**Supplementary Materials**

**Cryptosporidiosis modulates gut microbiome metabolism and the immune response in an infected host.**

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**1. Materials and methods**

1.1. Metabolome extraction and analysis

The homogenised samples prepared for metabolomic analysis were subjected to in-time derivatisation on an Agilent 7890B gas chromatography system with 5977B mass spectrometry detector fitted with an MPS autosampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany). Methoxyamine HCl (MOX) (20 mg/mL in pyridine) and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the samples in 20 µL and 40 µL volumes, respectively, by the autosampler. The samples were incubated at 37°C for 60 min and 30 min after the addition of MOX and BSTFA, respectively. After a 60-minute wait-time, samples were injected to the GC-MS system with the previously reported configurations and settings 18.

1.2. Quality control mix

A quality control (QC) mix containing 19 different polar and semi-polar metabolites was prepared as per Fiehn (2016). The QC mix consisted of 500 ng dried metabolites of valine, succinic acid, methionine, 4-hydroxyproline, salicylic acid, α-ketoglutaric acid, shikimic acid, citric acid, lysine, glucose, sucrose, chlorogenic acid, myristic acid, myristic acid-d27, stigmasterol, glucose (U13C6), L-glutamine (Amide-15N), palmitic acid (1-13C) and glycine (1-13C).

The metabolomics data, after Log10 normalisation, were then analysed using multivariate data analysis software SIMCA (version 16, Sartorius Stedim Biotech, Umeå, Sweden). Principal component analysis (PCA), the unsupervised model, was initially undertaken to find statistically significant differences between sample groups. This was followed by partial least square discriminate analysis (PLS-DA).

The PCA and PLS-DA model validity was determined by R2X, R2Y and Q2 values. The R2X and R2Y values define variation between X and Y variables of various components in the sample set and, Q2 gives predictability of the model 22. Furthermore univariate statistical tools such as volcano plots, enrichment analysis and pathway impacts were produced by MetaboAnalyst 4.0 19. The cut-off level for significant metabolites was a signal-to-noise (*S/N*) ratio of 50, a fold change of ≤ 0.5 (downregulation) or ≥ 2.0 (upregulation) and a Benjamini–Hochberg adjusted p-value of ≤ 0.05.

*1.3. Metaproteome extraction, LC-MS/MS analysis and data processing*

After the homogenisation and extraction, the tryptic peptides (100 ng) were desalted and concentrated with a trap column (PepMap100 C18 5 mm × 300 µm, 5 µm) and separated on a nano column (PepMap100 C18 150 mm × 75 µm, 2 µm) using an UltimateTM 3000 RSLC nano LC system, with mobile phases (A: water + 0.1% (*v/v*) formic acid; B: acetonitrile (80% *v/v*) + 0.08% (*v/v*) formic acid). The peptides were eluted using Solvent B at gradient s of 5 - 40% (0 - 60 min) and 40 - 99% (60 - 70 min). The eluted peptides were ionized with a Nanospray Flex Ion Source. The spray voltage was set to 2.3 kV and temperature of the heated capillary was set at 300°C. After ionization, mass spectra (MS1) and tandem mass spectra (MS/MS) analysis was performed using an Orbitrap Fusion MS. The MS survey scans of peptide precursors were performed in the Orbitrap detector and the scan range was 400 to 1500 *m/z* at resolution of 120 K (at 200 *m/z*). The target value of automatic gain control (AGC) was set as 4 × 105. The maximum injection time for the MS was 50 ms. MS/MS was performed on the most abundant precursors of charge states 2+ to 7+ with intensity greater than 1 × 105. They were isolated by the quadrupole with a window of 1.6 *m/z*. Fragmentation was achieved by high-energy collisional dissociation (HCD) with collision energy of 28%. Fragments were detected in the ion trap detector in rapid scan rate mode. The AGC target was 4 × 103, maximum injection time was 300 ms and the dynamic exclusion was 15 seconds. The instrument was set to run in top speed mode with a 3 second cycle for both the MS and MS/MS scans (***Note:*** *All instruments and parts of Liquid chromatography-High resolution mass spectrometry (LC-HR-MS) were sourced from Thermo Scientific Australia Pty Ltd, Scoresby, VIC, Australia*).

Protein Discoverer 2.2 (Thermo Scientific) and Sequest HT search engine were used to identify peptides/proteins and quantify relative abundance of proteins. The spectrum data was searched against the UniProt databases indicated below. Precursor mass tolerance was set to 10 ppm and product ions were searched at 0.6 Da. Three missed tryptic cleavages were allowed. Modification included Oxidation (+ 15.995 Da), Deamidation (+ 0.984 Da), Amidation (- 0.984Da), and Propionamidation (+ 71.037 Da). Peptide spectral match was validated using the Percolar algorithm, based on q-values and 1% False Discovery Rate (FDR). Relative abundance was calculated from precursor abundance intensity, normalized to total peptide amount (Ratio calculation based on summed abundance; ANOVA test based on individual proteins).

The proteomics data, after Log10 normalisation, were then analysed using multivariate data analysis software SIMCA (version 16, Sartorius Stedim Biotech, Umeå, Sweden). Principal component analysis (PCA), the unsupervised model, was initially undertaken to find statistically significant differences between sample groups. This was followed by partial least square discriminate analysis (PLS-DA). Uniprot databases used were *Mus musculus* (UP000000589) and various microbial databases (UP000242190, P000001218, UP000280657, UP000005219, UP000003081, UP000004459, UP000004596, UP000018901, UP000263263, UP000029920, UP000315402, UP000000927, UP000006599, UP000027129, UP000064844, UP000008178, UP000000625, UP000255233, UP000309428, UP000012589, UP000001377, UP000001415, UP000006000, UP000285063, UP000000439, UP000005561, UP000017429, UP000005384, UP000092574, UP000195817, UP000001031, UP000006657, UP000000333, UP000002938, UP000000559, UP000270830, UP000236311, UP000002037, UP000002311, and UP000006726) based on the genomic output.

The PCA and PLS-DA model validity was determined by R2X, R2Y and Q2 values. The R2X and R2Y values define variation between X and Y variables of various components in the sample set and, Q2 gives predictability of the model 22. Furthermore univariate statistical tools such as volcano plots, enrichment analysis and pathway impacts were produced by MetaboAnalyst 4.0 19. The cut-off level for significant proteins was a fold change of ≤ 0.5 (downregulation) or ≥ 2.0 (upregulation) and a Benjamini–Hochberg adjusted p-value of ≤ 0.05.

*1.4. Genomic extraction, analysis and processing*

Mouse faeces (13-32 mg) and intestinal washes (50 µl) of duodenum, jejunum, ileum, caecum and colon samples (all, n = 5) were homogenised for 4 cycles of 6,800 rpm × 20 s (Precellys® Evolution, Bertin Technologies, France) and extracted using the ZymoBiomics DNA miniprep kit (Zymo Research Corp., Irvin, CA, USA) following the manufacturer’s instructions.

Amplicons were generated from the V3 and V4 regions of 16S rRNA using gene-specific primers (in bold) with the appropriate adapter sequence for Illumina sequencing (in italics)

515f (5’- *TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***GTGCCAGCMGCCGCGGTAA** -3′) and

806rbc (5’-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***GGACTACHVGGGTWTCTAAT** -3’) (Integrated DNA Technologies, Inc., Coralville, IA, USA). Pooled DNA extracts were quantified (Qubit 3.0, ThermoFisher Scientific), and triplicate samples amplified using the following reaction mix: 10 ng DNA, 10 µl Platinum™ Hot Start PCR 2x Mastermix (Invitrogen™, Carlsbad, CA, USA), 0.5 µl of each 10 µM primer and water added to make the volume to 25 µl. The PCR conditions were 94°C/2 min, followed by 35 cycles of 94°C/30 s, 50°C/30 s and 72°C/1 min, and a final elongation step at 72°C/5 min.

Amplicon products were purified with 1.8x volume of Agencourt® AMPure XP beads (Beckman Coulter™, Brea, CA, USA) and suspended in 50 µl elution buffer (Qiagen, Hilden, Germany). Illumina index PCR was conducted following the Illumina amplicon sequencing protocol (94°C/2 min, followed by 8 cycles of 94°C/30 s, 55°C/30 s and 72°C/1 min, and a final elongation step at 72°C/5 min). Amplified products were re-purified with 1.12*x* volume of AMPure XP beads, size assessed as 456 bp on a Tape Station (Life Technologies, Carlsbad, CA, USA) using the High Sensitivity DNA screen tapes, quantified (Qubit 3.0) and pooled in equimolar concentrations (4nM). The purified library was sequenced and demultiplexed on an Illumina MiSeq using a v3 300 bp PE sequencing kit following the manufacturer’s protocol.

The 16s metagenomic analysis was of a pooled control and treatment sample to represent the maximum diversity present. Sequence processing was performed using QIIME 2 (Release no. 2019.7) pipeline 20 similar to previously reported methodology 15. Briefly, after checking the sequence quality 3 nucleotides were removed from left and right end of each sequence. The sequence length retained ranged from 284 – 319 bp with average 286 bp length. A total of 847 features were detected in all samples, represented by 65.39% retained sequences. Average feature count of the control sample group was 24188 and 24290 for sample treated with *Cryptosporidium* spiked diet. METAGENassist analysis 21 was performed to investigate the metabolic nature of the microbial community detected in each treatment group.

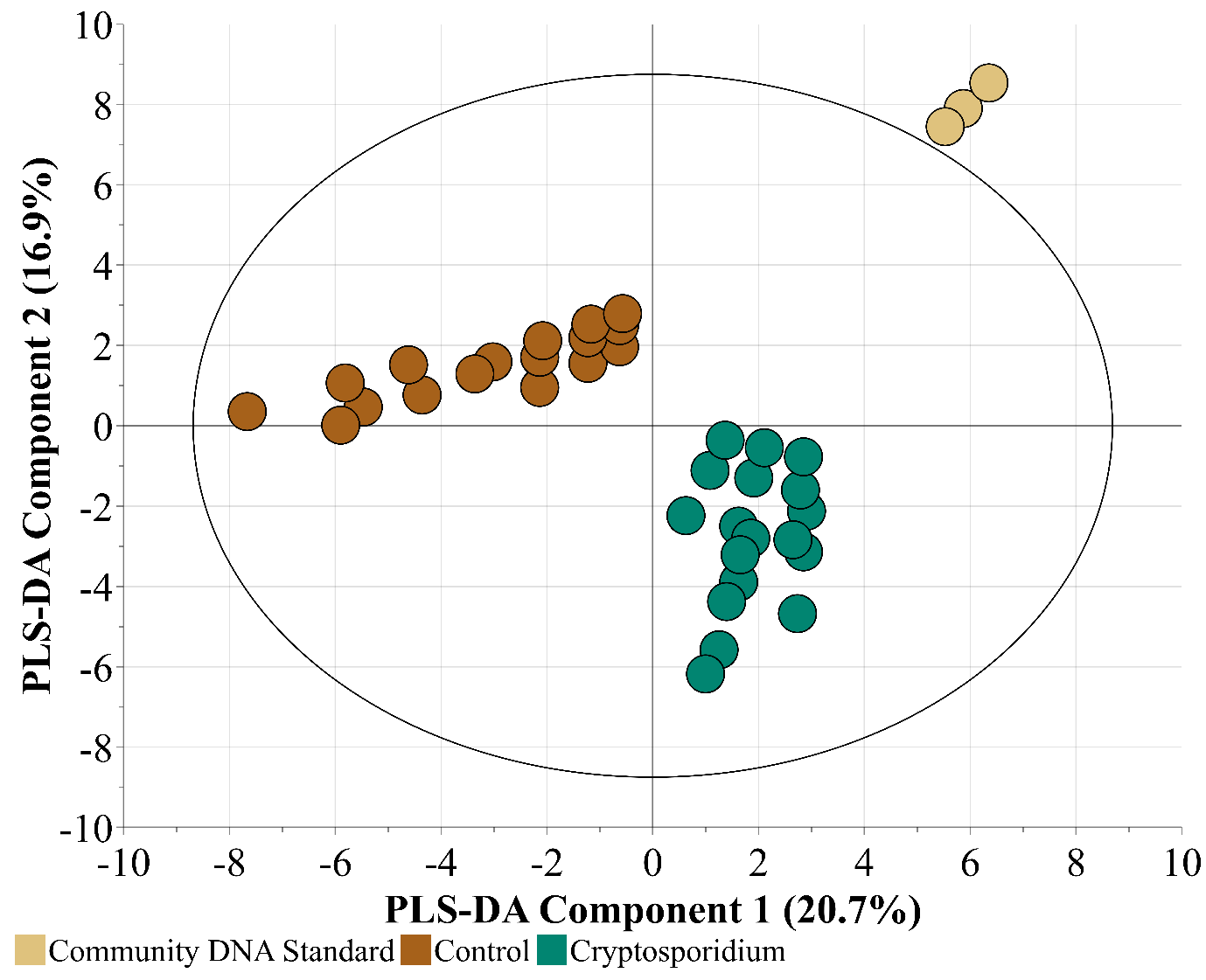
# **2. Results**

During cryptosporidiosis, most metabolites were depleted (FC < 0.5) in the small intestine. Primary depleted metabolites in the duodenum (Figure 3, Tables S3 – S5) included succinate, 6-hydroxy caproate, adenosine and inosine monophosphates, and glucose. In the jejunum, maleate, non-hexose sugars, and sugar acids (glucoheptonate and sedoheptulose) were depleted (Figure 2, Table S4). In the ileum, depleted metabolites included sorbose, erythrose phosphate, ribitol, glycerol, and gluconate, while shikimate, phenaceturate, and urea were elevated (Figure 2, Table S5). Overall, during cryptosporidiosis, more metabolites, especially sugars, sugar acids, and sugar alcohols, were depleted in the small intestine than elevated. Changes in fatty acid metabolism, especially of medium-chain and long-chain fatty acids (MCFAs and LCFAs), were observed in the intestine during infection. Although fatty acid oxidation and glycerolipid metabolism was observed in the duodenum, the latter was more prominent throughout the small intestine, indicated by a significant decrease of glycerol in the jejunum (FC = 0.07) and the ileum (FC = 0.04), and palmitate (FC = 0.26) and, palmitoleate (FC = 0.22) in the ileum.

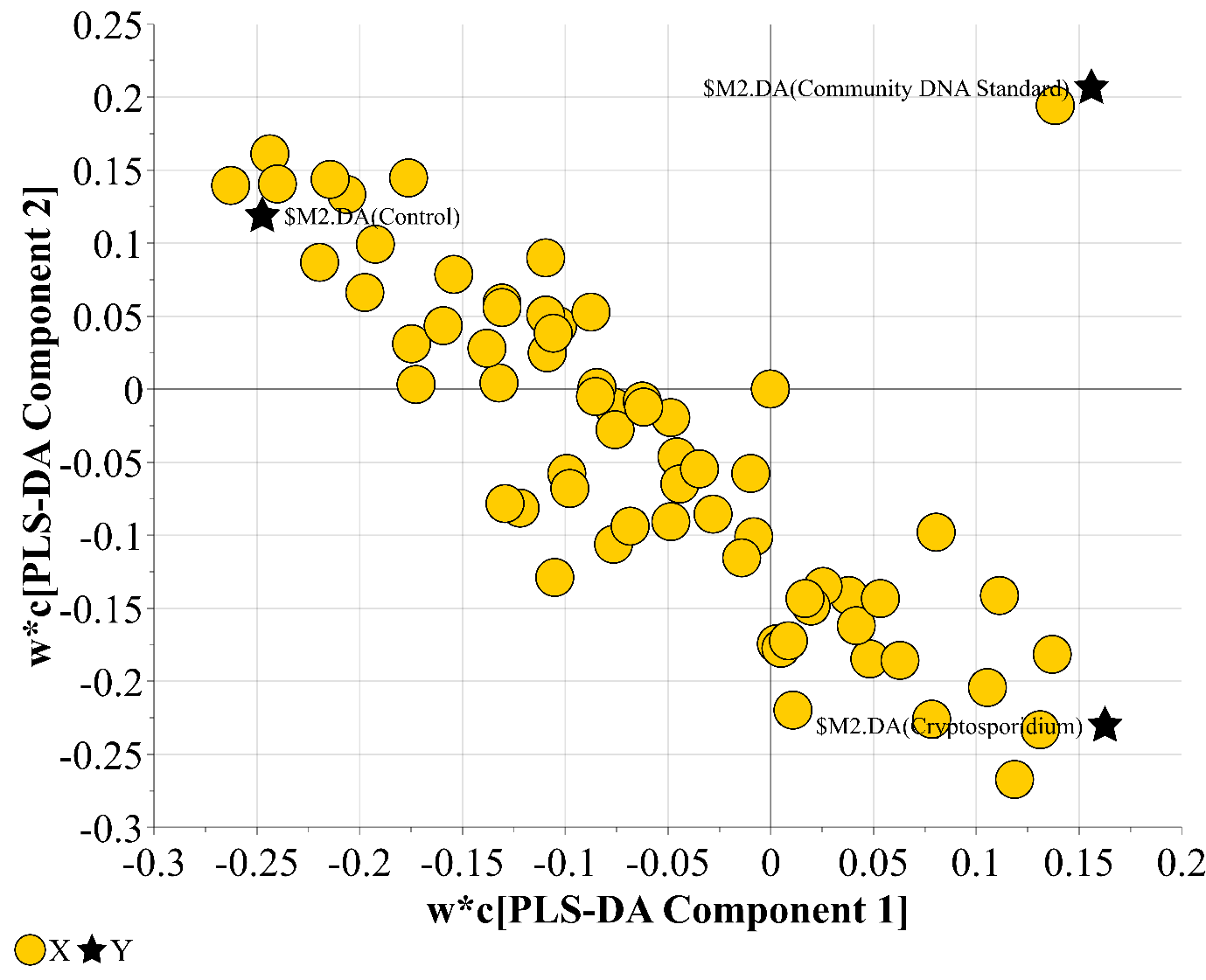
On the contrary, the number of elevated metabolites increased in the caecum and colon during infection. In the caecum, amino acids such as glycine, methionine, creatinine, tyrosine, alanine, lysine, and cysteine were elevated (Figure 2, Table S6). Other elevated metabolites in the colon included fatty and organic acids, such as malate, 3-aminoisobutyrate, fumarate, 3,4-dihydroxymandelate, and citrate (Figure 2, Table S7). Metabolic composition of the faeces was similar to that of the colon, with the addition of increased abundances of organic acids (Figure 2, Table S8).

Table S1. Statistical representation of data quality of 16S rRNA gene sequencing via the estimators of sequence diversity and richness across uninfected and *C. parvum* infected organ wash samples.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Experimental set** | **Sample ID** | **Average OTU count** | **Chao1** | **Ace** | **Shannon** | **Good’s Coverage** | **Simpson** |
| Uninfected | Faeces 0 dpi | 88,250 | 185.00 | 185.17 | 7.12 | 1.000 | 0.99 |
| Faeces 10 dpi | 155,743 | 156.33 | 156.33 | 6.38 | 1.000 | 0.98 |
| Duodenum | 148,152 | 232.20 | 232.32 | 7.24 | 1.000 | 0.99 |
| Jejunum | 162,266 | 151.00 | 151.00 | 6.54 | 1.000 | 0.98 |
| Ileum | 163,879 | 159.00 | 159.17 | 6.52 | 1.000 | 0.98 |
| Caecum | 100,420 | 187.25 | 187.33 | 6.65 | 1.000 | 0.98 |
| Colon | 153,308 | 130.00 | 130.22 | 6.04 | 1.000 | 0.97 |
| Cryptosporidiosis | Faeces 0 dpi | 127,391 | 153.00 | 153.00 | 6.48 | 1.000 | 0.98 |
| Faeces – 10 dpi | 112,841 | 132.50 | 131.68 | 6.04 | 0.999 | 0.98 |
| Duodenum | 158,630 | 123.00 | 122.76 | 6.10 | 0.999 | 0.98 |
| Jejunum | 154,824 | 188.14 | 188.51 | 6.99 | 1.000 | 0.99 |
| Ileum | 134,650 | 191.00 | 190.63 | 7.19 | 0.999 | 0.99 |
| Caecum | 106,048 | 322.33 | 322.30 | 7.95 | 1.000 | 0.99 |
| Colon | 155,536 | 277.00 | 275.82 | 7.50 | 0.999 | 0.99 |

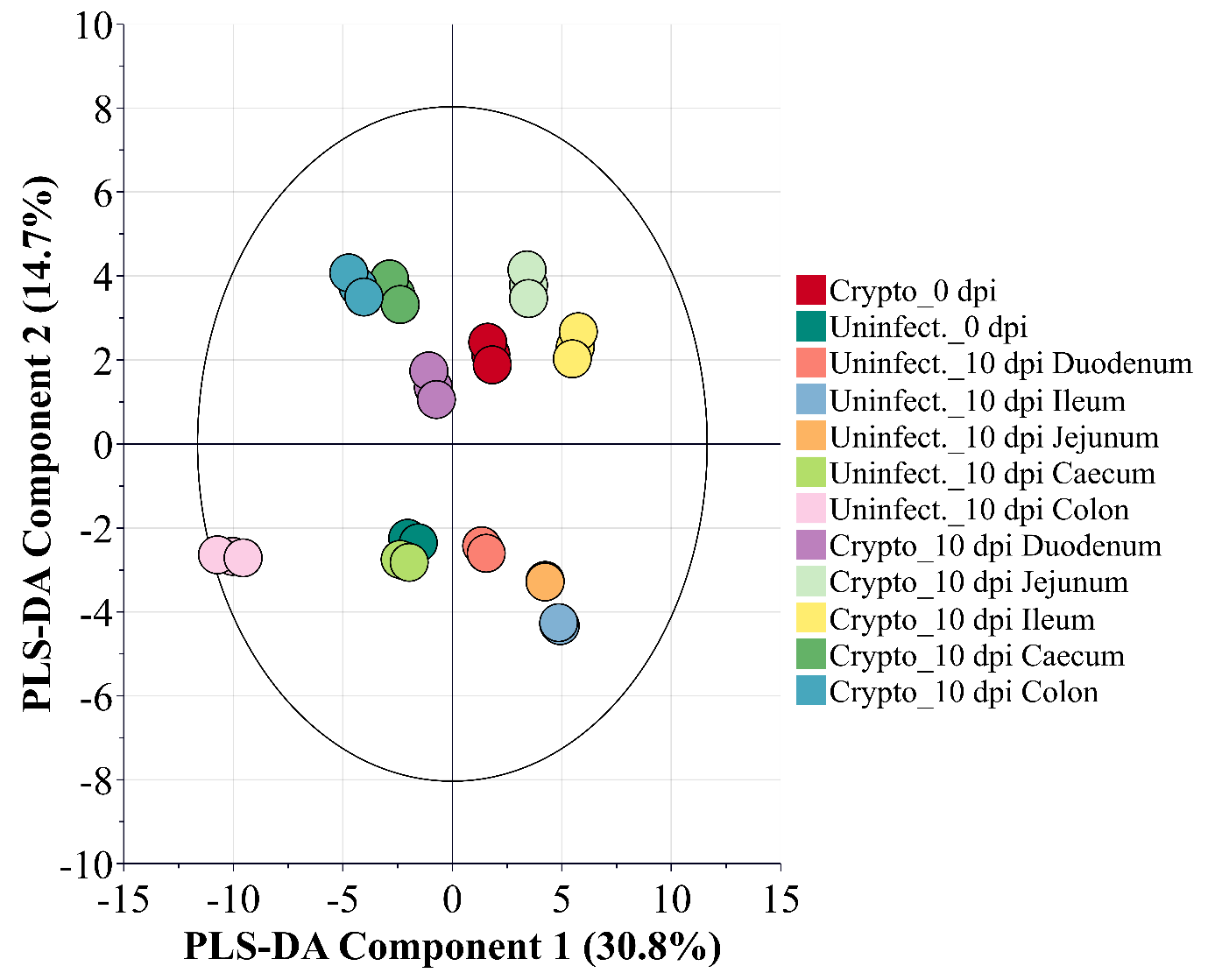


**(A)**



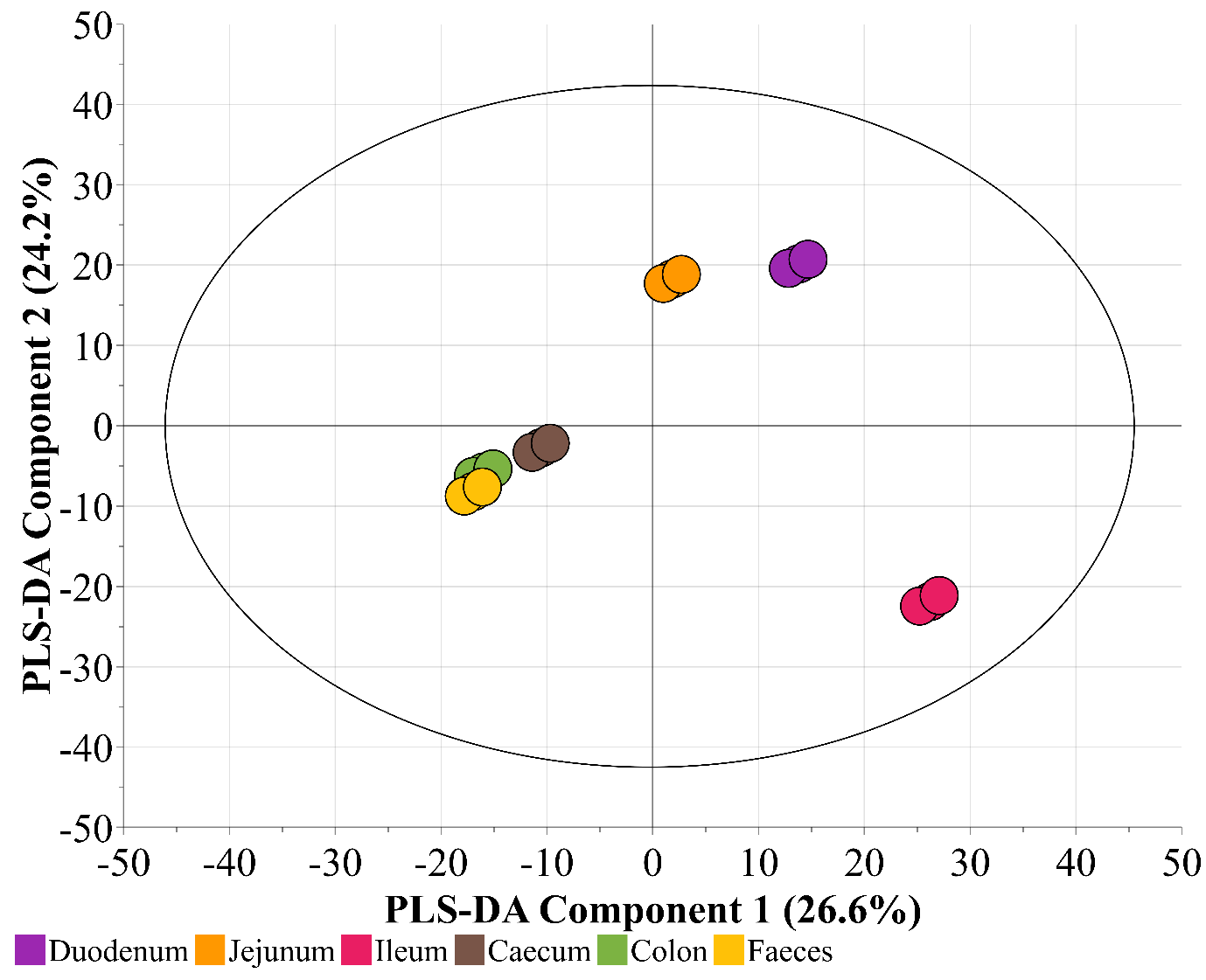
**(B)**

**(C)**

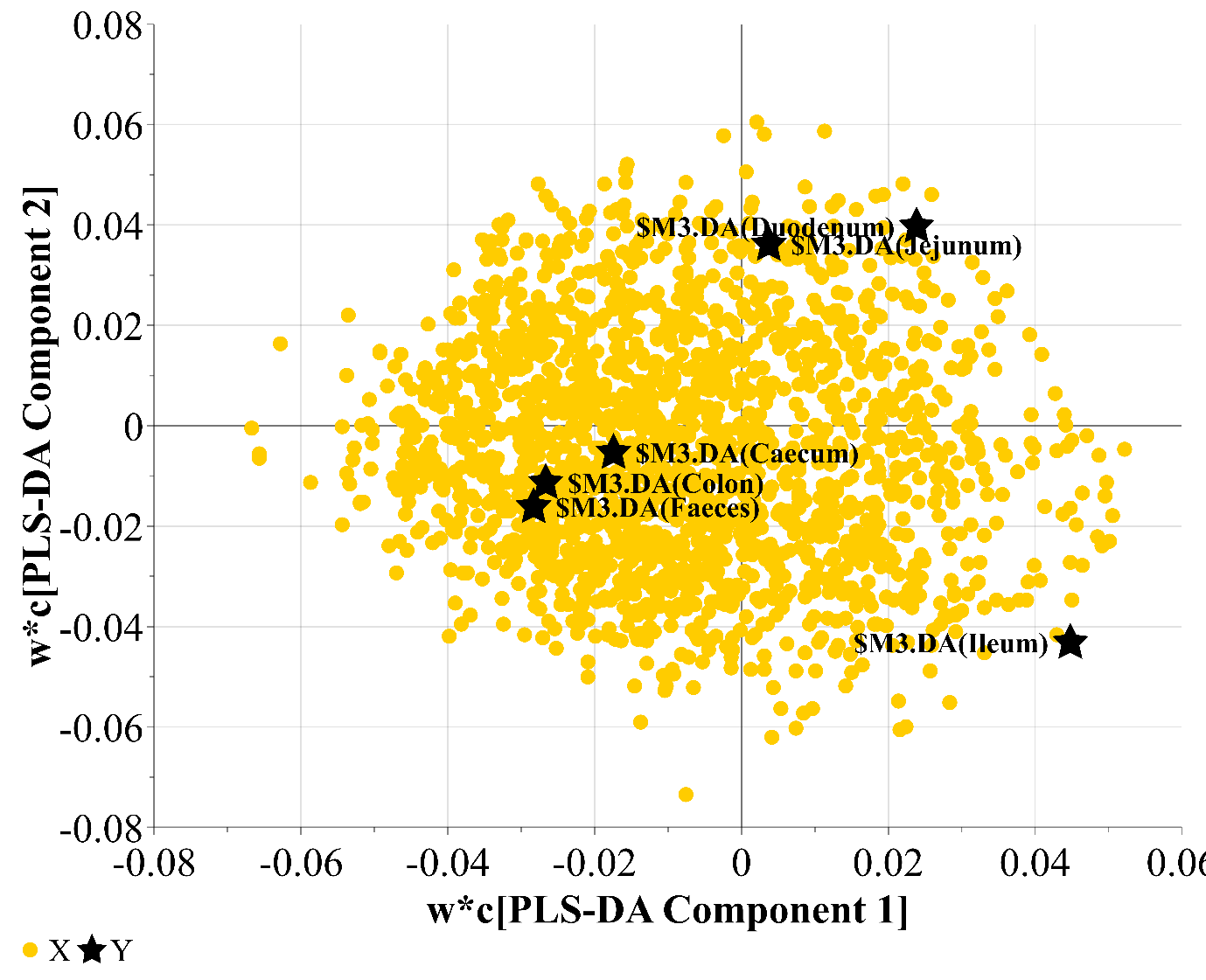


**(E)**

Figure S1. (A and B) PLS-DA score and loading scatter plots showing general discrimination between microbial spread across various sections of intestinal washes (R2X (cum) = 0.748, R2Y = 0.961, Q2 (cum) = 0.908) (Uninfect. = Uninfected samples, Crypto = *Cryptosporidium* infected’ samples); (C) Comparison of microbial distribution between 0 dpi and 10 dpi, (D) Ratio of major genera showing prominent changes during cryptosporidiosis infection compared to the uninfected mice, in mouse intestine regions. (E) PLS-DA score scatter plot of intestinal washes ((R2X (cum) = 0.961, R2Y = 0.884, Q2 (cum) = 0.618))



**(A)**



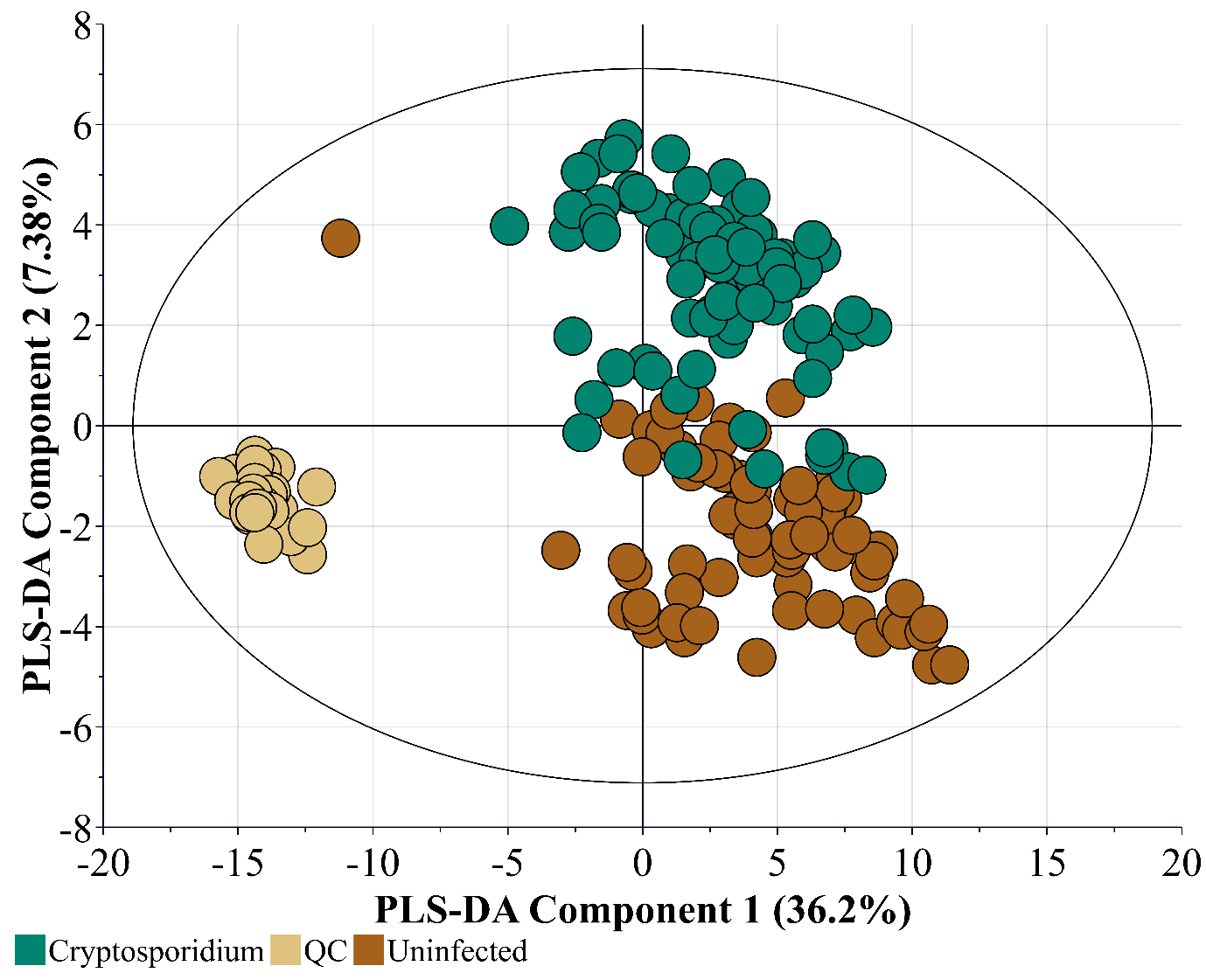
**(B)**

Figure S2. (A) PLS-DA score scatter plot and (B) PLS-DA loading scatter plots, for host protein expression in mouse gut during *Cryptosporidium* infection. R2X (cum) = 0.88, R2Y (cum) = 0.799, Q2 (cum) = 0.408. Note: The ellipse (Figure S2A) indicated the area of 95% confidence interval as determined by Hotelling T2 analysis.

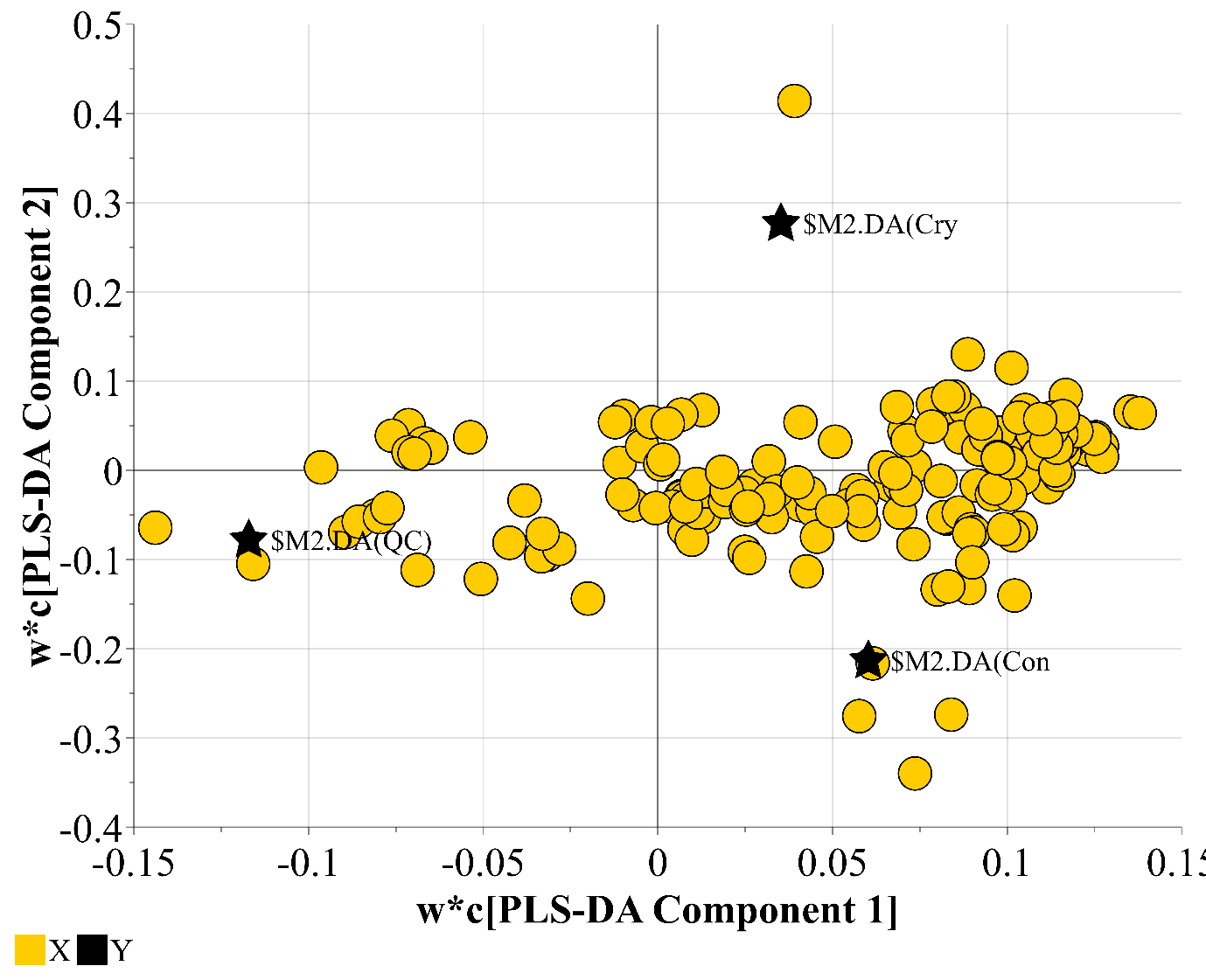
Table S2. Model fit (R2X and R2Y) and predictability (Q2) of metabolomic profile of all samples of *C. parvum* infected mice with respect to the uninfected mice, as analysed by the multivariate SIMCA analysis.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample | R2X | R2Y | Q2 |
| Faeces | 0.877 | 0.998 | 0.93 |
| Duodenum wash | 0.664 | 0.999 | 0.834 |
| Jejunum wash | 0.822 | 0.999 | 0.909 |
| Ileum wash | 0.719 | 0.997 | 0.957 |
| Caecum wash | 0.528 | 0.998 | 0.890 |
| Colon wash | 0.703 | 0.991 | 0.917 |
| Serum | 0.755 | 0.99 | 0.928 |
| Liver | 0.881 | 0.99 | 0.794 |

**Note:** Further related information can be found in Figures S1 – S7.



**(A)**



**(B)**

Figure S3. PLSDA plots of metabolic profile of various regions of intestine during cryptosporidiosis with respect to uninfected mice. The plots represent (A) PLS-DA Loading scatter for all samples (Uninfected, *C. parvum* infected and, QC = Quality Control). R2X (cum) = 0.736, R2Y (cum) = 0.981, Q2 (cum) = 0.888.

Table S3. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse duodenum during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in Metaboanalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Citric acid | 0.2359 | 0.0516 |
| Trans-4-hydroxy-L-proline | 0.1586 | 0.0611 |
| Gluconic acid lactone | 0.1362 | 0.0135 |
| Sedoheptulose | 0.1147 | 0.0351 |
| Glucoheptonic acid | 0.0955 | 0.0401 |
| Glycerol | 0.0766 | 0.0461 |
| Allantoin | 0.0686 | 7.59e-06 |
| O-acetylsalicylic acid | 0.0086 | 0.0007 |
| 6-hydroxy caproic acid | 8.81e-06 | 0.0001 |
| Succinic acid | 4.70e-07 | 0.0467 |

Table S4. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse jejunum during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in Metaboanalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Glycolic acid | 0.4544 | 0.0131 |
| L-mimosine | 0.3225 | 0.0349 |
| Oxalic acid | 0.2635 | 0.0094 |
| Gluconic acid lactone | 0.2613 | 0.6894 |
| α-D-Glucose | 0.1657 | 0.0494 |
| Maleic acid | 0.1517 | 0.0078 |
| Succinic acid | 0.1378 | 0.0368 |
| Allantoin | 0.0930 | 0.0138 |
| Glycerol | 0.0765 | 0.0439 |
| O-acetylsalicylic acid | 0.0442 | 0.0006 |
| Adenosine-5-monophosphate | 0.0005 | 0.05078 |
| 6-hydroxy caproic acid | 1.36e-05 | 4.86e-05 |

Table S5. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse ileum during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in Metaboanalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Compound\_116 | 38.8510 | 0.0002 |
| Shikimic acid | 32.6580 | 0.0001 |
| Gluconic acid lactone | 0.0552 | 0.0002 |
| Glycerol | 0.0410 | 0.0004 |
| Succinic acid | 0.0402 | 0.0317 |
| Ribitol | 0.0222 | 0.0010 |
| Allantoin | 0.0133 | 0.0009 |
| O-acetylsalicylic acid | 0.0033 | 0.0001 |
| Adenosine-5-monophosphate | 0.0020 | 0.0314 |
| 4-hydroxypyridine | 0.0006 | 0.0122 |
| D-erythrose-4-phosphate | 0.0004 | 0.0286 |
| Tagatose | 0.0001 | 0.0002 |
| L- sorbose | 0.0001 | 0.0005 |
| Glucoheptonic acid | 0.0001 | 0.0007 |
| α-D-Glucose | 0.0001 | 0.0008 |
| Sedoheptulose | 4.74e-05 | 0.0014 |
| 6-hydroxy caproic acid | 2.91e-05 | 0.0002 |

Table S6. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse caecum during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in Metaboanalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Cellobiose | 26982 | 3.51e-05 |
| Cycloleucine | 26112 | 0.0006 |
| L-glutamic acid | 26107 | 0.0006 |
| D-malic acid | 7157.8 | 3.67E-05 |
| Sophorose | 3645.1 | 0.0039 |
| Compound\_116 | 997 | 0.0001 |
| Shikimic acid | 8.4689 | 0.0045 |
| D-sphingosine | 5.4793 | 0.0240 |
| L- lactic acid | 2.7151 | 0.0186 |
| α ketoglutaric acid | 0.3294 | 0.0250 |
| 4-hydroxypyridine | 0.2818 | 0.0444 |
| L-lysine | 0.2672 | 0.0310 |
| Compound\_22 | 0.1940 | 0.0100 |
| L-tyrosine | 0.0847 | 0.0002 |
| Tagatose | 0.0834 | 0.0002 |
| Allantoin | 0.0646 | 0.0001 |
| Compound\_86 | 0.0048 | 0.0452 |
| O-acetylsalicylic acid | 0.0013 | 2.55e-06 |
| Creatinine | 0.0012 | 0.0001 |
| Inosine 5'-monophosphate | 9.11e-05 | 0.0444 |
| Adenosine-5-monophosphate | 9.11e-05 | 0.0444 |
| 6-hydroxy caproic acid | 1.00e-05 | 2.57e-06 |
| Succinic acid | 2.56e-06 | 0.0014 |

Table S7. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse colon during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR).*

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Cellobiose | 7152.5 | 2.14e-08 |
| Compound\_127 | 4169.9 | 2.03e-08 |
| D-malic acid | 2154.4 | 0.0005 |
| Compound\_122 | 3.9308 | 0.0049 |
| 3,4-dihydroxymandelic acid | 3.8245 | 0.0578 |
| Compound\_93 | 2.0142 | 0.0021 |
| Trans-aconitic acid | 0.3555 | 0.0887 |
| Trans-4-hydroxy-L-proline | 0.2142 | 0.0549 |
| Creatinine | 0.1640 | 0.0091 |
| L-lysine | 0.1591 | 0.0021 |
| Allantoin | 0.1449 | 0.0636 |
| D-mannose | 0.1167 | 0.0008 |
| Galactinol | 0.1087 | 0.0024 |
| Gluconic acid lactone | 0.1006 | 0.0005 |
| O-acetylsalicylic acid | 0.0255 | 1.52e-06 |
| Cholic acid | 0.0244 | 0.0035 |
| Allantoin | 0.0225 | 1.08e-07 |
| Glycine | 0.0206 | 0.0271 |
| L-methionine | 0.0015 | 4.04e-05 |
| L-tyrosine | 0.0005 | 0.0007 |
| Glucoheptonic acid | 7.97e-05 | 1.73e-08 |
| α-D-Glucose | 6.43e-05 | 1.73e-08 |
| Sedoheptulose | 5.57e-05 | 2.53e-08 |
| 6-hydroxy caproic acid | 3.20e-05 | 6.71e-08 |

Table S8. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in mouse faeces during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in Metaboanalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Cellobiose | 14994 | 0.0066 |
| Cycloleucine | 10128 | 0.0036 |
| L-glutamic acid | 10125 | 0.0036 |
| Compound\_127 | 7653 | 0.0094 |
| D-sphingosine | 6668.9 | 0.0599 |
| D-malic acid | 2787.8 | 0.0094 |
| Compound\_129 | 2535.7 | 0.0599 |
| Fumaric acid | 726.78 | 0.0134 |
| L-threonine | 105.98 | 0.0277 |
| Shikimic acid | 82.828 | 0.0066 |
| Compound\_121 | 18.134 | 0.0134 |
| D-ala-D-ala | 17.421 | 0.0322 |
| Adenosine | 9.458 | 0.0198 |
| Tartaric acid | 2.8516 | 0.0277 |
| Sedoheptulose | 0.1441 | 0.0293 |
| α-Glucose | 0.1378 | 0.0293 |
| Tagatose | 0.1118 | 0.0198 |
| Galactinol | 0.1064 | 0.0198 |
| L-tyrosine | 0.1033 | 0.0198 |
| Glucoheptonic acid | 0.1031 | 0.0198 |
| Gluconic acid lactone | 0.0764 | 0.0277 |
| L-methionine | 0.0746 | 0.0148 |
| Allantoin | 0.0465 | 0.0188 |
| Ribitol | 0.0178 | 0.0134 |
| Compound\_86 | 0.0121 | 0.0337 |
| 2-isopropylmalic acid | 0.0076 | 0.0277 |
| Glycine | 0.0008 | 0.0134 |
| Dehydroascorbic acid | 0.0006 | 0.0706 |
| Allantoin | 0.0003 | 0.0124 |
| Cholic acid | 0.0002 | 0.0198 |
| Compound\_22 | 0.0001 | 0.0148 |
| L- sorbose | 8.71e-05 | 0.0134 |
| Methyl-β-D-galactopyranoside | 3.88e-05 | 0.0153 |
| 6-hydroxy caproic acid | 1.98e-05 | 0.0198 |

Table S9. Signature host-response proteins expressed in mouse gut during cryptosporidiosis (combined output of duodenum: jejunum: ileum: caecum: colon: faeces) with respect to Giardiasis and UPEC infections when compared to the uninfected mice. The term cFC refers to log10 normalised combined fold change of statistically significant (adjusted p-value ≤ 0.05) proteins expressed.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Uniprot ID** | **Description** | **Crypto. (cFC)** | **P-value (FDR adj.)** | **Giardia (cFC)** | **P-value (FDR adj.)** | **UPEC (cFC)** | **P-value (FDR adj.)** |
| E9Q2D1 | Actin, cytoplasmic 1 (Fragment | 7.26 | 1.31e-16 |  |  | 6.02 | 3.94e-20 |
| E9Q606 | Actin, cytoplasmic 1 (Fragment) | 6.17 | 3.3e-09 | 1.07 | 0.0444 | 1.87 | 0.0037 |
| P68134 | Actin, alpha skeletal muscle | 5.37 | 5.06e-09 | 6.20 | 9.65e-18 |  |  |
| G3UZ07 | Actin, cytoplasmic 2 (Fragment) | 5.24 | 1.88e-07 |  |  | 1.56 | 0.0204 |
| E9Q5F4 | Actin, cytoplasmic 1 (Fragment) | 4.39 | 4.41e-08 |  |  | -1.45 | 0.0183 |
| P02535 | Keratin, type I cytoskeletal | 3.70 | 9.17e-06 |  |  | 1.57 | 0.0001 |
| A2A513 | Keratin, type I cytoskeletal 10 | 3.70 | 9.17e-06 |  |  | 1.57 | 0.0001 |
| P48962 | ADP/ATP translocase 1 | 3.59 | 1.7e-10 |  |  |  |  |
| A0A0U1RNK9 | Electron transfer flavoprotein subunit beta (Fragment) | 2.63 | 0.0036 |  |  |  |  |
| Q9DCW4 | Electron transfer flavoprotein subunit beta | 2.49 | 0.0012 |  |  |  |  |
| P11352 | Glutathione peroxidase 1 | 2.38 | 0.0001 | 0.98 | 0.0054 | 2.11 | 0.0001 |
| Q06890 | Clusterin | 2.35 | 0.0002 |  |  | 1.00 | 0.0478 |
| P63260 | Actin, cytoplasmic 2 | 2.33 | 7.47e-07 | -1.19 | 1.23e-06 | 0.80 | 0.0020 |
| P60710 | Actin, cytoplasmic 1 | 2.33 | 7.47e-07 | -1.19 | 1.23e-06 | 0.80 | 0.0020 |
| D3Z5E2 | Heat shock cognate 71 kDa protein (Fragment) | 2.32 | 0.0003 | -1.22 | 0.0097 |  |  |
| A0A1D5RM20 | Actin, alpha skeletal muscle (Fragment) | 2.30 | 0.0118 | 4.95 | 1.48e-12 |  |  |
| P43276 | Histone H1.5 | 2.27 | 0.0006 |  |  |  |  |
| A0A140LJ61 | Synemin | 2.21 | 0.0084 |  |  |  |  |
| Q9ET01 | Glycogen phosphorylase, liver form | 2.08 | 0.0012 | 1.87 | 0.0010 |  |  |
| Q3UEJ6 | Alpha-1,4 glucan phosphorylase | 2.08 | 0.0012 | 1.87 | 0.0010 |  |  |
| P31786 | Acyl-CoA-binding protein | 2.08 | 0.0006 |  |  | -1.29 | 0.0113 |
| Q4VWZ5 | Acyl-CoA-binding protein | 2.08 | 0.0006 |  |  | -1.29 | 0.0113 |
| P09411 | Phosphoglycerate kinase 1 | 2.07 | 0.0282 |  |  |  |  |
| E9Q1F2 | Actin, cytoplasmic 1 | 2.06 | 5.46e-06 | -1.33 | 9.79e-08 | 0.78 | 0.0016 |

Table S10. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in serum during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in MetaboAnalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| Compound\_110 | 10.525 | 0.0446 |
| Cellobiose | 6.2187 | 0.0222 |
| Trans-aconitic acid | 0.1039 | 0.0023 |
| Compound\_87 | 0.088 | 0.0089 |
| Palmitoleic acid | 0.0726 | 0.0145 |
| Compound\_53 | 0.0694 | 0.0192 |
| O-acetylsalicylic acid | 0.0573 | 0.0141 |
| Oleic acid | 0.047 | 0.0145 |
| Compound\_86 | 0.0454 | 0.0143 |
| Dehydroascorbic acid1 | 0.0324 | 0.0222 |
| Myristic acid | 0.0269 | 0.0089 |
| L-tyrosine | 0.0175 | 0.0089 |
| Compound\_32 | 0.002 | 0.0089 |
| Glycerol | 0.0017 | 0.0065 |
| Glycine | 0.0005 | 0.0145 |
| Cholic acid | 0.0001 | 0.0065 |
| Compound\_33 | 0.0001 | 0.0089 |

Table S11. Major upregulated (FC ≥ 2) and downregulated (FC ≤ 0.5) metabolites in liver during cryptosporidiosis with respect to the uninfected mice. ***Note:*** *The represented values were adjusted for Benjamini-Hochberg false discovery rate (FDR) in MetaboAnalyst 4.0 FDR adjustment tool*.

|  |  |  |
| --- | --- | --- |
| **Metabolite** | **Fold Change** | **P value (adjusted)** |
| L-mimosine | 11521 | 0.0123 |
| Oxalic acid | 536.45 | 0.0406 |
| L-fucose | 3.9177 | 0.0056 |
| Succinic acid | 0.2898 | 0.0046 |
| Compound\_32 | 0.1401 | 0.0226 |
| Glycerol | 0.1336 | 0.0225 |
| Compound\_33 | 0.0877 | 0.0154 |
| Compound\_25 | 0.0766 | 0.0154 |
| 6-hydroxy caproic acid | 0.0624 | 0.0157 |

Table S12. Signature host-response proteins expressed in mouse serum-liver during cryptosporidiosis with respect to the uninfected mice.

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| E9Q223 | Hemoglobin, β adult s chain (Fragment) OS=*Mus musculus* OX=10090 GN=Hbb-bs PE=1 SV=1 | 10.63 | 2.97E-11 |
| Q9CQ52 | Chymotrypsin-like elastase family member 3B OS=*Mus musculus* OX=10090 GN=Cela3b PE=1 SV=1 | 4.94 | 0.0001 |
| A0A0A6YWP4 | Complement factor H (Fragment) OS=*Mus musculus* OX=10090 GN=Cfh PE=1 SV=1 | 3.97 | 0.0008 |
| A0A2K6EDJ7 | Inter α-trypsin inhibitor, heavy chain 4 OS=*Mus musculus* OX=10090 GN=Itih4 PE=1 SV=1 | 3.91 | 0.0059 |
| Q8VCU2 | Glycosylphosphatidylinositol specific phospholipase D1 OS=*Mus musculus* OX=10090 GN=Gpld1 PE=1 SV=1 | 3.90 | 1.09E-05 |
| O70362 | Phosphatidylinositol-glycan-specific phospholipase D OS=*Mus musculus* OX=10090 GN=Gpld1 PE=1 SV=1 | 3.90 | 1.09E-05 |
| A0A0A6YXS8 | Antithrombin-III OS=*Mus musculus* OX=10090 GN=Serpinc1 PE=1 SV=1 | 3.70 | 0.0008 |
| Q9DBD0 | Inhibitor of carbonic anhydrase OS=*Mus musculus* OX=10090 GN=Ica PE=1 SV=1 | 3.67 | 0.0067 |
| D3YY36 | RIKEN cDNA 1300017J02 gene OS=*Mus musculus* OX=10090 GN=1300017J02Rik PE=1 SV=1 | 3.67 | 0.0067 |
| E9Q5L2 | Inter α-trypsin inhibitor, heavy chain 4 OS=*Mus musculus* OX=10090 GN=Itih4 PE=1 SV=1 | 3.65 | 0.0133 |

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| E9Q8Y5 | Clusterin (Fragment) OS=*Mus musculus* OX=10090 GN=Clu PE=1 SV=1 | 3.53 | 0.0002 |
| H3BK95 | Complement factor B (Fragment) OS=*Mus musculus* OX=10090 GN=Cfb PE=4 SV=1 | 3.51 | 0.0005 |
| B0R0E9 | Creatine kinase U-type, mitochondrial (Fragment) OS=*Mus musculus* OX=10090 GN=Ckmt1 PE=1 SV=1 | 3.27 | 0.0111 |
| F6W2T4 | Complement factor B (Fragment) OS=*Mus musculus* OX=10090 GN=Cfb PE=3 SV=1 | 3.21 | 0.0006 |
| H3BLB8 | Paraoxonase 1, isoform CRA\_c OS=*Mus musculus* OX=10090 GN=Pon1 PE=1 SV=1 | 3.18 | 0.0199 |
| G3X9T8 | Ceruloplasmin OS=*Mus musculus* OX=10090 GN=Cp PE=1 SV=1 | 3.11 | 0.0102 |
| E9Q9B8 | Clusterin (Fragment) OS=*Mus musculus* OX=10090 GN=Clu PE=1 SV=1 | 3.03 | 0.0099 |
| P08226 | Apolipoprotein E OS=*Mus musculus* OX=10090 GN=Apoe PE=1 SV=2 | 3.03 | 0.0113 |
| A2ARP5 | Creatine kinase U-type, mitochondrial (Fragment) OS=*Mus musculus* OX=10090 GN=Ckmt1 PE=1 SV=1 | 2.99 | 0.0041 |
| Q5ND35 | Α-2-antiplasmin (Fragment) OS=*Mus musculus* OX=10090 GN=Serpinf2 PE=1 SV=1 | 2.97 | 0.0043 |
| Q06890 | Clusterin OS=*Mus musculus* OX=10090 GN=Clu PE=1 SV=1 | 2.93 | 0.0011 |
| A0A0N4SVU1 | Predicted gene 7298 OS=*Mus musculus* OX=10090 GN=Gm7298 PE=4 SV=1 | 2.89 | 0.0040 |

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| A0A1B0GX15 | Apolipoprotein E (Fragment) OS=*Mus musculus* OX=10090 GN=Apoe PE=1 SV=1 | 2.89 | 0.0133 |
| P01029 | Complement C4-B OS=*Mus musculus* OX=10090 GN=C4b PE=1 SV=3 | 2.87 | 0.0181 |
| G3X977 | Inter-α trypsin inhibitor, heavy chain 2 OS=*Mus musculus* OX=10090 GN=Itih2 PE=1 SV=1 | 2.82 | 0.0127 |
| P28665 | Murinoglobulin-1 OS=*Mus musculus* OX=10090 GN=Mug1 PE=1 SV=3 | 2.82 | 0.0094 |
| G3UZM8 | Apolipoprotein E (Fragment) OS=*Mus musculus* OX=10090 GN=Apoe PE=1 SV=1 | 2.81 | 0.0254 |
| P11680 | Properdin OS=*Mus musculus* OX=10090 GN=Cfp PE=2 SV=2 | 2.76 | 0.0133 |
| A0A075B5P6 | Immunoglobulin heavy constant mu (Fragment) OS=*Mus musculus* OX=10090 GN=Ighm PE=1 SV=1 | 2.76 | 0.0119 |
| D3YUI3 | Pregnancy zone protein (Fragment) OS=*Mus musculus* OX=10090 GN=Pzp PE=1 SV=1 | 2.75 | 0.0268 |
| H7BWY6 | Retinol-binding protein 4 OS=*Mus musculus* OX=10090 GN=Rbp4 PE=1 SV=1 | 2.75 | 0.0185 |
| A0A075B6A0 | Immunoglobulin heavy constant mu (Fragment) OS=*Mus musculus* OX=10090 GN=Ighm PE=1 SV=2 | 2.74 | 0.0120 |
| D3Z2B2 | Kininogen-1 (Fragment) OS=*Mus musculus* OX=10090 GN=Kng1 PE=1 SV=8 | 2.72 | 0.0128 |
| A0A1B0GS57 | Hemopexin (Fragment) OS=*Mus musculus* OX=10090 GN=Hpx PE=1 SV=1 | 2.72 | 0.0084 |

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| P30275 | Creatine kinase U-type, mitochondrial OS=*Mus musculus* OX=10090 GN=Ckmt1 PE=1 SV=1 | 2.71 | 0.0053 |
| A0A0G2JGM6 | Vitamin D-binding protein (Fragment) OS=*Mus musculus* OX=10090 GN=Gc PE=1 SV=1 | 2.71 | 0.0106 |
| P01872 | Immunoglobulin heavy constant mu OS=*Mus musculus* OX=10090 GN=Ighm PE=1 SV=2 | 2.69 | 0.0133 |
| E9QP56 | Apolipoprotein C-III OS=*Mus musculus* OX=10090 GN=Apoc3 PE=1 SV=1 | 2.63 | 0.0094 |
| Q61838 | Pregnancy zone protein OS=*Mus musculus* OX=10090 GN=Pzp PE=1 SV=3 | 2.57 | 0.0253 |
| P09813 | Apolipoprotein A-II OS=*Mus musculus* OX=10090 GN=Apoa2 PE=1 SV=2 | 2.57 | 0.0013 |
| A0A0R4J0I1 | MCG1051009 OS=*Mus musculus* OX=10090 GN=Serpina3k PE=1 SV=1 | 2.55 | 0.0161 |
| P01865 | Ig γ-2A chain C region, membrane-bound form OS=*Mus musculus* OX=10090 GN=Igh-1a PE=1 SV=3 | 2.54 | 0.0297 |
| E9PYP1 | Carboxylic ester hydrolase OS=*Mus musculus* OX=10090 GN=Ces1a PE=1 SV=1 | 2.52 | 0.0341 |
| Q80X76 | Serine protease inhibitor A3F OS=*Mus musculus* OX=10090 GN=Serpina3f PE=1 SV=3 | 2.47 | 0.0333 |
| A0A0A6YXW6 | Immunoglobulin heavy constant α (Fragment) OS=*Mus musculus* OX=10090 GN=Igha PE=1 SV=1 | 2.44 | 0.0054 |
| P07309 | Transthyretin OS=*Mus musculus* OX=10090 GN=Ttr PE=1 SV=1 | 2.40 | 0.0121 |

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| E9Q499 | Serine protease inhibitor A3G (Fragment) OS=*Mus musculus* OX=10090 GN=Serpina3g PE=1 SV=8 | 2.36 | 0.0436 |
| Q61247 | Α-2-antiplasmin OS=*Mus musculus* OX=10090 GN=Serpinf2 PE=1 SV=1 | 2.34 | 0.0119 |
| P52430 | Serum paraoxonase/arylesterase 1 OS=*Mus musculus* OX=10090 GN=Pon1 PE=1 SV=2 | 2.31 | 0.0492 |
| P01027 | Complement C3 OS=*Mus musculus* OX=10090 GN=C3 PE=1 SV=3 | 2.30 | 0.0374 |
| Q01339 | Β-2-glycoprotein 1 OS=*Mus musculus* OX=10090 GN=Apoh PE=1 SV=1 | 2.25 | 0.0203 |
| H9H9R5 | Plasma kallikrein OS=*Mus musculus* OX=10090 GN=Klkb1 PE=1 SV=2 | 2.24 | 0.0366 |
| Q91X72 | Hemopexin OS=*Mus musculus* OX=10090 GN=Hpx PE=1 SV=2 | 2.21 | 0.0325 |
| Q07456 | Protein AMBP OS=*Mus musculus* OX=10090 GN=Ambp PE=1 SV=2 | 2.20 | 0.0407 |
| A0A0A6YX70 | Antithrombin-III (Fragment) OS=*Mus musculus* OX=10090 GN=Serpinc1 PE=1 SV=1 | 2.18 | 0.0251 |
| Q8K0C5 | Zymogen granule membrane protein 16 OS=*Mus musculus* OX=10090 GN=Zg16 PE=1 SV=1 | 2.16 | 0.0043 |
| P21614 | Vitamin D-binding protein OS=*Mus musculus* OX=10090 GN=Gc PE=1 SV=2 | 2.15 | 0.0186 |
| F7CJN9 | Transferrin (Fragment) OS=*Mus musculus* OX=10090 GN=Trf PE=4 SV=1 | 2.15 | 0.0360 |

|  |  |  |  |
| --- | --- | --- | --- |
| **UniProt ID** | **Description** | **Combined Log (FC)** | **P value**  **(FDR adj.)** |
| I7HPW5 | Β-2-glycoprotein 1 (Fragment) OS=*Mus musculus* OX=10090 GN=Apoh PE=1 SV=1 | 2.14 | 0.0116 |
| I7HJR3 | Β-2-glycoprotein 1 (Fragment) OS=*Mus musculus* OX=10090 GN=Apoh PE=1 SV=1 | 2.12 | 0.0282 |
| Q00897 | Α-1-antitrypsin 1-4 OS=*Mus musculus* OX=10090 GN=Serpina1d PE=1 SV=1 | 2.11 | 0.0355 |
| F7BAE9 | Transferrin (Fragment) OS=*Mus musculus* OX=10090 GN=Trf PE=1 SV=1 | 2.10 | 0.0366 |
| E9Q035 | Predicted gene 20425 OS=*Mus musculus* OX=10090 GN=Gm20425 PE=4 SV=1 | 2.06 | 0.0413 |
| Q921I1 | Serotransferrin OS=*Mus musculus* OX=10090 GN=Tf PE=1 SV=1 | 2.04 | 0.0418 |
| P01837 | Immunoglobulin kappa constant OS=*Mus musculus* OX=10090 GN=Igkc PE=1 SV=2 | 2.04 | 0.0071 |
| D6RGQ0 | Complement factor H OS=*Mus musculus* OX=10090 GN=Cfh PE=1 SV=1 | 2.02 | 0.0206 |

Table S13. Variable Importance in Projection (VIP) scores and impact importance of metabolites in small intestine (A: Jejunum, B: ileum) during cryptosporidiosiswith respect to the uninfected mice. VIP score ≥ 2 were considered significantly impacting the overall metabolic behaviour.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Metabolite** | **Duodenum** | **Jejunum** | **Ileum** | **Caecum** | **Colon** | **Faeces** |
| Glycerol | 5.77 | 5.93 | 6.06 | 4.79 | 5.81 | 2.78 |
| Succinate | 5.76 | 1.05 | 5.58 | 4.66 | 2.49 | 1.36 |
| Citrate | 5.45 | 1.15 | 0.63 | 5.53 | 6.31 | 0.97 |
| Orthophosphate | 5.25 | 6.62 | 3.73 | 5.58 | 7.67 | 0.06 |
| Glycolate | 3.13 | 3.98 | 2.24 | 2.26 | 3.73 | 0.00 |
| Glucose | 2.28 | 2.34 | 0.83 | 2.27 | 1.72 | 0.64 |
| Sedoheptulose | 2.04 | 0.89 | 0.52 | 1.83 | 0.40 | 0.41 |
| Methyl-beta-D-galactose | 1.79 | 0.15 | 0.56 | 0.27 | 0.43 | 0.43 |
| Cellobiose | 1.53 | 3.70 | 5.85 | 0.48 | 0.26 | 0.48 |
| Oxalate | 1.53 | 1.66 | 2.73 | 3.03 | 1.87 | 4.88 |
| Malonate | 1.41 | 1.30 | 2.25 | 2.66 | 0.94 | 4.13 |
| Lactate | 1.26 | 0.43 | 0.91 | 1.41 | 0.58 | 0.00 |
| Melezitose | 1.26 | 0.97 | 0.63 | 0.48 | 0.03 | 0.00 |
| Shikimate | 1.23 | 0.49 | 0.18 | 0.26 | 0.28 | 0.23 |
| Glucoheptonate | 1.19 | 0.54 | 0.36 | 0.98 | 0.28 | 0.30 |
| Glutamate | 0.79 | 0.30 | 0.49 | 2.68 | 0.48 | 4.53 |
| Urea | 0.68 | 0.91 | 0.71 | 1.00 | 0.80 | 1.97 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Metabolite** | **Duodenum** | **Jejunum** | **Ileum** | **Caecum** | **Colon** | **Faeces** |
| Sucrose | 0.64 | 1.19 | 0.38 | 0.04 | 0.04 | 0.11 |
| 6-hydroxy caproate | 0.63 | 0.53 | 0.84 | 1.16 | 0.70 | 0.80 |
| L-norleucine | 0.60 | 0.56 | 1.01 | 0.04 | 0.00 | 0.02 |
| 2-isopropylmalate | 0.57 | 0.05 | 0.10 | 0.31 | 0.32 | 0.16 |
| Tagatose | 0.56 | 0.12 | 0.20 | 0.27 | 0.09 | 0.17 |
| Sorbose | 0.54 | 0.11 | 0.20 | 0.23 | 0.11 | 0.20 |
| Sphingosine | 0.54 | 1.17 | 1.91 | 0.13 | 0.03 | 0.15 |
| Gluconate lactone | 0.49 | 0.23 | 0.24 | 0.28 | 0.22 | 0.18 |

A picture containing text, map

Description automatically generated

Figure S8. Predominant metabolic pathways in mouse gastrointestinal tract during cryptosporidiosis.