**Supplemental Methods**

**Cohort 1: The gut microbiome and post-COVID gastrointestinal symptoms**

***Study design***

This was a sub-study conducted within a parent double-blind randomized controlled trial with 1:1:1 assignment of patients to placebo, inulin 16 g/day, or inulin 32 g/day. The parent study (NCT03865706) tested whether inulin acts as a prebiotic to ameliorate gut colonization with multidrug-resistant organisms. Among prebiotic fiber options, inulin was selected because of prior studies demonstrating that it increases the relative abundance of anaerobes (e.g., F. prausnitzii) that are typically associated with a healthy state. At the start of the COVID-19 pandemic, the parent trial was paused because including COVID-19 patients within the larger trial cohort would have generated undesirable heterogeneity. Instead, this sub-study was initiated to test the impact of inulin on the gut microbiome among those with moderate or severe COVID-19 who required hospitalization but not ICU care.1 The parent study is funded by the U.S. Department of Defense (PR181960). Both the parent study and this sub-study were approved by the Columbia University Irving Medical Center (CUIMC) Institutional Review Board (IRB).

***Patient population***

Individuals ≥18 years old were eligible for the study if they were free from chronic GI symptoms at baseline, were hospitalized before June 1, 2020, and tested positive for SARS-CoV-2 by nasopharyngeal PCR (i.e., first wave of COVID-19). Additionally, patients needed to be enrolled within 24 hours of hospitalization and to have received broad-spectrum antibiotics within the 24 hours prior to enrollment. Antibiotics considered broad-spectrum included cephalosporins and β-lactam/β-lactamase combination antibiotics. Antibiotics were required for inclusion because antibiotics are the factor most strongly associated with depletion of the native gut microbiota; therefore, patients who receive antibiotics are most likely to benefit from therapies seeking to augment the native gut microbiota. Exclusion criteria were: inability to receive oral medications, hyponatremia with serum sodium <128 mEq/L (because the intervention was delivered in free water), CD4 count <200 109 cells/mL or absolute neutrophil count <500 cells/ µL, and limited goals of care (i.e., “Do Not Resuscitate” status). The complete trial protocol is published as an **Online** **Supplement**.

***Trial intervention***

Patients were 1:1:1 randomly assigned to placebo, inulin 16 g/day, or inulin 32 g/day. The trial intervention was diluted in 250 cc of free water and given in two divided doses for up to seven days or until hospital discharge. Inulin has a slightly sweet taste and non-sugar sweetener was added to mask the placebo.

***Biosamples***

Patients donated deep rectal swabs immediately prior to the study intervention (Day 0) and subsequently at Day 3, 7, 14, and 30 or until hospital discharge. Flocked nylon rectal swabs were performed by a study nurse with fecal soilage used to confirm adequate sampling and immediately flash-frozen at -80 C for storage.

***Bacterial genomic DNA extraction***

400 µL of trizol was added to each sample for SARS-CoV-2 inactivation and bacterial genomic DNA of fecal bacteria were extracted according to the manufacturer’s specifications.

***16S rRNA sequencing***

At the end of the study, samples were sequenced for the V4 hypervariable region of the 16S ribosomal RNA gene using a previously described custom dual-indexing protocol and the Illumina MiSeq platform.2 Sequencing data is available at the National Center for Biotechnology Information (BioProject PRJNA818796, accession numbers SRR18494293 to SRR18494314).

***OTU clustering and pathway analysis***

Raw sequencing reads were analyzed using a previously described pipeline.2 An operational taxonomic units (OTU) count per sample table was generated using USEARCH v11.0.6673 and taxonomies were assigned using the ribosomal database project (RDP) classifier trained with 16S rRNA training set 18.4 Samples with less than 1000 reads and features present in <50% of samples were filtered out. To estimate the abundance of functional pathways, OTU count matrix and OTU sequences were processed using the PICRUST2 pipeline (v2.4.2)5 which implements tools including HMMER, EPA-NG,6 GAPPA,7 CASTOR8 and MinPath.9 Features were analyzed by ALDEx2 using Kruskal-Wallis tests, a generalized linear model, and the Benjamini–Hochberg false discovery rate (FDR) correction to identify the features that were differentially abundant across experimental groups.

***Measurement of post-COVID gastrointestinal symptoms and other data***

Six months after COVID-19 diagnosis, subjects were contacted by telephone and asked questions evaluating the Rome IV criteria for IBS and completed the irritable bowel syndrome severity scoring system (IBS-SSS), although none of the patients met formal IBS criteria.10 The IBS-SSS was selected rather than competing instruments because it has been highly validated in non-COVID cohorts, and it was used to classify gastrointestinal symptoms as none/mild (IBS-SSS <50, which was approximately the median in the cohort), mild/moderate (IBS-SSS 50 to 200), or moderate/severe (>200, which was the 90th percentile in the cohort). Combined symptoms of anxiety/depression were reported using the EQ-5D-5L30 as a four-point Likert scale (no symptoms, mild, moderate, or severe), because none of the respondents were in the most severe level 5 category.

***Outcomes and statistical approach***

The primary outcome of interest was differences in tryptophan metabolism between patients based on the severity of gastrointestinal and/or mental health symptoms, with tryptophan metabolism assessed using PICRUSt results. Tryptophan metabolism was compared across categories based on (1) IBS-SSS score, (2) the study intervention (placebo vs. inulin 16 g/day vs. inulin 32 g/day); and (3) presence/severity of anxiety and depression. Tryptophan pathways were analyzed using a repeated-measures mixed model accounting for both between-subject effects (e.g., IBS-SSS category) and within-subject effects (sample time). Unsupervised analyses were also performed for differences in composition or function across groups; to enhance rigor, features were first tested for false discovery rate (FDR)-adjusted differences (p<0.05) and hits were then re-tested using the repeated-measures mixed model.

**Cohort 2: Plasma serotonin and post-COVID gastrointestinal symptoms**

***Patient population***

Individuals >18 years old were eligible for this study if they received a positive SARS-CoV-2 polymerase chain reaction test at Columbia University Irving Medical Center between April and November 2020. Additional inclusion criteria were: consent for participation in a COVID-19 biobanking study, donation of a randomly timed blood sample at the time of COVID-19 diagnosis, and completion of a follow-up questionnaire six months after COVID-19 diagnosis. From this biobank cohort of patients with mild-moderate COVID-19, forty patients were randomly selected including 20 patients with and 20 patients without gastrointestinal symptoms six months after COVID-19 (not matched). Severity of symptoms were defined using the NIH clinical spectrum of SARS-CoV-2 infection criteria.1 The biobank study, and this sub-study, were approved by the CUIMC IRB.

***Measurement of 5-HT***

Biobanked plasma was retrieved and tested for the absolute concentration of 5-HT (in pg/mL) using liquid chromatography (LC)-mass spectrometry (MS) with a spike-in internal standard.12 An integrated Waters LC-MS system was used with an ACUITY column, Xevo TQS MS, and Masslynx V4.2 for data acquisition and peak capture.

***Measurement of post-COVID gastrointestinal symptoms and other data***

Six months after COVID-19 diagnosis, patients participating in the COVID-19 biobank study received an electronic questionnaire broadly surveying a wide range of symptoms perceived by the patient to be new and COVID-related. Specifically, patients were asked to indicate the severity of any COVID-related symptoms on a scale from 1 to 5 (Very mild, mild, moderate, severe, very severe). The gastrointestinal symptoms assessed were diarrhea, constipation, abdominal pain. The mental health symptoms assessed were anxiety or sadness. Patients were also asked questions evaluating the Rome IV criteria for IBS. Using the survey responses, gastrointestinal and mental health symptoms (anxiety or sadness) were characterized as present or absent.

***Statistical approach***

5-HT concentrations were compared in patients with and without GI symptoms, with and without mental health symptoms, and in a factorial manner based on both GI and mental health symptoms. The Mann-Whitney U test was used to assess for differences between any two groups and the Kruskal-Wallis test for differences between more than two groups. STATA version 17.0 was used for all analyses and alpha < 0.05 was considered statistically significant.

References

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