SUPPLEMENTARY MATERIAL

SUPPLEMENTARY METHODS:

Bacterial strains:

C difficile DS1684 (ribotype 010, non-toxigenic strain) was used for chemostat experiments and mouse experiments. *C difficile* DS1684, *C difficile* CD630 (ribotype 012, virulent multidrug resistant strain), *C difficile* R20291 (ribotype 027, a hypervirulent strain), *Bacteroides uniformis*, *Bacteroides vulgatus*, and *Clostridium scindens* (DSM 5676) were used in batch culture experiments. *C difficile* ribotype 027 is a common ribotype in Europe and North America,[1-3] while ribotype 012 is one of the common ribotypes in mainland China.[4,5] CD630 and R20291 are genetically and phenotypically well-characterised and are good representatives of their ribotypes.[6] *B uniformis* and *B vulgatus* were isolated from the stool of a healthy male in his 30's using fastidious anaerobe agar (Lab M, Heywood, UK) or nutrient agar (Sigma-Aldrich, St. Louis, USA), respectively.

Chemostat model of CDI:

The working volume of each vessel was 235 ml and the growth medium feed was set to a retention time of 21 hours.[7,8] The composition of the growth medium consisted of a mixture of both soluble and insoluble starches, amino acids, peptides, proteins, vitamins, trace elements, and porcine gastric mucin (type II).[9] To mimic the gut environment cultures were maintained at a temperature of 37°C and a pH of 6.8, were gently agitated, and kept anaerobic by sparging with oxygen-free nitrogen gas. Chemostat cultures were sampled daily from each vessel and vessels were operated for 54 days post-inoculation. Chemostat culture samples were aliquoted and stored at -80°C for DNA extraction and mass spectrometry analysis. For NMR analysis, fresh chemostat culture was centrifuged at 20,000 x g and 4°C for 10 minutes, and the supernatant was aliquoted and stored at -80°C.

Design of chemostat experiments:

We induced CDI in our chemostat gut model following a modified version of the methods previously described by Freeman and colleagues (**Table 1**).[10] Briefly, chemostat cultures were grown for 24 days without experimental manipulation to allow the communities to stabilise. After sampling vessels on day 24 we added $7.8x10^6$ *C difficile* spores to each vessel to achieve an initial concentration of $3.3x10^4$ spores/mL.[11] On day 25 we added another dose of $7.8x10^6$ *C difficile* spores to both vessels, and clindamycin was added to both vessels at a final concentration of 33.9 mg/L every 12 hours for 7 days (from days 25-31). After stopping clindamycin dosing chemostat cultures were left to grow for 10 days without experimental manipulation (days 32-42). This was done to allow the perturbed microbial communities to stabilise, so we could more easily determine which bacteria or metabolites were altered by FMT, and which bacteria or metabolites were able to recover after antibiotic treatment in the absence of FMT. After sampling on day 42 we added a single dose of saline to VA (control vessel) and a single dose of FMT to VB (test vessel). Chemostat cultures were then left to grow for a further 12 days without further experimental manipulation to monitor the effects of FMT on the chemostat communities (days 43-54).

***C difficile* spore preparation:**

C difficile spores were prepared using previously described methods.[11] *C difficile* DS1684 was grown anaerobically on fastidious agar plates supplemented with 5% defibrinated horse blood (VWR, Radnor, USA) and incubated at 37°C for 7 days. The growth was removed from the plates using a sterile loop and resuspended in 1 mL sterile water. Next, 1 mL of 95% ethanol was mixed with the cell suspension and was incubated for 1 hour at room temperature. The cell suspension was then centrifuged at 3000 x g and resuspended in 1 mL sterile water. Spores were enumerated by preparing serial 10-fold dilutions in phosphate buffered saline (PBS) (Sigma-Aldrich) and plating the dilutions on Braziers Cycloserine, Cefoxitin Egg Yolk agar plates (containing Braziers CCEY agar base (Lab M), 250 mg/L cycloserine (VWR), 8

mg/L cefoxitin (Sigma-Aldrich), 8% egg yolk emulsion (SLS, Nottingham UK), 2% lysed defibrinated horse blood (VWR), and 5 mg/L lysozyme (Sigma-Aldrich)).[12] Plates were incubated anaerobically at 37°C for 48 hours and the number of colonies were enumerated.

Enumeration of *C difficile* counts from chemostat culture samples:

C difficile total viable counts and spore counts were quantified from fresh chemostat culture samples every other day starting 26 days post-inoculation. *C difficile* total viable counts (TVC) were enumerated from fresh chemostat culture samples by performing serial 10-fold dilutions in PBS and plating onto Brazier's Cycloserine, Cefoxitin Egg Yolk agar plates (as described above, with the addition of 2 mg/L moxifloxacin (VWR)) in triplicate using the Miles and Misra method.[13] *C difficile* spore counts were enumerated from alcohol-shocked chemostat culture samples by mixing an equal volume of fresh chemostat culture sample with 95% ethanol and incubating at room temperature for one hour. Samples were then centrifuged at 3000 x g and 4°C for 10 minutes and resuspended in PBS. Spores were then quantified by performing serial 10-fold dilutions in PBS and plating onto Brazier's Cycloserine, Cefoxitin Egg Yolk agar plates (as described above, without the addition of moxifloxacin) in triplicate using the Miles and Misra method. Plates were incubated anaerobically at 37°C for two days and colonies were enumerated.

Preparation and instillation of FMT:

Fresh faecal samples were placed into an anaerobic chamber within 5 minutes of defecation. FMT preparations were prepared by homogenising 10 g of stool in 100 mL of anaerobic 0.9% saline in a strainer stomacher bag (250 rpm for 1 min). We added 50 mL of anaerobic saline to VA (control vessel) and 50 mL of homogenised stool to VB (test vessel). For Run 1 and Run 2 the stool transplant was prepared from the stool of a healthy male donor in his 30's, and for Run 3 the stool transplant was prepared from the stool

of a healthy female donor in her 20's. Both individuals have been used as FMT donors to treat CDI patients in Imperial's FMT Programme (and therefore undergone the appropriate donor screening protocols), and had not taken antibiotics for at least 3 months prior to providing the stool sample.

DNA extraction:

DNA was extracted from 250 µL of chemostat culture using the PowerLyzer PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, USA) following the manufacturer's protocol, except that samples were lysed by bead beating for 3 min at speed 8 using a Bullet Blender Storm instrument (Chembio Ltd, St. Albans, UK). DNA was aliquoted and stored at -80°C until it was ready to be used.

16S rRNA gene qPCR:

16S rRNA gene qPCR data was used to determine the total bacterial biomass within each sample and was performed using extracted chemostat culture DNA to following a previously published protocol.[14] A total volume of 20 µL was used for each reaction and consisted of the following: 1x Platinum Supermix with ROX (Life Technologies, Carlsbad, USA), 1.8 µM BactQUANT forward primer (5'-CCTACGGGAGGCAGCA-3'), 1.8 µM BactQUANT reverse primer (5'-GGACTACCGGGTATCTAATC-3'), 225 nM probe ((6FAM) 5'-CAGCAGCCGCGGTA-3' (MGBNFQ)), PCR grade water (Roche, Penzberg, Germany), and 5 µL DNA. Each PCR plate included a standard curve using *E. coli* DNA (Sigma-Aldrich) (3-300,000 copies per reaction in 10-fold serial dilutions) as well as no template negative controls. All samples, standards, and controls were amplified in triplicate. Extracted DNA samples were diluted to ensure they fell within the standard curve. Amplification and real-time fluorescence detections were performed using the Applied Biosystems StepOnePlus Real-Time PCR System using the following PCR cycling conditions: 50 °C for 3 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. We used a paired t-test

to compare changes in log-transformed 16S rRNA gene copy number between samples at specific time points.

Pre-processing and analysis of 16S rRNA gene sequencing data:

We used the Mothur package (v1.35.1) to preprocess and analyse the resulting sequencing data following the MiSeq SOP Pipeline.[15] We used the Silva bacterial database for sequence alignments (www.arb-silva.de/) and the RDP database reference sequence files for classification of sequences using the Wang method.[16] We determined the Operational Taxonomic Unit (OTU) taxonomies (phylum to genus) using the RDP MultiClassifier script. We resampled and normalised data to the lowest read count in Mothur (9527 reads per sample), which resulted in greater than 99.4% coverage within each sample. We used 16S rRNA gene qPCR data and the following formula to express our 16S rRNA gene sequencing data as absolute abundances (instead of relative abundances):

$$Absolute\ abundance\ of\ taxa = relative\ abundance\ of\ taxa \times \left(\frac{16S\ rRNA\ gene\ copy\ number\ in\ sample}{highest\ 16S\ rRNA\ gene\ copy\ number\ in\ sample\ set}\right)$$

The Shannon diversity index (H'), Pielou evenness index (J'), and richness (total number of bacterial taxa observed, S_{obs}) were calculated using the vegan library[17] within the R statistical package.[18]

Stream plots were prepared by plotting the absolute abundance of 16S rRNA gene sequencing data (biomass-corrected) over time (OTU-level, coloured by phylum). This was accomplished using the streamgraph function within the streamgraph library (v0.8.1) within R.[19]

^{1}H-NMR spectroscopy sample preparation:

Chemostat culture supernatants were randomized and defrosted at room temperature for 1 hour. Once samples were defrosted supernatants were centrifuged at 20,000 x g and 4°C for 10 minutes. Next, 400 µL of chemostat culture supernatant was mixed with 250 µL of sodium phosphate buffer solution

(28.85 g Na_2HPO_4 (Sigma-Aldrich), 5.25 g NaH_2PO_4 (Sigma-Aldrich), 1 mM TSP (Sigma-Aldrich), 3 mM NaN_3 (Sigma-Aldrich), deuterium oxide (Goss Scientific Instruments, Crewe, UK) to 1 L, pH 7.4)[20] and 600 µL was pipetted into a 5 mm NMR tube.

Confirmation of NMR metabolite identities using 1D-NMR with spike-in and 2D-NMR spectroscopy:

We used the statistical total correlation spectroscopy (STOCSY) analysis method to aid in the identification of metabolites in NMR spectra by determining correlations between intensities of the various peaks across the whole sample.[21] To further confirm if the peaks assigned to valerate and other metabolites were correct, we also conducted a two-dimensional NMR spectra (including ^1H–^1H TOCSY and ^1H–^1H COSY) for the chemostat culture supernatant and valerate standard using typical parameters to confirm the connectivity of the proton in the metabolites.[22,23]

For the valerate spike-in experiment one-dimensional ^1H NMR spectra were acquired as described in the ^1H-NMR spectroscopy methods section from the main text, except 64 scans were recorded into 65536 data points with a spectral width of 20 ppm. After normal 1D ^1H NOESY NMR acquisition, 10 µL of valerate standard (99%, 0.9 M in PBS buffer) (Fisher Scientific, Hampton, USA) was added into the sample. A one-dimensional spectrum was recorded again to see if the relevant peaks of valerate increased.

Data pre-processing and analysis of UPLC-MS bile acid data:

Quality control samples were prepared using a mixture of equal parts of the chemostat culture supernatants. We used the quality control samples as an assay performance monitor and to guide the removal of features with high variation.[24] We also spiked quality control samples with defined mixtures of bile acids to determine the chromatographic retention times of specific bile acids and to aid in metabolite identification (55 bile acid standards, including 36 non-conjugated bile acids, 12 tauro-conjugated bile acids, and 7 glyco-conjugated bile acids) (Steraloids, Newport, USA).

We converted the Waters raw data files to NetCDF format and extracted the data using XCMS (v1.50) package implemented within the R (v3.3.1) software. Dilution effects were corrected for using probabilistic quotient normalisation[25] and chromatographic features with high coefficient of variation (higher than 30% in the quality control samples) were excluded from further analysis.

Short AsyNchronous Time-series Analysis (SANTA):

SANTA is an automated pipeline that is implemented within R and controlled through a graphical user interface developed with Shiny. [26,27] This method analyses short time series by estimating trajectories as a smooth spline, and calculates whether time trajectories are significantly altered between different groups or over different time periods. SANTA was used to make the following comparisons: stabilisation period vs. clindamycin-dosing period, stabilisation period vs. post-clindamycin stabilisation period, and FMT-treated vs. saline-treated cultures during the treatment period. We used mean subtraction to eliminate between-run differences in metabolite concentrations that arose from differences in the stool used to seed the chemostat vessels. For each metabolite, we calculated the mean for all samples within the same chemostat run, then we subtracted the mean from all its values within the run.[28] Depending upon the time series being analysed, the number of degrees of freedom (df) to fit the spline model was chosen to avoid overfitting the data (df = 3-5). We report the p_{Dist} values, which uses the area between the mean group fitted curves to determine whether there is a difference between the two groups over time. Analysis used 1000 permutation rounds to calculate p-values and 1000 bootstrap rounds to calculate the 95% confidence bands. Reported p_{Dist} values are with Benjamini-Hochberg FDR correction, and $p < 0.05$ was considered significant.

Integration of 16S rRNA gene sequencing data and metabolite data:

We used regularised Canonical Correlation Analysis (rCCA) to correlate 16S rRNA gene sequencing data (genus level) with bile acid mass spectrometry or ^{1}H-NMR data from the same set of samples using the mixOmics library within R.[29] rCCA is an unsupervised method that maximises the correlation between the two data sets X and Y (information on the treatment groups is not taken into account in the analysis). We used the shrinkage method to determine the regularisation parameters. The plotIndiv function was used to generate unit representation plots, where each point on the scatter plot represents a single chemostat culture sample, and samples were projected into the XY-variate space. The plotVar function was used to generate correlation circle plots, where strong correlations between variables (correlations greater than 0.5) are plotted outside of the inner circle. Variables are represented through their projections onto the planes defined by their respective canonical variates. In this plot the variables projected in the same direction from the origin have a strong positive correlation, and variables projected in opposite directions form the origin have strong negative correlations. Variables with stronger correlations sit at farther distances from the origin.

***C difficile* germination batch cultures with taurocholic acid (TCA):**

To test the effects of TCA on *C difficile* germination we resuspended *C difficile* DS1684 spores in supplemented brain heart infusion broth with or without 1% TCA (Sigma-Aldrich) in triplicate.[30] The OD_{600} was measured immediately after inoculation of broths (time zero) and after an overnight incubation at 37°C in anaerobic chamber. A paired t-test was used to determine whether TCA affected *C difficile* germination.

***C difficile* vegetative growth batch cultures with TCA:**

To test the effects of TCA on *C difficile* vegetative growth we centrifuged an overnight culture of *C difficile* DS1684 at 3000 x g for 10 minutes and resuspended the cells in supplemented brain heart infusion

broth with or without 1% TCA (in triplicate). The OD_{600} was measured at time zero and cultures were incubated at 37°C in an anaerobic chamber. Additional OD_{600} measurements were taken at 2, 4, 6, and 8 hours post-inoculation, and the change in OD_{600} was plotted against time. A paired t-test was used to determine whether TCA affected vegetative growth during the exponential phase.

SUPPLEMENTARY RESULTS:

***C difficile* total viable counts and spore counts:**

There was no significant difference in *C difficile* TVC at the end of the clindamycin-dosing period compared to TVC immediately prior to administering FMT or saline treatment ($p>0.05$). There was also no significant difference in *C difficile* TVC in vessels assigned to receive FMT or saline treatment immediately prior to administering the treatment ($p>0.05$).

^{1}H-NMR spectroscopy:

Following FMT or saline treatment there were significant strong negative correlations between valerate and 5-aminovalerate (r_S=-0.76, p=5.27x10^{-6}), ethanol (r_S=-0.69, p=6.53x10^{-5}), and methanol (r_S=-0.78, p=3.11x10^{-6}).

Confirmation of valerate in chemostat culture supernatant by 1D- and 2D-NMR:

The chemical shifts for the 1D ^{1}H-NMR spectrum of 99% valerate standard were: 0.9 (t), 1.3 (dt), 1.46 (m), 2.2 (t) (**Figure S5A**). Overlay of the 1D ^{1}H-NMR spectrum of the valerate standard with the sample showed that each peak of the valerate standard is visible in the sample (**Figure S5B**). Overlay of 1D ^{1}H-NMR spectra of the sample before and after valerate spike-in showed that all the valerate peaks increased after spike-in (**Figure S5C**). For 2D-NMR analysis overlay of the ^{1}H-^{1}H COSY spectrum of the valerate standard with the sample showed that each peak of the valerate standard was present in the sample

spectrum (**Figure S6A**). Overlay of ^{1}H-^{1}H TOCSY spectrum of the valerate standard with the sample showed that each peak of the valerate standard was present in the sample spectrum (**Figure S6B**).

SUPPLEMENTARY DISCUSSION:

Valerate is not expected to be harmful to host gut cells, as exposure of gut organoids to 5 mM valerate did not result in cell death or cause significant alterations in gene expression (Drs. Lee Parry and Richard Brown of the European Cancer Stem Cell Research Institute, personal communication, 19 Dec. 2017). Moreover, mice that received 15 mM glycerol trivalerate in our study did not show any adverse reactions.

The bacterial enzyme 7-α-dehydroxylase is responsible for converting unconjugated primary bile acids CA and CDCA to the secondary bile acids DCA and LCA, respectively. DCA and LCA have been shown to inhibit *C difficile* vegetative growth at specific concentrations,[30,31] and a previous study has shown that DCA and LCA were depleted in pre-FMT samples from recurrent CDI patients, but were restored in post-FMT samples.[32] These findings led researchers to propose antibiotic exposure results in the loss of bacteria with 7-α-dehydroxylase activity, reducing DCA and LCA production and permitting *C difficile* vegetative growth. A study by Buffie and colleagues found that administration of *Clostridium scindens* (a bacterium with 7-α-dehydroxylase activity) was associated with resistance to *C difficile* by restoring the production of the secondary bile acids DCA and LCA.[33] However, in our study we found that the levels of DCA and LCA recovered in chemostat cultures following the cessation of clindamycin. While we did find strong negative correlations between *C difficile* TVC and the secondary bile acids DCA and LCA, recovery of these bile acids to pre-clindamycin levels was not enough to decrease vegetative *C difficile* counts in chemostat cultures. Indeed, while DCA can inhibit *C difficile* vegetative growth, it appears that DCA can also encourage spore germination at specified concentrations.[30] A better strategy to prevent CDI prior to antibiotic exposure would be to prevent germination altogether by degrading TCA, a potent pro-germinant, using bile salt hydrolase enzymes.

Our chemostat experiments more closely modelled the first episode of CDI and not recurrent CDI. In first episodes of CDI, human patients are exposed to *C difficile* spores while taking an inciting antibiotic (e.g. clindamycin). In our study clindamycin exposure was sufficient to deplete valerate and elevate levels of TCA, allowing *C difficile* spore germination and vegetative growth. We waited 10 days after stopping clindamycin dosing before administering the FMT preparation to allow the perturbed microbial communities to stabilise following the cessation of antibiotics. This delay allowed us to more easily determine which metabolites were altered only by FMT, and which metabolites were able to recover after antibiotic treatment (in the absence of FMT). This feature is a major advantage of performing chemostat studies, as it would be unethical to withhold treatment from recurrent CDI patients in human studies to determine which metabolites would recover in the absence of FMT. We found no significant difference in *C difficile* TVC at the end of the clindamycin-dosing period compared to TVC immediately prior to administering FMT or saline treatment (see Supplementary Results). This means the metabolites that recovered following the cessation of clindamycin dosing, but before FMT, did not affect the vegetative growth of *C difficile* (i.e. bile acids). However, we did see a significant decrease in *C difficile* TVC and spore counts after FMT. This decrease suggests that bacterial metabolites that decreased *C difficile* counts did not recover after stopping clindamycin, but only recovered with FMT (i.e. valerate).

Most human studies of CDI have focused on recurrent CDI, not first episode of CDI. In this study we showed that valerate was depleted in recurrent CDI patients pre-FMT, but was restored post-FMT. To our knowledge this is the first study to measure valerate in the stool of recurrent CDI patients pre- and post-FMT. A previous study by Weingarden and colleagues found that TCA was elevated in the stool of recurrent CDI patients pre-FMT, but was decreased post-FMT.[32] It is important to note that in these human studies recurrent CDI patients were taking vancomycin when pre-FMT samples were collected, and FMT was administered to recurrent CDI patients within 1-2 days of stopping vancomycin therapy. While we could have designed our chemostat experiments to also include a vancomycin dosing regimen, followed

by FMT administration 1-2 days later, broad-spectrum vancomycin therapy would have killed more gut bacteria and depleted the chemostat cultures of additional ecosystem functions that were not important for the establishment of the infection, leading to false positives once these functionalities were restored following FMT. In our chemostat experiments FMT was administered 10 days after stopping clindamycin and pre-FMT samples were collected immediately prior to FMT administration. Had we chosen to administer the FMT preparation within 1-2 days after stopping clindamycin we would expect TCA levels to decrease with FMT.

REFERENCES:

1. Wilcox MH, Shetty N, Fawley WN, *et al*. Changing epidemiology of *Clostridium difficile* infection following the introduction of a national ribotyping-based surveillance scheme in England. Clin Infect Dis 2012;55:1056-1063.
2. Waslawski S, Lo ES, Ewing SA, *et al*. *Clostridium difficile* ribotype diversity at six health care institutions in the United States. J Clin Microbiol 2013;51:1938-1941.
3. Davies KA, Longshaw CM, Davis GL, *et al*. Underdiagnosis of *Clostridium difficile* across Europe: the European, multicentre, prospective, biannual, point-prevalence study of *Clostridium difficile* infection in hospitalised patients with diarrhoea (EUCLID). Lancet Infect Dis 2014;14:1208-1219.
4. Huang H, Fang H, Weintraub A, *et al*. Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. Clin Microbiol Infect 2009;15:1170-1173.
5. Hawkey PM, Marriott C, Liu WE, *et al*. Molecular epidemiology of *Clostridium difficile* infection in a major Chinese hospital: an underrecognized problem in Asia? J Clin Microbiol 2013;51:3308-3313.
6. Dawson LF, Valiente E, Donahue EH, *et al*. Hypervirulent *Clostridium difficile* PCR-ribotypes exhibit resistance to widely used disinfectants. PLoS One 2011;6:e25754.

7. Allison C, McFarlan C, MacFarlane GT. Studies on mixed populations of human intestinal bacteria grown in single-stage and multistage continuous culture systems. Appl Environ Microbiol 1989;55:672-678.

8. Duncan SH, Scott KP, Ramsay AG, *et al*. Effects of alternative dietary substrates on competition between human colonic bacteria in an anaerobic fermentor system. Appl Environ Microbiol 2003;69:1136-1142.

9. McDonald JA, Schroeter K, Fuentes S, *et al*. Evaluation of microbial community reproducibility, stability and composition in a human distal gut chemostat model. J Microbiol Methods 2013;95:167-174.

10. Freeman J, Baines SD, Saxton K, *et al*. Effect of metronidazole on growth and toxin production by epidemic *Clostridium difficile* PCR ribotypes 001 and 027 in a human gut model. J Antimicrob Chemother 2007;60:83-91.

11. Freeman J, O'Neill FJ, Wilcox MH. Effects of cefotaxime and desacetylcefotaxime upon *Clostridium difficile* proliferation and toxin production in a triple-stage chemostat model of the human gut. J Antimicrob Chemother 2003;52:96-102.

12. Crowther GS, Chilton CH, Todhunter SL, *et al*. Comparison of planktonic and biofilm-associated communities of *Clostridium difficile* and indigenous gut microbiota in a triple-stage chemostat gut model. J Antimicrob Chemother 2014;69:2137-2147.

13. Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. J Hyg (Lond) 1938;38:732-749.

14. Liu CM, Aziz M, Kachur S, *et al*. BactQuant: an enhanced broad-coverage bacterial quantitative real-time PCR assay. BMC Microbiol 2012;12:56-2180-12-56.

15. Kozich JJ, Westcott SL, Baxter NT, *et al*. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol 2013;79:5112-5120.

16. Wang Q, Garrity GM, Tiedje JM, *et al*. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261-5267.

17. Oksanen J, Blanchet FG, Friendly M, *et al*. vegan: Community Ecology Package. R package version 2.4-3.; 2017. Available at: https://CRAN.R-project.org/package=vegan, 2017.

18. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.; 2015. Available at: https://www.R-project.org/, 2017.

19. Rudis B. streamgraph: streamgraph is an htmlwidget for building streamgraph visualizations. R package version 0.8.1.; 2015. Available at: http://github.com/hrbrmstr/streamgraph, 2017.

20. Beckonert O, Keun HC, Ebbels TM, *et al*. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nat Protoc 2007;2:2692-2703.

21. Cloarec O, Dumas ME, Craig A, *et al*. Statistical total correlation spectroscopy: an exploratory approach for latent biomarker identification from metabolic ^{1}H NMR data sets. Anal Chem 2005;77:1282-1289.

22. Liu Z, Wang L, Zhang L, *et al*. Metabolic Characteristics of 16HBE and A549 Cells Exposed to Different Surface Modified Gold Nanorods. Adv Healthc Mater 2016;5:2363-2375.

23. Dai H, Xiao C, Liu H, *et al*. Combined NMR and LC-DAD-MS analysis reveals comprehensive metabonomic variations for three phenotypic cultivars of Salvia Miltiorrhiza Bunge. J Proteome Res 2010;9:1565-1578.

24. Sangster T, Major H, Plumb R, *et al*. A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. Analyst 2006;131:1075-1078.

25. Veselkov KA, Vingara LK, Masson P, *et al*. Optimized preprocessing of ultra-performance liquid chromatography/mass spectrometry urinary metabolic profiles for improved information recovery. Anal Chem 2011;83:5864-5872.

26. Chang W, Cheng J, Allaire JJ, *et al*. shiny: Web Application Framework for R. R package version 1.0.3.; 2017. Available at: https://CRAN.R-project.org/package=shiny, 2017.

27. Wolfer A. SANTA-App: Interactive package for Short AsyNchronous Time-series Analysis (SANTA) in R, implemented in Shiny; 2017. Available at: https://github.com/adwolfer/SANTA-App, 2017.
28. **Gratton J**, **Phetcharaburanin J**, Mullish BH, *et al*. Optimized Sample Handling Strategy for Metabolic Profiling of Human Feces. Anal Chem 2016;88:4661-4668.
29. Le Cao K, Rohart F, Gonzalez I, *et al*. mixOmics: Omics Data Integration Project. R package version 6.1.2.; 2017. Available at: https://CRAN.R-project.org/package=mixOmics, 2017.
30. Sorg JA, Sonenshein AL. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J Bacteriol 2008;190:2505-2512.
31. Thanissery R, Winston JA, Theriot CM. Inhibition of spore germination, growth, and toxin activity of clinically relevant *C difficile* strains by gut microbiota derived secondary bile acids. Anaerobe 2017;45:86-100.
32. **Weingarden AR**, **Chen C**, Bobr A, *et al*. Microbiota transplantation restores normal fecal bile acid composition in recurrent *Clostridium difficile* infection. Am J Physiol Gastrointest Liver Physiol 2014;306:G310-9.
33. Buffie CG, Bucci V, Stein RR, *et al*. Precision microbiome reconstitution restores bile acid mediated resistance to *Clostridium difficile*. Nature 2015;517:205-208.

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SUPPLEMENTARY FIGURE LEGENDS:

Figure S1: Stream plots showing the OTU abundances in each chemostat culture over time. Each stream of colour represents an OTU, and streams are grouped by phylum: *Bacteroidetes* (blue), *Firmicutes* (green), *Proteobacteria* (orange), *Verrucomicrobia* (purple), unclassified (grey), and *C difficile* (red). The

width of the stream represents the OTU abundance at each time point. The dotted box indicates the clindamycin-dosing period, while the dotted vertical line indicates the day of FMT or saline dosing.

Figure S2: Diversity of bacterial communities cultured in chemostat vessels (VA= saline-treated cultures, dashed line; VB= FMT-treated cultures, solid line). **(A)** Shannon diversity index (H'), **(B)** Richness (S_{obs}), **(C)** Pielou's evenness index (J'). The shaded grey box indicates the clindamycin-dosing period, while the vertical dotted line indicates the day of FMT or saline dosing. SANTA analysis with Benjamini-Hochberg FDR was used to compare the following: steady state cultures to clindamycin-treated cultures, steady state cultures to post-clindamycin cultures, and FMT-treated cultures to saline treated cultures.

Figure S3: ^{1}H-NMR metabolites that changed following clindamycin treatment and with FMT (VA= saline-treated cultures, dashed line; VB= FMT-treated cultures, solid line). **(A)** butyrate, **(B)** acetate, **(C)** isobutyrate, and **(D)** isovalerate. The shaded grey box indicates the clindamycin-dosing time period, while the vertical dotted line indicates the day of FMT or saline dosing. SANTA analysis with Benjamini-Hochberg FDR was used to compare the following: steady state cultures to clindamycin-treated cultures, steady state cultures to post-clindamycin cultures, and FMT-treated cultures to saline treated cultures.

Figure S4: Regularized CCA (rCCA) model correlating 16S rRNA gene sequencing data (genus-level) and ^{1}H-NMR metabolite data. **(A)** The representation of units (a.k.a. samples) for the first two canonical variates showing the correlations between variables before (grey), during (blue), and after (orange) the clindamycin-dosing period. "A" represents samples collected from VA and "B" represents samples from VB. **(B)** Correlation circle plot showing strong correlations between variables before, during, and after the clindamycin-dosing period. Metabolites are shown in blue and bacterial genera are shown in orange. *Clostridium* cluster XI (the clostridial cluster that includes *C difficile*) is shown in a black box. **(C)** The representation of units (a.k.a. samples) for the first two canonical variates showing the correlations between variables following FMT (blue) or saline (orange) treatment. "A" represents samples collected from VA (saline-treated cultures) and "B" represents samples from VB (FMT-treated cultures). **(D)**

Correlation circle plot showing strong correlations between variables following FMT or saline treatment. Metabolites are shown in blue and bacterial genera are shown in orange. *Clostridium* cluster XI (the clostridial cluster that includes *C difficile*) is shown in a black box.

Figure S5: 1D ^{1}H-NMR to confirm the identity of valerate in chemostat culture supernatants. **(A)** 1D ^{1}H-NMR spectrum of valerate standard (blue). **(B)** Overlay of 1D ^{1}H-NMR spectrum of valerate standard (blue) with sample spectrum (red). Each peak of the valerate standard is visible in the sample spectrum. **(C)** Overlay of 1D ^{1}H-NMR spectra of sample before (blue) and after (red) valerate spike-in. All the peaks proposed to belong to valerate increased following spike in with valerate standard (green).

Figure S6: 2D ^{1}H-NMR to confirm the identity of valerate in chemostat culture supernatants. **(A)** Overlay of the ^{1}H-^{1}H COSY spectrum of valerate standard (blue) with sample spectrum (red). Each peak of the valerate standard is visible in the sample spectrum. **(B)** Overlay of the ^{1}H-^{1}H TOCSY spectrum of valerate standard (blue) with sample spectrum (red). Again, each peak of the valerate standard is visible in the sample spectrum.

Figure S7: Overlay of ^{1}H-^{1}H COSY sample spectrum (blue) and ^{1}H-^{1}H TOCSY sample spectrum (red) to confirm the identity of other metabolites found in chemostat culture supernatants.

Figure S8: Statistical total correlation spectroscopy (STOCSY). **(A)** 5-aminovalerate STOCSY spectrum obtained by correlating all points in the spectra with the 5-aminovalerate resonance at 3.019 ppm. Peak clusters with high correlations (*) correspond to positions where we expected to see peaks for 5-aminovalerate. **(B)** Succinate STOCSY spectrum obtained by correlating all points in the spectra with the succinate resonance at 2.408 ppm. No other peaks had high correlations with the peak at 2.408, confirming this peak belonged to succinate.

Figure S9: Bile acids that changed following clindamycin treatment (VA= saline-treated cultures, dashed line; VB= FMT-treated cultures, solid line). **(A)** taurodeoxycholic acid (TDCA), **(B)** glycocholic acid (GCA), **(C)** glycodeoxycholic acid (GDCA), **(D)** glycochenodeoxycholic acid (GCDCA), **(E)** chenodeoxycholic acid

(CDCA), and **(F)** ursodeoxycholic acid (UDCA). The shaded grey box indicates the clindamycin-dosing period, while the vertical dotted line indicates the day of FMT or saline dosing. Steady state cultures were compared to clindamycin-treated cultures using SANTA analysis with Benjamini-Hochberg FDR.

Figure S10: Regularized CCA (rCCA) model correlating 16S rRNA gene sequencing data (genus-level) and bile acid data. **(A)** The representation of units (a.k.a. samples) for the first two canonical variates showing the correlations between variables before (grey), during (blue), and after (orange) the clindamycin-dosing period. "A" represents samples collected from VA and "B" represents samples from VB. **(B)** Correlation circle plot showing strong correlations between variables before, during, and after the clindamycin-dosing period. Bile acids are shown in blue and bacterial genera are shown in orange. *Clostridium* cluster XI (the clostridial cluster that includes *C difficile*) is shown in a black box.

Figure S11: TCA is required for *C difficile* spore germination, but has no effect on *C difficile* vegetative growth. **(A)** *C difficile* spores were incubated supplemented brain heart infusion broth in the presence and absence of 1% TCA and grown overnight. There was a significant increase in *C difficile* germination in the presence of TCA (*** $p<0.001$). **(B)** *C difficile* vegetative cells were inoculated into supplemented brain heart infusion broth in the presence and absence of 1% TCA. There were no significant differences in the growth of *C difficile* in the presence or absence of TCA at any time point in the growth curve. Growth of *C difficile* in the broths was quantified by taking OD_{600} measurements using a plate spectrometer. Error bars represent the mean ± standard deviation.

Figure S12: Schematic of key metabolite interactions with *C difficile* during health, CDI, and recurrent CDI. Initial antibiotic treatment decreases the diversity of the gut microbiota, killing bacteria that produce valerate and bile salt hydrolase (an enzyme that degrades TCA). This results in increased levels of TCA and decreased levels of valerate, allowing for *C difficile* spore germination and vegetative growth (respectively). Treatment for CDI (vancomycin/metronidazole) decreases *C difficile* vegetative cells, but *C difficile* spores remain and microbial community diversity remains low. Again, this antibiotic exposure

results in an environment with high TCA and low valerate, allowing the remaining *C difficile* spores to germinate and grow once vancomycin/metronidazole is stopped. When a patient receives FMT for recurrent CDI (usually within 1-2 days of stopping suppressive antibiotics) valerate levels (and valerate producing bacteria) and bile salt hydrolase levels (and bile salt hydrolysing bacteria) are restored, resulting in an environment that inhibits both *C difficile* germination and vegetative growth.

Figure S1

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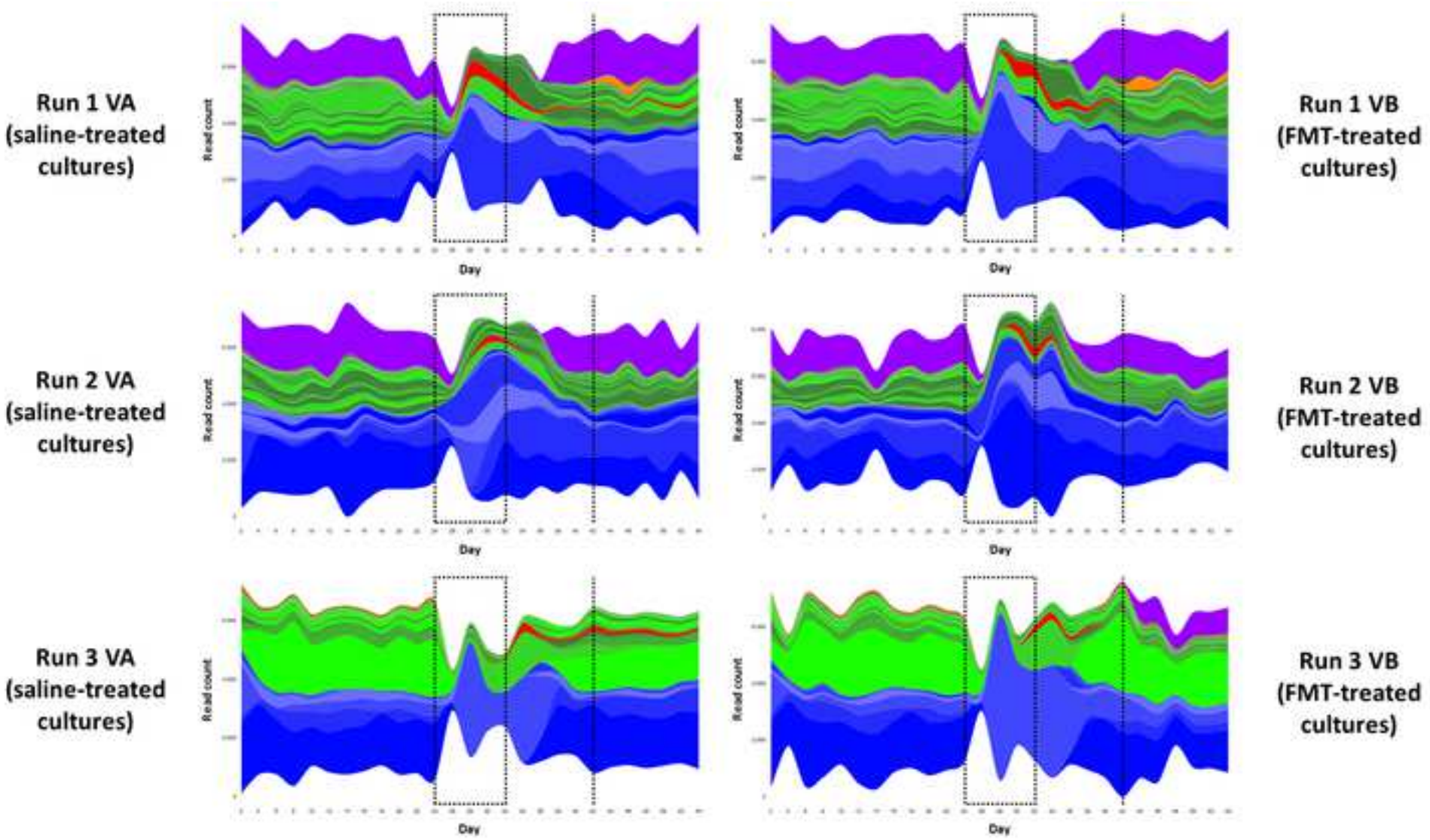

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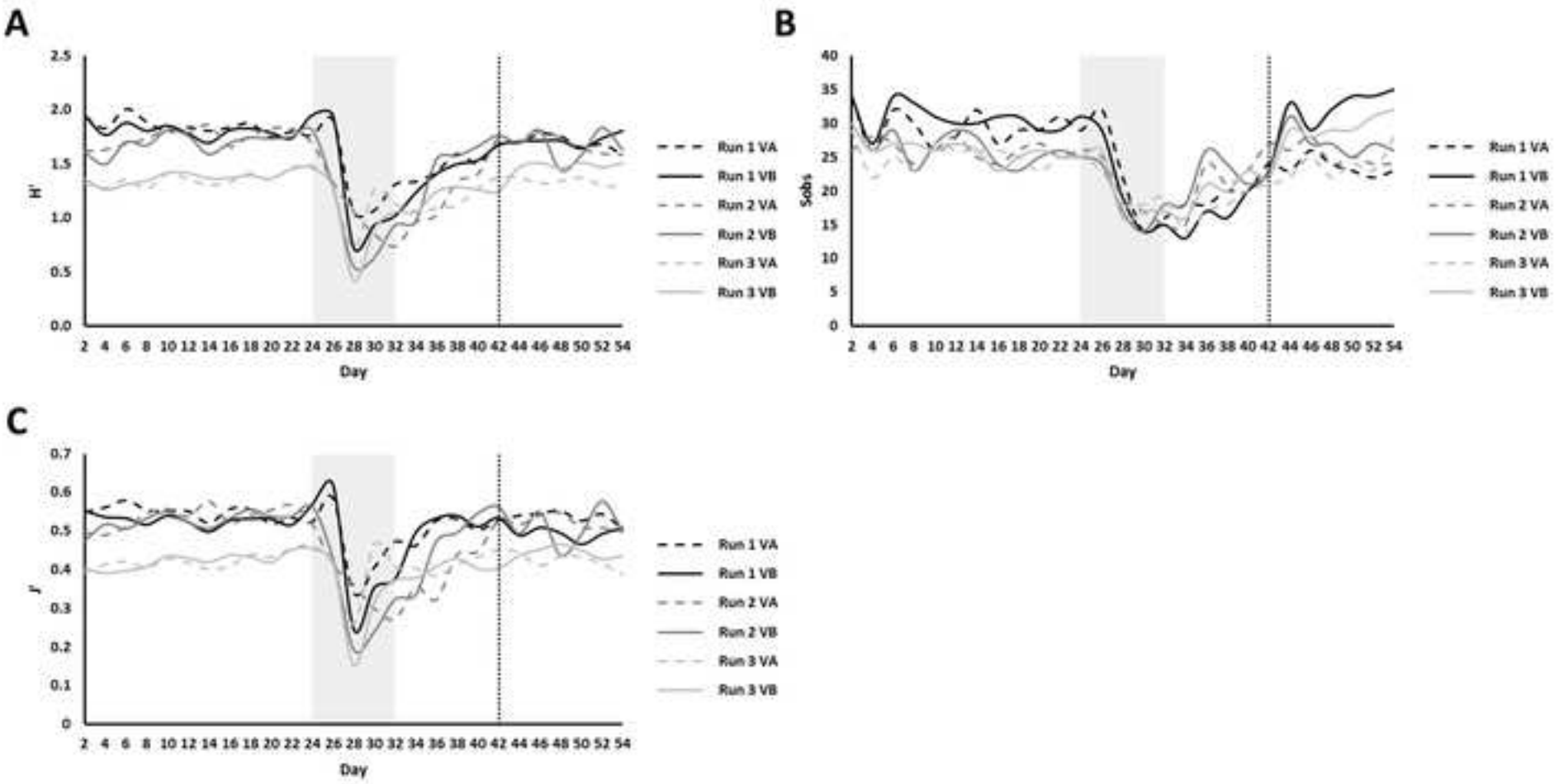

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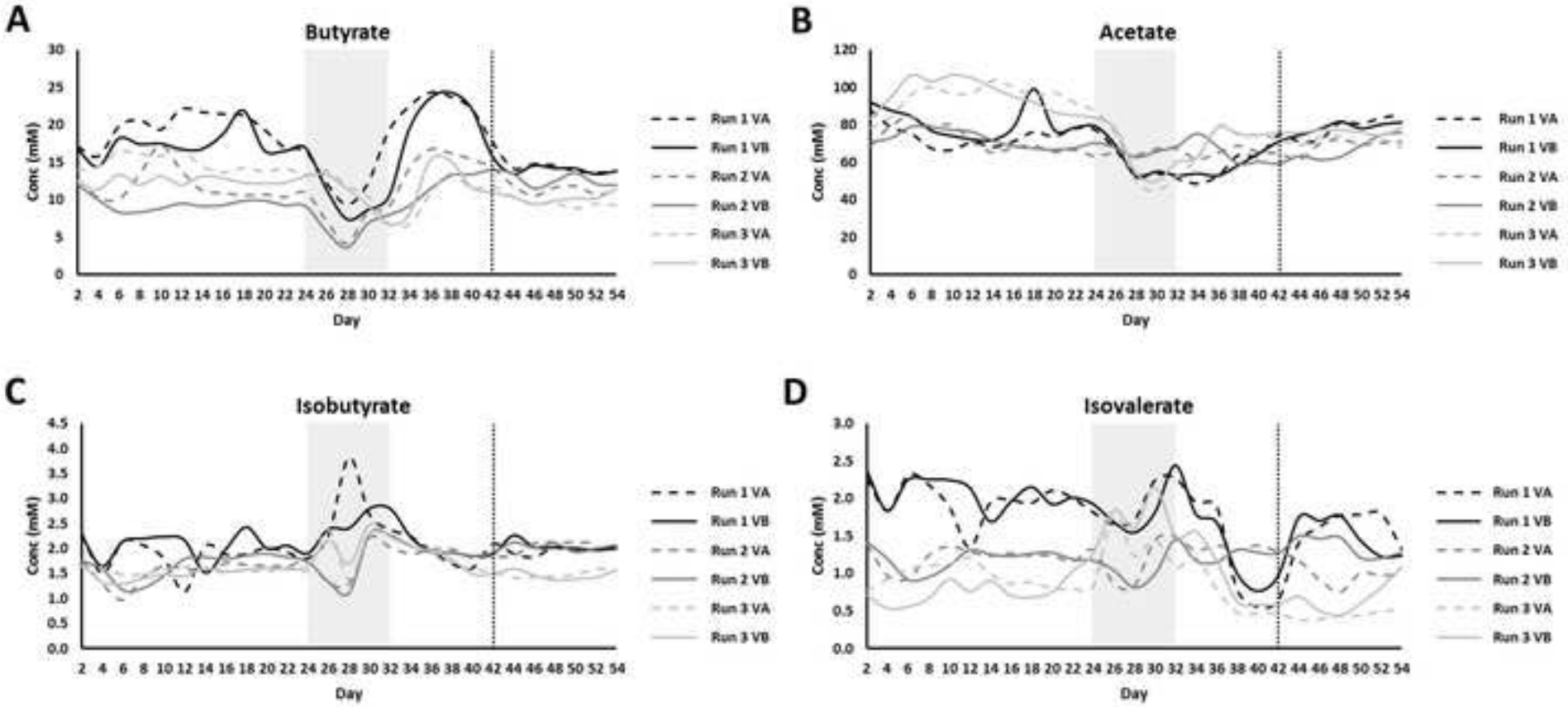

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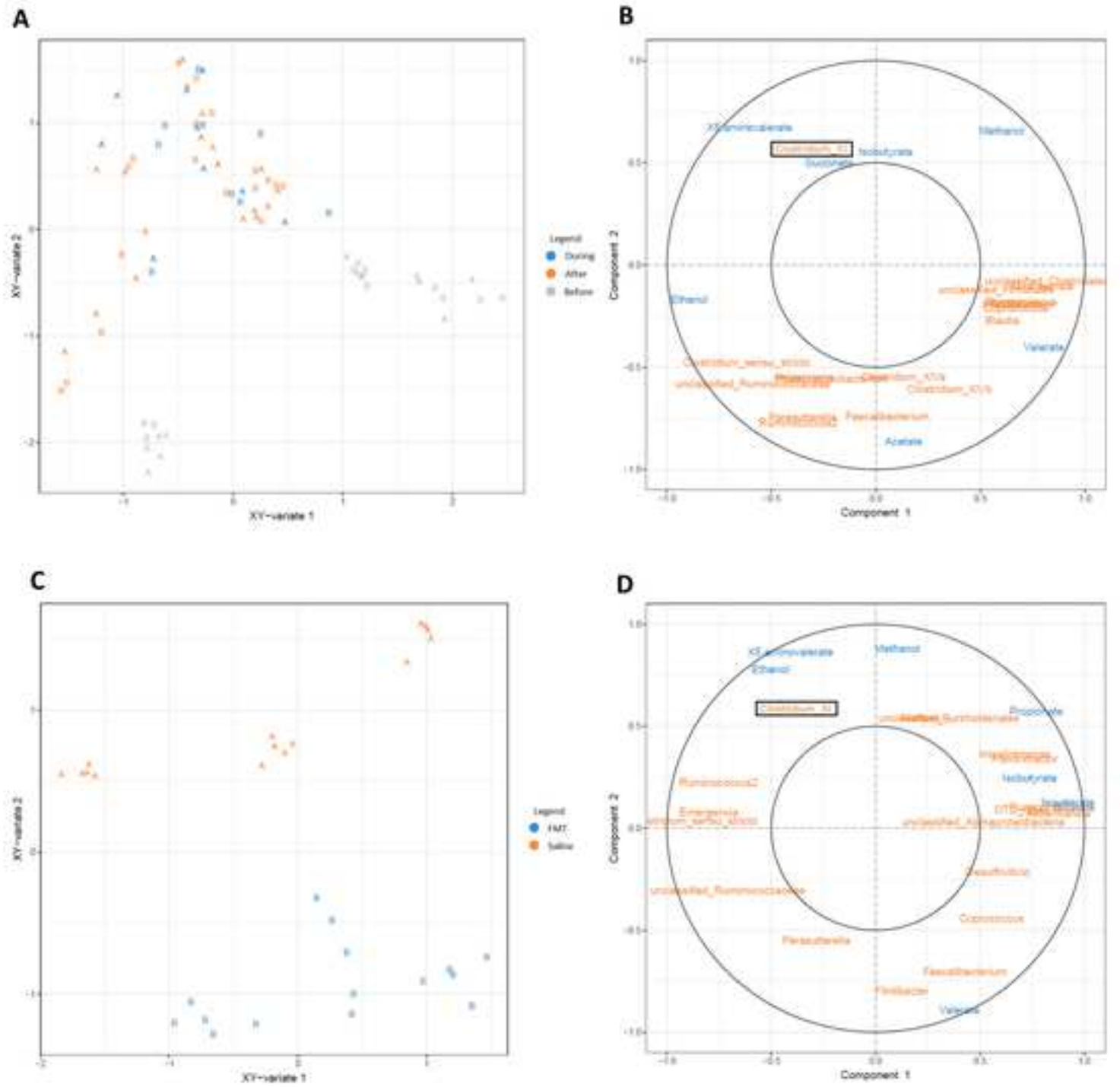

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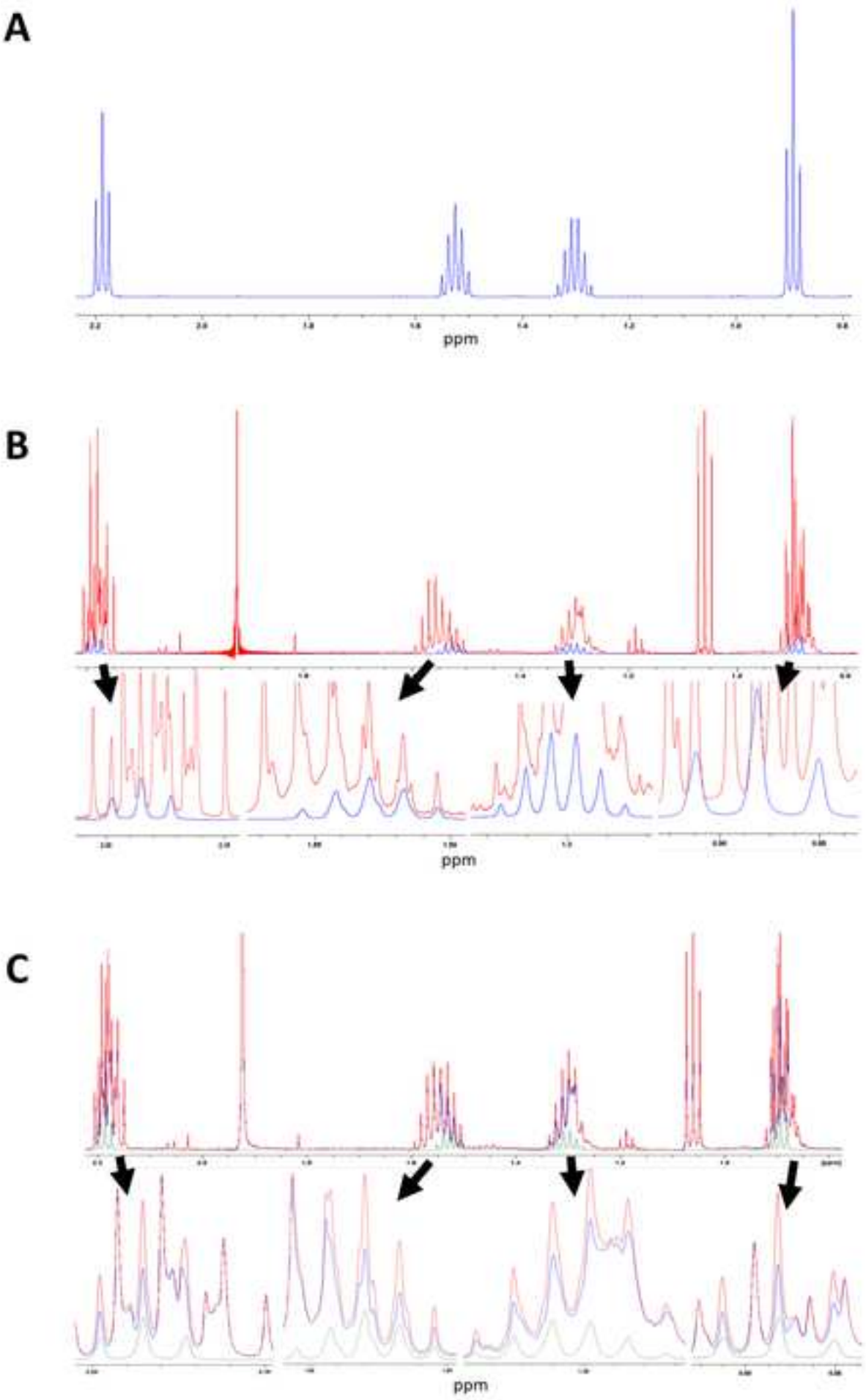

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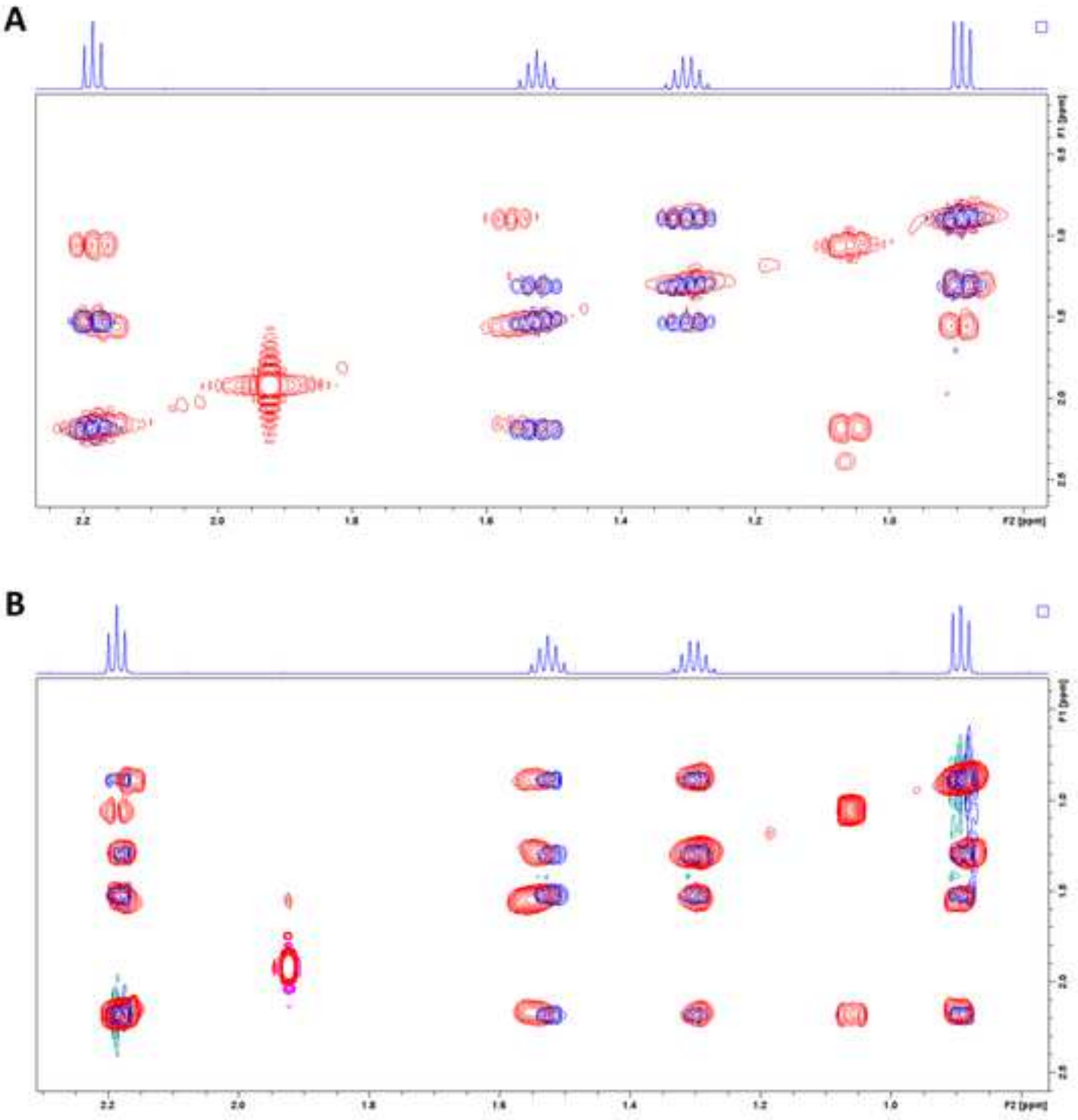

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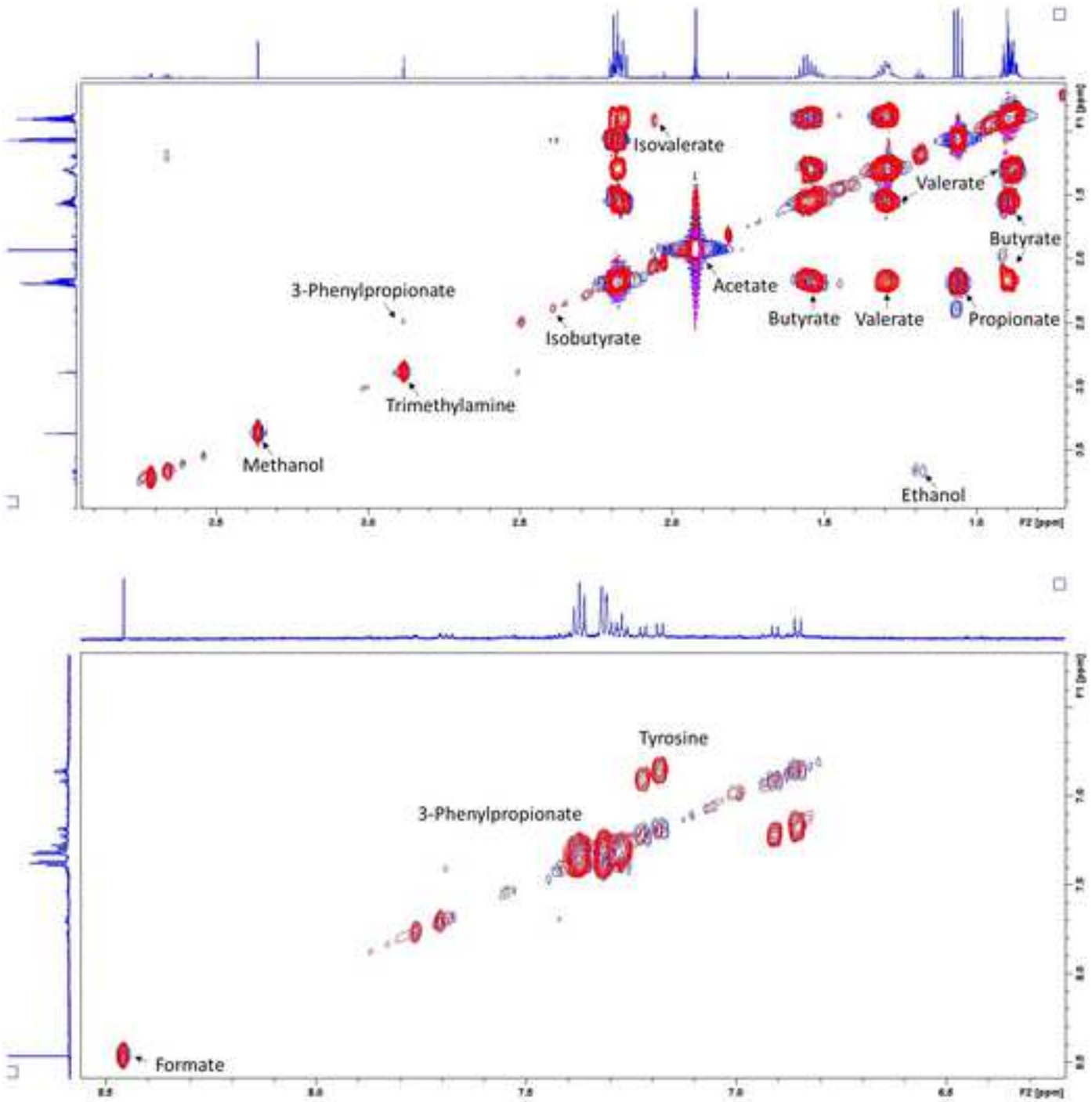

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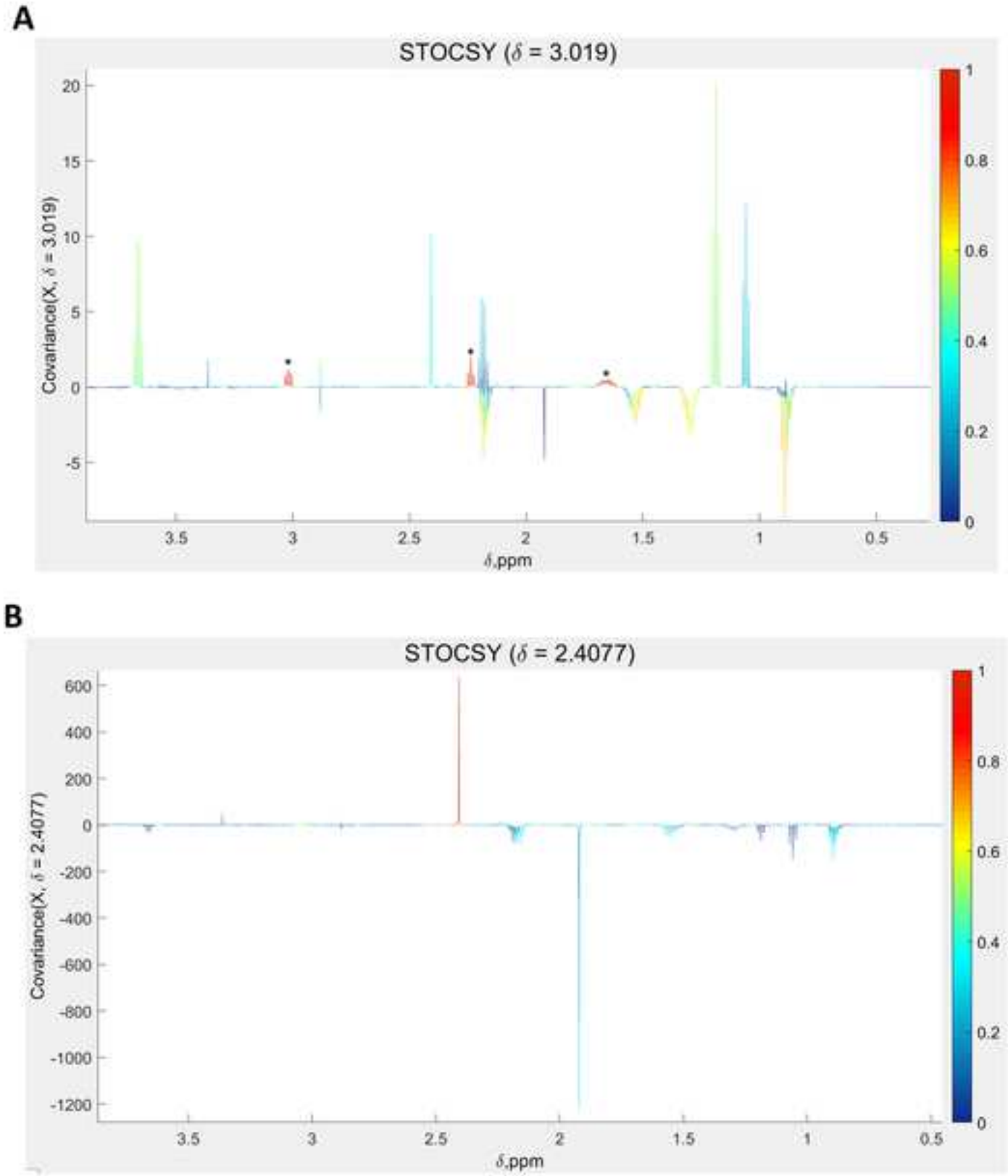

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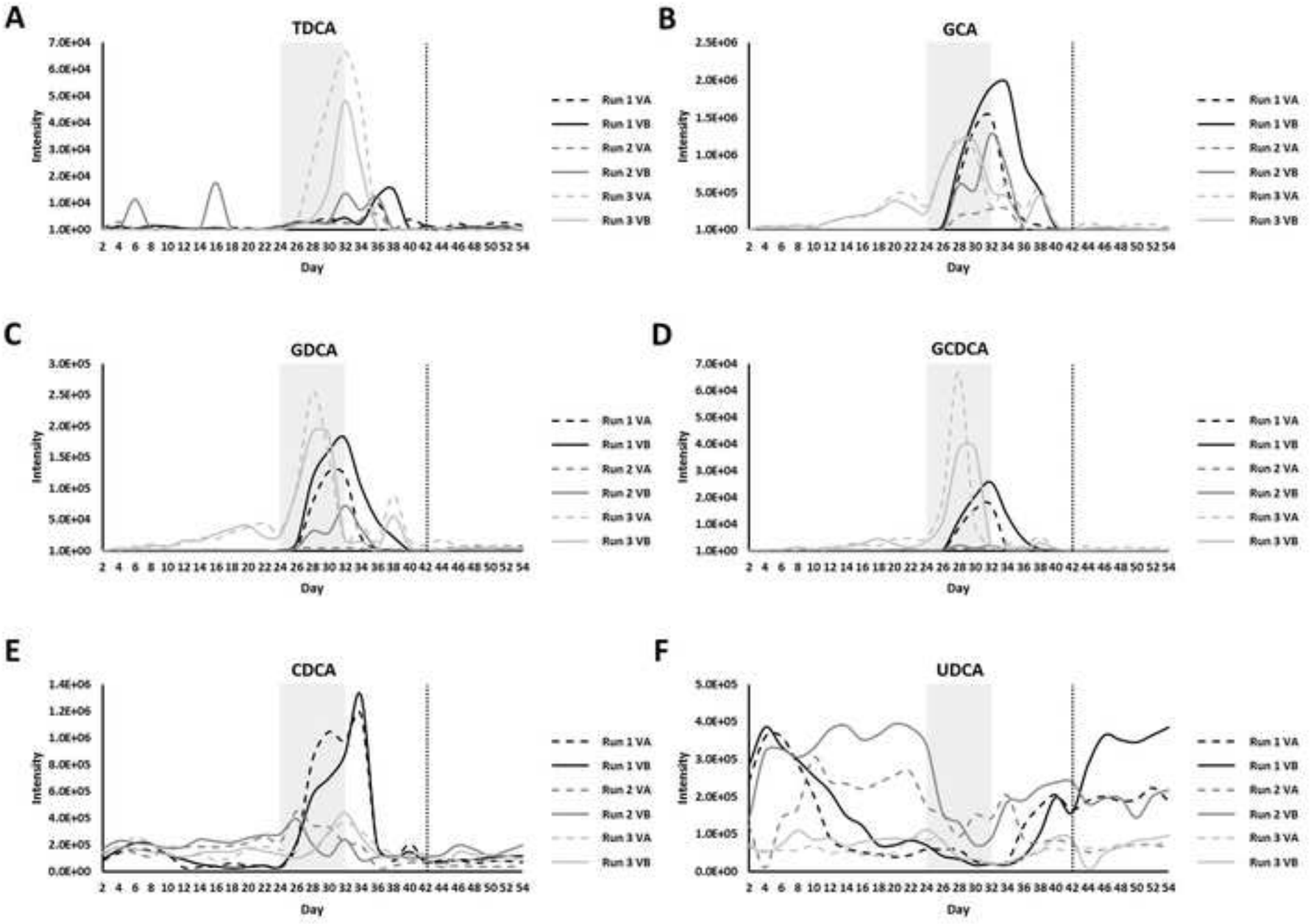

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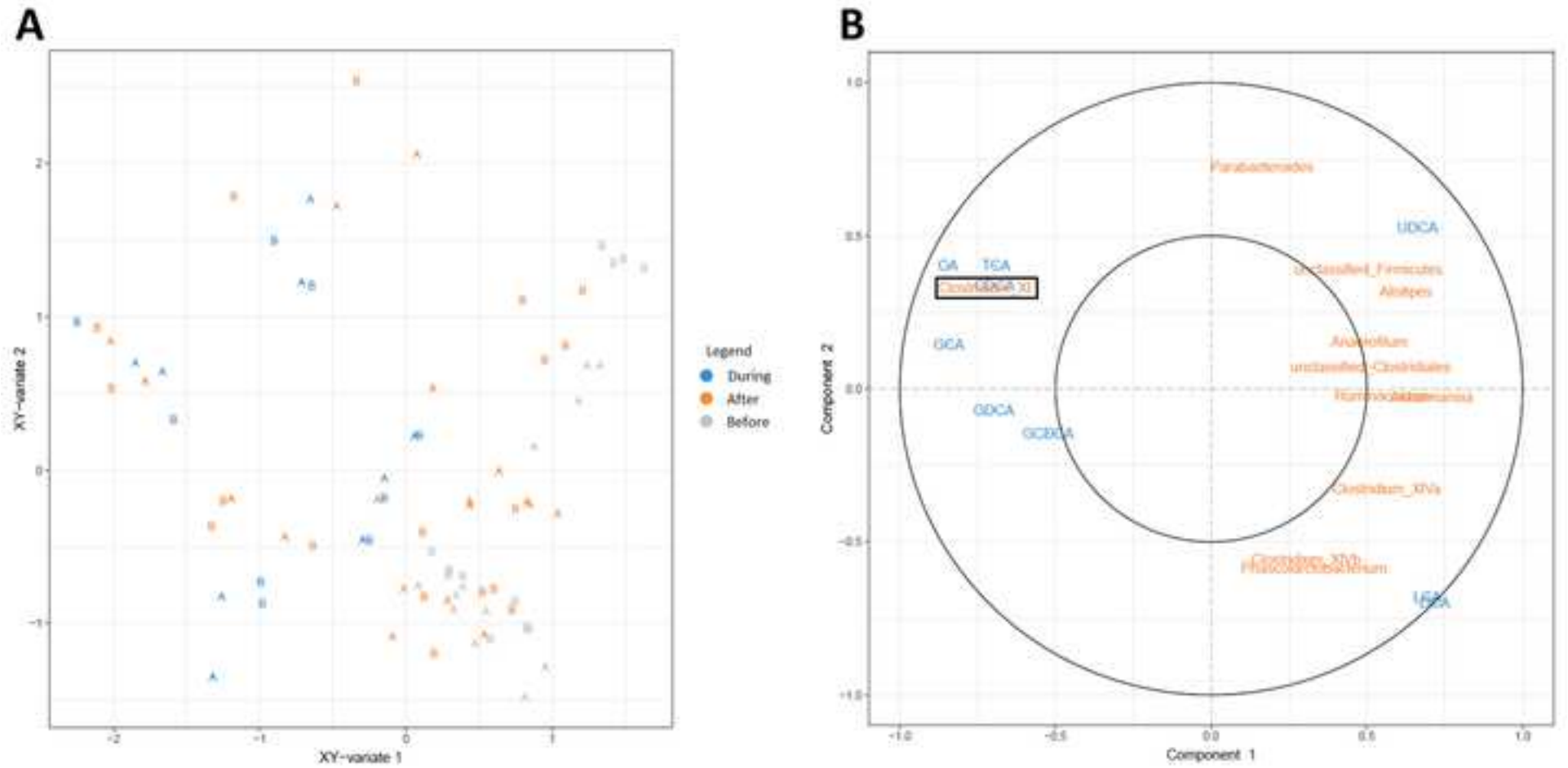

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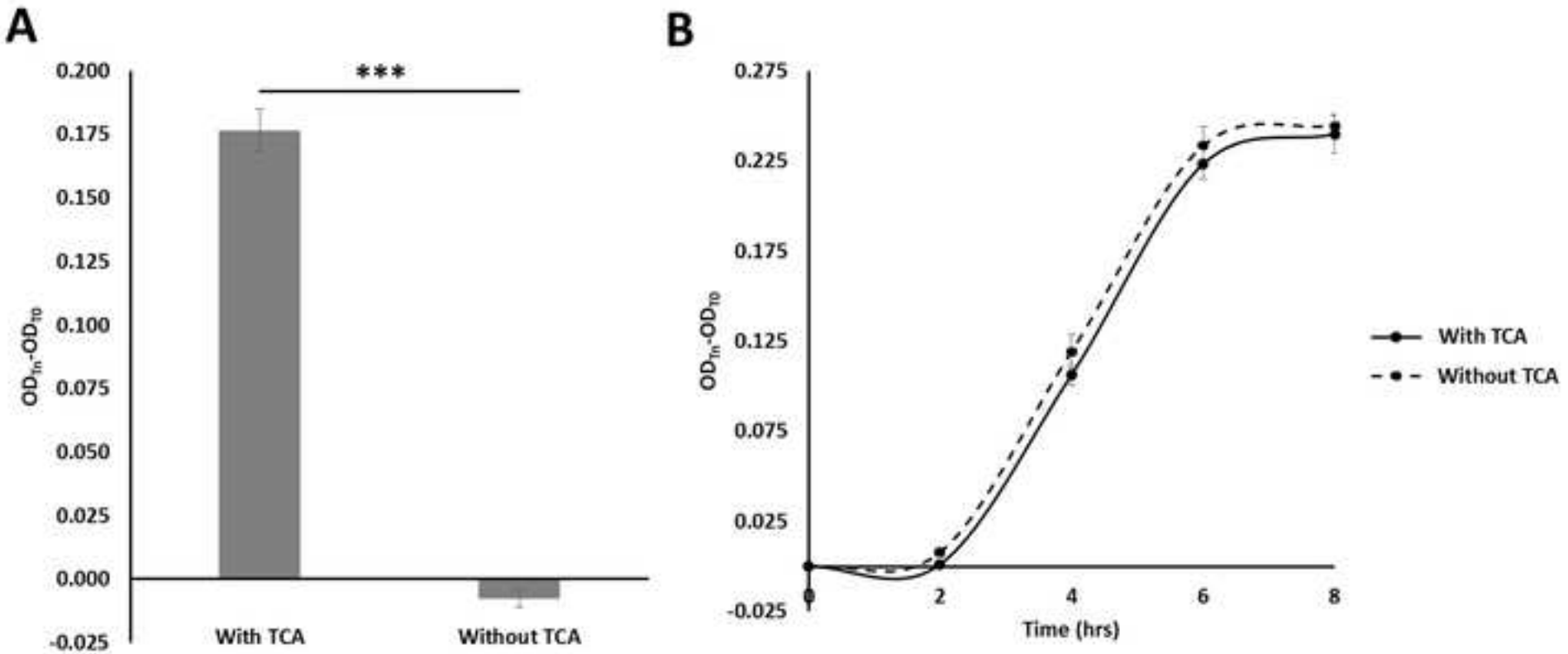

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