Cell Reports, Volume *42*

Supplemental information

Microbiota-produced indole metabolites disrupt mitochondrial function and inhibit *Cryptosporidium parvum* growth

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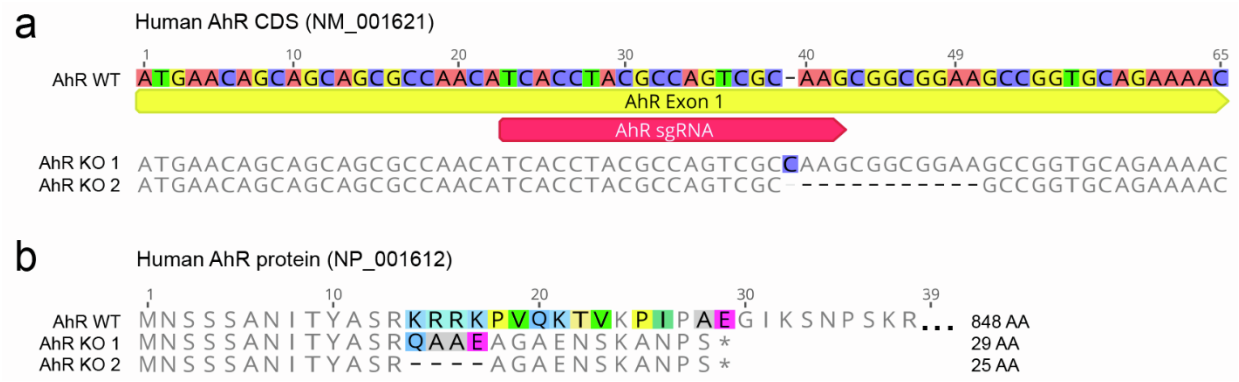
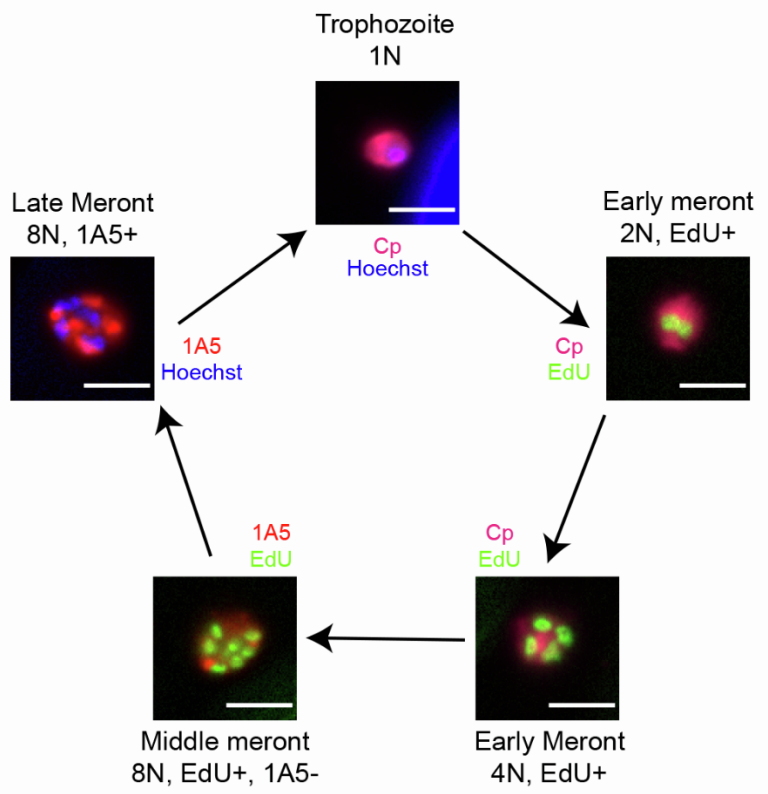
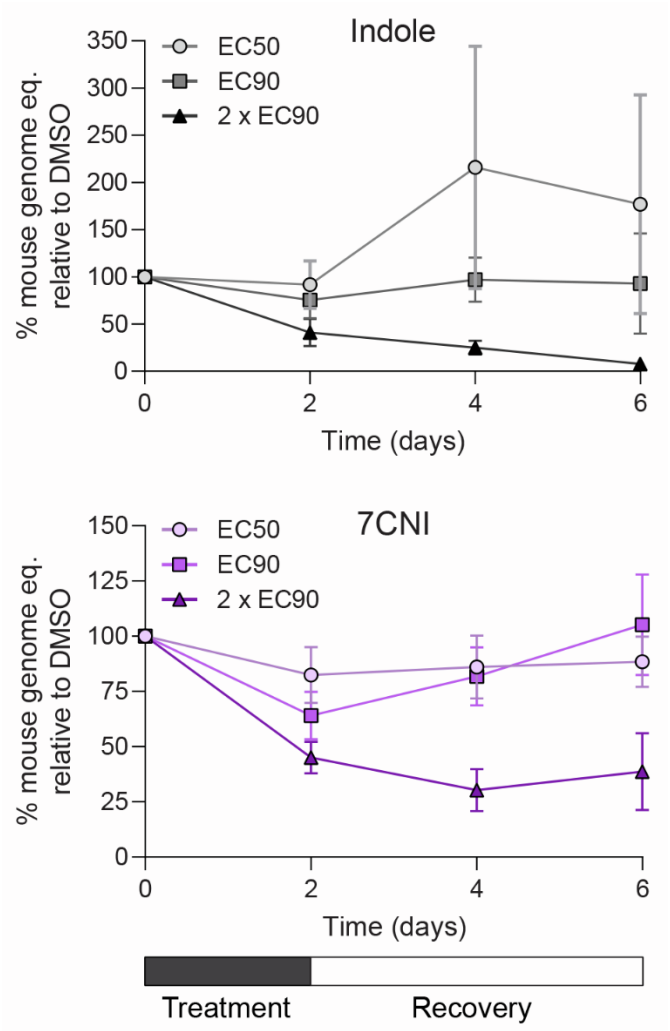


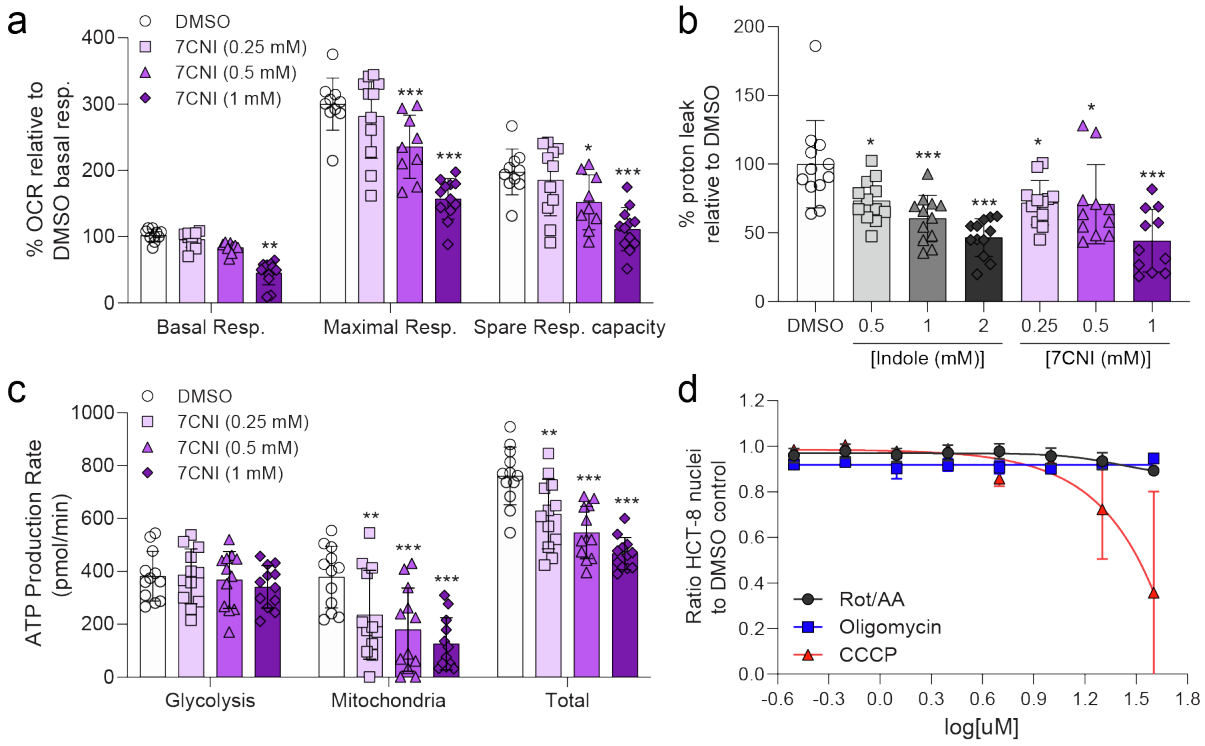
Supplementary Fig. 1: Mutations in the AhR gene introduced by CRISPR/Cas9 cause frameshift mutations resulting in truncated proteins. a) Nucleotide alignment of exon 1 for the wild type (WT) human AhR gene (NM_001621) with sequences from two mutated HCT-8 cell lines, one with a single bp insertion highlighted in blue (AhR KO 1) and the other with an 11 bp deletion (AhR KO 2). b) Amino acid (AA) alignment of the first 38 residues of the WT human AhR protein (NP_001612) with the predicted truncated protein sequences for the AhR KO lines. Graphics were created in Geneious v.2021.1.



Supplementary Fig. 2: **Staging *C. parvum* cell cycle progression.** Asexual stages were defined based on the number of nuclei (N), EdU incorporation and/or labeling of mature merozoites with monoclonal antibody 1A5, as follows: trophozoites had 1 nucleus, early meronts had either 2 or 4 EdU+ nuclei, middle meronts had 8 EdU+ nuclei but no 1A5 labeling, and mature meronts had 8 nuclei with 1A5 labeling.

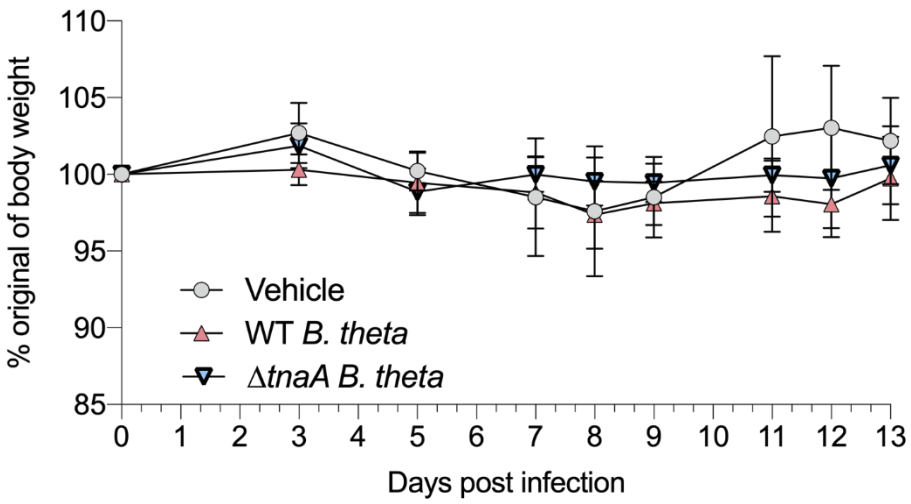


Supplementary Fig. 3: High concentrations of indole and 7-cyanoindole are toxic to host cells in an air-liquid interface (ALI) transwell culture system. Washout experiments in *Cp*-infected ALI cultures treated with 1% DMSO or indole at EC_{50} (577 µM), EC_{90} (1,894 µM) or 2 x EC_{90} (3,788 µM); or 7CNI at EC_{50} (379 µM), EC_{90} (688 µM) or 2 x EC_{90} (1,376 µM) for 48 h before washout. Mouse genome equivalents (eq.) were normalized to the DMSO control at each time point. Data plotted represents means ± s.d. of six replicates (three technical replicates from two independent experiments).

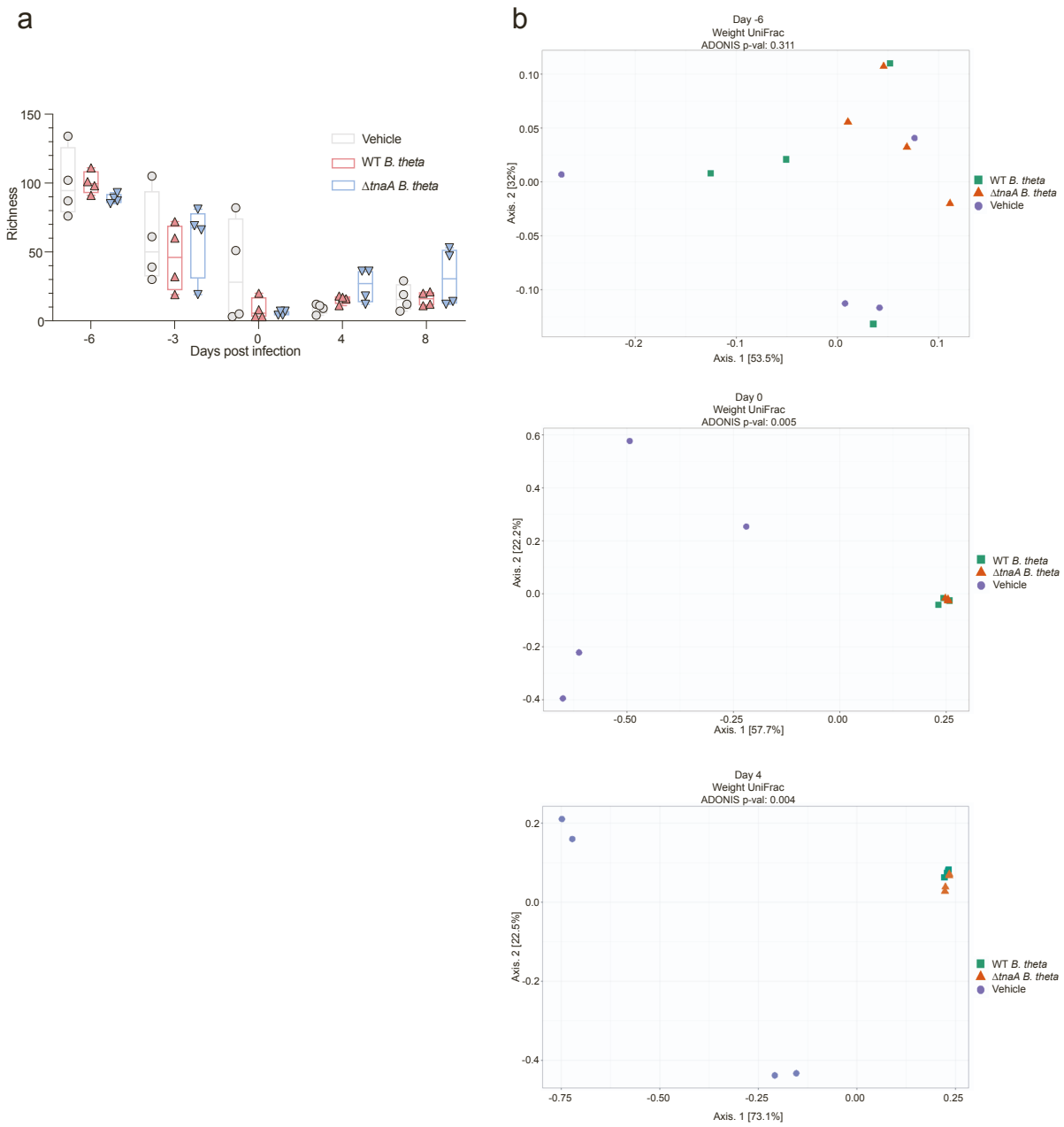


Supplementary Fig. 4: Indoles impair host mitochondrial ATP production but not through proton leak. a) Metabolic analysis using the Seahorse XF Cell Mito Stress Test kit on HCT-8 cells treated for 18 h with 1% DMSO or 7-cyanoindole (7CNI, 0.25 mM, 0.5 mM or 1 mM). Data calculated as a percentage of the oxygen consumption rate (OCR) for each well relative to the mean basal OCR of DMSO control cells for that experiment. Spare respiratory capacity = maximal respiratory rate – basal respiratory rate for each well. Data plotted represents mean ± s.d. of 12 replicates (six technical replicates from two independent experiments). Differences between *%* OCR for each indole concentration vs the DMSO control for each measurement were analyzed with a two-way ANOVA followed by a Dunnett's test for multiple comparisons. **P* < 0.05, ****P* < 0.001. b) Metabolic analysis using the Seahorse XF Cell Mito Stress Test kit on HCT-8 cells treated for 18 h with 1% DMSO, indole (0.5 mM, 1 mM or 2 mM) or 7CNI (0.25 mM, 0.5 mM or 1 mM). Data calculated as a percentage of the proton leak for each well relative to the mean proton leak of DMSO control cells for that experiment. Data plotted represents mean ± s.d. of 12 replicates (six technical replicates from two independent experiments). Differences between *%* proton leak for each indole/7CNI concentration vs the DMSO control were analyzed with a one-way ANOVA followed by a Dunnett's test for multiple comparisons. **P* < 0.05, ****P* < 0.001. c) Metabolic analysis using the Seahorse XF Real-time ATP Rate assay on HCT-8 cells treated for 18 h with 1% DMSO or 7CNI (0.25 mM, 0.5 mM or 1 mM). Data plotted represents mean ± s.d. of ATP production rate (pmol/min)

produced by glycolysis, the mitochondria, or total ATP (glycolysis + mitochondrial ATP rates) for 12 replicates (six technical replicates from two independent experiments). For each source of ATP, differences between ATP production rate for each 7CNI concentration vs the DMSO control were analyzed with a two-way ANOVA followed by a Dunnett's test for multiple comparisons. $^{**}P < 0.01$, $^{***}P < 0.001$. c) d) Ratio of host nuclei relative to DMSO control in HCT-8 cells after 24 h treatment with serial dilutions of mitochondrial Complex I and III inhibitors rotenone and antimycin A, respectively (Rot/AA); ATP synthase inhibitor oligomycin; or proton gradient uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Inhibition curves were calculated for each compound using a nonlinear regression curve fit with six replicates (three technical replicates from two independent experiments) per concentration.

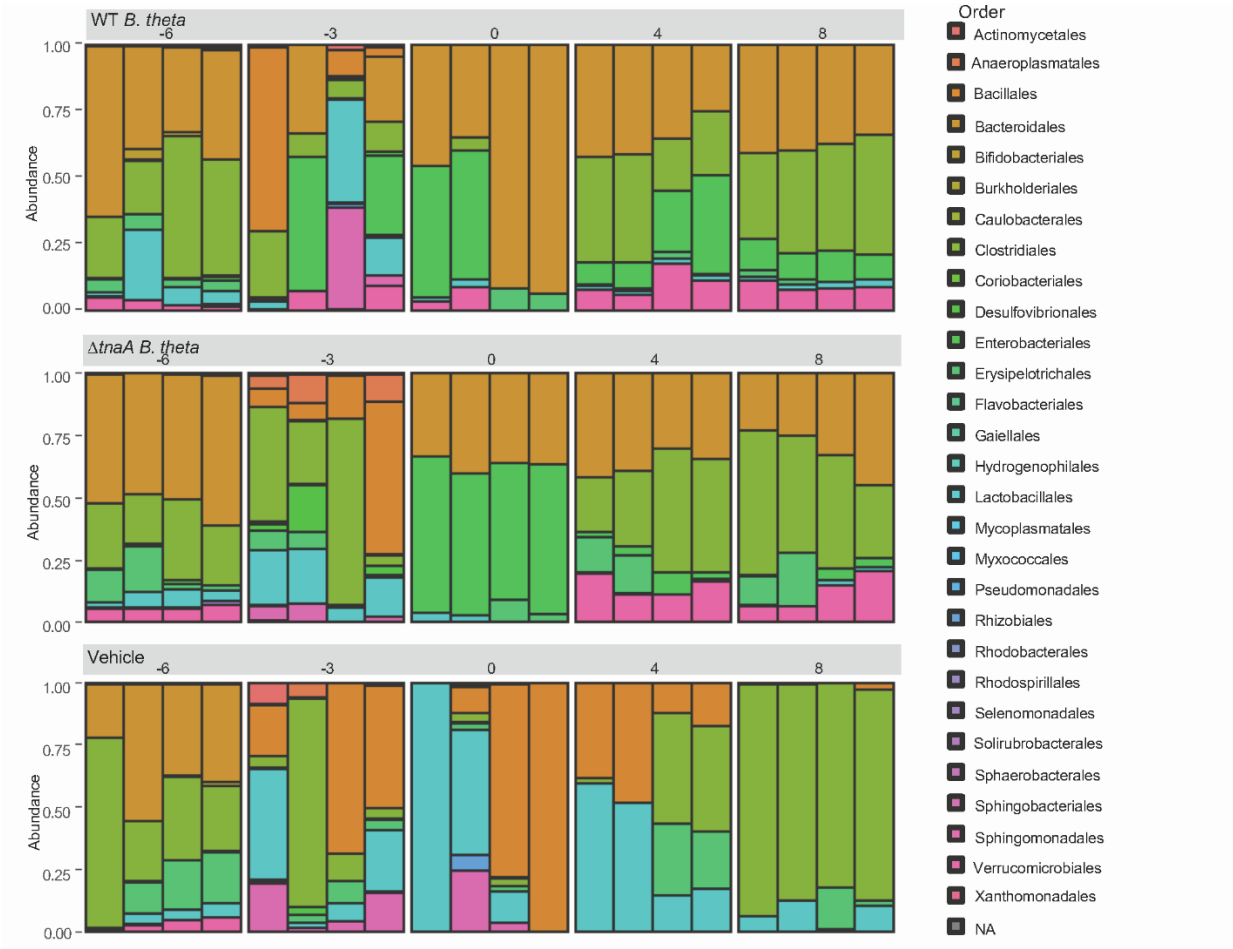


Supplementary Fig. 5: Reconstitution of indole-producing bacteria followed by challenged with *Cp* does not affect the body weight of GKO mice. Percent of original body weight plotted as means ± S.D. Statistical analysis performed using two-way ANOVA followed by a Dunnett's test for multiple comparisons. All of the comparisons between WT *B. theta* group and *ΔtnaA B. theta* group showed no significant difference.



Supplementary Fig. 6: Alpha and beta diversity analyses of 16S rRNA gene V4 sequencing data. a) Richness (observed amplicon sequence variants) for each group of mice at the indicated days post-infection. Statistical analyses comparing vehicle to WT *B. theta* group or *ΔtnaA B. theta* group on individual days were performed with a two-way ANOVA followed by a Dunnett's test for multiple comparisons. All of the comparisons showed no significant difference. b) Beta diversity analyses for each group of mice at the indicated dpi were conducted using weighted UniFrac distances. Results were

analyzed using Permutational Multivariate Analysis of Variance (ADONIS). Data plotted represents 4 mice per treatment group sampled at -6 dpi, 0 dpi and 4 dpi.



Supplementary Fig. 7: 16S rRNA gene profiling of commensal microbiota in fecal pellets. Order level composition for each group of mice at the indicated days post-infection. Data represent 4 mice per treatment group sampled over time. Prior to antibiotics treatment, Bacteroidales were the major component of the microbiota. Following antibiotics treatment and reconstitution, Bacteroidales (exclusively comprised of *B. theta*) were prominent taxa in mice administered wild type (WT) *B. theta* or the *ΔtnaA* mutant *B. theta*. In contrast, *Bacteroidales* were absent in mice administered the vehicle control.