

SEX-DEPENDENT PERSISTENT GUT MICROBIOTA DYSBIOSIS IN COVID-19 SURVIVORS: A 16S rRNA METAGENOMIC STUDY FROM ERBIL, IRAQ

BRADOSTY, S. W.^{1,2} – HASAN, A. H.^{2,3*} – HAMAD, S. W.⁴ – RASOOL, S. H.⁵ – SHEKHANY, B.⁶ – ABDULLAH, M. O.² – SHAIKH, F. K.⁷ – SUZERGOZ, F.¹

¹*Department of Biology, Faculty of Arts and Sciences, Harran University, Sanliurfa, Turkey*

²*Department of Medical Laboratory Analysis, College of Applied Sciences, Cihan University-Erbil, Iraq*

³*Department of Medical Microbiology, Faculty of Science and Health, Koya University, Koya 44023, Kurdistan Region– F.R. Iraq*

⁴*Department of Field Crops and Medicinal Plants, College of Agricultural Engineering Sciences, Salahaddin University-Erbil, Erbil, Kurdistan Region, Iraq*

⁵*Department of Medical Laboratory Technology, Shaqlawa Technical College, Erbil Polytechnic University, Erbil, Kurdistan Region, Iraq*

⁶*Department of Medical Laboratory Technology, Soran Technical College, Erbil Polytechnic University, Erbil 44001, Iraq*

⁷*College of Biosciences and Technology, Pravara Institute of Medical Sciences (Deemed to be University), Loni 413736, Maharashtra, India*

**Corresponding author
e-mail: ayad.hasan@koyauniversity.org*

(Received 24th Jul 2025; accepted 15th Oct 2025)

Abstract. Using 16S rRNA metagenomic sequencing of stool samples, this study examined the long-term changes in gut microbiota among COVID-19 survivors (n = 30) in comparison to healthy controls (n = 10). With 2,667,410 high-quality reads examined, survivors (21 females, 9 males) showed different microbial signatures than controls (7 females, 3 males). There were 926 operational taxonomic units (OTUs) in all, and survivors, particularly women, had a greater number of distinct OTUs. Females displayed slightly higher species richness (p = 0.051), despite alpha diversity metrics (Shannon, Simpson) did not differ significantly (p > 0.05). According to unweighted UniFrac, beta diversity showed notable changes in the microbial composition (p = 0.0001). According to taxonomic profiling, survivors had higher levels of *Bifidobacterium pseudocatenulatum* and *Lactobacillus ruminis* and lower levels of *Faecalibacterium prausnitzii*, which suggests weakened gut health. According to functional predictions made using PICRUSt, survivors had immune-related pathway enrichment, whereas healthy controls had higher carbohydrate metabolism. There were sex-specific trends, with male survivors having an excess of *Mycobacterium* and female survivors having an excess of *Collinsella*. Healthy females had higher levels of *Prevotella copri*, which has been associated with plant-based diets. Overall, the results show that after COVID-19 recovery, there is a persistent, sex-specific dysbiosis of the gut microbiome, marked by changes in taxa and functional profiles. These findings highlight the need for more research on the role of sex in influencing post-infection microbial resilience as well as microbiome-mediated mechanisms in long-term COVID.

Keywords: *microbiome resilience, post-viral immune modulation, opportunistic pathogens, sex-specific dysbiosis, functional metagenomics*

Introduction

The human gut microbiome is made up of trillions of microorganisms. It helps with immune functions and metabolism. It also contributes to the host's resistance to pathogens (Lloyd-Price et al., 2019; Khan et al., 2024). Recent studies show that alterations in gut microbiota composition, also known as dysbiosis, are associated with a range of illnesses, including inflammatory bowel disease (IBD), metabolic diseases and neurological diseases (Santana et al., 2022; Shen et al., 2025). SARS-CoV-2 is now thought to cause considerable and prolonged changes to gut microbiota. COVID-19 is affecting gut health, as scientists think that gut microbiota and viruses affect each other bidirectionally (Zuo et al., 2020; Yeoh et al., 2021; Rocchi et al., 2022). While SARS-CoV-2 mainly infects the respiratory tract, it also affect other organs (Zhang et al., 2020; Kopańska et al., 2022). The gastrointestinal system may also present as an entryway for the virus through the angiotensin-converting enzyme 2 (ACE2) receptor that occurs in high quantities in the gut epithelium (Gu et al., 2020; Shirbhate et al., 2021; Jami et al., 2023). Microbial interaction can cause inflammation of the gut, change gut permeability, and alter microbial composition. According to research, COVID-19 patients showed a reduction in microbial diversity, depletion of beneficial commensals (*Faecalibacterium prausnitzii*) and enrichment of opportunistic pathogens (Gou et al., 2021). These alterations can last long after the acute phase of infection, contributing to post-acute sequelae of COVID-19 (PASC), otherwise called long COVID (Liu et al., 2021). Sex differences further complicate this relationship (Bai et al., 2022; Russell et al., 2024).

Age, developmental stage, and hormone regulation are biological factors that have a significant impact on lysosomal activity, which is essential for immunological responses and neuromodulation. These elements also affect the makeup and activity of the gut microbiota (Aggarwal et al., 2023). For example, testosterone is frequently linked to a higher abundance of pro-inflammatory taxa, while estrogen has been demonstrated to improve gut barrier integrity and promote the growth of anti-inflammatory microbial species (Bordag et al., 2015). The variations in microbiome profiles between men and women during infection and during the recovery period following infection may be explained by these hormonal effects. There is a notable knowledge gap regarding the ways in which biological sex affect microbial resilience and recovery after infection, as the majority of microbiome studies pertaining to COVID-19 have not stratified analyses by sex.

Taking this into consideration, the current study conducts a comparative sex-stratified analysis of the gut microbiota composition of COVID-19 survivors. Through 16S rRNA sequencing, the study of bacterial diversity, taxonomic shifts, and functional pathways was performed on 30 survivors (21 females, 9 males) and 10 healthy controls (7 females, 3 males) from Erbil, Iraq. Our objectives are threefold. To find out the difference in the gut microbiota composition in COVID survivors and controls. To evaluate the functionally related implications of dysbiosis, including the deviations of metabolic and immune-related pathways. To look for potential markers of post-COVID dysbiosis for personal recovery strategies.

Materials and methods

Study design and sampling

A total of 40 stool samples were collected in Erbil, Iraq, including 30 from COVID-19 survivors (21 females and 9 males) and 10 from healthy controls (7 females and 3

males). The participants, aged between 20 and 60 years, were excluded if they had chronic diseases, a history of SARS-CoV-2 vaccination, or gastrointestinal disorders. Recovery in survivors and COVID-19 negativity in controls were verified through RT-qPCR and serological assays.

The cohort was stratified into four groups based on sex and COVID-19 history: Group A (n = 21): Female post-COVID-19 participants (A1, A2, A4, A7, A8, A10, A12, A13, A15, A17, A18, A20, A21, A22, A23, A24, A25, A27, A28, A30). Group B (n = 9): Male post-COVID-19 participants (A3, A5, A6, A9, A11, A14, A16, A19, A26). Group C (n = 7): Female healthy controls (C1, C3, C4, C5, C8, C9, C10). Group D (n = 3): Male healthy controls (C2, C6, C7).

Stool metagenomic DNA extraction

Total DNA was extracted directly from stool using a bead-beating protocol and column purification using the DNeasy PowerSoil Pro Kit (QIAGEN, 2023). DNA quality was confirmed using Nanodrop and agarose gel electrophoresis.

Library construction and sequencing

The 16S rRNA V4 region was amplified using fusion primers 319F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Libraries were sequenced using the DNBSeg G400 PE300 platform. PCR conditions and purification followed established protocols (Caporaso et al., 2012). Only libraries that met the required criteria were selected for sequencing on the DNBSeg G400 PE300 platform (Macrogen, Korea), based on their insert size, as shown in (Fig. 1).



Figure 1. Experiment workflow

Bioinformatics and statistical analysis

The fecal samples underwent metagenomic sequencing and were categorized into four distinct groups, Group A (this group comprises twenty-one fecal samples collected from female individuals who survived COVID-19), Group B (consisting of nine fecal samples collected from male individuals who survived COVID-19, Group C (comprised of seven fecal samples collected from healthy female individuals serving as controls) and Group D (this group includes fecal samples obtained from three healthy male individuals). Reads were filtered, merged, and clustered into OTUs at 97% similarity using USEARCH. Chimeras were removed using UCHIME. Alpha and beta diversity were analyzed using Mothur and R software (Schloss et al., 2009). Taxonomic annotation was performed via the RDP database.

Results

We compared the gut microbiota of COVID-19 survivors (n = 30) to healthy controls (n = 10) using 16S rRNA sequencing analysis. We found substantial changes in

composition and function. The community (21 women and 9 men survivors; 7 women and 3 men controls) had different post-COVID recovery-linked microbial signatures (Fig. 2).

After quality controls, we selected 2,667,410 high-quality clean reads from 2,735,549 paired reads (Table 1).

By employing these stringent filters, we resulted in solid downstream analyses, according to Bokulich et al. (2013). OTUs similar at 97% were clustered, yielding 926 OTUs (1,944,898 tags) (Table 2; Fig. 3).

Table 1. Data filtering summary of 16S rRNA samples

Sample	Reads length (bp)	Raw data (Mbp)	N base (%)	Poly base (%)	Low quality (%)	Clean data (Mbp)	Data utilization ratio (%)	Raw reads	Clean reads	Remove primer reads	Read utilization ratio (%)
A1	300:300	39.763	2.326	0.01	0.084	37.5	94.31	719792	681392	681392	94.66
A10	300:300	39.8	2.288	0.003	0.093	37.5	94.22	718502	679992	679992	94.64
A11	300:300	39.463	1.965	0.004	0.065	37.5	95.03	712022	678002	678002	95.22
A12	300:300	39.672	2.204	0.001	0.064	37.5	94.53	715122	677042	677042	94.67
A13	300:300	38.825	1.313	0.001	0.035	37.5	96.59	698292	674502	674502	96.59
A14	300:300	39.634	2.135	0.007	0.058	37.5	94.62	720932	684782	684782	94.99
A15	300:300	39.437	1.877	0.008	0.071	37.5	95.09	716382	683592	683592	95.42
A16	300:300	39.596	2.131	0.003	0.045	37.5	94.71	716622	680682	680682	94.98
A17	300:300	39.674	2.283	0.005	0.035	37.5	94.52	716462	678712	678712	94.73
A18	300:300	39.696	2.241	0.006	0.065	37.5	94.47	715462	678192	678192	94.79
A19	300:300	39.492	1.949	0.008	0.063	37.5	94.96	710912	677552	677552	95.31
A2	300:300	39.816	2.353	0.006	0.094	37.5	94.18	720062	680622	680622	94.52
A20	300:300	39.908	2.432	0.005	0.087	37.5	93.97	728932	685802	685802	94.08
A21	300:300	40.538	3.206	0.007	0.108	37.5	92.51	738132	684022	684022	92.67
A22	300:300	40.946	3.735	0.012	0.094	37.5	91.59	753882	691512	691512	91.73
A23	300:300	40.869	3.483	0.005	0.144	37.5	91.76	751742	691942	691942	92.04
A24	300:300	40.805	3.329	0.013	0.138	37.5	91.9	747452	690572	690572	92.39
A25	300:300	40.922	3.559	0.015	0.145	37.5	91.64	748002	687772	687772	91.95
A26	300:300	39.743	2.194	0.001	0.086	37.5	94.36	726012	687762	687762	94.73
A27	300:300	39.025	1.518	0.003	0.067	37.5	96.09	711562	684142	684142	96.15
A28	300:300	38.943	1.362	0.002	0.071	37.5	96.3	707562	682542	682542	96.46
A29	300:300	38.404	0.682	0.002	0.068	37.5	97.65	705562	691382	691382	97.99
A3	300:300	39.605	2.055	0.006	0.1	37.5	94.69	715312	679782	679782	95.03
A30	300:300	38.448	0.865	0.003	0.058	37.5	97.54	705002	688462	688462	97.65
A4	300:300	39.58	2.152	0.002	0.055	37.5	94.75	714532	677512	677512	94.82
A5	300:300	39.315	1.799	0.004	0.072	37.5	95.38	707762	676782	676782	95.62
A6	300:300	39.54	2.099	0.002	0.053	37.5	94.84	720352	685012	685012	95.09
A7	300:300	39.47	1.916	0.002	0.084	37.5	95.01	717852	684692	684692	95.38
A8	300:300	39.81	2.28	0.011	0.109	37.5	94.2	720562	682402	682402	94.7
A9	300:300	39.735	2.22	0.012	0.1	37.5	94.38	717892	680902	680902	94.85
C1	300:300	39.412	1.868	0.004	0.106	37.5	95.15	720362	687612	687612	95.45
C10	300:300	41.025	3.423	0.005	0.196	37.5	91.41	745712	686962	686962	92.12
C2	300:300	39.88	2.42	0.007	0.093	37.5	94.03	727072	686772	686772	94.46
C3	300:300	40.1	2.512	0.012	0.092	37.5	93.52	730422	687602	687602	94.14
C4	300:300	39.57	2.053	0.001	0.087	37.5	94.77	719532	684332	684332	95.11
C5	300:300	39.711	2.234	0.001	0.084	37.5	94.43	720382	681532	681532	94.61
C6	300:300	38.962	1.331	0.001	0.088	37.5	96.25	714952	690382	690382	96.56
C7	300:300	39.511	1.95	0.002	0.097	37.5	94.91	723982	689482	689482	95.23
C8	300:300	39.6	2.052	0.005	0.098	37.5	94.7	722652	687022	687022	95.07
C9	300:300	39.566	2.024	0.005	0.078	37.5	94.78	720462	685812	685812	95.19

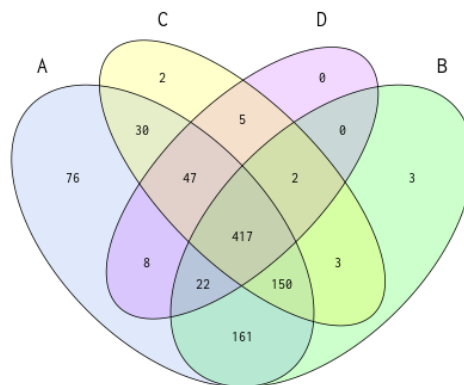


Figure 2. Venn diagram illustrating the distribution of shared and unique operational taxonomic units (OTUs) among four groups: Survived Females (A), Survived Males (B), Healthy Control Females (C), and Healthy Control Males (D). The largest overlap was observed between Survived Females and Survived Males, sharing 750 OTUs, while Healthy Control Males exhibited the fewest unique OTUs (0). A total of 417 OTUs were common across all four groups, representing a conserved core gut microbiota

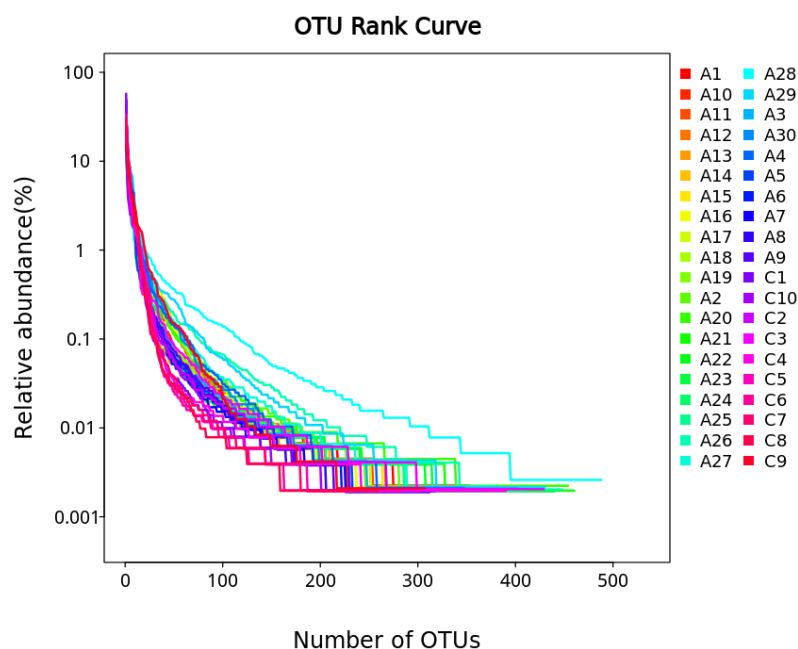


Figure 3. OTU rank-abundance distribution across study samples

Fig. 3 illustrates the rank-abundance curves of OTUs for the four study groups. The y-axis represents the log-transformed relative abundance of OTUs, while the x-axis denotes OTU rank in descending order of abundance. Each curve is color-coded to represent a specific sample. Group A (n = 21): Female post-COVID-19 participants (A1, A2, A4, A7, A8, A10, A12, A13, A15, A17, A18, A20, A21, A22, A23, A24, A25, A27, A28, A30). Group B (n = 9): Male post-COVID-19 participants (A3, A5, A6, A9, A11, A14, A16, A19, A26). Group C (n = 7): Female healthy controls (C1, C3, C4, C5, C8, C9, C10). Group D (n = 3): Male healthy controls (C2, C6, C7). The OTU rank-abundance distribution showed three clusters that did not strictly align with the designated study

groups, likely reflecting inter-individual variability in gut microbiota due to differences in diet, lifestyle, and host factors.”

Table 2. Tag counts and OTU numbers for individual samples across groups

Sample name	Tag number	OTU number	Sample name	Tag number	OTU number
A1	49559	401	A28	38452	488
A10	51681	311	A29	46456	430
A11	44353	353	A3	46384	347
A12	45321	373	A30	48771	331
A13	48503	373	A4	48206	347
A14	46551	340	A5	50485	338
A15	45981	342	A6	51810	317
A16	46379	337	A7	52748	312
A17	47239	357	A8	50747	295
A18	50280	384	A9	49859	296
A19	45404	349	C1	50101	368
A2	48335	387	C10	51656	302
A20	44734	454	C2	48495	403
A21	50732	460	C3	48937	429
A22	50739	436	C4	51361	390
A23	51735	440	C5	51716	282
A24	46391	419	C6	50398	243
A25	50657	410	C7	50172	259
A26	49201	448	C8	50952	259
A27	45600	382	C9	47817	307

Interestingly, female survivors displayed the highest unique OTU count of 76 OTUs while healthy males showed no unique OTUs (*Fig. 4*).

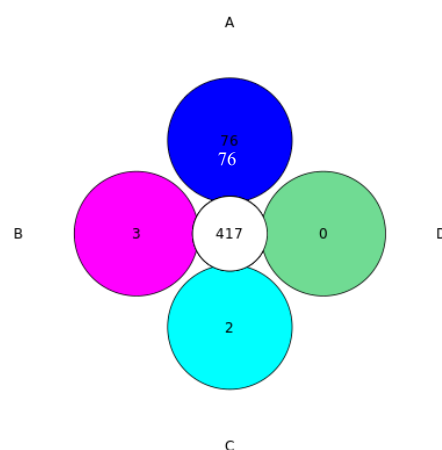


Figure 4. Microbial diversity across study groups. The figure displays the distribution of microbial diversity indices among the four study groups based on 16S rRNA sequencing data. Numerical values (e.g., 76, 3, 417, 2, 0) represent diversity indices for individual samples or mean values within groups. Higher index values indicate greater microbial richness and evenness. The presence of “0” suggests an absence or extremely low diversity in the corresponding group, potentially indicative of severe gut dysbiosis. Group-wise comparison reveals marked differences in microbial diversity levels

There were no significant differences in the Shannon ($p = 0.382$) or Simpson ($p = 0.506$) alpha diversity metrics between the groups, as shown in (Fig. 5).

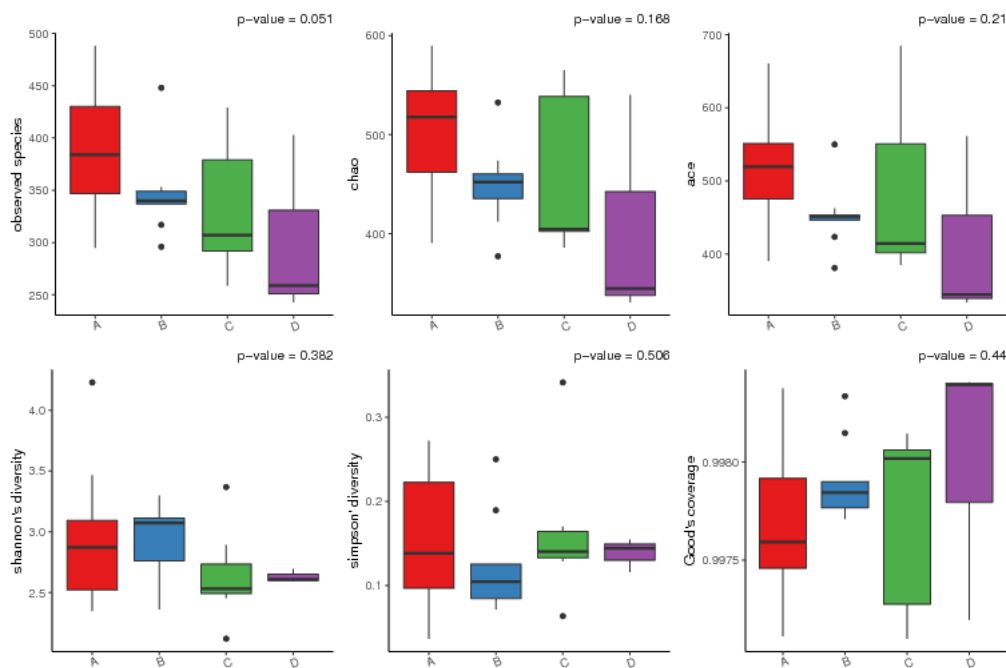


Figure 5. Comparison of alpha diversity estimates across study groups. Boxplots represent alpha diversity metrics, Shannon, Simpson, Chao1, Observed Species, ACE, and Good's Coverage for the four study groups. Each metric reflects a different aspect of microbial richness and evenness. Statistical comparisons using Kruskal-Wallis and Mann-Whitney U tests reveal no significant differences in alpha diversity among the groups ($p > 0.05$), as indicated by the p-values shown above each comparison

However, females that survived tended to have a higher richness as measured by the observed species ($p = 0.051$). According to beta diversity analysis based on unweighted UniFrac ($p = 0.0001$, Fig. 6), there was distinct separation between the post-infection dysbiotic microbiome from the healthy microbiome than from the Bray-Curtis metric ($p = 0.532$, Fig. 7).

The tree illustrates the phylogenetic relationships among fecal samples from COVID-19 survivors (Groups A and B) and healthy controls (Groups C and D). Constructed using Unweighted UniFrac distances, the tree reflects microbial community similarities, with branch clustering indicating phylogenetic proximity. Closely clustered samples share greater microbial similarity, while longer branch lengths indicate greater divergence among microbiota compositions.

The heatmap illustrates pairwise Bray-Curtis dissimilarity scores among fecal microbiome samples from four groups: Group A (female COVID-19 survivors), Group B (male COVID-19 survivors), Group C (healthy female controls), and Group D (healthy male controls). The color gradient, ranging from 0 (high similarity) to 1 (high dissimilarity), represents the degree of difference between microbial communities. Clustering patterns reveal intra- and inter-group relationships, with tighter clustering indicating higher similarity in microbiome profiles.

Taxonomic profiling identified key shifts in microbial composition (Fig. 8).

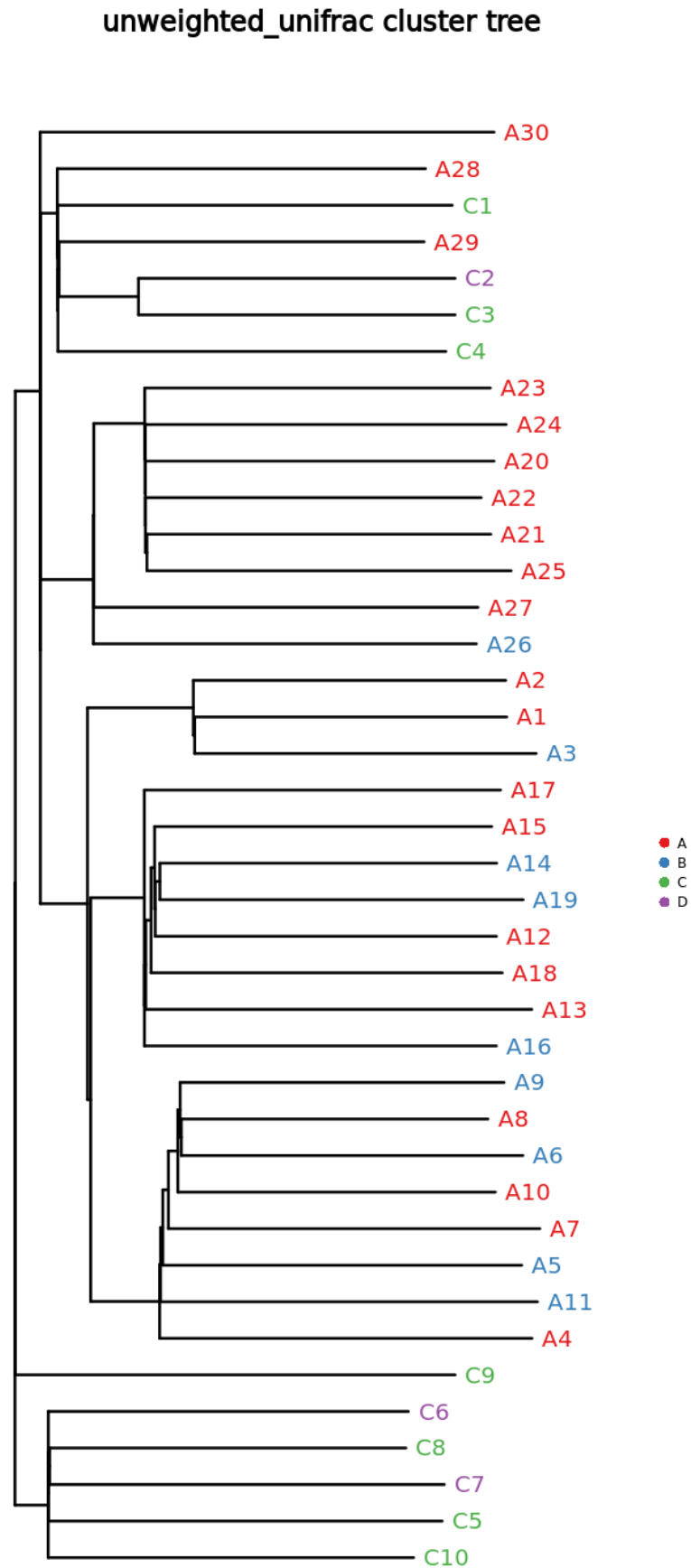


Figure 6. Phylogenetic tree based on unweighted UniFrac analysis of fecal microbiota

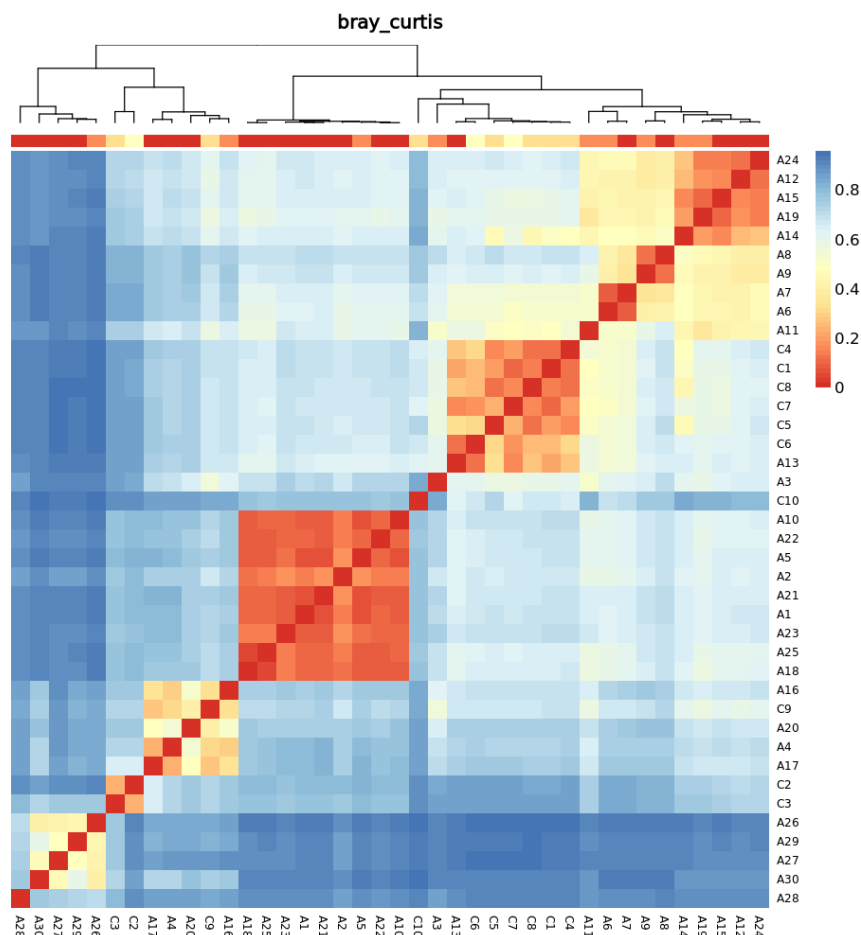


Figure 7. Heatmap of Bray-Curtis dissimilarity matrix for gut microbiome profiles across study groups

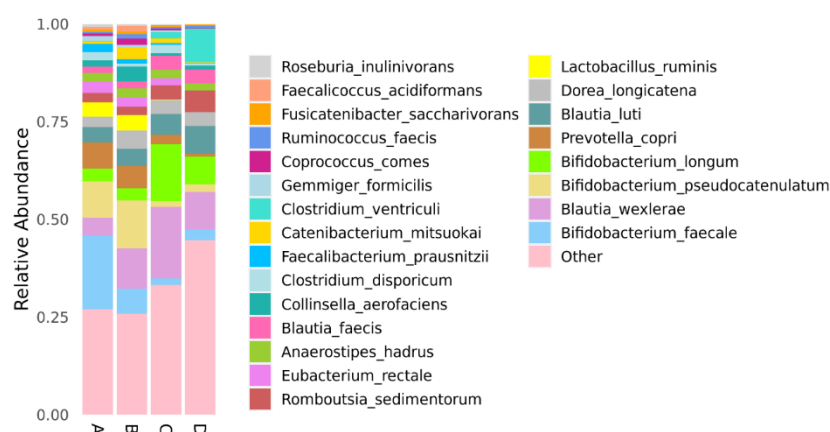


Figure 8. Relative abundance of bacterial taxa across study groups. The bar plot depicts the proportional representation of dominant microbial species in female COVID-19 survivors (Group A), male COVID-19 survivors (Group B), healthy female controls (Group C), and healthy male controls (Group D). Taxa are ranked according to their relative abundance, with “Other” indicating species present at very low abundances. Notable differences are observed in key taxa such as *Bifidobacterium*, *Lactobacillus*, and *Faecalibacterium* between COVID-19 survivors and healthy controls

Bifidobacterium species were significantly enriched in the survivors (A: 9.28%, B: 12.36% vs C: 1.47%, D: 1.99%; Table 3).

Table 3. Relative abundance (%) of bacterial taxa in study groups (A, B, C, D)

Taxon	A	B	C	D
<i>Roseburia_inulinivorans</i>	0.00806226	0.00462565	0.00217848	0.00033542
<i>Faecalicoccus_acidiformans</i>	0.00522379	0.01440433	0.00026096	0.00037568
<i>Fusicatenibacter_saccharivorans</i>	0.00641644	0.00690246	0.00561071	0.00263643
<i>Ruminococcus_faecis</i>	0.00438162	0.01189519	0.00406762	0.00649381
<i>Coprococcus_comes</i>	0.00571941	0.01493172	0.00359108	0.00050984
<i>Gemmiger_formicilis</i>	0.01353781	0.00679095	0.00541782	0.00067756
<i>Clostridium_ventriculi</i>	0.00109491	0.00021607	0.015099	0.08641197
<i>Catenibacterium_mitsuokai</i>	0.00665438	0.03030951	0.01268225	0.00212659
<i>Faecalibacterium_prausnitzii</i>	0.02007667	0.01136548	0.00355988	0.00105323
<i>Clostridium_disporicum</i>	0.02142927	0.00696752	0.02185568	0.00613826
<i>Collinsella_aerofaciens</i>	0.01624596	0.03803906	0.00785159	0.01003589
<i>Blautia_faecis</i>	0.01604258	0.01736419	0.03543428	0.03521954
<i>Anaerostipes_hadrus</i>	0.02117751	0.02384847	0.02093096	0.01775065
<i>Eubacterium_rectale</i>	0.03044822	0.02386008	0.01845181	0.00087881
<i>Romboutsia_sedimentorum</i>	0.02336733	0.02151822	0.03613774	0.0554389
<i>Lactobacillus_ruminis</i>	0.03680049	0.03915656	0.00022692	0.00014759
<i>Dorea_longicatena</i>	0.02682682	0.0470255	0.03687525	0.03491094
<i>Blautia_luti</i>	0.03907621	0.04320139	0.05295002	0.07060678
<i>Prevotella_copri</i>	0.06703151	0.05801694	0.02390367	0.007433
<i>Bifidobacterium_longum</i>	0.03352957	0.03045587	0.14574516	0.07078791
<i>Bifidobacterium_pseudocatenulatum</i>	0.0927565	0.12362636	0.0146962	0.01990407
<i>Blautia_wexlerae</i>	0.04445796	0.10263321	0.18359902	0.09581055
<i>Bifidobacterium_faecale</i>	0.18974159	0.06399009	0.01623362	0.02702848
Other	0.26990118	0.25885518	0.33264027	0.4472881

Bifidobacterium pseudocatenulatum ($\log_{10} = 0.97$ in females, 1.09 in males; Fig. 9) showed trends similar to those observed for immunomodulatory taxa in COVID-19.

Conversely, *Faecalibacterium prausnitzii* decreased in survivors (Group A: 2.01% vs. controls: 0.36%), a butyrate-producing bacterium involved in gut barrier function. Male survivors also showed higher levels of *Lactobacillus ruminis* (12.6% in A11 and A14; Fig. 10), possibly reflecting sex-specific microbial patterns.

The survivors' microbiomes were enriched for defense mechanisms ($\log_2FC = 1.8$) and signal transduction pathways (Fig. 11), as revealed by PICRUST-based functional analysis, indicating enhanced immune-related functional features.

Healthy individuals exhibited higher carbohydrate metabolism activity (Fig. 12), which is characteristic of a stable gut ecosystem.

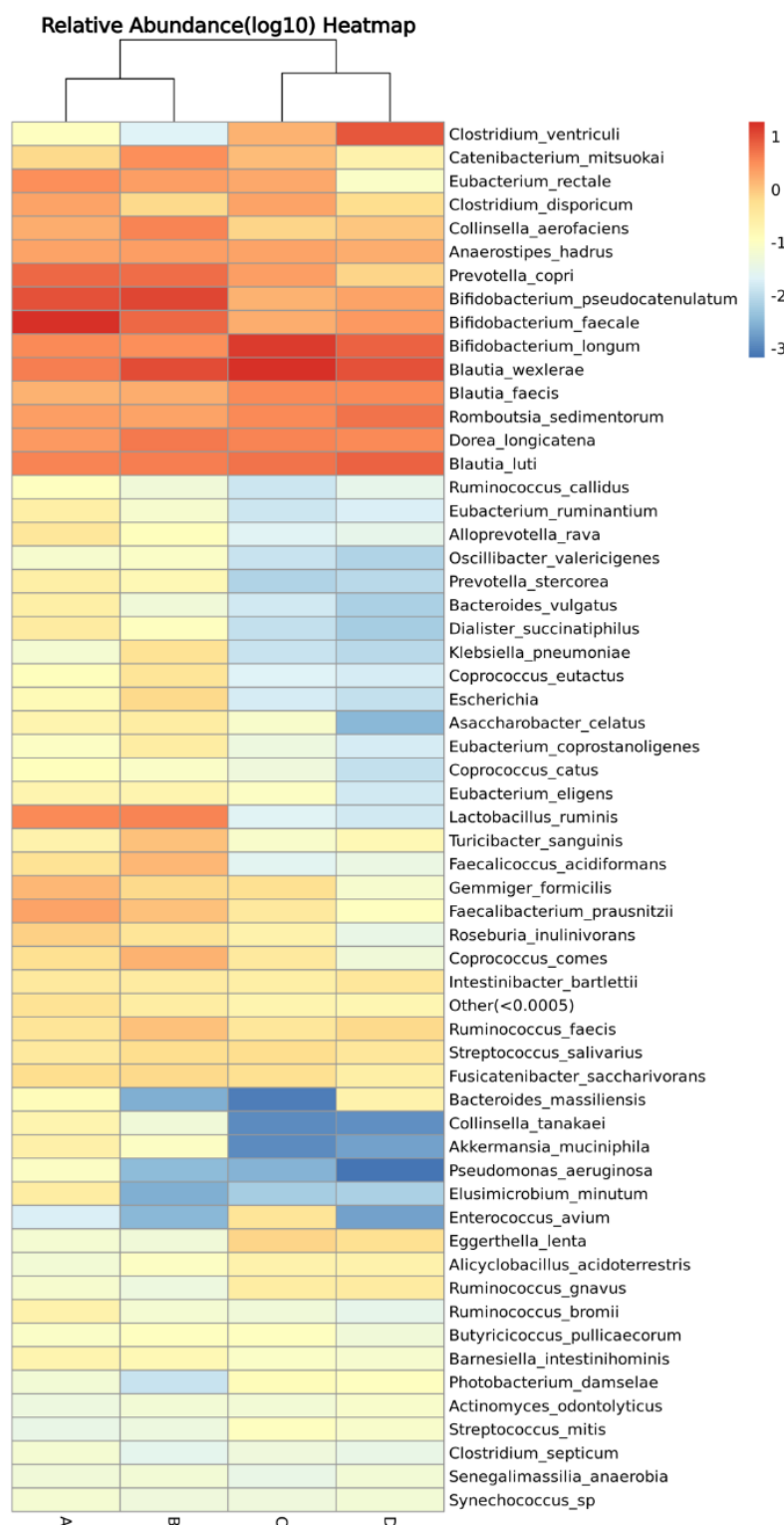


Figure 9. Log₁₀-transformed relative abundance of bacterial species across study groups. The heatmap shows the distribution of taxa in female COVID-19 survivors (Group A), male COVID-19 survivors (Group B), healthy female controls (Group C), and healthy male controls (Group D). Female survivors were enriched in *Bifidobacterium* species (*B. pseudocatenulatum*, *B. longum*, *B. faecale*), suggesting a compensatory response post-infection, whereas male survivors had higher *Blautia* species (*B. wexlerae*, *B. luti*). *Prevotella copri* was more abundant in healthy female controls, likely reflecting diet-associated microbial composition

The most abundant functional categories across all groups were associated with core microbial processes, including translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism, and amino acid transport and metabolism. These functions are essential for microbial growth and maintaining gut homeostasis. Further insights were discovered through the following analyses: NMDS showed partial overlap between groups (Fig. 13), with female survivors forming the most distinct cluster (MRPP $p = 0.017$; Table 4).

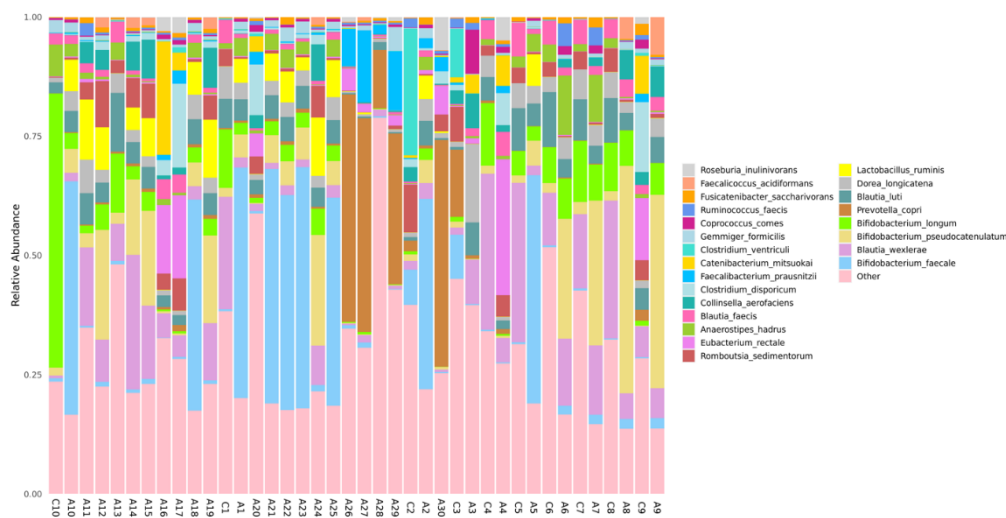


Figure 10. Relative abundance of bacterial species in individual fecal samples. Each bar represents one participant, with colors denoting different species. “Other” indicates taxa present at very low abundances. The y-axis shows relative abundance (0–1.0) and the x-axis shows individual samples

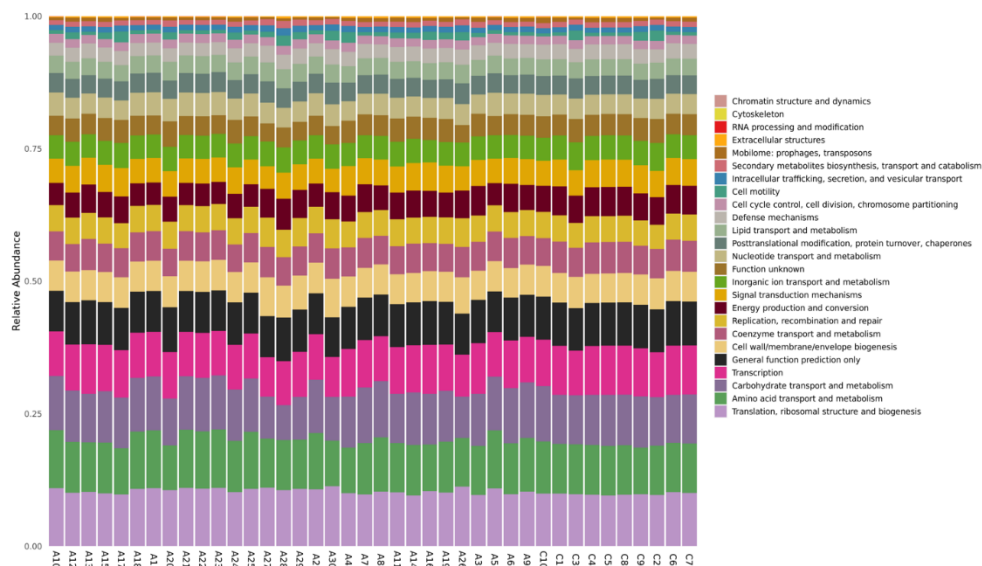


Figure 11. Relative abundance of COG functional categories at Level 2 across all samples.

This barplot shows the distribution of microbial functions. The dominant functions are Translation, ribosomal structure and biogenesis, Carbohydrate transport and metabolism, and Amino acid transport and metabolism. Trends grouped by specific groups visualize functional transition after COVID-19

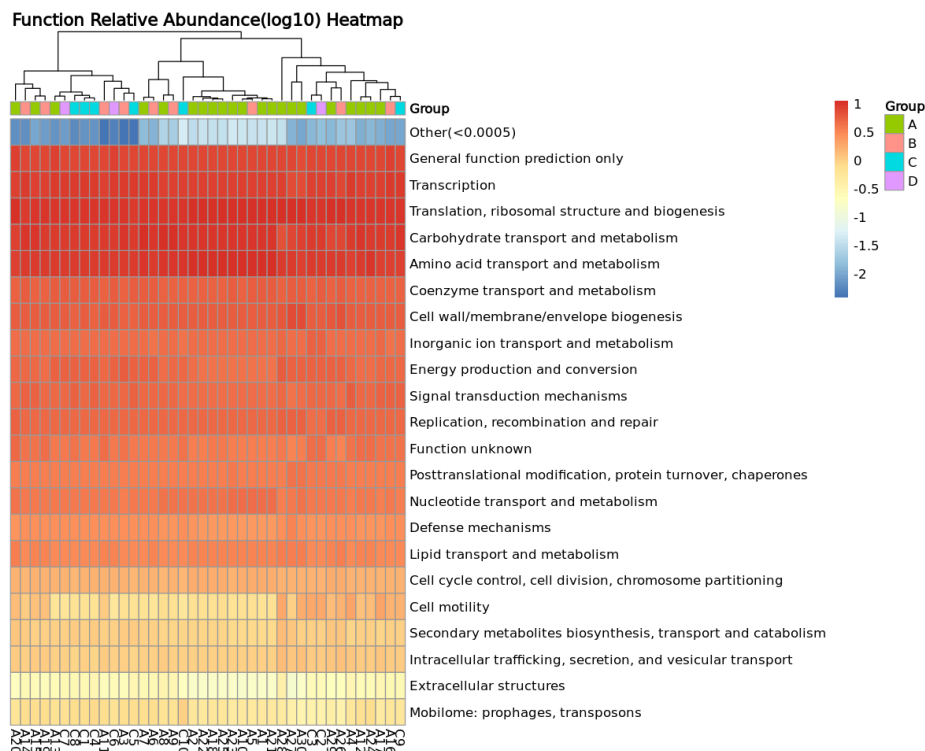


Figure 12. Heatmap of $\log_{10}$ -transformed COG functional abundances across study groups. Functional categories include carbohydrate metabolism, amino acid transport and metabolism, translation, and ribosomal structure. Healthy individuals (Groups C and D) show slightly higher carbohydrate metabolism activity compared with COVID-19 survivors (Groups A and B), suggesting a trend toward a stable gut functional profile

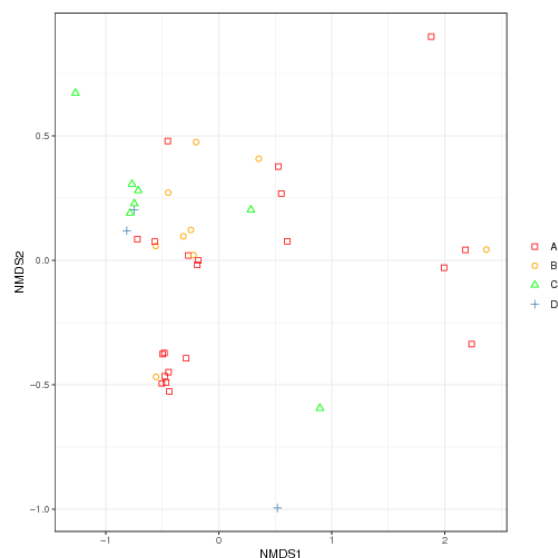


Figure 13. The NMDS plot (stress value) displays the dissimilarity (or similarities) of microbial communities of the four groups: Group A (female COVID-19 survivors, red), Group B (male COVID-19 survivors, blue), Group C (healthy female controls, green), Group D (healthy male controls, purple). Individual samples are represented by points, and clustering patterns reflect microbiota similarities. The two main dimensions of variation are represented by axes NMDS1 and NMDS2

Table 4. Group-wise dissimilarity comparison using MRPP with Bray-Curtis distance

Group	Distance	Observed delta	Expected delta	P value
A-B-C-D	Bray	0.6385	0.6712	0.017

PLS-DA highlighted sex-specific separation (Fig. 14), indicating gender-driven differences in microbiome composition. LEfSe analysis identified *Collinsella* as a biomarker in female survivors (LDA = 4.5) and *Mycobacterium* in male survivors (Fig. 15), suggesting distinct mechanisms of dysbiosis between sexes.

The rarefaction curves confirmed that sampling was sufficient (Fig. 16), and the visualization in the heat map (Fig. 17) and the cladogram (Fig. 18) confirms that the pattern by sex was maintained.

The findings of the present study demonstrate that the gut microbiome of COVID-19 survivors exhibits persistent sex-specific alterations, characterized by enrichment of immunomodulatory taxa (*Bifidobacterium*), opportunistic pathogens (*Mycobacterium*), depletion of anti-inflammatory species (*Faecalibacterium*), and a shift in metabolic pathways toward stress response rather than homeostasis. In contrast, healthy female controls (Group C) showed higher levels of *Prevotella copri*, a taxon associated with diets rich in plant-based foods, which may reflect dietary influences on microbial composition. These results highlight the role of sex in shaping long-term post-COVID microbiome alterations and underscore the need for further investigation into the functional consequences, particularly regarding potential contributions to long COVID symptoms.

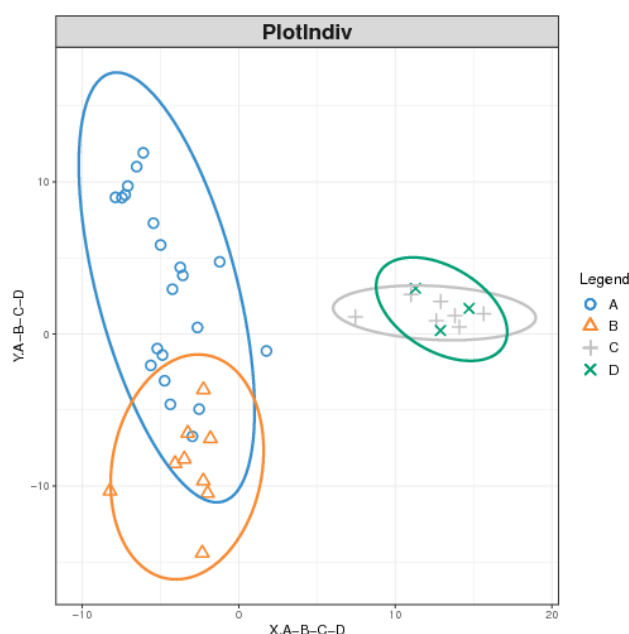


Figure 14. PLS-DA score plot of gut microbiota composition across groups. The two-dimensional score plot shows the first two latent components (A-B-C-D) and (V-A-B-C-D) of the PLS-DA model that best separates the four cohorts: female COVID-19 survivors (group A, $n = 21$), male COVID-19 survivors (group B, $n = 9$), healthy female controls (group C, $n = 7$), and healthy male controls (group D, $n = 3$). Clustering patterns imply different microbial profiles between groups and indicate 95% confidence intervals. The axes represent linear combinations of microbial taxa which contribute most to the discrimination of the group, while the percentage shows how much variance is explained

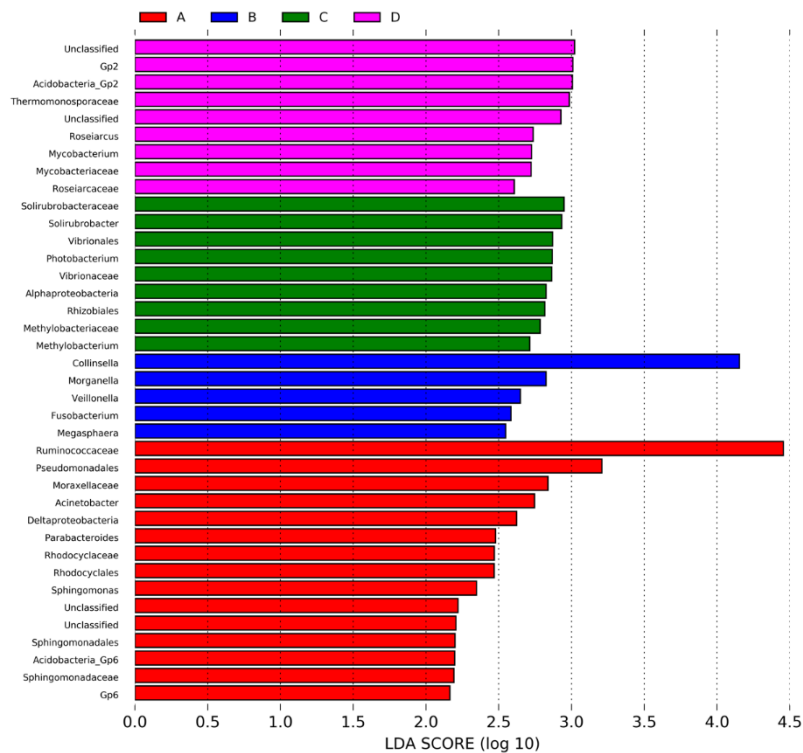


Figure 15. LEfSe analysis showing differentially abundant microbial taxa among the four study groups (A: female COVID-19 survivors, B: male COVID-19 survivors, C: healthy female controls, D: healthy male controls). The histogram shows the LDA scores (log10) of the taxa with significant differences seen ($p < 0.05$, LDA score > 2.0). Taxa enriched in each group are color-coded accordingly

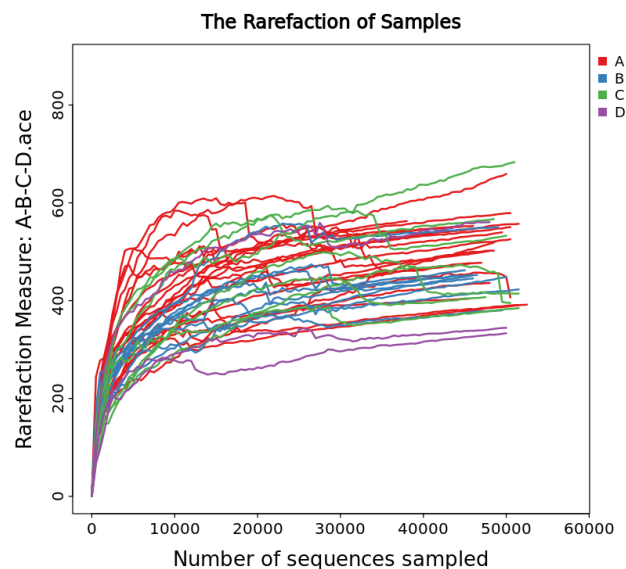


Figure 16. Microbial diversity shown for study groups by rarefaction curves. The curves show how the estimated richness (y-axis) of the four groups (A, B, C, D) changes as the number of sequences (axis x) sampled increases from 200 to 800 sequences. As for Groups A and B, survivors of COVID-19 are represented. Groups C and D are healthy controls. The shape of each curve provides insight into the sampling completeness, as well as the slope and level-off point, indicating differences in microbial community structure

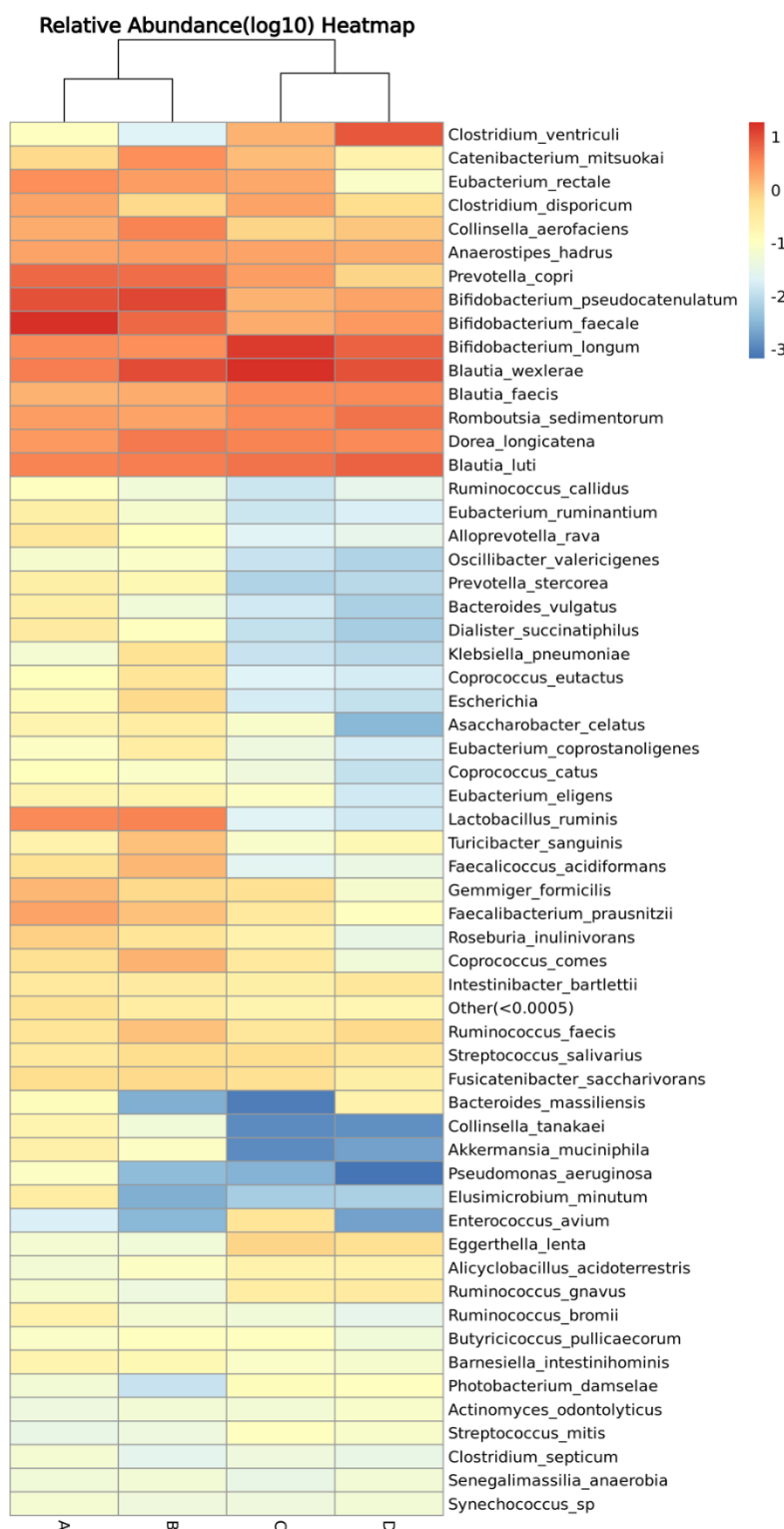


Figure 17. Log₁₀-transformed relative microbial abundance across study groups. The heatmap illustrates group-level patterns of bacterial taxa in female COVID-19 survivors (Group A), male COVID-19 survivors (Group B), healthy female controls (Group C), and healthy male controls (Group D). Warmer colors (red) indicate higher abundances, cooler colors (blue) indicate lower abundances. Female survivors show enrichment of *Bifidobacterium* species (*B. pseudocatenulatum*, *B. longum*, *B. faecale*), whereas male survivors show higher *Blautia* spp. (*B. wexlerae*, *B. luti*)

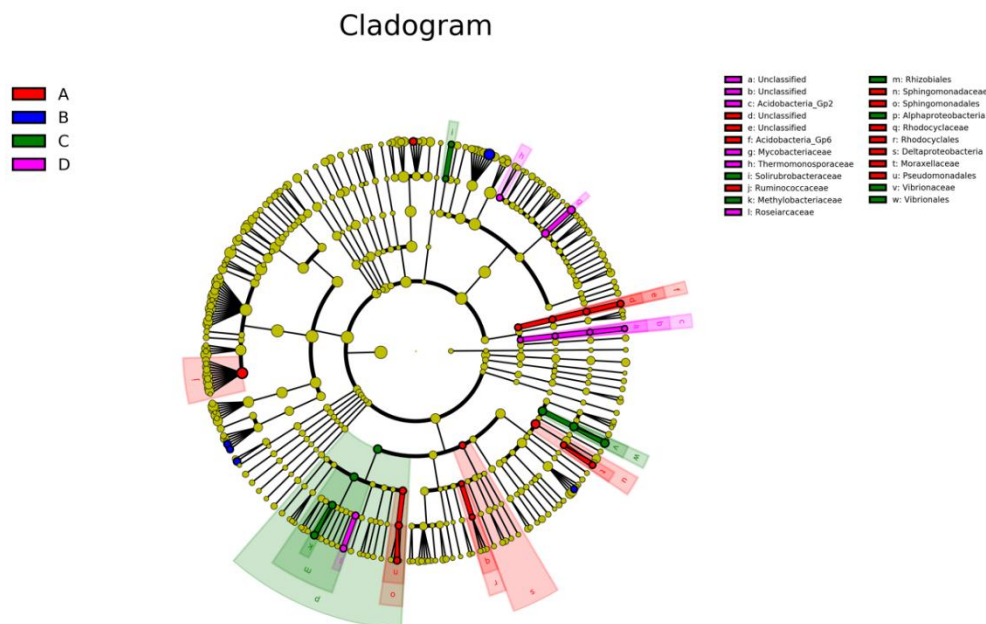


Figure 18. Cladogram showing the phylogenetic distribution of microbial taxa for four groups of samples, Group A: female COVID-19 survivors ($n = 21$), Group B: Male COVID-19 survivors ($n = 9$), Group C: Healthy female controls ($n = 7$) and Group D: Healthy male controls ($n = 3$). Coloring the branches indicates group association, and the labelled taxa are important discriminators

Discussion

Research on COVID-19 survivors shows significant alterations in gut microbiota (Mendes de Almeida et al., 2023; Zhang et al., 2023). Using high-throughput 16S rRNA sequencing, we identified major differences in microbial diversity, taxonomic composition, and functional potential between male and female survivors and their respective controls. Evidence is mounting that SARS-CoV-2 infection has long-term effects on the gut, with persistent dysbiosis observed months after recovery (Xiao et al., 2023; Xie et al., 2025). Post-COVID dysbiosis has been associated with long-term damage (Giannos and Prokopidis, 2022).

Interestingly, female survivors displayed the highest unique OTU count of 76 OTUs, while healthy males showed no unique OTUs, highlighting sex-specific microbiome resilience. This observation parallels previous findings showing subtle but steady changes in diversity after COVID-19 recovery (Liu et al., 2021).

We observed an increase in beneficial bacteria, particularly *Bifidobacterium pseudocatenulatum* ($\log_{10} = 0.97$ in females, 1.09 in males), in both male and female survivors, which may act as a compensatory mechanism to counteract inflammation and enhance mucosal healing (Astbury et al., 2020). This replacement effect did not occur equally across sexes. Conversely, there was a decrease in *Faecalibacterium prausnitzii* (A: 2.01% vs C: 0.36%), a butyrate-producer critical for gut barrier function (Gou et al., 2021). Male survivors exhibited elevated levels of *Lactobacillus ruminis* (12.6% in A11 and A14), suggesting sex-specific recovery patterns and potentially less efficient microbiome restoration (Haro et al., 2016).

Functional predictions using PICRUSt revealed that survivors' microbiomes were enriched for defense mechanisms ($\log_2FC = 1.8$) and signal transduction pathways,

indicative of ongoing immune-related adaptations post-infection (Gou et al., 2021). Healthy controls showed higher activity in carbohydrate metabolism, characteristic of stable gut ecosystems (Lloyd-Price et al., 2019). Across all groups, the most abundant functional categories were core microbial processes, including translation, ribosomal structure and biogenesis, carbohydrate transport and metabolism, and amino acid transport and metabolism, which are essential for microbial growth and homeostasis (Lloyd-Price et al., 2019).

PLS-DA highlighted sex-specific separation, supporting the concept of gender-driven differences in microbial composition (Zuo et al., 2020). LEfSe analysis identified *Collinsella* as a female survivor biomarker (LDA = 4.5) and *Mycobacterium* as a male biomarker, suggesting differential dysbiosis mechanisms between sexes.

The findings of this study demonstrate that the gut microbiome of COVID-19 survivors exhibits persistent sex-specific alterations, characterized by enrichment of immunomodulatory taxa (*Bifidobacterium*), opportunistic pathogens (*Mycobacterium*), depletion of anti-inflammatory species (*Faecalibacterium*), and a shift in metabolic pathways toward stress response rather than homeostasis. Healthy female controls (Group C) exhibited higher levels of *Prevotella copri*, likely reflecting dietary influences on microbial composition. These results are consistent with and expand upon prior studies regarding the mediation of sex in long-term post-COVID microbiome alterations (Yeoh et al., 2021; Liu et al., 2021). Further study is required to assess functional consequences, especially concerning contributions to long COVID symptoms.

The microbial patterns observed support previous reports of post-COVID dysbiosis (Yeoh et al., 2021; Liu et al., 2021), while also refining our understanding of how sex impacts recovery. Female survivors demonstrated greater microbial richness and unique taxa, suggesting better resilience and potential return to eubiotic states. In contrast, males showed enrichment of potentially pro-inflammatory or opportunistic taxa and lower diversity, indicating susceptibility to prolonged dysbiosis and long-term complications.

Despite high-quality data processing and robust analytical pipelines, limitations include the small number of male controls, which may affect generalizability. Additionally, the cross-sectional design precludes causal inference, emphasizing the need for longitudinal studies to assess microbiome dynamics from acute infection to full recovery.

Conclusions

This study demonstrates that COVID-19 survivors exhibit long-term, sex-specific alterations in gut microbiota composition. Using 16S rRNA sequencing, we observed dysbiosis characterized by reduced microbial diversity, depletion of beneficial bacteria such as *Faecalibacterium prausnitzii*, and enrichment of immunomodulatory taxa, including *Bifidobacterium pseudocatenulatum* and *Collinsella aerofaciens*. Female survivors displayed greater microbial richness and higher unique OTU counts, whereas male survivors showed increased colonization by potentially pro-inflammatory opportunistic taxa such as *Mycobacterium*. Functional analysis revealed that survivors' microbiomes were enriched in defense and stress response pathways, whereas healthy controls had higher carbohydrate and energy metabolism activity. These sex-specific differences are likely influenced by hormonal and immune factors. Overall, SARS-CoV-2 infection may trigger persistent gut microbial alterations that impact immunity, metabolism, and potentially contribute to chronic health conditions. Our findings

highlight the importance of sex-stratified interventions, including microbiome-based therapies, to support post-COVID recovery. Future longitudinal studies should investigate host-microbial interactions to better understand post-infectious dysbiosis and guide targeted therapeutic strategies.

Author contributions. SWB, AHH and FS conducted and coordinated and wrote the main manuscript. SWH, SHR, MOA assisted in sample collection and data analysis. BS and FKS provided major edits to the manuscript. All authors reviewed the manuscript.

Financial support. None.

Conflict of interests. The authors declare no competing interests.

Human ethical approval. The Koya University Faculty of Health and Science-Koya Institutional Ethics Committee gave its approval to this study on May 11, 2023 (Approval Number: 1005202).

Consent to participate declaration. Prior to sample collection, all participants provided written informed consent. All procedures performed in studies involving human participants were in accordance with the ethical standards of the Koya University Faculty of Health and Science-Koya Institutional Ethics Committee and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

- [1] Aggarwal, N., Kitano, S., Puah, G. R. Y., Kittelmann, S., Hwang, I. Y., Chang, M. W. (2023): Microbiome and human health: current understanding, engineering, and enabling technologies. – Chem. Rev. 123(1): 31-72. <https://doi.org/10.1021/acs.chemrev.2c00431>.
- [2] Astbury, S., Atallah, E., Vijay, A., Aithal, G. P., Grove, J. I., Valdes, A. M. (2020): Lower gut microbiome diversity and higher abundance of proinflammatory genus *Collinsella* are associated with biopsy-proven nonalcoholic steatohepatitis. – Gut Microbes 11(3): 569-580. <https://doi.org/10.1080/19490976.2019.1681861>.
- [3] Bai, F., Tomasoni, D., Falcinella, C., Barbanotti, D., Castoldi, R., Mulè, G., Augello, M., Mondatore, D., Allegrini, M., Cona, A., Tesoro, D., Tagliaferri, G., Viganò, O., Suardi, E., Tincati, C., Beringheli, T., Varisco, B., Battistini, C. L., Piscopo, K., Vegni, E. ... Monforte, A. D. (2022): Female gender is associated with long COVID syndrome: a prospective cohort study. – Clin. Microbiol. Infect. 28(4): 611.e9-611.e16. <https://doi.org/10.1016/j.cmi.2021.11.002>.
- [4] Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., Mills, D. A., Caporaso, J. G. (2013): Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. – Nat. Methods 10(1): 57-59.
- [5] Bordag, N., Klie, S., Jürchott, K., Vierheller, J., Schiewe, H., Albrecht, V., Tonn, J. C., Schwartz, C., Schichor, C., Selbig, J. (2015): Glucocorticoid (dexamethasone)-induced metabolome changes in healthy males suggest prediction of response and side effects. – Sci. Rep. 5: 15954. <https://doi.org/10.1038/srep15954>.
- [6] Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., Knight, R. (2012): Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. – Proc. Natl. Acad. Sci. U.S.A. 108 (Supplement 1): 4516-4522.
- [7] Giannos, P., Prokopenidis, K. (2022): Gut dysbiosis and long COVID-19: feeling gutted. – Journal of Medical Virology 94(7): 2917-2918. <https://doi.org/10.1002/jmv.27684>.

- [8] Gou, W., Fu, Y., Yue, L., Chen, G., Cai, X., Shuai, M., Xu, F., Yi, X., Chen, H., Zhu, Y., Guo, Y. (2021): Gut microbiota may underlie the predisposition of healthy individuals to COVID-19. – medRxiv. <https://doi.org/10.1101/2021.03.24.21254103>.
- [9] Gu, S., Chen, Y., Wu, Z., Chen, Y., Gao, H., Lv, L., Guo, F., Zhang, X., Luo, R., Huang, C., Lu, H. (2020): Alterations of the gut microbiota in patients with COVID-19 or H1N1 influenza. – *Clinical Infectious Diseases* 73(5): e136–e143.
- [10] Haro, C., Rangel-Zúñiga, O. A., Alcalá-Díaz, J. F., Gómez-Delgado, F., Pérez-Martínez, P., Delgado-Lista, J., Quintana-Navarro, G. M., Landa, B. B., Navas-Cortés, J. A., Tena-Sempere, M., Ordovás, J. M. (2016): Intestinal microbiota is influenced by gender and body mass index. – *PLoS One* 11(5): e0154090.
- [11] Hirayama, M., Nishiwaki, H., Hamaguchi, T., Ito, M., Ueyama, J., Maeda, T., Kashiwara, K., Tsuboi, Y., Ohno, K. (2021): Intestinal Collinsella may mitigate infection and exacerbation of COVID-19 by producing ursodeoxycholate. – *PLoS One* 16(11): e0260451. <https://doi.org/10.1371/journal.pone.0260451>.
- [12] Human Microbiome Project Consortium (2012): Structure, function and diversity of the healthy human microbiome. – *Nature* 486(7402): 207-214.
- [13] Jami, G., Ataee, M., Esmaili, V., Chamani, S., Rezaei, A., Naghizadeh, A. (2023): Characterization of the angiotensin-converting enzyme 2 (ACE2), the main receptor for the SARS-CoV-2 virus. – *Am. J. Clin. Exp. Immunol.* 12(3): 24-44.
- [14] Khan, I. M., Nassar, N., Chang, H., Khan, S., Cheng, M., Wang, Z., Xiang, X. (2024): The microbiota: a key regulator of health, productivity, and reproductive success in mammals. – *Front. Microbiol.* 15: 1480811. <https://doi.org/10.3389/fmicb.2024.1480811>.
- [15] Kopańska, M., Barnas, E., Błajda, J., Kuduk, B., Łagowska, A., Banaś-Ząbczyk, A. (2022): Effects of SARS-CoV-2 inflammation on selected organ systems of the human body. – *Int. J. Mol. Sci.* 23(8): 4178. <https://doi.org/10.3390/ijms23084178>.
- [16] Liu, F., Ye, S., Zhu, X., He, X., Wang, S., Zhang, J., Ma, Y., Yang, L., Zhou, Q., Xie, W., Zheng, H. (2021): Gut microbiota dynamics in a prospective cohort of patients with post-acute COVID-19 syndrome. – *Microbiome* 9(1): 1-18.
- [17] Lloyd-Price, J., Arze, C., Ananthakrishnan, A. N., Schirmer, M., Avila-Pacheco, J., Poon, T. W., Andrews, E., Ajami, N. J., Bonham, K. S., Brislawn, C. J., Casero, D. (2019): Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. – *Nature* 569(7758): 655-662.
- [18] Martín, R., Miquel, S., Chain, F., Natividad, J. M., Jury, J., Lu, J., Sokol, H., Theodorou, V., Bercik, P., Verdu, E. F., Langella, P., Bermúdez-Humarán, L. G. (2015): *Faecalibacterium prausnitzii* prevents physiological damages in a chronic low-grade inflammation murine model. – *BMC Microbiol.* 15: 67. <https://doi.org/10.1186/s12866-015-0400-1>.
- [19] Martín, R., Rios-Covian, D., Huillet, E., Auger, S., Khazaal, S., Bermúdez-Humarán, L. G., Sokol, H., Chatel, J. M., Langella, P. (2023): *Faecalibacterium*: a bacterial genus with promising human health applications. – *FEMS Microbiol. Reviews* 47(4): fuad039. <https://doi.org/10.1093/femsre/fuad039>.
- [20] Mendes de Almeida, V., Engel, D. F., Ricci, M. F., Cruz, C. S., Lopes, Í. S., Alves, D. A., d'Auriol, M., Magalhães, J., Machado, E. C., Rocha, V. M., Carvalho, T. G., Lacerda, L. S. B., Pimenta, J. C., Aganetti, M., Zuccoli, G. S., Smith, B. J., Carregari, V. C., da Silva Rosa, E., Galvão, I., Dantas Cassali, G., Garcia, C. C., Teixeira, M. M., André, L. C., Ribeiro, F. M., Martins, F. S., Saia, R. S., Costa, V. V., Martins-De-Souza, D., Hansbro, P. M., Marques, J. T., Aguiar, E. R. G. R., Vieira, A. T. (2023): Gut microbiota from patients with COVID-19 cause alterations in mice that resemble post-COVID symptoms. – *Gut Microbes* 15(2): 2249146. <https://doi.org/10.1080/19490976.2023.2249146>.
- [21] QIAGEN (2023): DNeasy PowerSoil Pro Kit handbook. – QIAGEN. <https://www.qiagen.com> (accessed 15 March 2023).
- [22] Rocchi, G., Giovanetti, M., Benedetti, F., Borsetti, A., Ceccarelli, G., Zella, D., Altomare, A., Ciccozzi, M., Guarino MPL (2022): Gut microbiota and COVID-19: potential

- implications for disease severity. – *Pathogens* 11(9): 1050. <https://doi.org/10.3390/pathogens11091050>.
- [23] Russell, S. J., Parker, K., Lehoczk, A., Lieberman, D., Partha, I. S., Scott, S. J., Phillips, L. R., Fain, M. J., Nikolich JŽ (2024): Post-acute sequelae of SARS-CoV-2 infection (Long COVID) in older adults. – *GeroScience* 46(6): 6563-6581. <https://doi.org/10.1007/s11357-024-01227-8>.
- [24] Santana, P. T., Rosas, S. L. B., Ribeiro, B. E., Marinho, Y. de Souza HSP (2022): Dysbiosis in inflammatory bowel disease: pathogenic role and potential therapeutic targets. – *Int. J., Mol. Sci.* 23(7): 3464. <https://doi.org/10.3390/ijms23073464>.
- [25] Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W. (2009): Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. – *Appl. Environ. Microbiol.* 75(23): 7537-7541.
- [26] Shen, Y., Fan, N., Ma, S. X., Cheng, X., Yang, X., Wang, G. (2025): Gut microbiota dysbiosis: pathogenesis, diseases, prevention, and therapy. – *MedComm* 6(5): e70168. <https://doi.org/10.1002/mco2.70168>.
- [27] Shirbhate, E., Pandey, J., Patel, V. K., Kamal, M., Jawaid, T., Gorain, B., Kesharwani, P., Rajak, H. (2021): Understanding the role of ACE-2 receptor in pathogenesis of COVID-19 disease: a potential approach for therapeutic intervention. – *Pharmacol. Rep.* 73(6): 1539-1550. <https://doi.org/10.1007/s43440-021-00303-6>.
- [28] Singh, V., Lee, G., Son, H., Koh, H., Kim, E. S., Unno, T., Shin, J. H. (2023): Butyrate producers, “The Sentinel of Gut”: Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. – *Front. Microbiol.* 13: 1103836. <https://doi.org/10.3389/fmicb.2022.1103836>.
- [29] Xiao, Z., Pan, M., Li, X., Zhao, C. (2023): Impact of SARS-CoV2 infection on gut microbiota dysbiosis. – *Microbiome Res. Rep.* 3(1): 7. <https://doi.org/10.20517/mrr.2023.48>.
- [30] Xie, Q., Ni, J., Guo, W., Ding, C., Wang, F., Wu, Y., Zhao, Y., Zhu, L., Xu, K., Chen, Y. (2025): Two-year follow-up of gut microbiota alterations in patients after COVID-19: from the perspective of gut enterotype. – *Microbiol. Spectr.* 13(5): e0277424. <https://doi.org/10.1128/spectrum.02774-24>.
- [31] Yeoh, Y. K., Zuo, T., Lui, G. C., Zhang, F., Liu, Q., Li, A. Y., Chung, A. C., Cheung, C. P., Tso, E. Y., Fung, K. S., Chan, V. (2021): Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. – *Gut* 70(4): 698-706.
- [32] Zhang, F., Lau, R. I., Liu, Q., Su, Q., Chan, F. K. L., Ng, S. C. (2023): Gut microbiota in COVID-19: key microbial changes, potential mechanisms and clinical applications. – *Nat. Rev. Gastroenterol. Hepatol.* 20(5): 323-337. <https://doi.org/10.1038/s41575-022-00698-4>.
- [33] Zhang, Y., Geng, X., Tan, Y., Li, Q., Xu, C., Xu, J., Hao, L., Zeng, Z., Luo, X., Liu, F., Wang, H. (2020): New understanding of the damage of SARS-CoV-2 infection outside the respiratory system. – *Biomed. Pharmacother.* 127: 110195. <https://doi.org/10.1016/j.biopha.2020.110195>.
- [34] Zhou, B., Pang, X., Wu, J., Liu, T., Wang, B., Cao, H. (2023): Gut microbiota in COVID-19: new insights from inside. – *Gut Microbes* 15(1): 2201157. <https://doi.org/10.1080/19490976.2023.2201157>.
- [35] Zuo, T., Zhan, H., Zhang, F., Lui, G. C., Wan, Y., Chung, A. C., Cheung, C. P., Chen, N., Lai, C. K., Tsang, J. O., Ching, J. Y. (2020): Alterations in gut microbiota of patients with COVID-19 during the time of hospitalisation. – *Gastroenterology* 159(3): 944-955.e8.