Multi-tissue analysis exploring disruption of the gut-brain axis caused by bacterial infection and treatment

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1. Introduction

The 'gut-brain axis' is considered to be a bi-directional communication pathway between gut microbiota and the brain. In this study, *Clostridioides difficile* infection (CDI) has been selected as a model to study gut disruption in the gut-brain axis caused by a bacterial pathogen and treatments. Metronidazole - an antibiotic that can transfer between the blood brain barrier and known to have a negative effect on gut microbiome - was compared to faecal microbiota transplantation (FMT). High resolution LC-MS/MS metabolic profiling data was acquired from mouse brain tissue, caecum tissue and faecal gut content collected post-mortem from each treatment group using reverse phase and HILIC separation and data independent acquisition MS/MS.

2. Methods

2.1 Experimental design

Thirteen week old male C57BL/6 mice were treated for four days with an antibiotic cocktail and administered an intraperitoneal injection of clindamycin 10 mg kg^{-1} on day six. One group was left uninfected, while other groups received non-toxinogenic (TCDA-, TCDB-) CDI on day 7. Of the three groups infected, one remained untreated while the other groups received a 10-day course of either metronidazole or 10% faecal water in drinking water, prepared from faecal samples of uninfected controls. Brain tissue, caecum tissue and faecal gut content samples collected post-mortem were extracted in MeOH:IPA:H_2O 1:1:2 v/v/v in a ratio of 3:1 tissue:solvent. Following sonication, homogenisation and centrifugation, supernatants were collected, and pellets were re-extracted in MeOH:MTBE 1:3 v/v. Supernatants were evaporated to dryness and reconstituted in methanol (reverse phase) or 90% acetonitrile (HILIC) for analysis. Aliquots of these samples were pooled to make QCs.

Reverse phase LC Separation (cycle time 35 mins)

- Acquity C18 BEH (2.1x100mm 1.7µm); 50ºC, flow rate 0.4 mL/min
- Binary gradient: water + 0.1% formic acid; acetonitrile + 0.1% formic acid

HILIC LC Separation (cycle time 18 mins)

- Shim-pack Velox HILIC (2.1x100mm 2.7µm); 40ºC, flow rate 0.3 mL/min
- Binary gradient; water + 10mM ammonium formate 0.1% formic acid, and acetonitrile:water+ 10mM ammonium formate 0.1% formic acid [92:8]

Mass Spectrometry Detection. QTOF LCMS-9030; external mass calibration; ESI+/-

- MS mass scan m/z 65-1010; 100 msecs
- DIA-MS/MS mass scans m/z 40-1000; 33 msecs for each precursor isolation window; isolation width 35 Da; collision energy spread 5-55V. Scan cycle time 0.99 second

Data processing.

- Targeted processing of DIA-MS/MS data used LabSolutions Insight with automated metabolite identification against in-house MS/MS libraries of authentic standards where available.

2.2 Metabolite identification

- Metabolites were identified by comparison to an in-house MS/MS library of authentic standards for metabolomics Standards Initiative (MSI) level 1 (Figure 1) or by comparison to online repositories such as MassBank, mzCloud and LipidMaps for MSI level 2. The in-house library included accurate mass spectra for lipid distributions (LPCs, LPEs MGs, fatty acids, fatty acid conjugates, fatty amides) and polar metabolites (nucleotides, amino acids, purines, pyrimidines).

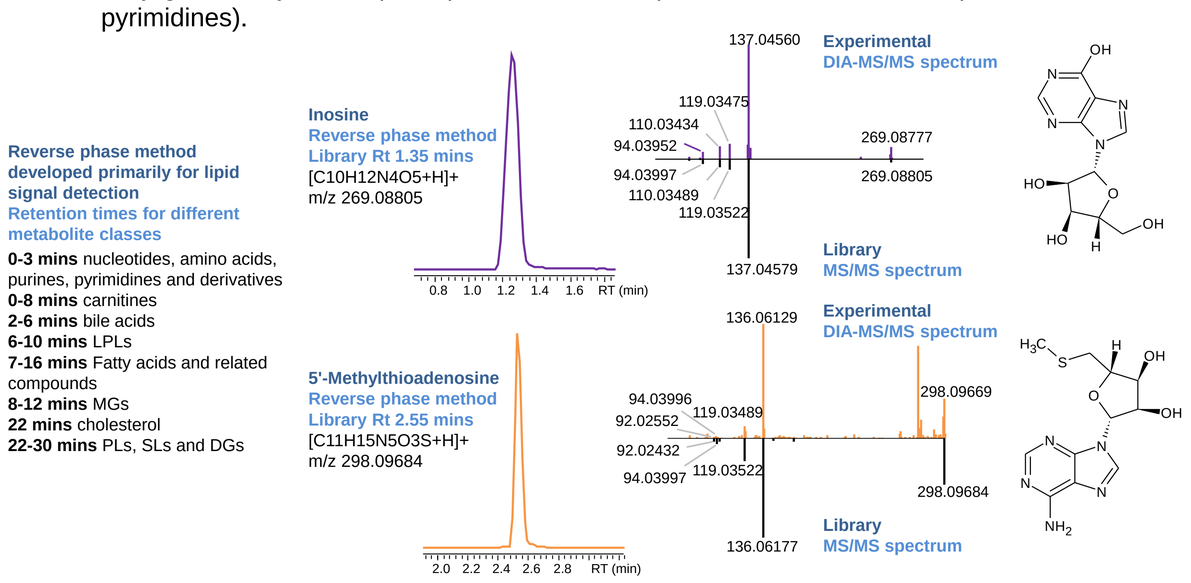

Figure 1. To increase reporting confidence particularly for early eluting features, MS/MS product ion spectra were matched against an in-house MS/MS data repository acquired with authentic standards.

3. Results

3.1 Targeted effects of different treatments in the brain

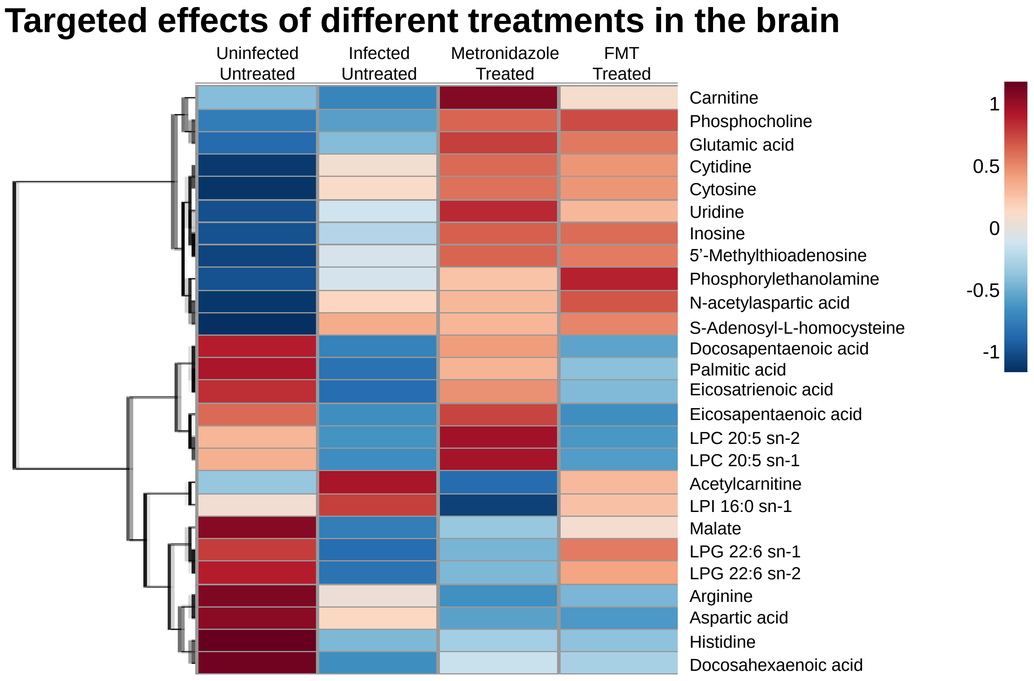

Figure 2. Heatmap created using MetaboAnalyst showing 26 significant phenotypic differences in brain extracts from uninfected mice and mice infected with *Clostridium difficile* that were left untreated or treated with metronidazole or treated FMT. Significance determined by ANOVA $p<0.05$ (FDR corrected). Metabolites marked with * are those identified to MSI level 1 by comparison to authentic standards.

- In brain extracts, infection and treatments had an additive effect on increasing the relative concentrations of nucleosides including cytidine, cytosine, inosine, uridine and 5'-methylthioadenosine as well as glutamic acid (the most abundant free amino acid in the brain and major mediator of excitatory signals) and N-acetylaspartate (highly abundant brain metabolite).
- Aspartic acid, arginine and histidine were decreased by infection and treatments in the brain.
- Fatty acids (except DHA) were reduced by infection and restored only with metronidazole treatment.

3.2 Targeted effects of different treatments in the gut

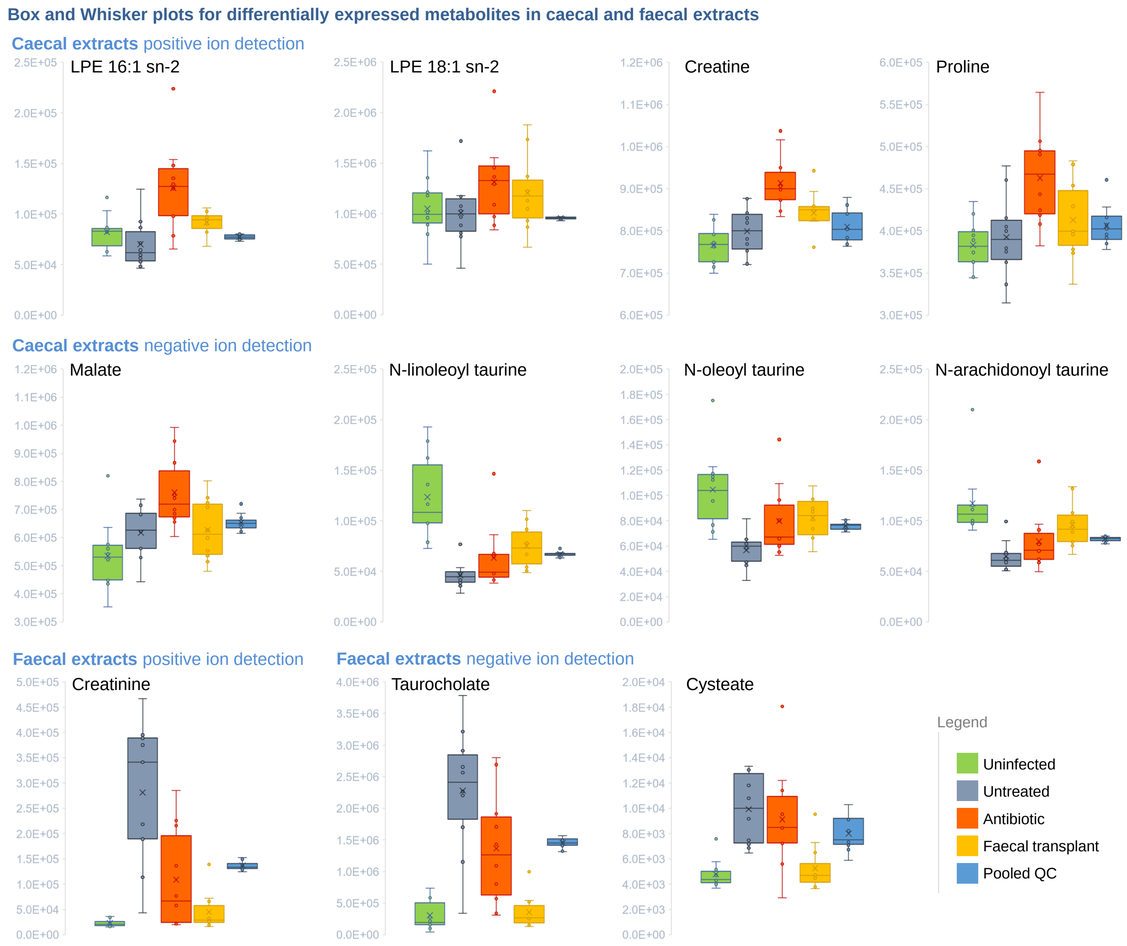

Figure 3. Box and whisker plots highlighting differentially expressed metabolites in caecal and faecal extracts infected with CDI and treated with metronidazole or FMT.

- In caecal extracts LPE 16:1 and LPE 18:1 (both sn-2 isoforms), malate, proline and creatine were all significantly increased following the antibiotic metronidazole treatment.
- Taurocholate, creatinine and cysteate significantly increased in faecal gut content following infection but reverted to the concentration of the uninfected controls with FMT treatment.
- The bacterial pathogen infection lowered the concentration of N-acyl taurines (N-linoleoyl taurine, N-arachidonoyl taurine, N-oleoyl taurine) in caecal tissue but this effect was reversed to some extent following FMT.

3.3 Increasing confidence in metabolite detection

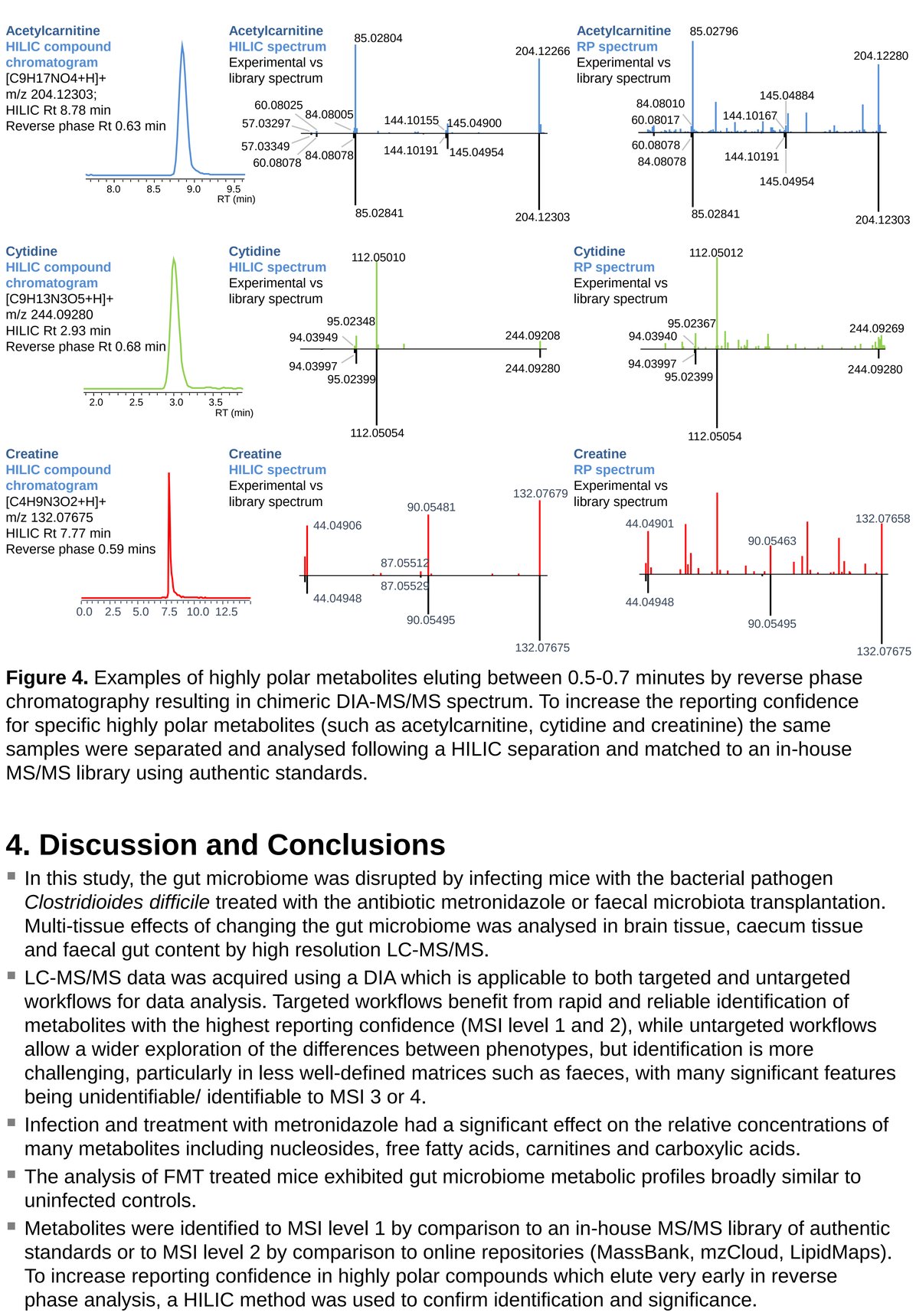

Figure 4. Examples of highly polar metabolites eluting between 0.5-0.7 minutes by reverse phase chromatography resulting in chimeric DIA-MS/MS spectrum. To increase the reporting confidence for specific highly polar metabolites (such as acetylcarnitine, cytidine and creatinine) the same samples were separated and analysed following a HILIC separation and matched to an in-house MS/MS library using authentic standards.

4. Discussion and Conclusions

- In this study, the gut microbiome was disrupted by infecting mice with the bacterial pathogen *Clostridioides difficile* treated with the antibiotic metronidazole or faecal microbiota transplantation. Multi-tissue effects of changing the gut microbiome was analysed in brain tissue, caecum tissue and faecal gut content by high resolution LC-MS/MS.
- LC-MS/MS data was acquired using a DIA which is applicable to both targeted and untargeted workflows for data analysis. Targeted workflows benefit from rapid and reliable identification of metabolites with the highest reporting confidence (MSI level 1 and 2), while untargeted workflows allow a wider exploration of the differences between phenotypes, but identification is more challenging, particularly in less well-defined matrices such as faeces, with many significant features being unidentifiable/ identifiable to MSI 3 or 4.
- Infection and treatment with metronidazole had a significant effect on the relative concentrations of many metabolites including nucleosides, free fatty acids, carnitines and carboxylic acids.
- The analysis of FMT treated mice exhibited gut microbiome metabolic profiles broadly similar to uninfected controls.
- Metabolites were identified to MSI level 1 by comparison to an in-house MS/MS library of authentic standards or to MSI level 2 by comparison to online repositories (MassBank, mzCloud, LipidMaps). To increase reporting confidence in highly polar compounds which elute very early in reverse phase analysis, a HILIC method was used to confirm identification and significance.