Gut microbiota changes are detected in asymptomatic very young children with SARS-CoV-2 infection

We read with great interest the recent article by Yeoh *et al*, demonstrating an altered stool microbiome composition in patients with COVID-19 compared with controls, with greater dysbiosis correlating with elevated inflammatory markers.[1] Additionally, dysbiosis was seen after disease resolution.[1]

To our knowledge, gut microbiome studies in young children with COVID-19 have not been reported. Critically, the developing gut microbiome of very young children differs from adults and establishes immune and inflammatory pathways.[2 3] Moreover, children with COVID-19 can subsequently develop autoimmune and autoinflammatory diseases including Multisystem Inflammatory Syndrome in Children (MIS-C)[4 5], which may in part be microbiome mediated, given recent findings by Yeoh *et al*.[1] It is difficult to study this in young children, as many with SARS-CoV-2 infection are asymptomatic and rarely tested.[6]

To address this, knowing that SARS-CoV-2 can be detected in stool,[7] we used an established study collecting longitudinal stool samples from before and throughout the pandemic to investigate the prevalence and associated microbiome changes of SARS-CoV-2 in very young children. We ran the CDC 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel assay on 769 serial stool samples from 595 children aged 0–24 months collected from February 2020 to February 2021. The prevalence of SARS-CoV-2 in faeces was 1.7% (13 samples from 13 separate children) with prevalence at <2 days and 2, 6, 12 and 24 months of 0% (0/1), 0% (0/21), 2.6% (4/156), 2.0% (7/357) and 0.9%,(2/234), respectively. Prevalence by month is shown in online supplemental figure 1A, with the first positive sample detected 31 days before the first reported case of COVID-19 regionally. No samples were positive in controls collected prior to the pandemic in 2019 (n=97 samples from 66 individuals). Of 13 positive children, 12 were asymptomatic with no personal or family history of SARS-CoV-2 (table 1A). Of 13 children, 1 was symptomatic with COVID-19 diagnosed 21 days before stool was collected. Hispanic ethnicity was associated with stool positivity (61.5% in positive samples vs 23.4% in negative samples, p=0.006 (χ^2), table 1A). This study may underestimate prevalence rates as stool positivity may be lower than respiratory samples.

We successfully sequenced the SARS-CoV-2 genome from all positive samples (full methods in online supplemental data), with variant identification achieved for five samples (online supplemental figure 1B). We performed V4 16S rRNA gene sequencing on samples using DADA2 and the SILVA database for microbiome taxonomic profiling. We compared microbiomes using a 1:2 case–control match, controlled for ethnicity, age, delivery mode, gestational age, gender and recent antibiotic use (table 1B). Differential species abundance testing was performed using DESeq2 contrasting the SARS-CoV-2 positive and control samples. We found a significantly different relative abundance of taxa (adjusted p<0.05) between positive and control samples (all significantly different taxa at a species level shown in figure 1). Notably, we found a decreased abundance of *Bifidobacterium bifidum* and *Akkermansia muciniphila* in positive samples, both of which are linked to protection against

Table 1 (A) Characteristics of overall cohort and (B) characteristics of matched cohort for microbiome analysis

(A) Characteristics of overall cohort	Negative (n=582)	Positive (n=13)	P Value
Hispanic Ethnicity Missing=26	130 (23.4%)	8 (61.5%)	0.006
Household member diagnosed with SARS-CoV-2 Missing=51	19 (3.6%)	1 (7.7%)	0.4
Childcare outside of the home during quarantine Missing=63	104 (20.0%)	2 (15.4%)	0.7
Household with essential worker during quarantine Missing=58	269 (51.3%)	9 (69.2%)	0.2
(B) Characteristics of matched cohort for microbiome analysis	**Negative (n=26)**	**Positive (n=13)**	**P value**
Sex			
Male	12 (46.2%)	6 (46.2%)	1
Female	14 (53.8%)	7 (53.8%)	
Delivery Mode			
Caesarean Section	12 (46.2%)	6 (46.2%)	1
Vaginal Delivery	14 (53.8%)	7 (53.8%)	
Ethnicity			
Hispanic	16 (61.5 %)	8 (61.5 %)	1
Non-Hispanic	10 (38.5 %)	5 (38.5 %)	
Age at time of sample:			
6 months	8 (30.8 %)	4 (30.8 %)	1
12 months	14 (53.8 %)	7 (53.8%)	
24 months	4 (15.4 %)	2 (15.4 %)	
Gestational age			
Full term	26 (100%)	13 (100 %)	1
Preterm	0 (0%)	0 (0%)	
Recent antibiotic use			
Yes	8 (30.8 %)	4 (30.8 %)	1
No	18 (69.2 %)	9 (69.2 %)	

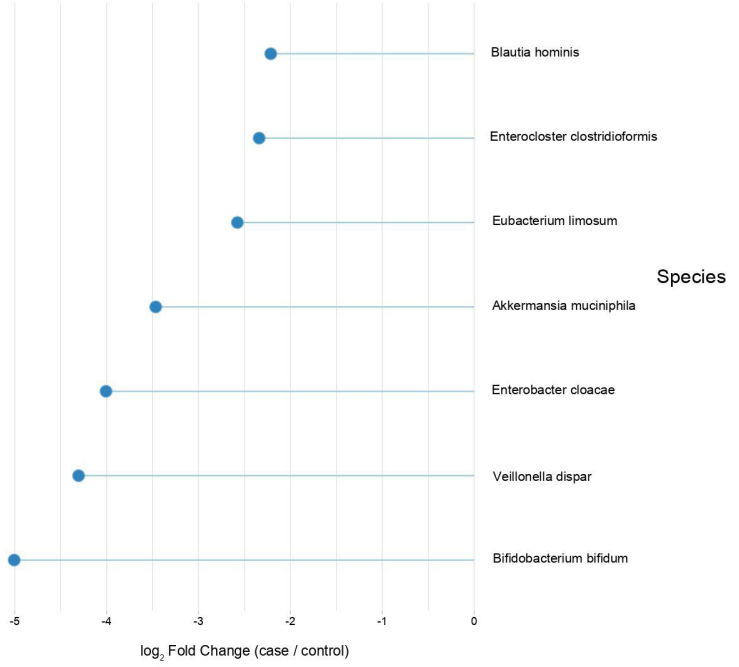


Figure 1 Significantly differentially abundant species between SARS-CoV-2 positive infants and controls identified using DESeq2. Negative $\log_2$ fold changes indicate a lower abundance of these species in SARS-CoV-2 positive samples relative to controls.

inflammation.[8 9] *Bifidobacterium* are also pioneering colonisers of the gut microbiota and have immunomodulatory properties.[10] *Bifidobacterium bifidum* was found to be inversely correlated with disease severity in adults.[1] While Yeoh *et al*[1] saw differences in beta diversity, our microbiome changes may be less robust compared with those symptomatic patients, with no differences seen in alpha or beta diversity. Detection of changes may also be limited by sample size.

We show that microbiome changes are detectable even in asymptomatic infants infected with SARS-CoV-2. Of relevance, there is a decrease in anti-inflammatory taxa, similar to that seen in symptomatic adults. The impact of this on the developing microbiome, and subsequent immune and inflammatory responses is unknown, but deserves further exploration given the risk of development of autoimmune and auto-inflammatory conditions in children with COVID-19.

Lydia Nashed,[1] Jyoti Mani,[2] Sahel Hazrati,[3] David B Stern,[4] Poorani Subramanian ,[4] Lisa Mattei,[5] Kyle Bittinger,[5] Weiming Hu,[5] Shira Levy,[1,6] George L Maxwell,[3] Suchitra K Hourigan [1,6]

[1]Inova Children's Hospital, Inova Health System, Falls Church, Virginia, USA
[2]Pediatric Gastroenterology, Children's National Health System, Washington, District of Columbia, USA
[3]Women's Service Line, Inova Health System, Falls Church, Virginia, USA
[4]Bioinformatics and Computational Biosciences Branch, NIAID, Bethesda, Maryland, USA
[5]Division of Gastroenterology, Hepatology and Nutrition, Children's Hospital of Philadelphia Pediatrics Residency Program, Philadelphia, Pennsylvania, USA
[6]Laboratory of Host Immunity and Microbiome, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA

Correspondence to Dr Suchitra K Hourigan, NIAID, Bethesda, Maryland, USA; suchitra.hourigan@nih.gov

Twitter David B Stern @TheDBStern and Suchitra K Hourigan @DrSuchiHourigan

Contributors LN, SL, GM and SKH conceptualised and designed the study. LN, JM, SL and SH designed data collection instruments and collected and interpreted subject's clinical data. SH conducted epidemiological analyses. DS and PS conducted microbiome analysis. LM conducted laboratory design and analysis. KB and WH conducted viral genome analysis. LN and SH drafted the initial manuscript. All authors reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

Funding This work was supported in part by a National Institute of Child Health and Human Development K23 award (No. K23HD099240, Hourigan) and by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under the Intramural Research Programme (Hourigan) and BCBB Support Services Contract HHSN316201300006W/HHSN27200002 to MSC (Subramanian, Stern). This work was also supported by an Inova Health System Seed Grant (Hourigan, Nashed). Inova expresses its appreciation to the Fairfax County in VA, which has supported Inova's research projects with annual funding from its Contributory Fund (Fund 10030, Maxwell).

Disclaimer The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Competing interests None declared.

Patient consent for publication Consent obtained from parent(s)/guardian(s)

Ethics approval This study has been approved by the WCG institutional review board (IRB# 20120204). Written informed consent was obtained from all participants/guardians prior to collecting stool samples. Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

► Additional supplemental material is published online only. To view, please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2021-326599).

To cite Nashed L, Mani J, Hazrati S, *et al*. *Gut* 2022;**71**:2371–2373.

Received 17 November 2021
Accepted 28 December 2021
Published Online First 8 February 2022

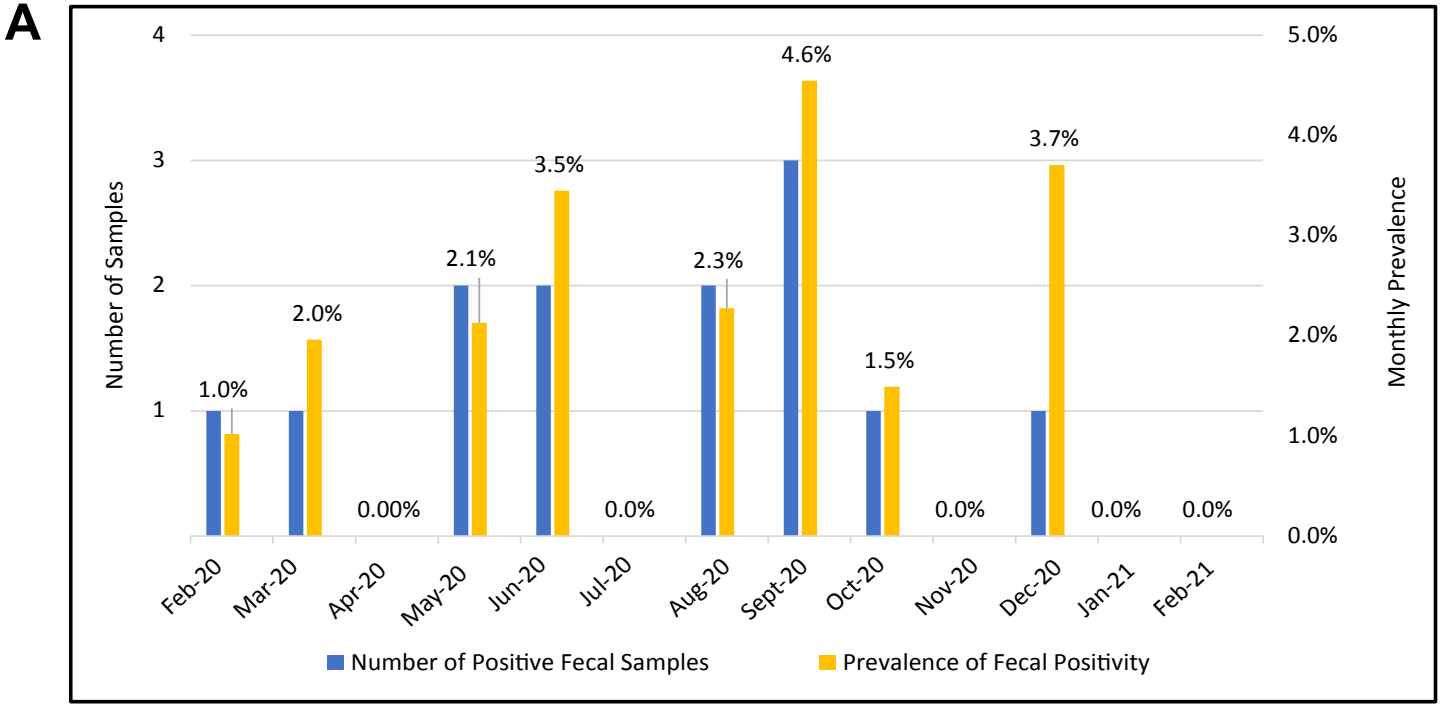
Gut 2022;**71**:2371–2373. doi:10.1136/gutjnl-2021-326599

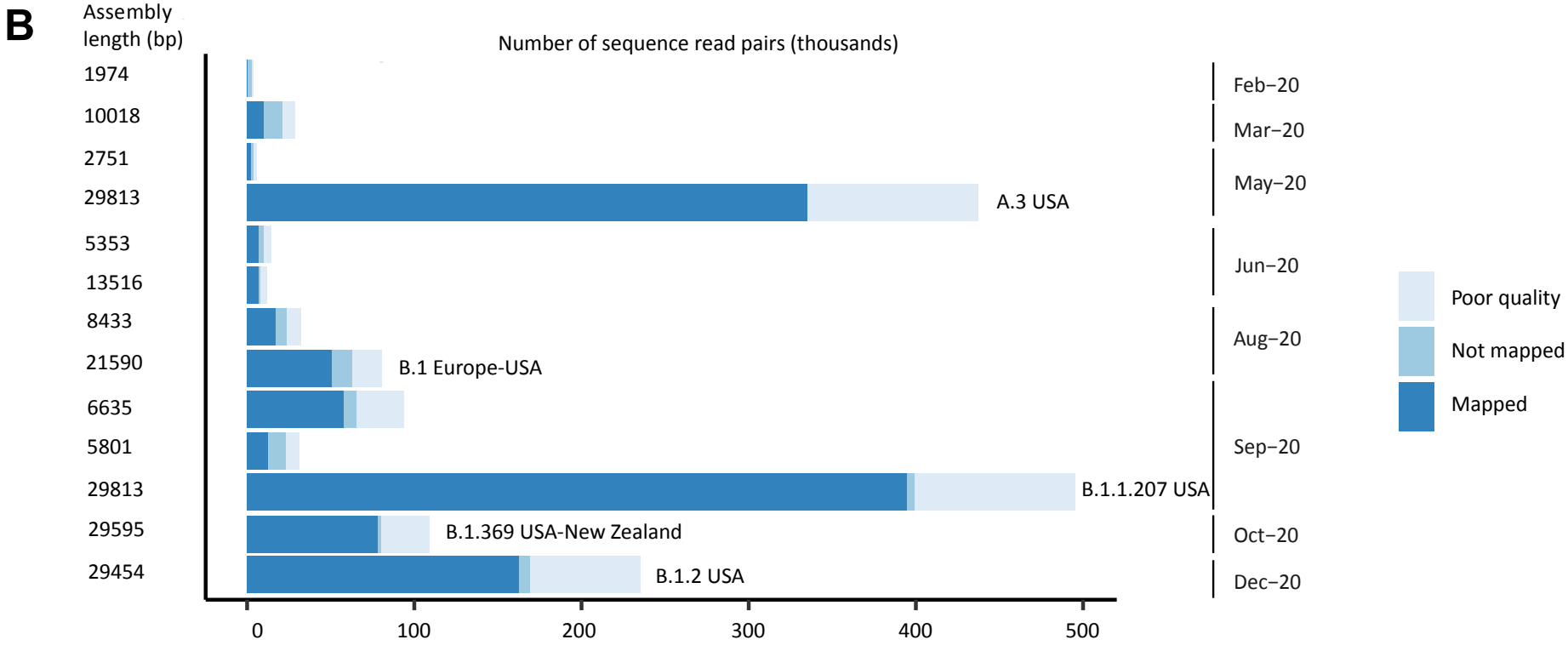
ORCID iDs
Poorani Subramanian http://orcid.org/0000-0003-4371-7290
Suchitra K Hourigan http://orcid.org/0000-0003-2656-5694

REFERENCES

1 Yeoh YK, Zuo T, Lui GC-Y, *et al*. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut* 2021;70:698–706.

2 Yatsunenko T, Rey FE, Manary MJ, *et al*. Human gut microbiome viewed across age and geography. *Nature* 2012;486:222–7.
3 Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and disease. *Cell Res* 2020;30:492–506.
4 Galeotti C, Bayry J. Autoimmune and inflammatory diseases following COVID-19. *Nat Rev Rheumatol* 2020;16:413–4.
5 Francino MP. Early development of the gut microbiota and immune health. *Pathogens* 2014;3:769–90.
6 Dong Y, Mo X, Hu Y, *et al*. Epidemiology of COVID-19 among children in China. *Pediatrics* 2020;145. doi:10.1542/peds.2020-0702. [Epub ahead of print: 16 Mar 2020].
7 van Doorn AS, Meijer B, Frampton CMA, *et al*. Systematic review with meta-analysis: SARS-CoV-2 stool testing and the potential for faecal-oral transmission. *Aliment Pharmacol Ther* 2020;52:1276–88.
8 Al-Sadi R, Dharmaprakash V, Nighot P, *et al*. *Bifidobacterium bifidum* Enhances the Intestinal Epithelial Tight Junction Barrier and Protects against Intestinal Inflammation by Targeting the Toll-like Receptor-2 Pathway in an NF-κB-Independent Manner. *Int J Mol Sci* 2021;22:8070.
9 Earley H, Lennon G, Balfe Áine, *et al*. The abundance of Akkermansia muciniphila and its relationship with sulphated colonic mucins in health and ulcerative colitis. *Sci Rep* 2019;9:15683.
10 Ruiz L, Delgado S, Ruas-Madiedo P, *et al*. Bifidobacteria and their molecular communication with the immune system. *Front Microbiol* 2017;8:2345.


A
Number of Samples
Monthly Prevalence
4
3
2
1
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
1.0%
2.0%
0.00%
2.1%
3.5%
0.0%
2.3%
4.6%
1.5%
0.0%
3.7%
0.0%
0.0%
Feb-20
Mar-20
Apr-20
May-20
Jun-20
Jul-20
Aug-20
Sept-20
Oct-20
Nov-20
Dec-20
Jan-21
Feb-21
Number of Positive Fecal Samples
Prevalence of Fecal Positivity


B
Assembly length (bp)
Number of sequence read pairs (thousands)
1974
10018
2751
29813
5353
13516
8433
21590
6635
5801
29813
29595
29454
A.3 USA
B.1 Europe-USA
B.1.1.207 USA
B.1.369 USA-New Zealand
B.1.2 USA
0
100
200
300
400
500
Feb–20
Mar–20
May–20
Jun–20
Aug–20
Sep–20
Oct–20
Dec–20
Poor quality
Not mapped
Mapped

Supplementary Bioinformatics Methods

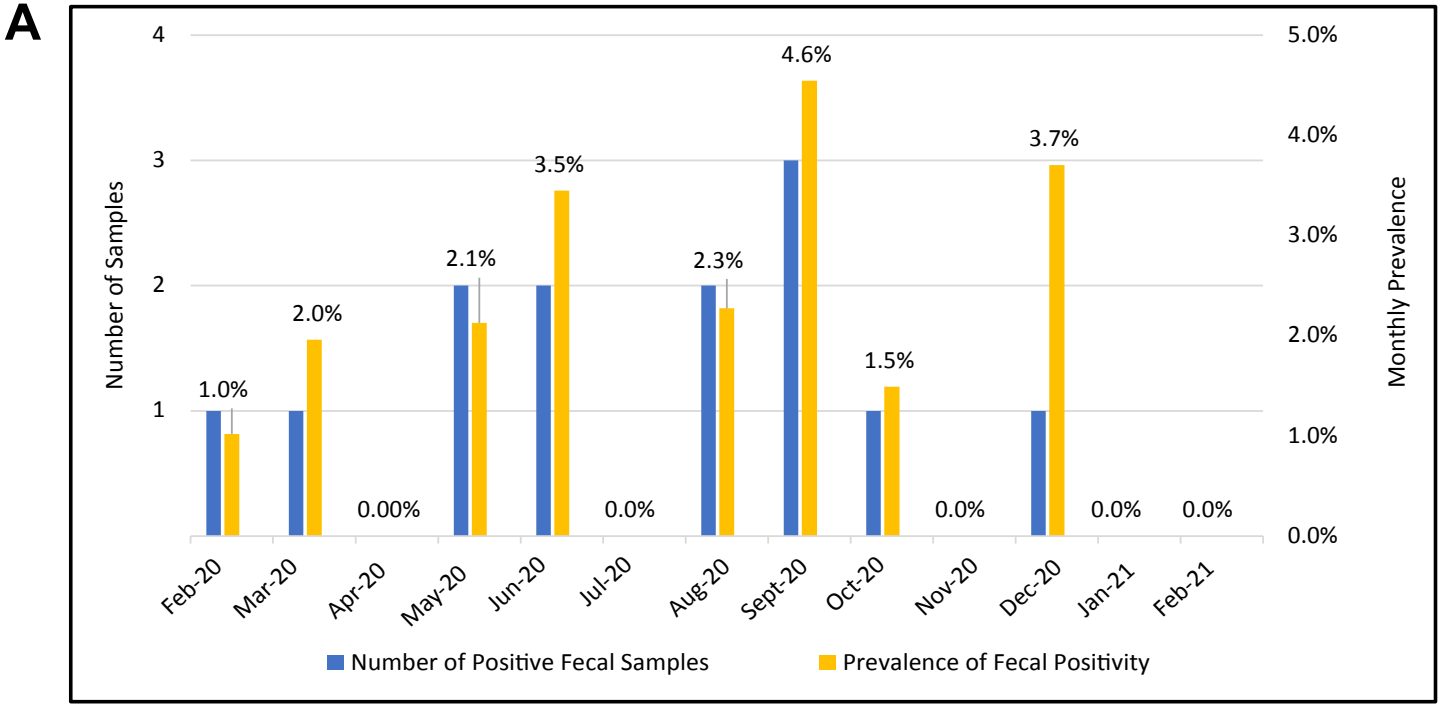
For the 16S ribosomal RNA gene sequence analysis, read pairs were trimmed and filtered for quality with BBDuk v38.90 [1] and then processed with DADA2 v1.20.0 [2] for denoising, merging, and taxonomic assignment of the resulting amplicon sequence variants (ASV) with rdp and the SILVA v138.1 database.[3] ASVs unable to be identified by SILVA were further queried against NCBI's 16S ribosomal RNA database using BLAST+ v2.12.0. Differential species abundance testing was performed using DESeq2 [4] contrasting the SARS-CoV-2 positive and control samples, accounting for the paired sampling design with p-values adjusted by the Benjamini-Hochberg method for multiple comparisons. ASV counts were aggregated at the species level and averaged for each pair of matched controls prior to testing.

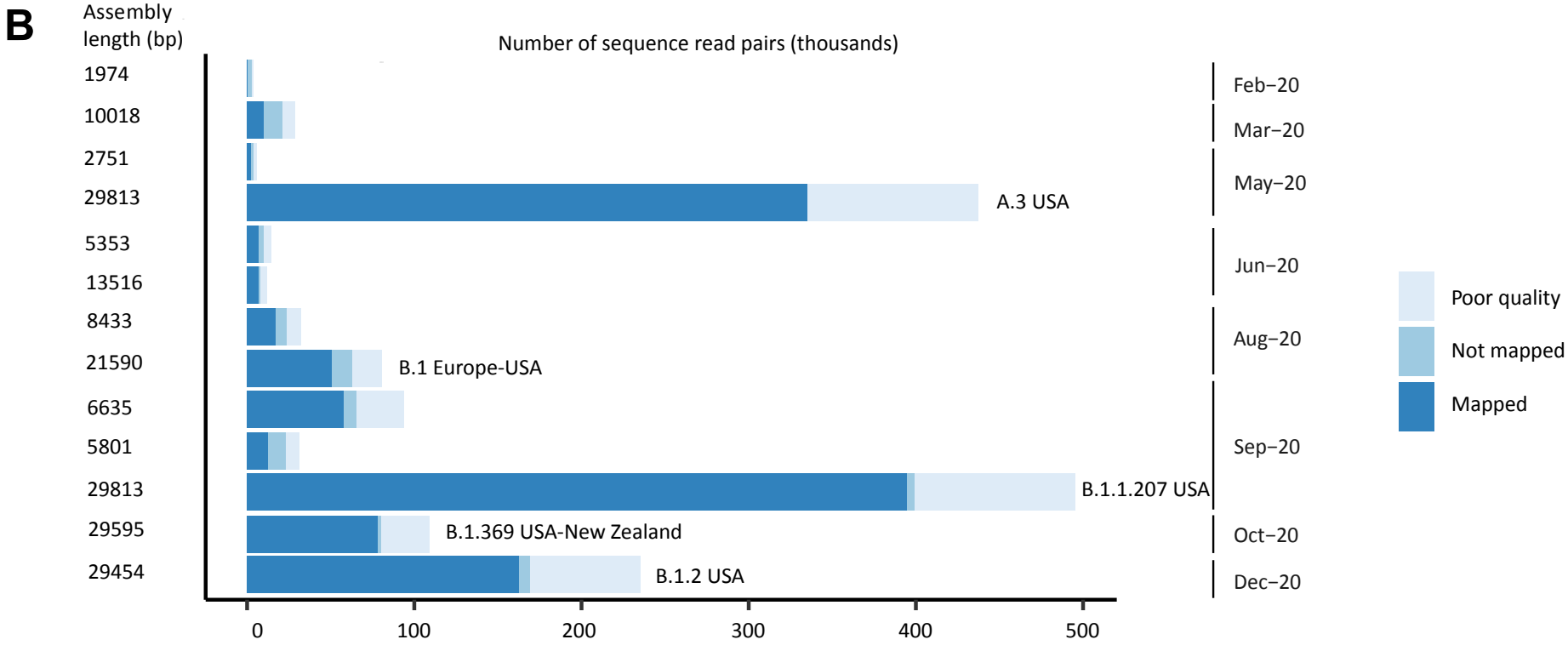
For SARS-CoV-2 genome sequencing, RNA from the stool was extracted using the QIAamp Viral RNA Mini Kit 250 (Qiagen), and amplified following the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel method. The amplified RNA was transformed to cDNA and sequenced on 2x75bp Nextseq platform. For genome assembly, raw reads were trimmed and quality filtered using BBDuk v38.90 [1] and assembled with a reference-mapping-based approach using the Wuhan-Hu-1 reference genome. Additional details of the bioinformatic pipeline can be found here: https://github.com/TheDBStern/viral-assembly-variant-calling.

References

1. Bushnell B. BBTools. DOE Joint Genome Institute [accessed November 16th 2021.
2. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *Isme j* 2017;11(12):2639-43. doi: 10.1038/ismej.2017.119 [published Online First: 2017/07/22]

3. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41(Database issue):D590-6. doi: 10.1093/nar/gks1219 [published Online First: 2012/11/30]
4. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550. doi: 10.1186/s13059-014-0550-8 [published Online First: 2014/12/18]


A
Number of Samples
Monthly Prevalence
4
3
2
1
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
1.0%
2.0%
0.00%
2.1%
3.5%
0.0%
2.3%
4.6%
1.5%
0.0%
3.7%
0.0%
0.0%
Feb-20
Mar-20
Apr-20
May-20
Jun-20
Jul-20
Aug-20
Sept-20
Oct-20
Nov-20
Dec-20
Jan-21
Feb-21
Number of Positive Fecal Samples
Prevalence of Fecal Positivity


B
Assembly length (bp)
Number of sequence read pairs (thousands)
1974
10018
2751
29813
5353
13516
8433
21590
6635
5801
29813
29595
29454
A.3 USA
B.1 Europe-USA
B.1.1.207 USA
B.1.369 USA-New Zealand
B.1.2 USA
0
100
200
300
400
500
Feb–20
Mar–20
May–20
Jun–20
Aug–20
Sep–20
Oct–20
Dec–20
Poor quality
Not mapped
Mapped

Supplementary Bioinformatics Methods

For the 16S ribosomal RNA gene sequence analysis, read pairs were trimmed and filtered for quality with BBDuk v38.90 [1] and then processed with DADA2 v1.20.0 [2] for denoising, merging, and taxonomic assignment of the resulting amplicon sequence variants (ASV) with rdp and the SILVA v138.1 database.[3] ASVs unable to be identified by SILVA were further queried against NCBI's 16S ribosomal RNA database using BLAST+ v2.12.0. Differential species abundance testing was performed using DESeq2 [4] contrasting the SARS-CoV-2 positive and control samples, accounting for the paired sampling design with p-values adjusted by the Benjamini-Hochberg method for multiple comparisons. ASV counts were aggregated at the species level and averaged for each pair of matched controls prior to testing.

For SARS-CoV-2 genome sequencing, RNA from the stool was extracted using the QIAamp Viral RNA Mini Kit 250 (Qiagen), and amplified following the CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel method. The amplified RNA was transformed to cDNA and sequenced on 2x75bp Nextseq platform. For genome assembly, raw reads were trimmed and quality filtered using BBDuk v38.90 [1] and assembled with a reference-mapping-based approach using the Wuhan-Hu-1 reference genome. Additional details of the bioinformatic pipeline can be found here: https://github.com/TheDBStern/viral-assembly-variant-calling.

References

1. Bushnell B. BBTools. DOE Joint Genome Institute [accessed November 16th 2021.
2. Callahan BJ, McMurdie PJ, Holmes SP. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *Isme j* 2017;11(12):2639-43. doi: 10.1038/ismej.2017.119 [published Online First: 2017/07/22]

3. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 2013;41(Database issue):D590-6. doi: 10.1093/nar/gks1219 [published Online First: 2012/11/30]
4. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15(12):550. doi: 10.1186/s13059-014-0550-8 [published Online First: 2014/12/18]