849	C00408	**1.33E-04**	**0.043**	0.784	0.230	0.536	0.374	0.553
Proline (2TMS)	99.80	145742	C00148	0.291	0.676	0.458	0.724	0.516	**0.009**	**1.24E-04**
Glycine (3TMS)	97.24	750	C00037	0.283	**0.008**	0.529	0.899	0.177	0.068	**0.003**
Succinic acid (2TMS)	96.69	1110	C00042	0.636	**0.017**	0.352	0.163	**0.049**	**0.009**	0.450
Glyceric acid (3TMS)	95.06	439194	C00258	0.713	0.604	0.212	0.973	0.096	0.100	**0.020**
Uracil (2TMS)	94.93	1174	C00106	0.503	0.113	0.508	0.617	0.944	**0.028**	**0.009**
Pyrazine (2TMS)	85.71	9261	C02018	0.825	0.440	0.642	0.186	0.725	0.058	**0.010**
Fumaric acid (2TMS)	96.93	444972	C00122	0.304	0.091	0.080	0.561	0.096	**1.37E-04**	**5.87E-04**

Thymine (2TMS)	78.49	1135	C00178	0.720	**0.013**	0.879	0.592	0.128	0.086	**0.035**
Threonine (3TMS)	96.88	6288	C00188	0.548	**0.037**	0.822	0.886	0.107	0.084	0.385
Methionine (1TMS)	99.18	6137	C00073	0.303	0.977	0.387	0.756	0.584	0.062	**0.008**
3-Deoxytetronic acid (3TMS)	96.43	192742	-	0.817	**0.007**	0.994	0.227	0.744	0.140	**0.019**
Aspartic acid (2TMS)	98.31	5960	C00049	**0.047**	0.866	0.676	0.347	0.212	0.393	0.892
Beta-Alanine (3TMS)	94.49	239	C00099	0.366	0.956	**0.016**	0.926	0.406	**0.003**	**3.12E-04**
3,4-Dihydroxybutanoic acid (3TMS)	93.99	150929	-	0.767	0.315	0.827	0.161	0.742	0.135	**0.017**
Homoserine (3TMS)	96.40	12647	C00263	**0.015**	0.192	**0.008**	0.482	**0.009**	**0.022**	**6.21E-04**
2-Piperidone (2TMS)	89.68	12665	-	0.075	0.281	0.219	0.437	**0.001**	0.163	**0.027**
Aminomalonic acid (3TMS)	96.22	100714	C00872	0.075	0.188	0.108	0.084	0.151	0.189	**8.51E-04**
Malic acid (3TMS)	94.36	525	C00711	0.202	0.894	0.516	0.139	0.758	0.097	**0.030**
Parabanic acid (2TMS)	98.68	67126	-	0.550	**0.005**	0.874	**0.021**	**0.017**	**0.001**	**0.022**
N-Acetylmethionine (2TMS)	97.45	6180	C02712	0.706	**1.39E-05**	**0.015**	0.759	**0.023**	**0.005**	**0.010**
Pyroglutamic acid (2TMS)	89.74	7405	C01879	0.884	**0.016**	0.192	**0.017**	0.568	0.246	0.063
4-Aminobutanoic acid (3TMS)	97.00	119	C00334	0.626	**0.002**	**0.025**	0.070	0.102	0.705	**0.014**
Phenylalanine (1TMS)	99.49	6140	C00079	0.469	**0.018**	0.155	0.988	0.735	**0.019**	**0.024**
Cysteine (3TMS)	90.48	5862	C00097	**0.018**	0.592	0.514	0.263	0.757	**0.006**	**0.001**
Threonic acid (4TMS)	88.10	151152	C01620	0.266	0.631	0.377	0.667	**0.038**	**0.010**	**0.031**

2-Oxo-Glutaric acid (2TMS)	77.45	51	C00026	0.914	**0.036**	0.806	0.617	0.156	0.089	0.386
Asparagine (2TMS)	74.67	6267	C00152	**0.042**	0.732	0.979	0.617	0.279	**0.004**	0.904
Butanoic acid (2TMS)	78.84	264	C00246	0.290	0.633	0.111	0.462	0.241	0.177	**0.026**
Ornithine (3TMS)	98.39	6262	C00077	0.074	0.624	0.323	0.759	0.355	**0.007**	0.587
Glutamic acid (3TMS)	89.74	33032	C00025	0.222	0.538	0.216	0.741	0.422	**0.008**	0.201
Gluconic acid (5TMS)	88.49	10690	C00257	0.662	0.165	0.151	0.212	**0.047**	**0.004**	**1.99E-04**
Adenine (2TMS)	72.10	190	C00147	0.727	**3.10E-05**	0.072	0.526	0.056	0.127	**0.007**
Arabinose (4TMS)	97.99	439195	C00259	0.094	**0.005**	0.401	0.179	**1.65E-04**	0.108	**3.33E-04**
Glycerol-3-phosphate (4TMS)	91.18	439162	C00093	0.114	0.177	0.152	0.857	0.758	**0.018**	**0.001**
Ribofuranose (4TMS)	97.45	5779	C00121	0.534	0.985	**0.005**	0.540	0.337	**0.029**	**0.001**
Ribonic acid (4TMS)	79.49	5460677	C01685	**0.025**	0.731	0.371	0.218	0.328	**0.014**	0.052
Ethanolaminephosphate (4TMS)	84.24	1015	C00346	0.081	**0.024**	0.338	0.327	0.094	**6.89E-04**	**7.01E-04**
L-Val-L-leu(1TMS)	99.00	352039	-	0.962	**0.011**	0.093	0.394	0.490	**4.03E-04**	0.309
Hypoxanthine (2TMS)	95.94	790	C00262	**0.040**	0.243	**4.77E-08**	0.525	0.925	0.237	**0.025**
Fructofuranose (5TMS)	97.04	439709	C02336	0.199	0.170	**0.002**	0.392	0.531	0.654	0.792
Citric acid (4TMS)	95.52	311	C00158	**0.001**	**1.12E-09**	**4.1E-10**	**2.69E-10**	**1.00E-06**	**5.33E-05**	**8.34E-07**
Citrulline (4TMS)	97.68	9750	C00327	0.597	0.223	0.666	0.353	0.319	**2.48E-04**	0.206
N-alpha-Acetyl-L-Lysine (3TMS)	97.88	192590	C12989	0.250	**0.039**	0.353	0.311	0.831	**0.021**	**0.004**
Arabino-Hexonic acid (5TMS)	91.65	152990	-	0.121	0.272	**0.002**	0.681	0.912	**0.011**	**0.005**

Fructose (5TMS)	98.38	5984	C01496	**5.95E-05**	0.906	0.896	0.828	0.691	**0.022**	**0.017**
Tyrosine (2TMS)	99.62	6057	C00082	0.091	**0.014**	0.090	0.737	0.484	**0.015**	0.149
Mannose (5TMS)	97.84	18950	C00159	0.179	**0.010**	**0.013**	**0.018**	0.933	**0.049**	0.424
Glucose (5TMS)	96.42	5793	C00031	0.135	**0.007**	**0.009**	**0.033**	0.235	**0.031**	**0.025**
Lysine (4TMS)	98.55	5962	C00047	**8.58E-05**	0.197	0.535	0.175	0.752	0.124	0.159
Lactulose (8TMS)	85.37	11333	C07064	**0.023**	**0.007**	**0.006**	0.231	0.143	**1.54E-04**	**9.98E-05**
Inositol (6TMS)	84.08	892	C00137	0.141	0.298	0.139	0.298	0.108	**0.013**	**0.036**
Hexadecanoic acid (1TMS)	94.08	985	C00249	0.123	**1.41E-05**	0.870	0.844	**0.003**	**0.041**	0.472
Myo-Inositol (6TMS)	94.89	892	C00137	0.858	0.733	0.177	0.810	0.801	0.127	**0.001**
Decanedioic acid (2TMS)	87.47	5192	C08277	0.165	**6.61E-04**	0.350	0.993	0.736	**0.022**	**3.39E-04**
Tryptophan (3TMS)	99.00	6305	C00078	**4.02E-04**	**9.55E-05**	0.880	0.122	0.580	**0.027**	0.217
Octadecanoic acid (1TMS)	93.59	5281	C01530	0.626	**2.05E-04**	0.970	0.523	**0.006**	0.074	0.335
Allose (5TMS)	98.21	12285879	C01487	0.158	0.995	0.772	0.282	**0.016**	0.348	0.463
Myristic acid (1TMS)	72.90	11005	C06424	0.891	0.966	**0.006**	0.096	0.505	**0.001**	**1.12E-04**
Uridine (3TMS)	97.38	6029	C00299	**0.044**	0.783	**0.043**	0.545	0.753	**0.004**	0.543
Sucrose (8TMS)	91.65	5988	C00089	0.425	0.141	0.723	0.761	0.130	**0.003**	0.129
Cellobiose (8TMS)	97.82	10712	C06422	0.369	0.365	0.528	0.421	0.486	**0.002**	0.114
Trehalose (8TMS)	93.68	7427	C01083	0.125	0.434	0.487	0.878	0.125	**0.004**	**0.012**

* The *p*-values were calculated by comparing the normalized integrated peak areas of each metabolite from control and CBS treatment groups, using two-tailed *t*-test. The *p*-values highlighted in red or green represent the metabolites being up- or down-regulated by CBS at the specific time point.

Table S4 qRT-PCR primers used in this study.

Gene ID	Gene		Sequence (5'-3')
HP0026	*gltA*	For	TTGTCATTGGCCGAACCGTA
		Rev	ACTTGTCTTGGGCGTGTGAT
HP0027	*icd*	For	AGGCACAGAGAGGCTAGTGA
		Rev	AACGCCCCTTCGGTGTATTT
HP0779	*acnB*	For	AAACGCTCCTCCCAGTCAAG
		Rev	AGTGGAAAAGACGACCGCAT
HP0086	*mqo*	For	TTGCCTTTGGCTCAGAGCAT
		Rev	GGCTGCAAAAGGGAGTTTGG
HP0192	*frdA*	For	GGGCATTGATCCGGCTGATA
		Rev	CTTGCCCGTATTCAGGCTCA
HP0191	*frdB*	For	CCTGACGAAGCCCAAGAAGT
		Rev	AGGGCGCATGAGTTTAGTCC
HP0193	*frdC*	For	GCTATTGTGAGCGTGGTTGC
		Rev	ACAAGCTCGTATCGCCATGT
HP0073	*ureA*	For	TGCGGCTGAATTGATGCAAG
		Rev	GGGTATGCACGGTTACGAGT
HP0072	*ureB*	For	GTGGCGGTAAAACCCTGAGA
		Rev	TCAGTAGCAGGACCTACGCT
HP0070	*ureE*	For	ACCAGGCAAGGCAAAGACAT
		Rev	CGCTCTTGGCTTGGATGTGA
HP0069	*ureF*	For	TCAATGATGCGGTGTTCCCT
		Rev	GGAGGGCGCTTTCATAGGTT
HP0068	*ureG*	For	ATTCGGTGATGCCACGAGAG
		Rev	AGGTTATCGCCTCCGCTTTC
HP0067	*ureH*	For	AATTCAACCGCTTGCACACC
		Rev	ATGCATTCTCGCACACCAGA
	16S rRNA	For	GGAGTACGGTCGCAAGATTAAA
		Rev	CTAGCGGATTCTCTCAATGTCAA

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