Gut microbiome remains stable following COVID-19 vaccination in healthy and immuno-compromised individuals

Rebecca H. Boston[1], Rui Guan[1], Sina Beier[1], Lajos Kalmar[1], Emily C. Horner[1], Nonantzin Beristain-Covarrubias[1], Juan Carlos Yam-Puc[1], Pehuén Pereyra Gerber[2,3], Luisa Faria[1], Anna Kuroshchenkova[1], Anna E. Lindell[1], Sonja Blasche[1], Andrea Correa-Noguera[4], Anne Elmer[5], Caroline Saunders[5], Areti Bermperi[5], Sherly Jose[5], Nathalie Kingston[6], CITIID-NIHR COVID-19 BioResource Collaboration, Sofia Grigoriadou[7], Emily Staples[1], Matthew S. Buckland[7,8], Sara Lear[4], Nicholas J. Matheson[2,3,4,9], Vladimir Benes[10], Christine Parkinson[4], James E. D. Thaventhiran[1,4,*] and Kiran R. Patil[1,*]

[1] Medical Research Council Toxicology Unit, University of Cambridge, Cambridge, UK.
[2] Cambridge Institute of Therapeutic Immunology and Infectious Disease (CITIID), University of Cambridge, Cambridge, UK
[3] Department of Medicine, University of Cambridge, Cambridge, UK
[4] Department of Clinical Immunology, Cambridge University NHS Hospitals Foundation Trust, Cambridge, UK
[5] NIHR Cambridge Clinical Research Facility, Cambridge, UK
[6] NIHR BioResource, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK
[7] Department of Clinical Immunology, Barts Health, London, UK
[8] UCL GOSH Institute of Child Health Division of Infection and Immunity, Section of Cellular and Molecular Immunology, London, UK
[9] NHS Blood and Transplant, Cambridge, UK
[10] European Molecular Biology Laboratory, Heidelberg, Germany
[*]Correspondence to: JEDT, jedt2@mrc-tox.cam.ac.uk; KRP, kp533@mrc-tox.cam.ac.uk.

Abstract

Objectives

The bidirectional interaction between the immune system and the gut microbiota is a key contributor to various host physiological functions. Immune-associated diseases such as cancer and autoimmunity, as well as the efficacy of immunomodulatory therapies, have been linked to microbiome variation. While COVID-19 infection has been shown to cause microbial dysbiosis, it remained unknown whether the inflammatory response associated with vaccination also impacts the microbiota.

Design

Here, we investigate the temporal impact of COVID-19 vaccination on the gut microbiome in healthy and immuno-compromised individuals; the latter included patients with primary immunodeficiency and cancer patients on immunomodulating therapies, assessed using shotgun metagenomic sequencing.

Results

We find that the gut microbiome remained stable post-vaccination irrespective of diverse immune status, vaccine response, and microbial composition spanned by the cohort. The stability is evident at all evaluated levels including phylum, species, and functional capacity.

Conclusion

Our results show the resilience of the gut microbiome to host immune changes triggered by COVID-19 vaccination and suggest minimal, if any, impact on microbiome-mediated processes. These findings encourage vaccine acceptance, particularly when contrasted with the significant microbiome shifts observed during COVID-19 infection.

Key Messages

What is already known on this topic

- COVID-19 infection induces marked gut microbiota dysbiosis.
- COVID-19 vaccines induce a strong inflammatory response which could therefore perturb the gut microbiota.
- The temporal impact of the COVID-19 vaccine on the gut microbiota remains unknown despite millions of doses being administered worldwide.

What this study adds

- Our study demonstrates that there is no evident impact of the COVID-19 vaccination on the gut microbiome in healthy participants as well as in immunocompromised individuals.
- No correlation was observed between the magnitude of the vaccine response and the composition of the gut microbiome.

How this study might affect research, practice or policy

- Our study provides reassurance of vaccine safety promoting the uptake of the COVID-19 vaccine.

Introduction

In the first 30 months of the pandemic, there have been reported to be almost 800 million PCR confirmed cases of COVID-19 infection and approaching 7 million related deaths globally[1]. To reduce this severity, vaccines were deployed with the aim of promoting anti-SARS-CoV-2 immunity, with almost 13.5 billion vaccine doses administered globally, 150 million of which were administered in the United Kingdom[1]. Yet, continued COVID-19 transmission remains of concern[2] with one of the reasons being vaccine hesitancy[3]. Thus, data helping to understand holistic effects of vaccination will have a profound impact on the public health management of the ongoing pandemic.

The SARS-CoV-2 mRNA and viral vector vaccines induce a strong immune response through the promotion of both innate and adaptive immunity against the spike protein[4]. Of note, there has been reported to be a promotion of inflammatory cytokines IFNy, IL-15 and IL-6 secretion in response to vaccination[5] each known to impact intestinal epithelial architecture and mucosal immunity[6, 7, 8]. The sequestration of the spike mRNA by antigen-presenting cells aims to limit the spread into systemic circulation, however the SARS-CoV-2 spike protein was also found in blood plasma[9], potentially leading to inflammation at different sites of the body other than the site of vaccination including that of the gut. This led us to hypothesize that the systemic immune response to the SARS-CoV-2 vaccination may impact the gut microbiota.

Numerous studies have analysed the gut microbiome during COVID-19 infection[10], finding notable depletion in both commensal bacterial, such as *Bacteroides* and *Bifidobacterium spp*. and beneficial *Lachnospiraceae,* coupled with increased abundances of opportunistic pathogens such as *Streptococcus* and *Clostridium hathawayi*; this indicates a marked dybiosis induced by COVID-19 infection. Yet since the initiation of the vaccination programme against COVID-19, very few studies have addressed the impact of the vaccination on the gut microbiome[11,12,13,14]. Previous work has addressed the link between the gut microbiome and vaccine immunogenicity. Primarily, baseline abundances of certain bacterial species before the first vaccine dose have been correlated with a defined end point of vaccine efficacy, typically a vaccine-related readout, such as virus neutralisation or spike-specific antibody titres.

Yet, two open questions remain: how the gut microbiome is affected by COVID-19 vaccination in the days following vaccination when the inflammatory response is at its highest, and are any immediate changes in the gut microbiome maintained or resolved once humoral immunity has been initiated. Addressing this knowledge-gap could help understand the extent and the nature of reciprocal links between the gut microbiome and systemic

immunity in the context of vaccination. We therefore sought to analyse the gut microbiome of patients receiving doses of the COVID-19 vaccines to decipher whether there were any notable, characteristic changes in the gut microbiome in either healthy or immunocompromised individuals [Table 1]. The immunocompromised patients we recruited cancer patients receiving immune checkpoint modulating therapies and patients with primary immunodeficiencies. These cohorts consist of patients with impaired immunity resulting from either therapeutic intervention or monogenic defects in immunoregulatory genes (CTLA4, NFKB1, CD40L), respectively.

This presented us with the unique opportunity to elucidate whether the COVID-19 vaccines alter the gut microbiome in the absence of complete, functional immunity and subsequent impairments of the regulation of the gut microbiome.

Methods

Study recruitment and ethics

Participants volunteered and were enrolled to one of 3 cohorts, healthy controls, cancer patients which presented with either melanoma or renal malignancies, or primary immunodeficient patients with defined mutations in key immunoregulatory genes as well as patients with clinical presentation aligning to that of defined primary immunodeficiency, including antibody deficiency [Table 1]. Almost all patients in each cohort received BNT162b2 Pfizer vaccine, aside from one patient at second dose receiving AstraZeneca, and 4 patients receiving Moderna at the third [Table 1]. Patients were excluded if presenting with positive COVID-19 serology or if presenting in hospital with clinal symptoms/features related to their disease which may influence the physiological response to the COVID-19 vaccination. The research was conducted in accordance with the principles of Good Clinical Practice and following approved protocols of the NIHR National Bioresource. Samples were collected with the written informed consent of all study participants under the NIHR National BioResource - Research Tissue Bank (NBR-RTB) ethics (REC:17/EE/0025. The patients and healthy controls were consented under the East of England Cambridge South national research ethics committee (REC) reference: 13/EE/0325).

Participant sampling

Participant samples were anonymised by clinical staff prior to sample delivery to the research lab. Peripheral blood and fecal samples were collected longitudinally over the course of up to three doses of the vaccines against COVID-19 (sample coverage varied across doses), spanning the course of 2021. Peripheral blood mononuclear cells (PBMCs) were extracted from blood samples using density gradient centrifugation, stored temporarily

at -80°C, before being transferred to long-term storage in liquid nitrogen. Serum was isolated from peripheral blood via centrifugation and stored at -80°C until required. Fecal samples were collected at 3 timepoints around each vaccine dose: pre-dose (94% of samples taken within 3 days prior to vaccination, the remaining 3 samples taken up to 14 days prior), acute (day 2 or 3 after vaccination) or late (day 16–28 after vaccination). Fecal matter was collected in OMNIgene•GUT kits (DNA Genotek, Canada) whereby samples are stored in a stabilizing, inactivating solution. Samples were transported to the lab and homogenized upon arrival before being stored at -80°C until required.

Fecal DNA extraction and sequencing

DNA was extracted from fecal samples using QIAamp® PowerFecal® Pro DNA kits (Qiagen). Samples were thawed and approximately 250mg of fecal sample was lysed via bead beating. According to the kit protocol, the sample was then cleaned of non-DNA organic and inorganic material, then washed using ethanol. DNA was eluted into 10mM Tris and quantified using the Qubit™ 1X dsDNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, UK) using the Qubit™ fluorometer (Thermo Fisher Scientific, UK). DNA at a concentration of 10mg/uL was sent for sequencing. Shotgun metagenomic sequencing was performed with Illumina NextSeq 2000 sequencing platform using paired-end reads of 150bp in length.

Shotgun metagenomic analysis

Raw sequencing data was pre-processed with PRINSEQ++[18] in paired read mode, quality trimming to a minimal Phred score of 30 in a window of 15 bases and removing reads of less than 75 bp length after trimming. Additionally, host contamination was removed by mapping against the GRCh38 reference human genome using Bowtie2[19] (v2.4.5) and removing any mapped reads from the dataset. Raw, trimmed and filtered reads were checked for quality using FastQC[20]. From the remaining read pairs, taxonomic profiling was determined using mOTUs3[21] profiler. For functional profiling, the remaining read pairs after filtering were assembled using metaSPAdeS[22] with a *k*-mer size of 55. The resulting scaffolds were filtered for at least 200 bp length and weighted by their average coverage of the filtered reads to enable quantitative analysis. The remaining scaffolds were aligned to the EggNOG database[23] (downloaded on 2022/04/08) using DIAMOND (v2.0.13). Microbiome analysis was performed in R using phyloseq[24] and vegan[25] packages. Differential abundance analysis was performed using DESeq2[26].

Serological assessment of immune response to COVID-19 vaccines

Serum samples were thawed, heat-inactivated at 56°C for 30 mins and measured for the dilution of serum that reduces viral activity by 50% (NT_{50}); the method for which has been

previously described[15]. For anti-SARS-CoV-2 specific IgG antibodies, we utilised a previously described method[16,17], in which Luminex bead sets are covalently coupled to the recombinant SARS-CoV-2 proteins nucleocapsid protein (NCP), spike (S) and receptor-binding domain (RBD) to quantify antibody levels.

Statistical analysis

Wilcoxon tests with multiple testing correction using the false discovery rate were deployed throughout, using pairwise comparison where appropriate. Mixed effect linear modelling was performed using lmer4 in R. Correlation was determined using Spearman's Rank coefficient.

Results

The composition of gut microbiome is not altered by vaccination against COVID-19

To investigate the impact of the COVID-19 vaccines on the gut microbiome, shotgun metagenomic sequencing was performed on a total of 239 fecal samples from 59 patients from our three cohorts (43 healthy control, 160 cancer and 36 primary immunodeficient patient samples). Samples were taken over the course of 3 vaccine doses, at one of three vaccine timepoints pre-dose (before vaccination), acute (2–3 days after vaccination) or late (16–28 days after vaccination) for each vaccine dose [Fig1A]. Pre-dose sampling provides a baseline assessment of the gut microbiome prior to vaccination, whilst acute samples provide the opportunity to assess the effect of vaccination on the gut microbiome at the height of the initial inflammatory response, and late samples allow a determination of any resolution or maintenance of acute effects.

We noted significant differences in the alpha diversities of samples taken from our cohorts [Supplementary Figure 1A], however when assessing samples taken at different vaccine timepoints from within each cohort, we did not observe any significantly differences [Figure 1B]. To further, when assessing samples from the same patient at different vaccine timepoints using a paired sample analysis approach, we also did not see any significant differences in the alpha diversities of our patient samples [Supplementary Figure 1B]. This indicates that the COVID-19 vaccine is not affecting the existing diversity of the gut microbiome, despite the distinct microbial diversity between the patient cohorts.

We next used principal component analysis to visualize the beta-diversity of our microbiome composition data that includes the abundance of all detected operational taxonomic units (OTUs) [Fig1C]. The principal components (PCs) describe the largest variation components in the dataset, representing shifts in microbiome composition and potentially reflecting to the abundance changes of bacterial species between the samples. The first 5 principal

components were responsible for the 2.9%, 2.4%, 2.1%, 2.0% and 1.9% of variation in the data respectively, and were further analysed using mixed effect linear models with multiple input variables from our available metadata. In our linear models we asked whether the vaccination timepoint of the samples in each cohort could improve the explained variance of the PCs when compared to a baseline model describing the explained variance using patient samples as the grouping variable. We found that there was no significant improvement on the baseline model [Supplementary Figure 1C]. This suggests that the variance we see in our samples is not a signature of the COVID-19 vaccines, rather those of the patients presenting with different microbiome compositions.

Next, we asked whether the COVID-19 vaccines induce any changes in the phylum-level composition of the gut microbiome and profiled the relative abundance of taxa at the phylum level across all samples, [Fig1D] observing variation in our patient samples. Moreover, when comparing the top 6 most prevalent phyla, no significant differences were observed between vaccine timepoints despite significant differences in these phyla between cohorts [Fig1E] [Supplementary Figure 1D]. We similarly observed no significant differences when using the paired sample analysis [Supplementary Figure 1E]. This demonstrates that the COVID-19 vaccines do not alter the composition of the gut microbiome irrespective of the unique compositions found in our cohort samples.

COVID-19 vaccination does not induce species level changes in the gut microbiome

We next sought to analyse differentially abundant microbial species between vaccine timepoints using DESeq2. All cohorts were analysed independently for the abundance changes in samples taken at each vaccine timepoint with the most differentially abundant species presented in a representative heatmap of log2 fold-change in abundance. For the cancer cohort, when assessing the change in abundance of these top differential responding bacterial species between samples taken pre-dose and acutely, unsupervised clustering does not demonstrate evident grouping [Fig2A]. Among all the species, only two were significantly increased in acute samples compared to pre-dose samples, *Klebsiella pneumoniae* and *Butyrivibrio crossotus* found in 11 [p = 1.01e-24] and 5 samples [p = 7.63e-12] out of the 160 cancer patient samples respectively [Fig2B]. The former is only representative in a quarter of the cohort, only melanoma patients, and within those has an average relative abundance of 0.7% [Fig2C]; the latter in 2 renal cancer patients, representing on average 3% of the relative abundance. Considering approximately 2500 species are represented across all patient samples, change in 2 low-abundant and sparsely represented species signifies negligible changes. Similar findings were seen for our other two cohorts, healthy controls and primary immunodeficient patients [Supplementary Figure

2]. When performing paired sample analysis in the cancer cohort using DESeq2, we find that no significantly altered species between pre-dose and acute, or pre-dose and late samples; the same is demonstrated for the healthy controls. Samples from one primary immunodeficient patient showed a significant reduction in *Enterobacter sp.* in an acute sample compared to pre-dose. This demonstrates that on a species level there is no unified, biologically relevant change in abundance of microbial species induced by the COVID-19 vaccines.

As the differential abundance analysis considers the change in abundance of all species irrespective of their relative abundance within each sample, we were curious whether there were any noticeable changes in the most abundant species found within each patient cohort that could be attributed to the vaccine timepoints. There was no significant difference in any of the most abundant 15 species in samples taken at any of the three vaccine timepoints [Fig2D], representing on average 47% of the relative abundance of the species within patient samples in the cancer cohort, 50% within the healthy controls and 53% within the primary immunodeficient patient cohort. This indicates that we see no effect of the vaccine on the species occupying the highest proportion of the abundant microbial species. Thus, on a species level there is no unified, biologically relevant change in abundance of microbial species induced by the COVID-19 vaccines.

There was considerable concern both at the time of the initial vaccine programme, and to this day, on the safety of COVID-19 vaccines. Given that, we sought to observe if there was any differential outgrowth of bacterial species that have been associated to various immune-related diseases, including gastric cancer and autoimmunity, as well as metabolic and neurological diseases. Although our study cannot address long-term outgrowth of bacterial associated to these diseases, we are able to highlight if there is any temporal, acute changes in these species which is still of physiological relevance. *Faecalibacterium prausnitzii,* which is reported to be reduced in both gastric cancers, autoimmunity, and Crohn's disease[27], showed no significant abundance changes at the vaccine timepoints in our cohorts [Fig2E]. We also found no presence of *Helicobacter pylori*, which is strongly associated with the initiation and development of gastric cancers[28]. *Akkermansia muciniphila,* found to be increased in obesity[29] and correlated with response rates to immune checkpoint blockade therapies in various malignancies[30], was not altered by vaccination in all three cohorts [Fig2F]. In studies of Alzheimer's, *Escherichia coli* has been demonstrated to promote neurodegeneration[31], in our samples we did not see significant difference induced by the vaccine in any of our cohorts [Fig2G]. This supports that the COVID-19 vaccine does not promote the change in abundance of microbes that are associated with

various immune-related diseases within our patient cohorts and is indicative of no greater risk of the aforementioned diseases as a result of COVID-19 vaccination.

The magnitude of the response to the COVID-19 vaccines is not correlated with gut microbiome diversity

Within the current literature, a few studies have reported changes in the gut microbiome that correlate with vaccine efficacy[12,13,14], so we sought to determine whether the gut microbiome composition was related to the magnitude of the COVID-19 vaccine response. We performed an assessment of vaccine efficacy using a live-virus neutralisation assay, as a predictive measure of vaccine protection[31], to assess whether vaccine efficacy had correlation with microbial diversity.

When taking the neutralising capacity of patient serum at both second dose [Fig3A] and third dose [Fig3B], we asked whether the Shannon diversity of gut microbiome at different vaccine timepoints, correlated with neutralisation. We did not see any correlation between diversity and vaccine efficacy in any of our patient cohorts; the same is true for the quantity of anti-spike IgG antibodies [Supplementary Figure 3]. This indicates that the magnitude of the immune response was not correlated with the gut microbiome diversity in our patient cohorts, thus suggesting that improved efficacy of the vaccine does not come at a cost of microbial disturbance.

The gut microbiome functional capacity was not affected by COVID-19 vaccines

Having investigated the composition and relative abundance of the microbial species that constitute the gut microbiome, we next sought to investigate whether the functional capacity of the microbial species was altered by the COVID-19 vaccines. Using the EggNOG database, we assigned functional annotations to the sequenced metagenomes. The highest level of functional annotation depicts three functional groups, cellular processes and signalling, information storage and processing, and metabolism. In these, we did not see any significant differences between the vaccine timepoints within our cohorts [Fig4A]; similar to taxonomic data presented earlier, when combining samples from within the same cohorts, there are significant changes [Supplementary Figure 4A].

We next observed the abundance of the 22 defined functional groups in the next functional annotation level down in the separate vaccine timepoints within each patient cohort [Fig4B]. Representative graphs of the most abundant functional annotations within each of the

highest three level functional levels remained unchanged after vaccination within our patient cohorts [Fig4C], the same is true for the remaining 19 (Supplementary Figure 4B].

At the lowest functional annotation level, we interrogated the abundance of cluster of orthologous genes (COGs) at separate vaccine timepoints within our cohorts. Remarkably, only two COGs, out of a possible 2142 presented in our patient samples, were significantly different as a result of the COVID-19 vaccines, in control samples COG2243 Precorrin-2 c20-methyltransferase between acute and late vaccine samples ($p = 0.04$ [FDR]), and COG4750 cytidylyltransferase choline kinase between pre-dose and late samples in cancer patients ($p = 0.03$ [FDR]); not a single COG was found to be significantly altered in primary immunodeficient patient samples as a result of the vaccine. This demonstrates that the functional annotations of the gut microbiome are not altered by the administration of the COVID-19 vaccines.

Discussion

To our knowledge, this study is the first to assess the gut microbiome composition in response to the COVID-19 vaccines across multiple doses and at multiple timepoints with samples taken pre-dose, acutely and late following vaccination. The study is also the first to assess the effect of the COVID-19 vaccines on the gut microbiome in cancer patients and in patients with inborn errors of immunity associated with severe immune dysregulation. As sampling across the cohorts varied throughout, we opted to combine the three vaccine doses and assess vaccine timepoints or samples from within each cohort. This allowed us to better observe the influence of the COVID-19 vaccines in these contexts.

The relative abundance of microbes within the gut microbiome has more recently been assessed with vaccine immunogenicity including that of vaccines against SARS-CoV-2 virus. The first reported study of the gut microbiome in COVID-19 vaccinated patients correlated vaccine immunogenicity of the inactivated virus, CoronaVac, and the mRNA vaccine encoding spike protein, BNT162b2 vaccine, with the baseline abundance of gut *Bifidobacterium adolescentis* and *Roseburia faecis* respectively[12]. We did not observe changes in these bacterial species. Another study investigated the variability of the COVID-19 vaccine response to the gut microbiome by correlating RNAseq data with microbial abundance using 16S rRNA gene amplicon sequencing[14]. They identified several differentially abundant taxa between high- and low-antibody responders and high- and low-T-cell responders. In the context of immunocompromised cohorts, a previous study assessed patients with inflammatory bowel disease[13] well known to be characterised by gut microbiome dysbiosis[33], who were receiving anti-TNF immunomodulators. Their study did

not demonstrate changes in diversity in above geometric mean vaccine responders but found *Bilophila* abundance correlated to an improved response. They also assessed the fecal metabolome showing that various metabolites were associated with a better vaccine response; several metabolites were also associated with a reduced response. What these studies have in common is associating microbiome composition to vaccine immunogenicity, however these studies have not addressed temporal changes that can be seen in the gut microbiome in the days following vaccination, whereas our longitudinal study design affords us the opportunity to do so.

In our patient cohorts, we did not find any significant effect on the diversity of the gut microbiome following COVID-19 vaccination despite considerable differences between the cohorts. In line with the studies investigating the gut microbiome of patients with primary immunodeficiencies[34], we also observed decreased diversity in our cohort compared to control samples. These patients have been demonstrated to exhibit increased gut permeability with higher rates of bacterial translocation[35], perhaps indicative that there is bidirectional permeability of systemic immunity affecting the gut microbiome. In our study, we find no influence on microbiome variation after vaccination in patients at genetically determined persistent state of immune dysregulation.

In melanoma patients, the presence of species bacteria species from the *Actinobacteria* and *Firmicutes* phylum have been associated with better responses to immune checkpoint blockade therapies[36, 37, 38] An *Akkermansia muciniphilia* signature was also found in renal cancer patients responding better to immune checkpoint blockade therapy[39,40]. While cancer progression is reported to be linked to gut microbiome composition and its derived metabolites, these associations vary between cancer types[41]. We saw wide compositional variation within our cancer cohort samples, perhaps due to the wide range of disease presentation and treatment included in our patients. Our study did not consider factors such diet and medications known to affect the gut microbiome[42]. Nevertheless, this is not critical as our analysis suggests stability as opposed to specific changes and post-hoc power calculation indicates sufficient power against false negatives (for effect size Cohen's d = 0.55, estimated power = 0.8). Cancer patients' response to vaccination depend significantly on cancer type, for example antibody-related immune responses in solid cancers are better than in haematological cancers[43]. It is therefore notable that in an immunologically diverse cohort of individuals, with varied vaccine responses, we did not observe any effect of the COVID-19 vaccination on the gut microbiome, indicating stability irrespective of pre-dose composition.

While we did not observe any changes at any taxonomic level or functional capacity, we cannot rule out genetic changes at mutational levels that may alter the microbiota function.

An independent functional validation such as metabolomics to look for bacterial derived short-chain fatty acids, tryptophan and bile acid metabolites known to mediate microbiome-host interactions[44] could be utilised to assess this. Sampling from the PID cohort was limited reflecting the rarity of the individuals within the general population. Despite this, we were able to recruit patients characterised by monogenic defects in both intrinsic and extrinsic B-cell aetiologies, thus representing multiple facets within the rare PID population. Further, we were unable to sample all patients at all vaccine timepoints across all vaccine doses. Nevertheless, our findings still bare relevance as we assess patient cohorts individually and where possible the paired sample data analysis aligns with the overall findings.

Although the global vaccination efforts have controlled the spread of the SARS-CoV-2 virus, there were still reported to be 2.6 million new cases within the past month[1], highlighting that prevention of disease through vaccination is still relevant for public health. Considering the measurable impact of common life factors such as alcohol consumption, meat intake and commonly used medications on the microbiota[45], our study finds that the vaccination has negligible, if any, impact on microbiome-mediated processes. The contrast is even starker when considering large microbiome changes have been reported for COVID-19 infection[10]. Our findings shows that the gut microbiome remains stable post-vaccination and provides an additional reassurance towards promoting vaccine uptake.

Data availability

Sequencing data will be uploaded to the European Genome-phenome Archive, whilst code will be available on GitHub: https://github.com/RHBoston/COVID-19_Vaccination_GM.

Acknowledgements

We are grateful to the clinical staff at Addenbrookes hospital who facilitated patient recruitment and sample collection, and the members of the Thaventhiran and Patil lab for help in processing the samples.

Competing interests

The authors declare no competing interests.

Funding declaration

This work was funded by the UK Medical Research Council (project numbers MC_UU_00025/11 and MC_UU_00025/12).

References

[1] World Health Organization. Available: https://covid19.who.int [Accessed 30th August 2023].

[2] Lind, M.L., *et al.* Evidence of leaky protection following COVID-19 vaccination and SARS-CoV-2 infection in an incarcerated population. *Nat Commun* **14**, 5055 (2023).

[3] Carrieri V, Guthmuller S, Wübker A. Trust and COVID-19 vaccine hesitancy. Sci Rep. 2023 Jun 7;13(1):9245.

[4] Bellamkonda N, et al. Immune Response to SARS-CoV-2 Vaccines. *Biomedicines*. 2022 Jun 21;10(7):1464.

[5] Bergamaschi C, et al. Systemic IL-15, IFN-γ, and IP-10/CXCL10 signature associated with effective immune response to SARS-CoV-2 in BNT162b2 mRNA vaccine recipients. *Cell Rep*. 2021;36(6):109504.

[6] Nava P, et al. Interferon-gamma regulates intestinal epithelial homeostasis through converging beta-catenin signaling pathways. *Immunity*. 2010;32(3):392-402.

[7] Pagliari D, et al. The role of IL-15 in gastrointestinal diseases: a bridge between innate and adaptive immune response. *Cytokine Growth Factor Rev*. 2013;24(5):455-466.

[8] Wu S, et al. Interleukin-6 absence triggers intestinal microbiota dysbiosis and mucosal immunity in mice. *Cytokine*. 2022;153:155841.

[9] Röltgen K, et al. Immune imprinting, breadth of variant recognition, and germinal center response in human SARS-CoV-2 infection and vaccination. *Cell*. 2022 Mar 17;185(6):1025-1040.e14.

[10] Rocchi G, et al. Gut Microbiota and COVID-19: Potential Implications for Disease Severity. *Pathogens*. 2022; 11(9):1050.

[11] Leung JSM. Interaction between gut microbiota and COVID-19 and its vaccines. *World J Gastroenterol.O* 2022 Oct 28;28(40):5801-5806.

[12] Ng SC, et al. Gut microbiota composition is associated with SARS-CoV-2 vaccine immunogenicity and adverse events. Gut. 2022 Jun;71(6):1106-1116.

[13] Alexander JL, et al. The gut microbiota and metabolome are associated with diminished COVID-19 vaccine-induced antibody responses in immunosuppressed inflammatory bowel disease patients. *EBioMedicine*. 2023 Feb;88:104430.

[14] Hirota M, et al. Human immune and gut microbial parameters associated with inter-individual variations in COVID-19 mRNA vaccine-induced immunity. *Commun Biol*. 2023 Apr 20;6(1):368.

[15] Gerber PP, et al. A protease-activatable luminescent biosensor and reporter cell line for authentic SARS-CoV-2 infection. PLoS Pathog. 2022 Feb 10;18(2):e1010265.

[16] Xiong X, et al. A thermostable, closed SARS-CoV-2 spike protein trimer. Nat Struct Mol Biol. 2020 Oct;27(10):934-941.

[17] Collier DA, et al. Age-related immune response heterogeneity to SARS-CoV-2 vaccine BNT162b2. Nature. 2021 Aug;596(7872):417-422.

[18] Cantu VA, Sadural J, Edwards R. PRINSEQ++, a multi-threaded tool for fast and efficient quality control and preprocessing of sequencing datasets. PeerJ Preprints. 2019. 7:e27553v1

[19] Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012 Mar 4;9(4):357-9.

[20] Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online]. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[21] Ruscheweyh, HJ., Milanese, A., Paoli, L. et al. Cultivation-independent genomes greatly expand taxonomic-profiling capabilities of mOTUs across various environments. Microbiome 10, 212 (2022).

[22] Nurk S, et al. metaSPAdes: a new versatile metagenomic assembler. Genome Res. 2017 May;27(5):824-834.

[23] Huerta-Cepas J, et al. eggNOG 5.0: a hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res. 2019 Jan 8;47(D1):D309-D314.

[24] McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE (2013). 8(4): e61217.

[25] Oksanen J, et al. vegan: Community Ecology Package_. R package version 2.6-4, (2022). <https://CRAN.R-project.org/package=vegan>.

[26] Love, M.I., Huber, W., Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2 Genome Biology (2014). 15(12):550

[27] Islam MZ, et al. Reproducible and opposing gut microbiome signatures distinguish autoimmune diseases and cancers: a systematic review and meta-analysis. Microbiome. 2022 Dec 9;10(1):218.

[28] Polk DB, Peek RM Jr. Helicobacter pylori: gastric cancer and beyond. Nat Rev Cancer. 2010 Jun;10(6):403-14.

[29] Aoun A, Darwish F, Hamod N. The Influence of the Gut Microbiome on Obesity in Adults and the Role of Probiotics, Prebiotics, and Synbiotics for Weight Loss. Prev Nutr Food Sci. 2020 Jun 30;25(2):113-123.

[30] Shi Z, Li H, Song W, Zhou Z, Li Z, Zhang M. Emerging roles of the gut microbiota in cancer immunotherapy. Front Immunol. 2023 Feb 22;14:1139821.

[31] Bairamian D, et al. Microbiota in neuroinflammation and synaptic dysfunction: a focus on Alzheimer's disease. Mol Neurodegener. 2022 Mar 5;17(1):19.

[32] Khoury DS, Cromer D, Reynaldi A, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. *Nat Med*. 2021;27(7):1205-1211.

[33] Qiu P, et al. The Gut Microbiota in Inflammatory Bowel Disease. Front Cell Infect Microbiol. 2022 Feb 22;12:733992.

[34] Castagnoli R, et al. Gut Microbiota-Host Interactions in Inborn Errors of Immunity. Int J Mol Sci. 2021 Jan 31;22(3):1416.

[35] Ho HE, et al. Circulating bioactive bacterial DNA is associated with immune activation and complications in common variable immunodeficiency. JCI Insight. 2021 Oct 8;6(19):e144777.

[36] Routy B, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science. 2018 Jan 5;359(6371):91-97.

[37] Matson V, et al. The commensal microbiome is associated with anti-PD-1 efficacy in metastatic melanoma patients. Science. 2018 Jan 5;359(6371):104-108.

[38] Frankel AE, et al. Metagenomic Shotgun Sequencing and Unbiased Metabolomic Profiling Identify Specific Human Gut Microbiota and Metabolites Associated with Immune Checkpoint Therapy Efficacy in Melanoma Patients. Neoplasia. 2017 Oct;19(10):848-855.

[39] Derosa L, et al. Gut Bacteria Composition Drives Primary Resistance to Cancer Immunotherapy in Renal Cell Carcinoma Patients. Eur Urol. 2020 Aug;78(2):195-206.

[40] Salgia NJ, et al. Stool Microbiome Profiling of Patients with Metastatic Renal Cell Carcinoma Receiving Anti-PD-1 Immune Checkpoint Inhibitors. Eur Urol. 2020 Oct;78(4):498-502.

[41] Yang Q, et al. A Review of Gut Microbiota-Derived Metabolites in Tumor Progression and Cancer Therapy. Adv Sci (Weinh). 2023 May;10(15):e2207366.

[42] Maier L, et al. Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature*. 2018;555(7698):623-628.

[43] Hiam-Galvez KJ, Allen BM, Spitzer MH. Systemic immunity in cancer. Nat Rev Cancer. 2021 Jun;21(6):345-359. doi: 10.1038/s41568-021-00347-z.

[44] Wang J, et al.. Gut-Microbiota-Derived Metabolites Maintain Gut and Systemic Immune Homeostasis. Cells. 2023 Mar 2;12(5):793.

[45] Lindell AE, Zimmermann-Kogadeeva M, Patil KR. Multimodal interactions of drugs, natural compounds and pollutants with the gut microbiota. *Nat Rev Microbiol*. 2022;20(7):431-443.

517 **Table 1.** Characteristics of the participants in this study.

518

Cohort	*Participants*	*Age*	*Vaccine type*	*Condition*	*Treatment*
Healthy Controls	6F 9M	28–59 Mean = 43.7	Vaccine doses, n = 20 90% Pfizer doses 10% Moderna doses	NA	NA
Immune checkpoint treated cancer patients (ICP)	9F 26M	39–86 Mean = 61.7	Vaccine doses, n = 70 97% Pfizer doses 3% Moderna doses	11 Metastatic Melanoma, 10 Adjuvant Melanoma, 5 Melanoma controls, 6 Metastatic Renal, 3 Renal controls	3 Nivolumab, 13 Pembrolizumab, 10 Ipilimumab + Nivolumab, 1 Ipilimumab + Pembrolizumab
Primary Immunodeficient patients (PID)	4F 5M	19–61 Mean = 41.1	Vaccine doses, n = 19 95% Pfizer doses 5% AstraZeneca doses	1 CD40L deficiency, 2 CTLA4 deficiency, 4 NFKB1 deficiency, 2 Undiagnosed condition	5 intravenous immunoglobulin, 3 Antibiotics

519
520

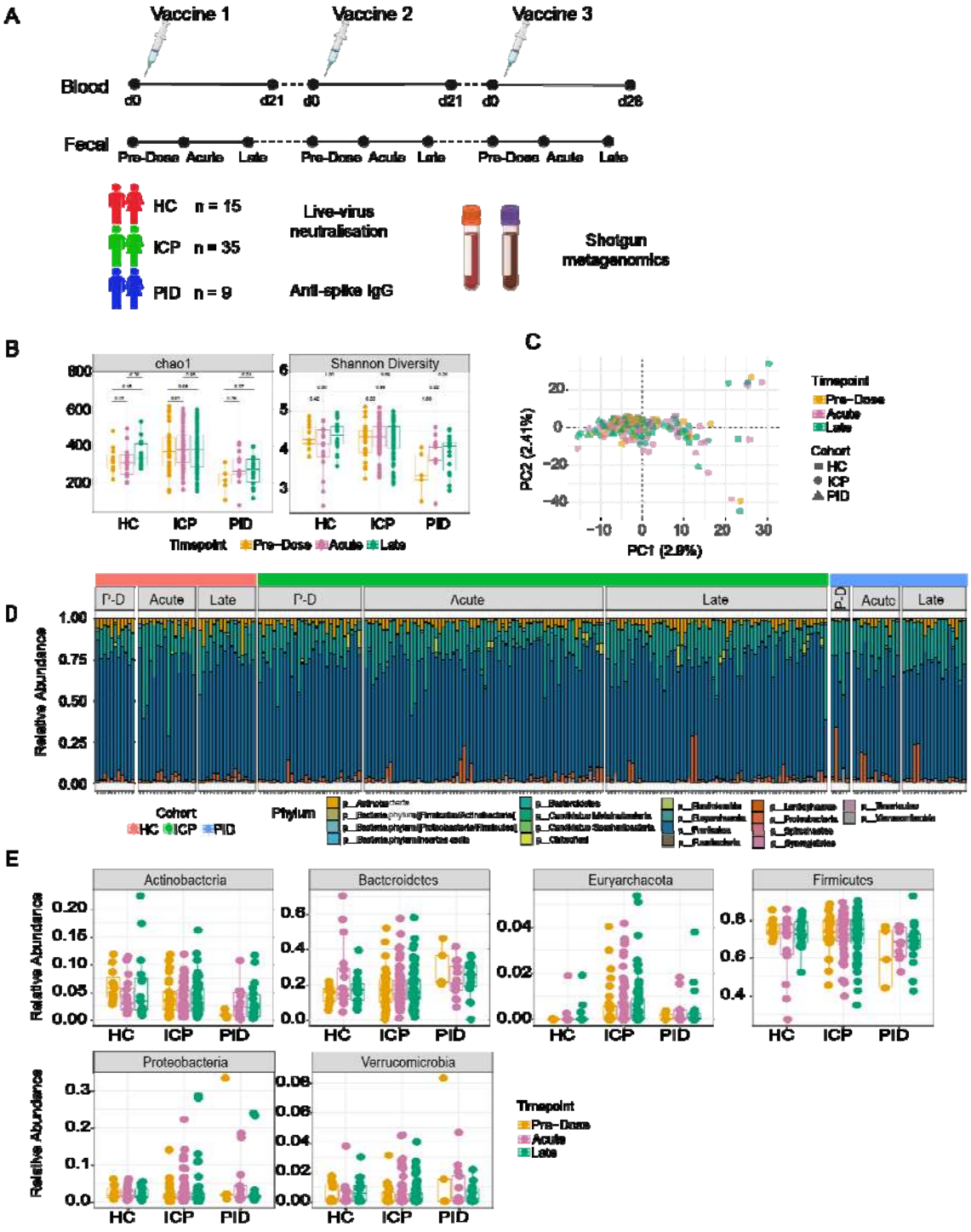

Figure 1. *(A) Study design. 59 patients were recruited for longitudinal analysis of the effect of the vaccines against COVID-19. Samples were assigned to one of three patient cohorts, healthy control (HC), immune-checkpoint therapy treated cancer patients (ICP), or patients with primary immunodeficiencies (PID). Blood samples were analysed for their live-virus neutralisation capacity and quantifying the amount of anti-spike IgG antibodies, whilst fecal samples were analysed with shotgun metagenomics for taxonomic and functional annotations. (B) Diversity measures of chao1*

and Shannon assessed in fecal samples taken from different vaccine timepoints, from within healthy control (HC), immune-checkpoint therapy treated cancer patients (ICP) and patients with primary immunodeficiencies (PID). Statistical testing performed using Wilcoxon test and adjusted for multiple testing using FDR correction. (C) Principal component (PC) analysis at the operational taxonomic unit level. Each dot represents a unique sample from within each cohort (shapes) taken at unique timepoints following vaccination (colours). (D) Relative abundance at the phyla taxonomic level depicted by colours of each of the bars, from samples taken from each of the cohorts (HC, ICP and PID), separated by the vaccine timepoints from which the sample was taken; PD = Pre-Dose, Acute and Late. (E) Relative abundance of the 6 most prevalent phyla in patient samples from within each of the cohorts and separated by the vaccine timepoint from which the sample was taken. Statistical testing performed using Wilcoxon test and adjusted for multiple testing using FDR correction.

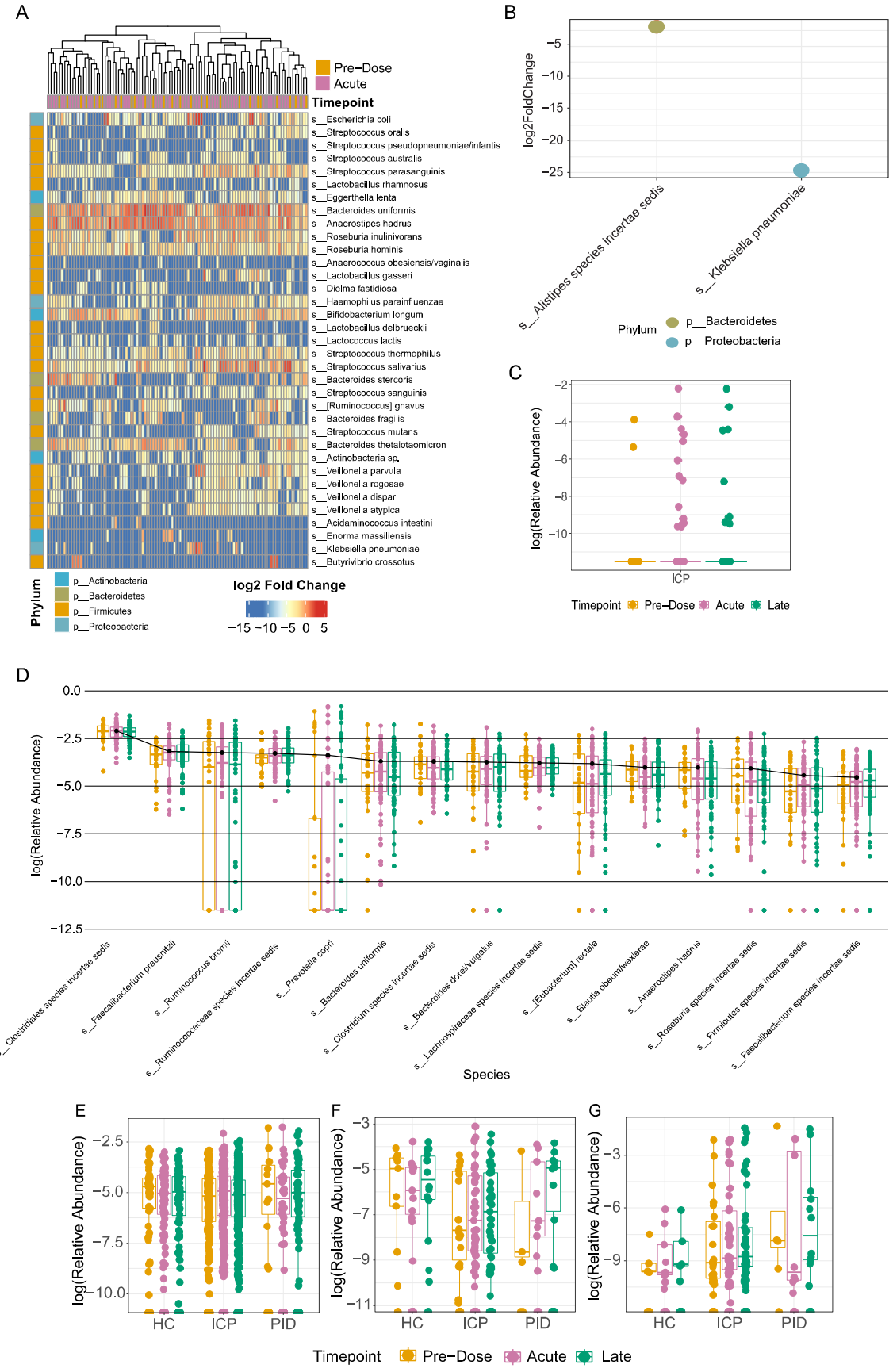

Figure 2. *Representative analysis of the cancer cohort (ICP) demonstrates minimal change attributable to the COVID-19 vaccines. (A) Differential abundance analysis using DESeq2 of the log2 fold-change of the top 35 differential species between samples taken at pre-dose and acutely after vaccination. (B) Log2 fold-change of the significant differential abundant species taken from the DESeq2 analysis. (C) Relative abundance of Klebsiella pneumoniae* in ICP cohort samples. *(D) Relative abundance of the top 15 abundant species within the ICP cohort taken at each of the vaccine timepoints. Relative abundance of various bacterial species correlated with immune-related diseases: Faecalibacterium prausnitzii (E), Akkermansia muciniphila (F) and Escherichia coli (G) within patient samples taken at each vaccine timepoint.*

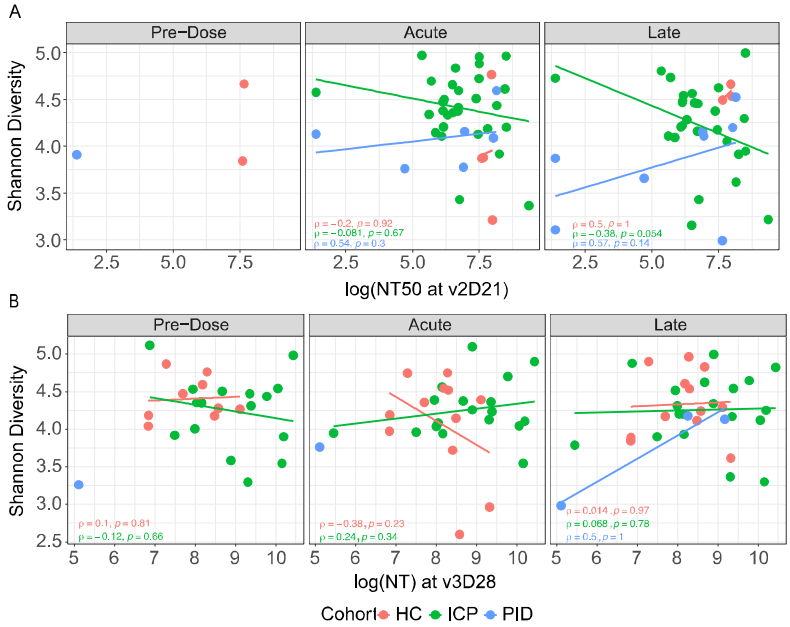

Figure 3. *Vaccine efficacy is not correlated with the gut microbiome composition. Live-virus neutralisation (NT) capacity assessed against Shannon diversity of fecal samples, each point represents a different sample taken at one of the three vaccine timepoints. Colours represent cohorts, within healthy control (HC), immune-checkpoint therapy treated cancer patients (ICP) and patients with primary immunodeficiencies (PID). Correlated vaccine response through NT capacity of patient serum taken at second dose (A) or third dose (B). rho and p values from Spearman's Rank correlation testing displayed.*

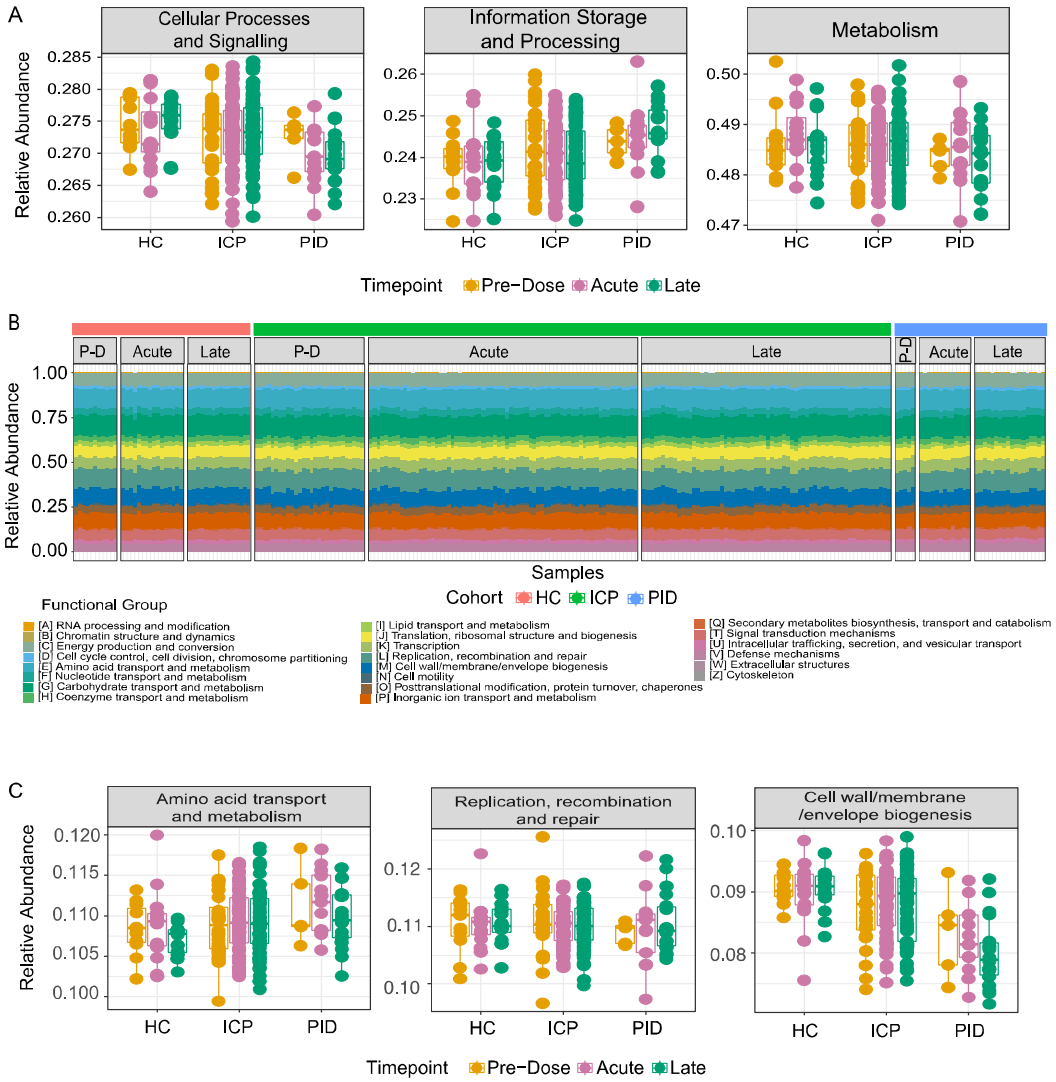

Figure 4. *Functional capacity of microbiome samples using EGGNOG database are not altered by the COVID-19 vaccines. (A) The relative abundance of the highest functional annotation level within patient samples at different vaccine timepoints in each of our patient cohorts. (B) Functional composition depicted by colours of each of the bars, from samples taken from each of the cohorts (HC, ICP and PID), separated by the vaccine timepoints from which the sample was taken; PD = Pre-Dose, Acute and Late. (C) Relative abundance of the 3 most abundant functional annotations in our patient samples from within each of our patient cohorts and separated by the vaccine timepoint from which the sample was taken. Statistical testing performed using Wilcoxon test and adjusted for multiple testing using FDR.*