Gut microbiome and dietary fibre intake strongly associate with IgG function and maturation following SARS-CoV-2 mRNA vaccination

The first study to investigate potential associations between gut microbiota composition and SARS-CoV-2 vaccine immunogenicity was recently published in *Gut*.[1] This study demonstrated a statistically significant reduction in alpha diversity and a shift in gut microbiota composition following BNT162b2 vaccination, characterised by reductions in Actinobacteriota, *Blautia, Dorea, Adlercreutzia, Asacchaobacter, Coprococcus, Streptococcus, Collinsella* and *Ruminococcus* spp and an increase in *Bacteroides cacaae* and *Alistipes shahii*. Our prospective observational study (n=52; figure 1A, online supplemental table S1) similarly showed a shift in gut microbiota after the first BNT162b2 vaccine dose (p=0.016; online supplemental figure S1A), including a reduction in Actinobacteria, *Blautia* spp (p<0.01; figure 1B), and alpha diversity (p=0.078; online supplemental figure S1B). Our data support the findings by Ng *et al*,[1] reinforcing the link between SARS-CoV-2 mRNA vaccine immunogenicity and the gut microbiota.

Ng *et al*[1] also identified strong associations between baseline gut microbiota composition and serological IgG responses to BNT162b2 vaccination. After stratifying participants as low or high vaccine responders, they showed higher abundances of *Eubacterium rectale, Roseburia faces, Bacteroides thetaiotaomicron* and *Bacteroides spp OM05-12* were associated with stronger BNT162b2 vaccine responses. Correspondingly, in our cohort, several baseline bacterial taxa significantly differed between participants with low versus high BNT162b2 vaccine responses. Specifically, we observed higher baseline counts of *Prevotella*, *Haemophilus, Veillonella* and *Ruminococcus gnavus* taxa in participants with higher RBD and Spike competitive binding antibody and IgG levels (p<0.01; figure 1C). Additional studies are needed to ascertain the clinical significance of these findings. However, together with Ng *et al*,[1] these findings further support that differences in microbiome composition and/or function modulate antibody responses to SARS-CoV-2 vaccination. Interestingly, a study recently discovered that SARS-CoV-2 specific T cells can also cross-react with microbial peptides from commensals (including *Prevotella* spp) and undefined faecal lysates.[2] Considering that individuals with milder COVID-19 showed higher frequencies of cross-reactive SARS-CoV-2 T cells[3] it is plausible that microbe-based stimulation of SARS-CoV-2-reactive T or B cells could modulate SARS-CoV-2 vaccine responses. Consistent with Ng *et al*[1] we demonstrated an association between a gut microbiome signature at baseline and SARS-CoV-2 vaccine immunogenicity; however, the specific bacterial

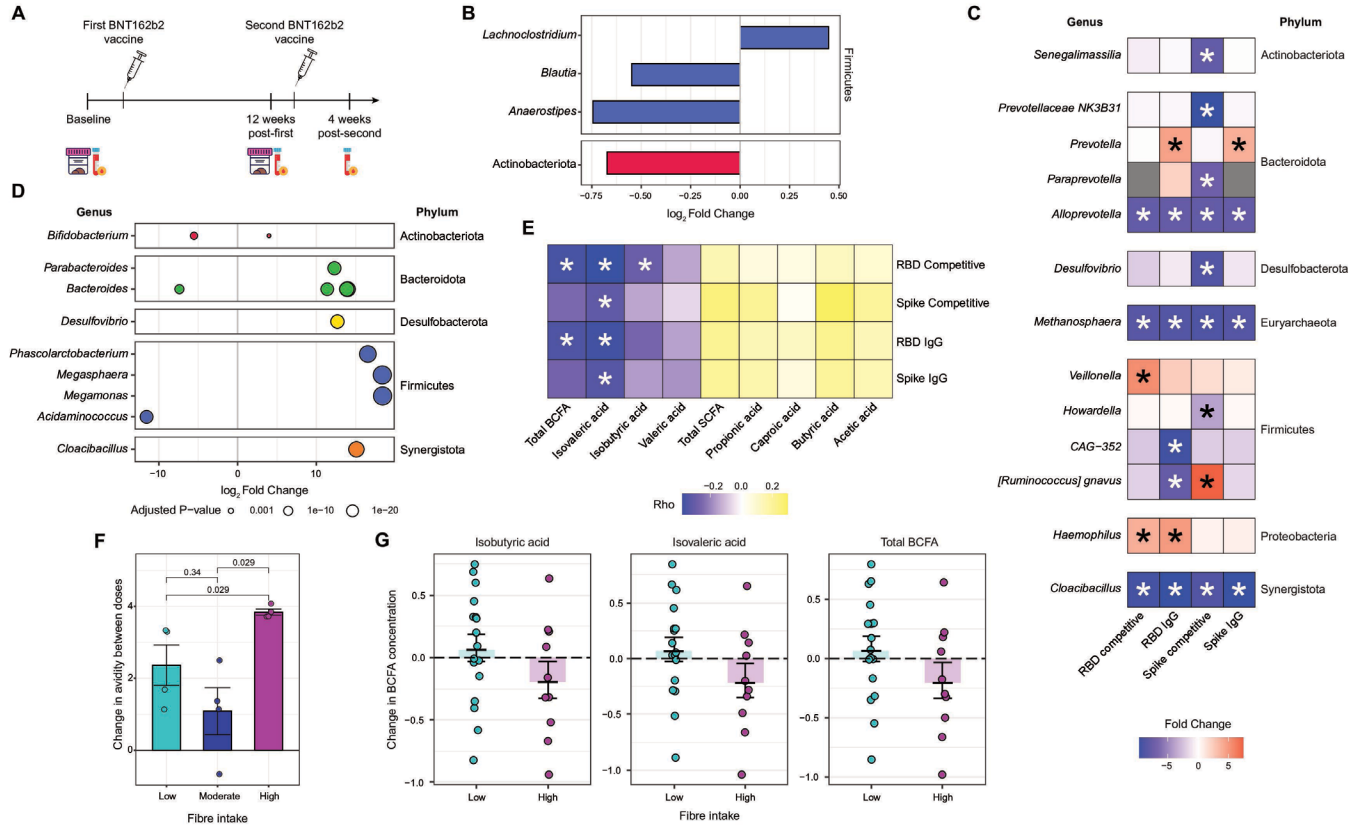

Figure 1 Impact of the mRNA SARS-CoV-2 BNT162b2 vaccine on gut microbiota composition as well as the gut microbiome and dietary factors that affect BNT162b2 vaccine response. (A) Schematic of the study design and blood and stool sample collection timepoints. (B) A significant reduction in Actinobacteriota (p<0.0001), *Anaerostipes* (p=0.00161) and *Blautia* (p=0.00103) and an increase in *Lachnoclostridium* (p=0.00179) were observed after the first BNT162b2 vaccine. (C) Heat-map depicting several significantly higher (red) or lower (blue) (*p<0.05; Wald test) baseline microbial counts in high (quartile 4) vs low (quartile 1) BTN162b2 vaccine responders across several immune parameters. (D) Several gut microbiota species were significantly positively or negatively associated (p<0.01; Wald test) with higher total relative fractional avidity after the second dose of the BNT162b2 vaccine in a subset of participants (n=15). Refer to online supplemental table S2 for the specific species names and p values. (E) Baseline total branched-chain fatty acid (BCFA) concentrations were negatively correlated with RBD IgG (p=0.02, r=−0.35; Spearman rank) and Spike IgG levels (p=0.017, r=−0.36; Spearman rank), isovaleric acid concentrations were negatively associated with RBD IgG (p=0.015, r=−0.37; Spearman rank) and competitive binding antibody (p=0.026, r=−0.34; Spearman rank), and Spike IgG (p=0.013, r=−0.38; Spearman rank) and competitive binding antibody levels (p=0.034, r=−0.32; Spearman rank), and baseline isobutyric acid concentrations were negatively associated with Spike IgG levels (p=0.047, r=−0.29; Spearman rank). (F) High dietary fibre consumers had a significantly greater change in total relative fractional avidity from the first to second BNT162b2 dose compared with low and moderate dietary fibre consumers (p=0.029; Mann-Whitney). (G) High dietary fibre consumers experienced a reduction in total BCFAs (p=0.164, adjusted t-test), isovaleric (p=0.189, adjusted t-test) and isobutyric acid (p=0.213 adjusted t-test) concentrations (−0.186 to −0.198 and −0.177 mean fold change, respectively) after BNT162b2 vaccination, whereas low dietary fibre consumers experienced an increase in total BCFAs, isovaleric and isobutyric acid concentrations (0.08, 0.08 and 0.08 mean fold change, respectively).

taxa associated with vaccine responses differed between cohorts. This is possibly due to differences in geography (Hong Kong vs Canada), dietary habits and/or microbiome sampling/analysis methods.

Moving beyond antibody levels, we explored the associations between BNT162b2 vaccine-induced antibody avidity maturation (ie, the ratio of low to high IgG antibody avidity to the Spike protein[4 5]) and specific gut microbiome signatures, in a subset of participants (n=15). Multiple bacterial taxa were negatively (ie, *Bifidobacterium bifidum*, *Acidaminococcus intestini*) or positively (ie, *Bifidobacterium animalis*, *Bacteroides plebeius*, *Bacteroides ovatus*) associated with enhanced antibody avidity (p<0.01; figure 1D), with several species having known immunomodulatory properties. Most notably, *Bacteroides ovatus* induces increased production of IgM and IgG antibodies specific to human cancer cells.[6] Additionally, *Bifidobacterium animalis* can significantly increase vaccine-specific IgG production after seasonal influenza vaccination.[7] Thus, these observations provide further evidence that gut bacterial species may enhance functional binding of IgG elicited by BNT162b2 vaccination.

We also examined the potential link between the functional capacity of the gut microbiome and habitual diets, with BNT162b2 vaccine response. Our data suggest that microbial-derived branched-chain fatty acids (BCFA) isovaleric and isobutyric acids, produced via protein fermentation, may reduce vaccine responses (p<0.05; figure 1E). BCFA concentrations are known to be higher in patients with immune-mediated conditions such as inflammatory bowel disease[8]; however, little is known about the mechanisms by which BCFAs modulate inflammation or antibody-mediated vaccine responses. Interestingly, *Megasphaera* spp, which were negatively associated with vaccine responses, are prominent isovaleric and isobutyric acid producers.[9] Future research is needed to explore the

potential impact of BCFAs on SARS-CoV-2 vaccine immunogenicity.

Lastly, no research has elucidated the role distinct dietary intakes have on SARS-CoV-2 vaccine responses. Therefore, we determined whether differing dietary fibre (microbial substrate) intakes affected IgG binding strength. We observed that the change in avidity between the first and second BNT162b2 dose was significantly greater in high fibre consumers (p=0.029; figure 1F). This further suggests that dietary fibre intakes may modulate BNT162b2 vaccine response maturation. Interestingly, high fibre consumers also experienced a reduction in total BCFAs post vaccination (p=0.164; figure 1G), signifying a potential mechanistic link between fibre intake, BCFA production and SARS-CoV-2 vaccine immunogenicity.

In summary, these data, while exploratory, validate findings from Ng *et al*,[1] reinforcing a potential link between the gut microbiome and SARS-CoV-2 mRNA vaccine antibody responses. This study further expands on these findings and shows, for the first time, that BCFA levels may negatively impact, while dietary factors, such as fibre intake, may enhance BNT162b2 immunogenicity. Studies are currently underway to explore the therapeutic benefit of microbiome-modulating interventions to enhance SARS-COV-2 vaccine immunogenicity.[10] Considering that the effectiveness of most SARS-CoV-2 vaccines are high, but relatively short-lived, especially in vulnerable age and medical groups, the gut microbiome could represent a simple, yet powerful way to optimise long-term protection or improve recovery after infection.

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Correction notice This article has been corrected since it published Online First. The first author's name has been corrected and figure 1 replaced.

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Contributors GRH, PML and BAV conceived and designed the study. LG coordinated patient recruitment and assisted with blood sample processing with AM. LG undertook the vaccine response experimental design, execution, and analyses. GRH assembled stool collection kits, undertook the dietary fibre analysis, and prepared stool samples for sequencing and short-chain fatty acid analysis. AS undertook the statistical and bioinformatic analyses. GRH wrote the manuscript with significant input from all co-authors.

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Competing interests None declared.

Patient consent for publication Not applicable.

Ethics approval This study was approved by the University of British Columbia Clinical Research Ethics Board (H20-01205).

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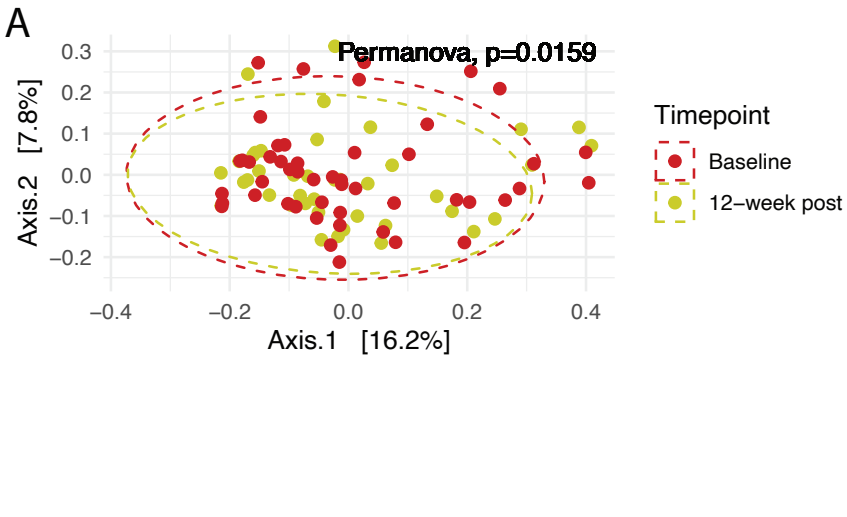
A
Permanova, p=0.0159
Axis.2 [7.8%]
Axis.1 [16.2%]
0.3
0.2
0.1
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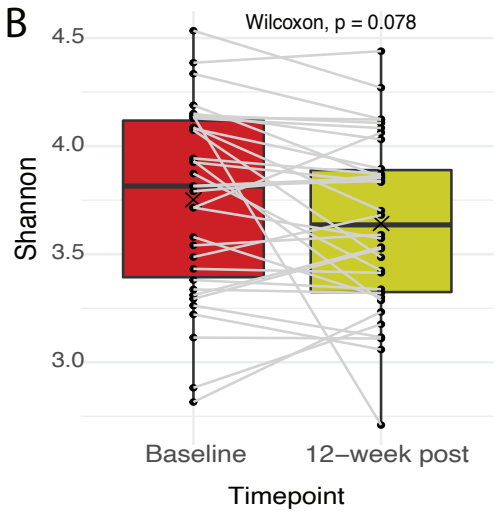
Timepoint
Baseline
12−week post


B
Wilcoxon, p = 0.078
Shannon
4.5
4.0
3.5
3.0
Baseline
12−week post
Timepoint

Supplementary Information

The gut microbiome and habitual dietary fibre intakes are associated with variable responses to the mRNA SARS-CoV-2 BNT162b2 vaccine

SUPPLEMENTARY MATERIALS AND METHODS

Study cohort

Uninfected healthcare workers aged ≥ 18 years were prospectively recruited at British Columbia Children's Hospital (Vancouver, Canada) between February and August 2021 prior to receiving two Pfizer-BioNtech BNT162b2 mRNA vaccine doses, approximately 4 months apart[1]. Participants who tested positive for SARS-CoV-2 by viral or Spike antibody testing of baseline samples or nucleocapsid antibody testing of any sample were excluded. Blood samples were collected at baseline (pre-vaccine), 12 weeks after 1st vaccination, and 4 weeks after 2nd Pfizer-BioNtech BNT162b2 mRNA vaccination. Stool samples were collected at baseline and 12 weeks after the 1st vaccine dose (**figure 1A**). This study was approved by the University of British Columbia Clinical Research Ethics Board (H20-01205). Informed e-consent for collection of blood and stool samples was obtained from all participants.

Due to the rapid initiation of SARS-CoV-2 vaccine programs for healthcare workers in British Columbia this research was particularly time sensitive as samples needed to be collected promptly prior to participants receiving their 1st dose of the BNT612b2 vaccine. Therefore, we did not have an opportunity to involve participants in the design or implementation of this study.

Participant characteristic and dietary intake data collection

Demographic data (e.g., age, sex) was collected from participants via a secure web-based application (REDCap). A standardized form was used to collect information from participants regarding antibiotic use prior to stool collection, significant changes in body weight or food intake over the previous year, whether participants were vegan or vegetarian and habitual dietary fibre intakes. Participants were classified as low (males <22 g/day, females <18 g/day), moderate (males 22 to 29.9 g/day, females 18 to 24.9 g/day), or high (males ≥30 g/day, females ≥25 g/day) dietary fibre consumers using a validated habitual dietary fibre intake food frequency questionnaire[2]. These participant characteristics were captured as they are known to modulate the gut microbiota and could potentially confound study results[3].

Serum sample analyses

SARS-CoV-2 spike and RBD IgG multiplex assay

Anti-SARS-CoV-2 spike and receptor-binding domain (RBD)-specific antibodies were quantified using a multiplexed electro-chemiluminescent assay (MSD, Rockvile, MD)[4]. Briefly, plates were blocked using 5% bovine serum albumin for 30 minutes and then washed. To the plate 50 μl of diluted serum, standards, and controls were added and incubated for 2 hours with shaking (700 rpm) at room temperature. After washing, 50 μl of SULFO-TAG™ anti-human IgG were added and incubated for 1 hour with shaking at room temperature. Plates were washed and, immediately after adding 150 μl of MSD GOLD read buffer, were read using MSD QuickPlex SQ120 plate reader and Methodical Mind software. Concentrations of anti-spike and anti-RBD antibodies (AU/ml) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

SARS-CoV-2 spike and RBD ACE-2 competition assay

ACE-2 competitive binding (U/ml) was quantified using the same MSD multiplex assay as described above. Plates were blocked, washed, and 25 μl of diluted sample, standard, and controls were added and incubated for 1 hour with shaking (700 rpm) at room temperature. Next, without washing, 25 μl of SULFO-TAG™ ACE-2 calibrator was added and incubated for another 1 hour at room temperature with shaking. After washing, plates were read using MSD GOLD read buffer on MSD QuickPlex SQ120 plate reader and software. Concentrations of competitive binding antibodies (U/mL) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

Anti-SARS-CoV-2 spike absolute and relative fractional avidity assay

Anti-spike absolute and relative fractional avidities were determined using a modified version of a previously described method[5,6]. Briefly, a stock concentration of ammonium thiocyanate (98.9% NH_4SCN, chaotrope; Milipore Sigma, cat # A1479; 4.0 mol/L) was prepared in 1X PBS and serially diluted to working solutions of 2.0, 1.0, 0.75, and 0.50 mol/L. Anti-SARS-CoV-2 spike ELISA plates (Invitrogen, cat # BMS2325) were washed and 6-fold diluted serum, standards, and controls were added with the final dilution in-plate. Plates were incubated for 30 minutes at 37°C without shaking then washed before adding chaotrope. Each sample was tested at 0, 0.50, 0.75, 1.0 and 2.0 M in the same plate by adding 100 μl of titrate or 1X PBS. To empty wells 100 μl of 1X PBS was added to prevent drying. Following a 30-minute incubation at 37°C,

plates were washed into a waste bucket, and 100 μl of anti-human HRP-conjugated IgG detection antibody were added. Plates were incubated for another 30 minutes at 37°C, washed, and incubated with 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) for 15 minutes in the dark at room temperature. To each well 100 μl of stop solution was added and plates were immediately read at 450nm using Bio-Rad iMark plate reader and MMP version 6.3 software. Concentration extrapolation (U/ml) was performed using the plate reader software and a 4-parameter logistic regression model. Untreated sample wells were used as the concentration of total IgG (U/ml). Values below detection limit were assigned ½ of the lowest back-calculated standard concentration. The limit of detection was determined as 2.5 standard deviations above the mean of lowest back-calculated standard concentration (182 U/ml).

Calculation of absolute and relative fractional avidity

Chaotrope concentrations were determined *a priori* during optimization for Pfizer BNT162b2 vaccinated serum. To calculate the absolute fractional avidity (AFA; U/ml), the difference in consecutive chaotrope conditions were obtained by the formula:

$$AFA_n = Y_n - Y_{n+1}$$

where AFA_n is the absolute fractional avidity of a given range of chaotrope, and Y_n and Y_{n+1} are concentrations of antibodies (U/ml) at consecutive chaotrope concentrations. Therefore, AFA_n quantifies the concentration of IgG released between chaotrope concentrations n and $n+1$. Where Y_n is the upper limit of chaotrope, the formula takes the form:

$$AFA_{upper} = Y_{upper}$$

The upper range of chaotrope was determined to be 2.0 M being the concentration eliminating the majority of detectable signal with good separation between vaccination statuses. The chaotrope ranges were described as very low (0 – 0.50M), low (0.5 – 0.75M), medium (0.75 – 1.0M), high (1.0 – 2.0M), and very high (2.0M).

To calculate relative fractional avidity (RFA; %), each AFA was divided by the total IgG levels in the untreated condition (0 mol/L NH_4SCN) and multiplied by 100%. Samples were retested at adjusted dilution factors if the sum of relative avidity profiles did not equal 100% of total IgG (**table S3**).

To quantify avidity as a single variable, we combined the RFA values taken at each chaotrope concentration using a principal component analysis (PCA), defining total relative fractional avidity (TRFA) using the loadings of PC1 as follows:

$TRFA = -0.533(RFA_{vlo}) + 0.0269(RFA_{lo}) + 0.477(RFA_{med}) + 0.492(RFA_{hi}) + 0.495(RFA_{vhi})$

Stool collection

Participants were provided with a stool sample collection kit and written instructions at their initial and twelve-week post 1st BNT162b2 vaccine clinic visits. Participants were asked to collect their stool samples within 24 hours of receiving the collection kits. The stool sample collection kits contained two DNA Genotek collection tubes (OM-200 for metagenomics and ME-200 for metabolomics), gloves, toilet liner, alcohol wipes, absorbent paper, and a pre-paid envelope. Stool collected in the OM-200 and ME-200 tubes can be stored at room temperature for 30 and 7 days, respectively. Participants were asked to mail their samples to the lab within 24 hours of stool collection. On arrival, samples were immediately aliquoted and stored at -80°C.

Short-chain fatty acid analysis

Stool samples, collected in DNA Genotek ME-200 tubes (80-95% ethanol), were thawed on ice, vortexed, and 700 µl was transferred to a Savant SPD131 DDA speedvac (Thermo Scientific) centrifugal vacuum concentrator and run at room temperature until virtually all the ethanol had evaporated. The samples were placed into 20 mL headspace autosample vials and acidified water, and deuterium labeled internal standards were added. The samples were then heated to 95°C while being mixed for 40 minutes. Once an equilibrium was reached, where the SCFA in the gas phase was directly proportional to the SCFA in the liquid mixture, the autosampler needle drew 0.5 mL of the gas phase from the vial and injected the gas into an Agilent 8890 gas chromatograph coupled with an Agilent 7010B mass spectrometer (GC-MS) system. The GC-MS was equipped with a CTC-PAL headspace system and Agilent FATWAX column (0.25mm ID X 0.25um phase thickness and 30m length). The carrier gas used was Helium at a flow rate of 1.25 mL/min and samples were injected with a 10:1 split ratio. The column oven was temperature programmed from 90°C to 230°C over 18 minutes with baseline separation of all SCFA. Calibration curves utilized authentic standards and deuterium labeled internal standards of acetic, propionic, butyric and caproic acids (Millipore Sigma or CDN isotopes). The GC-MS was operated in SRM mode and regression lines were calculated using quadratic fits with correlation coefficients of 0.995 to 0.9995.

Microbial gDNA extraction and quantification

In brief, stool samples, collected in DNA Genotek OM-200 tubes, were thawed on ice, vortexed, and a 250 µl aliquot of sample was transferred to the supplied bead beating tube. The samples were homogenized using a benchtop vortex with bead beating tube adaptor at 2500rpm for 10 minutes. The DNA was eluted

in 100 µl of elution buffer. DNA concentrations were quantified using the Quant-iT Picogreen dsDNA kit following manufacturer's instructions.

16S rRNA sequencing

The ThermoFisher MagMax microbiome nucleic acid ultra-isolation kit and semi-automated Kingfisher Duo Prime were used to extract microbial DNA from the stool samples following the manufacturer's instructions with minor modifications. 16S library preparation for individual samples were performed at the BC Children's Hospital Research Institute Gut4Health Microbiome Sequencing CORE, using the preferred method described by De Wolfe *et al*[7]. Briefly, the V4 region of the 16S rRNA gene was amplified with barcode primers containing the index sequences using a KAPA HiFi HotStart Real-time PCR Master Mix (Roche). PCR product amplification and concentration was monitored on a Bio-Rad CFT Connect Real-Time PCR system. Amplicon libraries were then purified, normalized, and pooled using the SequalPrep™ normalization plate (Applied Biosystems). The pooled library was further purified with Agencourt AMPure XP system (Beckman Coulter) following the manufacturer's protocol. Library concentrations were verified using a Qubit™ dsDNA high sensitivity assay kit (Invitrogen). The purified pooled libraries were submitted to the Sequencing and Bioinformatics Consortium at the University of British Columbia (Vancouver, Canada) for QC and sequencing on a single MiSeq v2 flow cell, to generate paired-end 250 bp reads. Raw base call data (bcl) were converted into FastQ format using the bcl2fastq conversion software from Illumina.

On average 24.67Mb (90,509 reads) of data was generated per sample. The raw 16S rRNA sequences have been deposited in the NCBI Short Read Archive (SRA), accession number PRJNA872852.

Bioinformatics

Raw .fastq files were processed using a custom script based on the R package DADA2 (version 1.20.0). Quality filtering was performed using the filterAndTrim function under the following criteria: 1) First 10 bp of each read removed; 2) Reads with expected error (EE = sum(10^(-Q/10)) greater than 2 were discarded. Following merging forward and reverse reads and removing chimeric sequences, a table of amplicon sequence variants (ASVs) was generated, with taxonomic assignment using the Silva database (version 138). For all downstream analyses except calculations of alpha diversity, amplicon sequence variants (ASVs) present in less than 5% of the samples were removed.

Data analyses

Alpha diversity of the samples was calculated with species richness and the Shannon index of diversity on raw counts of ASVs. To assess changes in alpha diversity between baseline and 12-weeks post vaccine, only participants with a sample from both time points were considered, and a Wilcoxon rank test was used to determine if the difference within participants was significantly less than zero. Overall composition of samples (beta diversity) was calculated by PCoA on a Bray-Curtis distance matrix. DESeq2 was used to identify differentially abundant taxonomic groups, using variance-stabilizing transformed counts, with significance determined by a Wald test, adjusted for multiple-inference using the Benjamini-Hochberg method. Associations between immune response variables and overall composition of the gut microbiota were assessed by PERMANOVA using the R package ADONIS. Associations between short chain fatty acids (SCFAs) and immune response variables was determined using spearman rank correlation analysis, Bonferroni corrected for multiple comparisons. To define participants as responders and non-responders, we binned the immune response variables into quartiles to group participants as the lowest response (quartile 1) or the highest response (quartile 4). Using these quartiles, differentially abundant taxa associated with immune response was assessed in the same method as above. To assess the association between SCFAs and fibre intake, we compared the change in SCFA concentration (on a $\log_{10}$ scale) from baseline to 12-weeks post vaccine in participants with low fibre intake to those with high fibre intake, determining significance using a Student's t-test.

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Supplementary figure legend

Figure S1 Changes in alpha and beta diversity from baseline to twelve-weeks post 1st BTN162b2 vaccine. (A) A significant shift in beta diversity occurred between baseline and post 1st BTN162b2 vaccine (p=0.0159; PERMANOVA). (B) A trend towards a significant reduction in Shannon alpha diversity was observed (p=0.07812; Wilcoxon rank test).

Supplementary tables – refer to separate document

Supplementary Table 1 Participant characteristics and sample collection information

Variable	BNT162b2 (n=52)
Age, years (median [IQR])	4 [32.3-54]
Female [Male]	46 [6]
Seropositivity to SARS-CoV-2 (Reactive [Non-reactive])	2 [50]
Blood sample collected post 1st vaccine (Yes [No])	47 [5]
Blood sample collected post 2nd vaccine (Yes [No])	18 [34]
Baseline stool samples collected (Yes [No])	48 [4]
Twelve-weeks post 1st vaccine stool samples collected (Yes [No])	41 [11]
Matching baseline and twelve-week stool samples collected (Yes [No])	38 [14]
Dietary fibre grouping (Low [Moderate] High) (n=41)	13 [14] 14
Total dietary fibre intake, g/day (mean [SD]) (n=41)	24.7 [11.9]
Vegetarian (Yes [No]) (n=41)	4 [37]
Antibiotics taken within 9 months of collecting stool (Yes [No]) (n=41)	3 [38]
Significant* change in weight or food intake over the past year (Yes [No]) (n=41)	7 [34]

IQR – inter quartile range, SD – standard deviation. * Significant body weight changes >10% weight gain or loss and significant changes in food intake included becoming vegetarian/vegan, stopped consuming gluten, dairy or sugar, increased fruit, or vegetable intake, etc.

Supplementary Table 2 Gut microbiota species significantly positively or negatively associated (p<0.01; statistical test) with higher avidity after the 2nd dose of the BNT162b2 vaccine (D2) in a subset of participants (n=15)

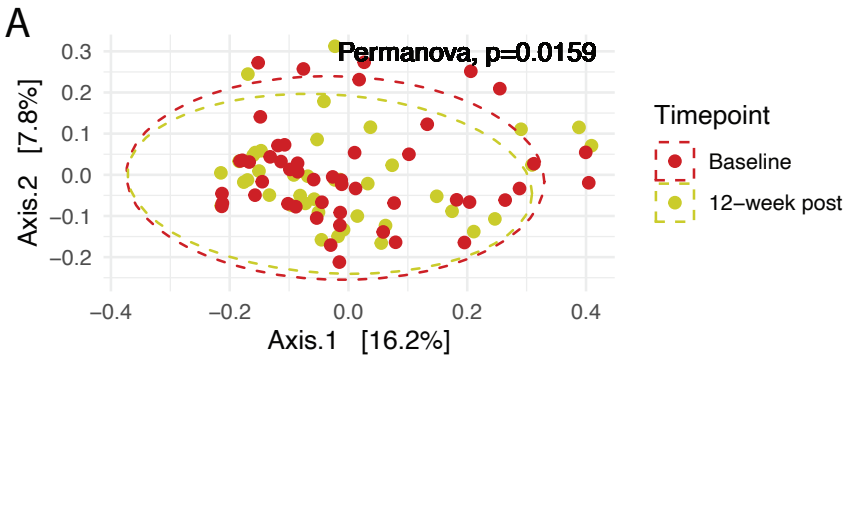
	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV_12	652.6828066	16.61603655	1.12976698	14.70748999	5.77085204873443E-49	4.0395964341141E-47	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Phascolarctobacterium	succinatutens
ASV_40	856.3116714	15.01679959	1.12976278	13.29199355	2.57621721167962E-40	1.20223469878382E-38	Bacteria	Synergistota	Synergistia	Synergistales	Synergistaceae	Cloacibacillus	evryensis
ASV_56	1163.66926110663	18.9073311	1.129781224	16.73539151	7.23796558640599E-63	1.01331518209684E-60	Bacteria	Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Selenomonadaceae	Megamonas	funiformis
ASV_90	299.9462157	-11.55809526	1.09781944	-10.52822972	6.40272464578869E-26	1.12047681301302E-24	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Acidaminococcus	intestini
ASV_104	165.2171921	-7.257755983	1.094064509	-6.633755071	3.27252692455385E-11	3.81794807864615E-10	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	massiliensis
ASV_123	100.8114371	-5.521510801	1.091821644	-5.057154555	4.25558338438288E-07	4.58293595241233E-06	Bacteria	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	bifidum
ASV_177	53.6415468	4.110286262	1.128616539	3.641880232	0.000270654	0.00270654	Bacteria	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	animalis
ASV_202	127.887441	14.00883584	1.12979702	12.39942714	2.63198463344399E-35	9.21194621705396E-34	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	plebeius
ASV_217	45.77577167	12.5267809	1.129873892	11.08688411	1.45259641590064E-28	3.38939163710149E-27	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Tannerellaceae	Parabacteroides	johnsonii
ASV_309	60.52674668	12.85841755	1.129833358	11.38080891	5.21140863375641E-30	1.45919441745179E-28	Bacteria	Desulfobacterota	Desulfovibrionia	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	fairfieldensis
ASV_328	27.36761572	11.33415571	1.123464443	10.08857537	6.20636856209842E-24	9.65435109659754E-23	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
ASV_384	36.43927284	12.11973529	1.129888243	10.72649031	7.64447009656451E-27	1.5288940193129E-25	Bacteria	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Eisenbergiella	tayi
ASV_635	21.05201209	11.23206477	1.138659715	9.864285715	5.9456105183706E-23	8.32385472571884E-22	Bacteria	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	Negativibacillus	massiliensis

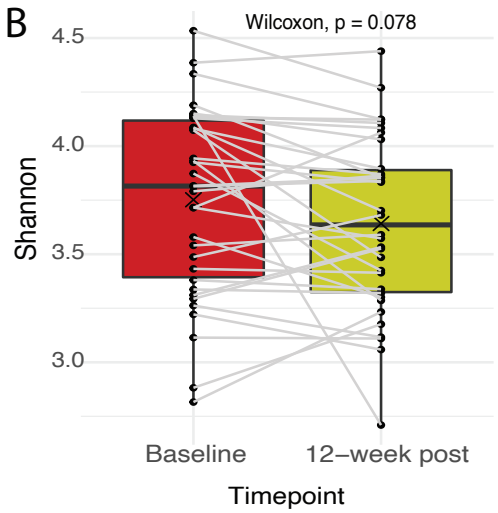
Supplementary Table 3 Equations used to calculate absolute (U/mL) and relative fractional avidity (%), and total absolute and relative fractional avidity indices

	Very low	Low	Medium	High	Very high
Absolute fractional avidity (U/mL)	$AFA_{vlo} = Y_0 - Y_{0.5}$	$AFA_{lo} = Y_{0.5} - Y_{0.75}$	$AFA_{med} = Y_{0.75} - Y_1$	$AFA_{hi} = Y_1 - Y_2$	$AFA_{vhi} = Y_2$
Total absolute fractional avidity index	$2 * AFA_{vhi} + 1 * AFA_{hi} + 0.25 * AFA_{med} + 0.25 * AFA_{lo} + 0.5 * AFA_{vlo}$				
Relative fractional avidity (%)	$RFA_{vlo} = \frac{AFA_{vlo}}{Y_0} * 100\%$	$RFA_{lo} = \frac{AFA_{lo}}{Y_0} * 100\%$	$RFA_{med} = \frac{AFA_{med}}{Y_0} * 100\%$	$RFA_{hi} = \frac{AFA_{hi}}{Y_0} * 100\%$	$RFA_{vhi} = \frac{AFA_{vhi}}{Y_0} * 100\%$
Total relative fractional avidity (TRFA)*	$TRFA = -0.533 * RFA_{vlo} + 0.0269 * RFA_{lo} + 0.477 * RFA_{med} + 0.492 * RFA_{hi} + 0.495 * RFA_{vhi}$				

AFA – absolute fractional avidity, RFA – relative fractional avidity

*Based on eigenvalue plot loadings of each avidity for PC1 of all avidity data


A
Permanova, p=0.0159
Axis.2 [7.8%]
Axis.1 [16.2%]
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.4
−0.2
0.0
0.2
0.4
Timepoint
Baseline
12−week post


B
Wilcoxon, p = 0.078
Shannon
4.5
4.0
3.5
3.0
Baseline
12−week post
Timepoint

Supplementary Information

The gut microbiome and habitual dietary fibre intakes are associated with variable responses to the mRNA SARS-CoV-2 BNT162b2 vaccine

SUPPLEMENTARY MATERIALS AND METHODS

Study cohort

Uninfected healthcare workers aged ≥ 18 years were prospectively recruited at British Columbia Children's Hospital (Vancouver, Canada) between February and August 2021 prior to receiving two Pfizer-BioNtech BNT162b2 mRNA vaccine doses, approximately 4 months apart[1]. Participants who tested positive for SARS-CoV-2 by viral or Spike antibody testing of baseline samples or nucleocapsid antibody testing of any sample were excluded. Blood samples were collected at baseline (pre-vaccine), 12 weeks after 1st vaccination, and 4 weeks after 2nd Pfizer-BioNtech BNT162b2 mRNA vaccination. Stool samples were collected at baseline and 12 weeks after the 1st vaccine dose (**figure 1A**). This study was approved by the University of British Columbia Clinical Research Ethics Board (H20-01205). Informed e-consent for collection of blood and stool samples was obtained from all participants.

Due to the rapid initiation of SARS-CoV-2 vaccine programs for healthcare workers in British Columbia this research was particularly time sensitive as samples needed to be collected promptly prior to participants receiving their 1st dose of the BNT612b2 vaccine. Therefore, we did not have an opportunity to involve participants in the design or implementation of this study.

Participant characteristic and dietary intake data collection

Demographic data (e.g., age, sex) was collected from participants via a secure web-based application (REDCap). A standardized form was used to collect information from participants regarding antibiotic use prior to stool collection, significant changes in body weight or food intake over the previous year, whether participants were vegan or vegetarian and habitual dietary fibre intakes. Participants were classified as low (males <22 g/day, females <18 g/day), moderate (males 22 to 29.9 g/day, females 18 to 24.9 g/day), or high (males ≥30 g/day, females ≥25 g/day) dietary fibre consumers using a validated habitual dietary fibre intake food frequency questionnaire[2]. These participant characteristics were captured as they are known to modulate the gut microbiota and could potentially confound study results[3].

Serum sample analyses

SARS-CoV-2 spike and RBD IgG multiplex assay

Anti-SARS-CoV-2 spike and receptor-binding domain (RBD)-specific antibodies were quantified using a multiplexed electro-chemiluminescent assay (MSD, Rockvile, MD)[4]. Briefly, plates were blocked using 5% bovine serum albumin for 30 minutes and then washed. To the plate 50 μl of diluted serum, standards, and controls were added and incubated for 2 hours with shaking (700 rpm) at room temperature. After washing, 50 μl of SULFO-TAG™ anti-human IgG were added and incubated for 1 hour with shaking at room temperature. Plates were washed and, immediately after adding 150 μl of MSD GOLD read buffer, were read using MSD QuickPlex SQ120 plate reader and Methodical Mind software. Concentrations of anti-spike and anti-RBD antibodies (AU/ml) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

SARS-CoV-2 spike and RBD ACE-2 competition assay

ACE-2 competitive binding (U/ml) was quantified using the same MSD multiplex assay as described above. Plates were blocked, washed, and 25 μl of diluted sample, standard, and controls were added and incubated for 1 hour with shaking (700 rpm) at room temperature. Next, without washing, 25 μl of SULFO-TAG™ ACE-2 calibrator was added and incubated for another 1 hour at room temperature with shaking. After washing, plates were read using MSD GOLD read buffer on MSD QuickPlex SQ120 plate reader and software. Concentrations of competitive binding antibodies (U/mL) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

Anti-SARS-CoV-2 spike absolute and relative fractional avidity assay

Anti-spike absolute and relative fractional avidities were determined using a modified version of a previously described method[5,6]. Briefly, a stock concentration of ammonium thiocyanate (98.9% NH_4SCN, chaotrope; Milipore Sigma, cat # A1479; 4.0 mol/L) was prepared in 1X PBS and serially diluted to working solutions of 2.0, 1.0, 0.75, and 0.50 mol/L. Anti-SARS-CoV-2 spike ELISA plates (Invitrogen, cat # BMS2325) were washed and 6-fold diluted serum, standards, and controls were added with the final dilution in-plate. Plates were incubated for 30 minutes at 37°C without shaking then washed before adding chaotrope. Each sample was tested at 0, 0.50, 0.75, 1.0 and 2.0 M in the same plate by adding 100 μl of titrate or 1X PBS. To empty wells 100 μl of 1X PBS was added to prevent drying. Following a 30-minute incubation at 37°C,

plates were washed into a waste bucket, and 100 μl of anti-human HRP-conjugated IgG detection antibody were added. Plates were incubated for another 30 minutes at 37°C, washed, and incubated with 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) for 15 minutes in the dark at room temperature. To each well 100 μl of stop solution was added and plates were immediately read at 450nm using Bio-Rad iMark plate reader and MMP version 6.3 software. Concentration extrapolation (U/ml) was performed using the plate reader software and a 4-parameter logistic regression model. Untreated sample wells were used as the concentration of total IgG (U/ml). Values below detection limit were assigned ½ of the lowest back-calculated standard concentration. The limit of detection was determined as 2.5 standard deviations above the mean of lowest back-calculated standard concentration (182 U/ml).

Calculation of absolute and relative fractional avidity

Chaotrope concentrations were determined *a priori* during optimization for Pfizer BNT162b2 vaccinated serum. To calculate the absolute fractional avidity (AFA; U/ml), the difference in consecutive chaotrope conditions were obtained by the formula:

$$AFA_n = Y_n - Y_{n+1}$$

where AFA_n is the absolute fractional avidity of a given range of chaotrope, and Y_n and Y_{n+1} are concentrations of antibodies (U/ml) at consecutive chaotrope concentrations. Therefore, AFA_n quantifies the concentration of IgG released between chaotrope concentrations n and $n+1$. Where Y_n is the upper limit of chaotrope, the formula takes the form:

$$AFA_{upper} = Y_{upper}$$

The upper range of chaotrope was determined to be 2.0 M being the concentration eliminating the majority of detectable signal with good separation between vaccination statuses. The chaotrope ranges were described as very low (0 – 0.50M), low (0.5 – 0.75M), medium (0.75 – 1.0M), high (1.0 – 2.0M), and very high (2.0M).

To calculate relative fractional avidity (RFA; %), each AFA was divided by the total IgG levels in the untreated condition (0 mol/L NH_4SCN) and multiplied by 100%. Samples were retested at adjusted dilution factors if the sum of relative avidity profiles did not equal 100% of total IgG (**table S3**).

To quantify avidity as a single variable, we combined the RFA values taken at each chaotrope concentration using a principal component analysis (PCA), defining total relative fractional avidity (TRFA) using the loadings of PC1 as follows:

$TRFA = -0.533(RFA_{vlo}) + 0.0269(RFA_{lo}) + 0.477(RFA_{med}) + 0.492(RFA_{hi}) + 0.495(RFA_{vhi})$

Stool collection

Participants were provided with a stool sample collection kit and written instructions at their initial and twelve-week post 1st BNT162b2 vaccine clinic visits. Participants were asked to collect their stool samples within 24 hours of receiving the collection kits. The stool sample collection kits contained two DNA Genotek collection tubes (OM-200 for metagenomics and ME-200 for metabolomics), gloves, toilet liner, alcohol wipes, absorbent paper, and a pre-paid envelope. Stool collected in the OM-200 and ME-200 tubes can be stored at room temperature for 30 and 7 days, respectively. Participants were asked to mail their samples to the lab within 24 hours of stool collection. On arrival, samples were immediately aliquoted and stored at -80°C.

Short-chain fatty acid analysis

Stool samples, collected in DNA Genotek ME-200 tubes (80-95% ethanol), were thawed on ice, vortexed, and 700 µl was transferred to a Savant SPD131 DDA speedvac (Thermo Scientific) centrifugal vacuum concentrator and run at room temperature until virtually all the ethanol had evaporated. The samples were placed into 20 mL headspace autosample vials and acidified water, and deuterium labeled internal standards were added. The samples were then heated to 95°C while being mixed for 40 minutes. Once an equilibrium was reached, where the SCFA in the gas phase was directly proportional to the SCFA in the liquid mixture, the autosampler needle drew 0.5 mL of the gas phase from the vial and injected the gas into an Agilent 8890 gas chromatograph coupled with an Agilent 7010B mass spectrometer (GC-MS) system. The GC-MS was equipped with a CTC-PAL headspace system and Agilent FATWAX column (0.25mm ID X 0.25um phase thickness and 30m length). The carrier gas used was Helium at a flow rate of 1.25 mL/min and samples were injected with a 10:1 split ratio. The column oven was temperature programmed from 90°C to 230°C over 18 minutes with baseline separation of all SCFA. Calibration curves utilized authentic standards and deuterium labeled internal standards of acetic, propionic, butyric and caproic acids (Millipore Sigma or CDN isotopes). The GC-MS was operated in SRM mode and regression lines were calculated using quadratic fits with correlation coefficients of 0.995 to 0.9995.

Microbial gDNA extraction and quantification

In brief, stool samples, collected in DNA Genotek OM-200 tubes, were thawed on ice, vortexed, and a 250 µl aliquot of sample was transferred to the supplied bead beating tube. The samples were homogenized using a benchtop vortex with bead beating tube adaptor at 2500rpm for 10 minutes. The DNA was eluted

in 100 µl of elution buffer. DNA concentrations were quantified using the Quant-iT Picogreen dsDNA kit following manufacturer's instructions.

16S rRNA sequencing

The ThermoFisher MagMax microbiome nucleic acid ultra-isolation kit and semi-automated Kingfisher Duo Prime were used to extract microbial DNA from the stool samples following the manufacturer's instructions with minor modifications. 16S library preparation for individual samples were performed at the BC Children's Hospital Research Institute Gut4Health Microbiome Sequencing CORE, using the preferred method described by De Wolfe *et al*[7]. Briefly, the V4 region of the 16S rRNA gene was amplified with barcode primers containing the index sequences using a KAPA HiFi HotStart Real-time PCR Master Mix (Roche). PCR product amplification and concentration was monitored on a Bio-Rad CFT Connect Real-Time PCR system. Amplicon libraries were then purified, normalized, and pooled using the SequalPrep™ normalization plate (Applied Biosystems). The pooled library was further purified with Agencourt AMPure XP system (Beckman Coulter) following the manufacturer's protocol. Library concentrations were verified using a Qubit™ dsDNA high sensitivity assay kit (Invitrogen). The purified pooled libraries were submitted to the Sequencing and Bioinformatics Consortium at the University of British Columbia (Vancouver, Canada) for QC and sequencing on a single MiSeq v2 flow cell, to generate paired-end 250 bp reads. Raw base call data (bcl) were converted into FastQ format using the bcl2fastq conversion software from Illumina.

On average 24.67Mb (90,509 reads) of data was generated per sample. The raw 16S rRNA sequences have been deposited in the NCBI Short Read Archive (SRA), accession number PRJNA872852.

Bioinformatics

Raw .fastq files were processed using a custom script based on the R package DADA2 (version 1.20.0). Quality filtering was performed using the filterAndTrim function under the following criteria: 1) First 10 bp of each read removed; 2) Reads with expected error (EE = sum(10^(-Q/10)) greater than 2 were discarded. Following merging forward and reverse reads and removing chimeric sequences, a table of amplicon sequence variants (ASVs) was generated, with taxonomic assignment using the Silva database (version 138). For all downstream analyses except calculations of alpha diversity, amplicon sequence variants (ASVs) present in less than 5% of the samples were removed.

Data analyses

Alpha diversity of the samples was calculated with species richness and the Shannon index of diversity on raw counts of ASVs. To assess changes in alpha diversity between baseline and 12-weeks post vaccine, only participants with a sample from both time points were considered, and a Wilcoxon rank test was used to determine if the difference within participants was significantly less than zero. Overall composition of samples (beta diversity) was calculated by PCoA on a Bray-Curtis distance matrix. DESeq2 was used to identify differentially abundant taxonomic groups, using variance-stabilizing transformed counts, with significance determined by a Wald test, adjusted for multiple-inference using the Benjamini-Hochberg method. Associations between immune response variables and overall composition of the gut microbiota were assessed by PERMANOVA using the R package ADONIS. Associations between short chain fatty acids (SCFAs) and immune response variables was determined using spearman rank correlation analysis, Bonferroni corrected for multiple comparisons. To define participants as responders and non-responders, we binned the immune response variables into quartiles to group participants as the lowest response (quartile 1) or the highest response (quartile 4). Using these quartiles, differentially abundant taxa associated with immune response was assessed in the same method as above. To assess the association between SCFAs and fibre intake, we compared the change in SCFA concentration (on a $\log_{10}$ scale) from baseline to 12-weeks post vaccine in participants with low fibre intake to those with high fibre intake, determining significance using a Student's t-test.

References

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Supplementary figure legend

Figure S1 Changes in alpha and beta diversity from baseline to twelve-weeks post 1st BTN162b2 vaccine. (A) A significant shift in beta diversity occurred between baseline and post 1st BTN162b2 vaccine (p=0.0159; PERMANOVA). (B) A trend towards a significant reduction in Shannon alpha diversity was observed (p=0.07812; Wilcoxon rank test).

Supplementary tables – refer to separate document

Supplementary Table 1 Participant characteristics and sample collection information

Variable	BNT162b2 (n=52)
Age, years (median [IQR])	4 [32.3-54]
Female [Male]	46 [6]
Seropositivity to SARS-CoV-2 (Reactive [Non-reactive])	2 [50]
Blood sample collected post 1st vaccine (Yes [No])	47 [5]
Blood sample collected post 2nd vaccine (Yes [No])	18 [34]
Baseline stool samples collected (Yes [No])	48 [4]
Twelve-weeks post 1st vaccine stool samples collected (Yes [No])	41 [11]
Matching baseline and twelve-week stool samples collected (Yes [No])	38 [14]
Dietary fibre grouping (Low [Moderate] High) (n=41)	13 [14] 14
Total dietary fibre intake, g/day (mean [SD]) (n=41)	24.7 [11.9]
Vegetarian (Yes [No]) (n=41)	4 [37]
Antibiotics taken within 9 months of collecting stool (Yes [No]) (n=41)	3 [38]
Significant* change in weight or food intake over the past year (Yes [No]) (n=41)	7 [34]

IQR – inter quartile range, SD – standard deviation. * Significant body weight changes >10% weight gain or loss and significant changes in food intake included becoming vegetarian/vegan, stopped consuming gluten, dairy or sugar, increased fruit, or vegetable intake, etc.

Supplementary Table 2 Gut microbiota species significantly positively or negatively associated (p<0.01; statistical test) with higher avidity after the 2nd dose of the BNT162b2 vaccine (D2) in a subset of participants (n=15)

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV_12	652.6828066	16.61603655	1.12976698	14.70748999	5.77085204873443E-49	4.0395964341141E-47	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Phascolarctobacterium	succinatutens
ASV_40	856.3116714	15.01679959	1.12976278	13.29199355	2.57621721167962E-40	1.20223469878382E-38	Bacteria	Synergistota	Synergistia	Synergistales	Synergistaceae	Cloacibacillus	evryensis
ASV_56	1163.66926110663	18.9073311	1.129781224	16.73539151	7.23796558640599E-63	1.01331518209684E-60	Bacteria	Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Selenomonadaceae	Megamonas	funiformis
ASV_90	299.9462157	-11.55809526	1.09781944	-10.52822972	6.40272464578869E-26	1.12047681301302E-24	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Acidaminococcus	intestini
ASV_104	165.2171921	-7.257755983	1.094064509	-6.633755071	3.27252692455385E-11	3.81794807864615E-10	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	massiliensis
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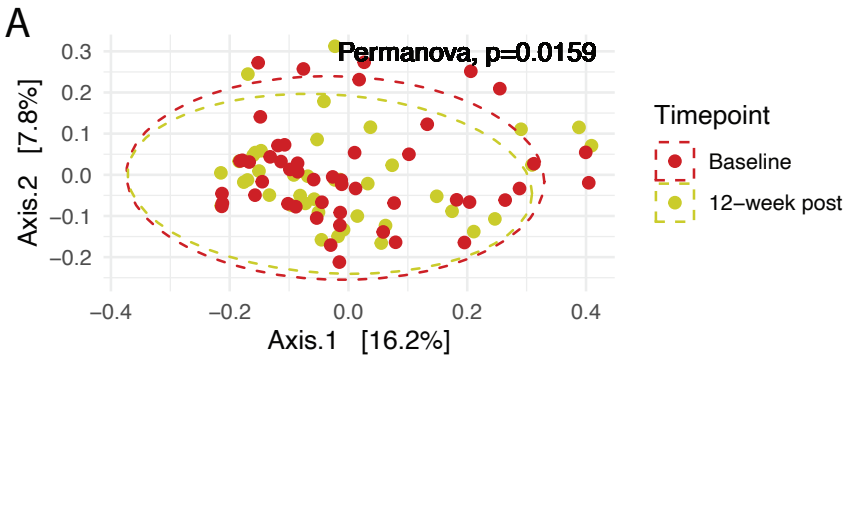
ASV_177	53.6415468	4.110286262	1.128616539	3.641880232	0.000270654	0.00270654	Bacteria	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	animalis
ASV_202	127.887441	14.00883584	1.12979702	12.39942714	2.63198463344399E-35	9.21194621705396E-34	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	plebeius
ASV_217	45.77577167	12.5267809	1.129873892	11.08688411	1.45259641590064E-28	3.38939163710149E-27	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Tannerellaceae	Parabacteroides	johnsonii
ASV_309	60.52674668	12.85841755	1.129833358	11.38080891	5.21140863375641E-30	1.45919441745179E-28	Bacteria	Desulfobacterota	Desulfovibrionia	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	fairfieldensis
ASV_328	27.36761572	11.33415571	1.123464443	10.08857537	6.20636856209842E-24	9.65435109659754E-23	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
ASV_384	36.43927284	12.11973529	1.129888243	10.72649031	7.64447009656451E-27	1.5288940193129E-25	Bacteria	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Eisenbergiella	tayi
ASV_635	21.05201209	11.23206477	1.138659715	9.864285715	5.9456105183706E-23	8.32385472571884E-22	Bacteria	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	Negativibacillus	massiliensis

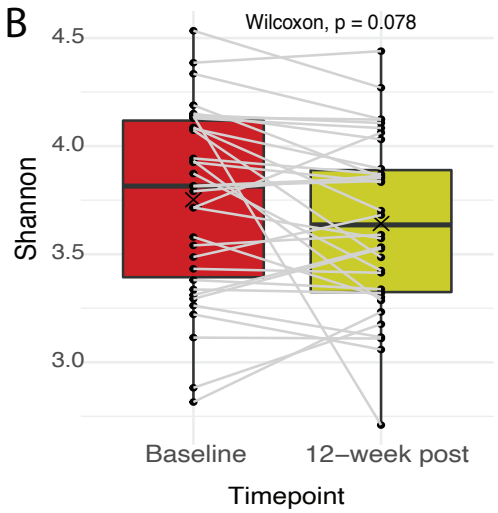
Supplementary Table 3 Equations used to calculate absolute (U/mL) and relative fractional avidity (%), and total absolute and relative fractional avidity indices

	Very low	Low	Medium	High	Very high
Absolute fractional avidity (U/mL)	$AFA_{vlo} = Y_0 - Y_{0.5}$	$AFA_{lo} = Y_{0.5} - Y_{0.75}$	$AFA_{med} = Y_{0.75} - Y_1$	$AFA_{hi} = Y_1 - Y_2$	$AFA_{vhi} = Y_2$
Total absolute fractional avidity index	$2 * AFA_{vhi} + 1 * AFA_{hi} + 0.25 * AFA_{med} + 0.25 * AFA_{lo} + 0.5 * AFA_{vlo}$				
Relative fractional avidity (%)	$RFA_{vlo} = \frac{AFA_{vlo}}{Y_0} * 100\%$	$RFA_{lo} = \frac{AFA_{lo}}{Y_0} * 100\%$	$RFA_{med} = \frac{AFA_{med}}{Y_0} * 100\%$	$RFA_{hi} = \frac{AFA_{hi}}{Y_0} * 100\%$	$RFA_{vhi} = \frac{AFA_{vhi}}{Y_0} * 100\%$
Total relative fractional avidity (TRFA)*	$TRFA = -0.533 * RFA_{vlo} + 0.0269 * RFA_{lo} + 0.477 * RFA_{med} + 0.492 * RFA_{hi} + 0.495 * RFA_{vhi}$				

AFA – absolute fractional avidity, RFA – relative fractional avidity

*Based on eigenvalue plot loadings of each avidity for PC1 of all avidity data


A
Permanova, p=0.0159
Axis.2 [7.8%]
Axis.1 [16.2%]
0.3
0.2
0.1
0.0
−0.1
−0.2
−0.4
−0.2
0.0
0.2
0.4
Timepoint
Baseline
12−week post


B
Wilcoxon, p = 0.078
Shannon
4.5
4.0
3.5
3.0
Baseline
12−week post
Timepoint

Supplementary Information

The gut microbiome and habitual dietary fibre intakes are associated with variable responses to the mRNA SARS-CoV-2 BNT162b2 vaccine

SUPPLEMENTARY MATERIALS AND METHODS

Study cohort

Uninfected healthcare workers aged ≥ 18 years were prospectively recruited at British Columbia Children's Hospital (Vancouver, Canada) between February and August 2021 prior to receiving two Pfizer-BioNtech BNT162b2 mRNA vaccine doses, approximately 4 months apart[1]. Participants who tested positive for SARS-CoV-2 by viral or Spike antibody testing of baseline samples or nucleocapsid antibody testing of any sample were excluded. Blood samples were collected at baseline (pre-vaccine), 12 weeks after 1st vaccination, and 4 weeks after 2nd Pfizer-BioNtech BNT162b2 mRNA vaccination. Stool samples were collected at baseline and 12 weeks after the 1st vaccine dose (**figure 1A**). This study was approved by the University of British Columbia Clinical Research Ethics Board (H20-01205). Informed e-consent for collection of blood and stool samples was obtained from all participants.

Due to the rapid initiation of SARS-CoV-2 vaccine programs for healthcare workers in British Columbia this research was particularly time sensitive as samples needed to be collected promptly prior to participants receiving their 1st dose of the BNT612b2 vaccine. Therefore, we did not have an opportunity to involve participants in the design or implementation of this study.

Participant characteristic and dietary intake data collection

Demographic data (e.g., age, sex) was collected from participants via a secure web-based application (REDCap). A standardized form was used to collect information from participants regarding antibiotic use prior to stool collection, significant changes in body weight or food intake over the previous year, whether participants were vegan or vegetarian and habitual dietary fibre intakes. Participants were classified as low (males <22 g/day, females <18 g/day), moderate (males 22 to 29.9 g/day, females 18 to 24.9 g/day), or high (males ≥30 g/day, females ≥25 g/day) dietary fibre consumers using a validated habitual dietary fibre intake food frequency questionnaire[2]. These participant characteristics were captured as they are known to modulate the gut microbiota and could potentially confound study results[3].

Serum sample analyses

SARS-CoV-2 spike and RBD IgG multiplex assay

Anti-SARS-CoV-2 spike and receptor-binding domain (RBD)-specific antibodies were quantified using a multiplexed electro-chemiluminescent assay (MSD, Rockvile, MD)[4]. Briefly, plates were blocked using 5% bovine serum albumin for 30 minutes and then washed. To the plate 50 μl of diluted serum, standards, and controls were added and incubated for 2 hours with shaking (700 rpm) at room temperature. After washing, 50 μl of SULFO-TAG™ anti-human IgG were added and incubated for 1 hour with shaking at room temperature. Plates were washed and, immediately after adding 150 μl of MSD GOLD read buffer, were read using MSD QuickPlex SQ120 plate reader and Methodical Mind software. Concentrations of anti-spike and anti-RBD antibodies (AU/ml) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

SARS-CoV-2 spike and RBD ACE-2 competition assay

ACE-2 competitive binding (U/ml) was quantified using the same MSD multiplex assay as described above. Plates were blocked, washed, and 25 μl of diluted sample, standard, and controls were added and incubated for 1 hour with shaking (700 rpm) at room temperature. Next, without washing, 25 μl of SULFO-TAG™ ACE-2 calibrator was added and incubated for another 1 hour at room temperature with shaking. After washing, plates were read using MSD GOLD read buffer on MSD QuickPlex SQ120 plate reader and software. Concentrations of competitive binding antibodies (U/mL) were extrapolated to a standard curve using a 4-parameter logistic regression model. Samples above the limit of detection were re-diluted at a higher dilution and retested until within the detectable range.

Anti-SARS-CoV-2 spike absolute and relative fractional avidity assay

Anti-spike absolute and relative fractional avidities were determined using a modified version of a previously described method[5,6]. Briefly, a stock concentration of ammonium thiocyanate (98.9% NH_4SCN, chaotrope; Milipore Sigma, cat # A1479; 4.0 mol/L) was prepared in 1X PBS and serially diluted to working solutions of 2.0, 1.0, 0.75, and 0.50 mol/L. Anti-SARS-CoV-2 spike ELISA plates (Invitrogen, cat # BMS2325) were washed and 6-fold diluted serum, standards, and controls were added with the final dilution in-plate. Plates were incubated for 30 minutes at 37°C without shaking then washed before adding chaotrope. Each sample was tested at 0, 0.50, 0.75, 1.0 and 2.0 M in the same plate by adding 100 μl of titrate or 1X PBS. To empty wells 100 μl of 1X PBS was added to prevent drying. Following a 30-minute incubation at 37°C,

plates were washed into a waste bucket, and 100 μl of anti-human HRP-conjugated IgG detection antibody were added. Plates were incubated for another 30 minutes at 37°C, washed, and incubated with 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) for 15 minutes in the dark at room temperature. To each well 100 μl of stop solution was added and plates were immediately read at 450nm using Bio-Rad iMark plate reader and MMP version 6.3 software. Concentration extrapolation (U/ml) was performed using the plate reader software and a 4-parameter logistic regression model. Untreated sample wells were used as the concentration of total IgG (U/ml). Values below detection limit were assigned ½ of the lowest back-calculated standard concentration. The limit of detection was determined as 2.5 standard deviations above the mean of lowest back-calculated standard concentration (182 U/ml).

Calculation of absolute and relative fractional avidity

Chaotrope concentrations were determined *a priori* during optimization for Pfizer BNT162b2 vaccinated serum. To calculate the absolute fractional avidity (AFA; U/ml), the difference in consecutive chaotrope conditions were obtained by the formula:

$$AFA_n = Y_n - Y_{n+1}$$

where AFA_n is the absolute fractional avidity of a given range of chaotrope, and Y_n and Y_{n+1} are concentrations of antibodies (U/ml) at consecutive chaotrope concentrations. Therefore, AFA_n quantifies the concentration of IgG released between chaotrope concentrations n and $n+1$. Where Y_n is the upper limit of chaotrope, the formula takes the form:

$$AFA_{upper} = Y_{upper}$$

The upper range of chaotrope was determined to be 2.0 M being the concentration eliminating the majority of detectable signal with good separation between vaccination statuses. The chaotrope ranges were described as very low (0 – 0.50M), low (0.5 – 0.75M), medium (0.75 – 1.0M), high (1.0 – 2.0M), and very high (2.0M).

To calculate relative fractional avidity (RFA; %), each AFA was divided by the total IgG levels in the untreated condition (0 mol/L NH_4SCN) and multiplied by 100%. Samples were retested at adjusted dilution factors if the sum of relative avidity profiles did not equal 100% of total IgG (**table S3**).

To quantify avidity as a single variable, we combined the RFA values taken at each chaotrope concentration using a principal component analysis (PCA), defining total relative fractional avidity (TRFA) using the loadings of PC1 as follows:

$TRFA = -0.533(RFA_{vlo}) + 0.0269(RFA_{lo}) + 0.477(RFA_{med}) + 0.492(RFA_{hi}) + 0.495(RFA_{vhi})$

Stool collection

Participants were provided with a stool sample collection kit and written instructions at their initial and twelve-week post 1st BNT162b2 vaccine clinic visits. Participants were asked to collect their stool samples within 24 hours of receiving the collection kits. The stool sample collection kits contained two DNA Genotek collection tubes (OM-200 for metagenomics and ME-200 for metabolomics), gloves, toilet liner, alcohol wipes, absorbent paper, and a pre-paid envelope. Stool collected in the OM-200 and ME-200 tubes can be stored at room temperature for 30 and 7 days, respectively. Participants were asked to mail their samples to the lab within 24 hours of stool collection. On arrival, samples were immediately aliquoted and stored at -80°C.

Short-chain fatty acid analysis

Stool samples, collected in DNA Genotek ME-200 tubes (80-95% ethanol), were thawed on ice, vortexed, and 700 µl was transferred to a Savant SPD131 DDA speedvac (Thermo Scientific) centrifugal vacuum concentrator and run at room temperature until virtually all the ethanol had evaporated. The samples were placed into 20 mL headspace autosample vials and acidified water, and deuterium labeled internal standards were added. The samples were then heated to 95°C while being mixed for 40 minutes. Once an equilibrium was reached, where the SCFA in the gas phase was directly proportional to the SCFA in the liquid mixture, the autosampler needle drew 0.5 mL of the gas phase from the vial and injected the gas into an Agilent 8890 gas chromatograph coupled with an Agilent 7010B mass spectrometer (GC-MS) system. The GC-MS was equipped with a CTC-PAL headspace system and Agilent FATWAX column (0.25mm ID X 0.25um phase thickness and 30m length). The carrier gas used was Helium at a flow rate of 1.25 mL/min and samples were injected with a 10:1 split ratio. The column oven was temperature programmed from 90°C to 230°C over 18 minutes with baseline separation of all SCFA. Calibration curves utilized authentic standards and deuterium labeled internal standards of acetic, propionic, butyric and caproic acids (Millipore Sigma or CDN isotopes). The GC-MS was operated in SRM mode and regression lines were calculated using quadratic fits with correlation coefficients of 0.995 to 0.9995.

Microbial gDNA extraction and quantification

In brief, stool samples, collected in DNA Genotek OM-200 tubes, were thawed on ice, vortexed, and a 250 µl aliquot of sample was transferred to the supplied bead beating tube. The samples were homogenized using a benchtop vortex with bead beating tube adaptor at 2500rpm for 10 minutes. The DNA was eluted

in 100 µl of elution buffer. DNA concentrations were quantified using the Quant-iT Picogreen dsDNA kit following manufacturer's instructions.

16S rRNA sequencing

The ThermoFisher MagMax microbiome nucleic acid ultra-isolation kit and semi-automated Kingfisher Duo Prime were used to extract microbial DNA from the stool samples following the manufacturer's instructions with minor modifications. 16S library preparation for individual samples were performed at the BC Children's Hospital Research Institute Gut4Health Microbiome Sequencing CORE, using the preferred method described by De Wolfe *et al*[7]. Briefly, the V4 region of the 16S rRNA gene was amplified with barcode primers containing the index sequences using a KAPA HiFi HotStart Real-time PCR Master Mix (Roche). PCR product amplification and concentration was monitored on a Bio-Rad CFT Connect Real-Time PCR system. Amplicon libraries were then purified, normalized, and pooled using the SequalPrep™ normalization plate (Applied Biosystems). The pooled library was further purified with Agencourt AMPure XP system (Beckman Coulter) following the manufacturer's protocol. Library concentrations were verified using a Qubit™ dsDNA high sensitivity assay kit (Invitrogen). The purified pooled libraries were submitted to the Sequencing and Bioinformatics Consortium at the University of British Columbia (Vancouver, Canada) for QC and sequencing on a single MiSeq v2 flow cell, to generate paired-end 250 bp reads. Raw base call data (bcl) were converted into FastQ format using the bcl2fastq conversion software from Illumina.

On average 24.67Mb (90,509 reads) of data was generated per sample. The raw 16S rRNA sequences have been deposited in the NCBI Short Read Archive (SRA), accession number PRJNA872852.

Bioinformatics

Raw .fastq files were processed using a custom script based on the R package DADA2 (version 1.20.0). Quality filtering was performed using the filterAndTrim function under the following criteria: 1) First 10 bp of each read removed; 2) Reads with expected error (EE = sum(10^(-Q/10)) greater than 2 were discarded. Following merging forward and reverse reads and removing chimeric sequences, a table of amplicon sequence variants (ASVs) was generated, with taxonomic assignment using the Silva database (version 138). For all downstream analyses except calculations of alpha diversity, amplicon sequence variants (ASVs) present in less than 5% of the samples were removed.

Data analyses

Alpha diversity of the samples was calculated with species richness and the Shannon index of diversity on raw counts of ASVs. To assess changes in alpha diversity between baseline and 12-weeks post vaccine, only participants with a sample from both time points were considered, and a Wilcoxon rank test was used to determine if the difference within participants was significantly less than zero. Overall composition of samples (beta diversity) was calculated by PCoA on a Bray-Curtis distance matrix. DESeq2 was used to identify differentially abundant taxonomic groups, using variance-stabilizing transformed counts, with significance determined by a Wald test, adjusted for multiple-inference using the Benjamini-Hochberg method. Associations between immune response variables and overall composition of the gut microbiota were assessed by PERMANOVA using the R package ADONIS. Associations between short chain fatty acids (SCFAs) and immune response variables was determined using spearman rank correlation analysis, Bonferroni corrected for multiple comparisons. To define participants as responders and non-responders, we binned the immune response variables into quartiles to group participants as the lowest response (quartile 1) or the highest response (quartile 4). Using these quartiles, differentially abundant taxa associated with immune response was assessed in the same method as above. To assess the association between SCFAs and fibre intake, we compared the change in SCFA concentration (on a $\log_{10}$ scale) from baseline to 12-weeks post vaccine in participants with low fibre intake to those with high fibre intake, determining significance using a Student's t-test.

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Supplementary figure legend

Figure S1 Changes in alpha and beta diversity from baseline to twelve-weeks post 1st BTN162b2 vaccine. (A) A significant shift in beta diversity occurred between baseline and post 1st BTN162b2 vaccine (p=0.0159; PERMANOVA). (B) A trend towards a significant reduction in Shannon alpha diversity was observed (p=0.07812; Wilcoxon rank test).

Supplementary tables – refer to separate document

Supplementary Table 1 Participant characteristics and sample collection information

Variable	BNT162b2 (n=52)
Age, years (median [IQR])	4 [32.3-54]
Female [Male]	46 [6]
Seropositivity to SARS-CoV-2 (Reactive [Non-reactive])	2 [50]
Blood sample collected post 1st vaccine (Yes [No])	47 [5]
Blood sample collected post 2nd vaccine (Yes [No])	18 [34]
Baseline stool samples collected (Yes [No])	48 [4]
Twelve-weeks post 1st vaccine stool samples collected (Yes [No])	41 [11]
Matching baseline and twelve-week stool samples collected (Yes [No])	38 [14]
Dietary fibre grouping (Low [Moderate] High) (n=41)	13 [14] 14
Total dietary fibre intake, g/day (mean [SD]) (n=41)	24.7 [11.9]
Vegetarian (Yes [No]) (n=41)	4 [37]
Antibiotics taken within 9 months of collecting stool (Yes [No]) (n=41)	3 [38]
Significant* change in weight or food intake over the past year (Yes [No]) (n=41)	7 [34]

IQR – inter quartile range, SD – standard deviation. * Significant body weight changes >10% weight gain or loss and significant changes in food intake included becoming vegetarian/vegan, stopped consuming gluten, dairy or sugar, increased fruit, or vegetable intake, etc.

Supplementary Table 2 Gut microbiota species significantly positively or negatively associated (p<0.01; statistical test) with higher avidity after the 2nd dose of the BNT162b2 vaccine (D2) in a subset of participants (n=15)

	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj	Kingdom	Phylum	Class	Order	Family	Genus	Species
ASV_12	652.6828066	16.61603655	1.12976698	14.70748999	5.77085204873443E-49	4.0395964341141E-47	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Phascolarctobacterium	succinatutens
ASV_40	856.3116714	15.01679959	1.12976278	13.29199355	2.57621721167962E-40	1.20223469878382E-38	Bacteria	Synergistota	Synergistia	Synergistales	Synergistaceae	Cloacibacillus	evryensis
ASV_56	1163.66926110663	18.9073311	1.129781224	16.73539151	7.23796558640599E-63	1.01331518209684E-60	Bacteria	Firmicutes	Negativicutes	Veillonellales-Selenomonadales	Selenomonadaceae	Megamonas	funiformis
ASV_90	299.9462157	-11.55809526	1.09781944	-10.52822972	6.40272464578869E-26	1.12047681301302E-24	Bacteria	Firmicutes	Negativicutes	Acidaminococcales	Acidaminococcaceae	Acidaminococcus	intestini
ASV_104	165.2171921	-7.257755983	1.094064509	-6.633755071	3.27252692455385E-11	3.81794807864615E-10	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	massiliensis
ASV_123	100.8114371	-5.521510801	1.091821644	-5.057154555	4.25558338438288E-07	4.58293595241233E-06	Bacteria	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	bifidum
ASV_177	53.6415468	4.110286262	1.128616539	3.641880232	0.000270654	0.00270654	Bacteria	Actinobacteriota	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium	animalis
ASV_202	127.887441	14.00883584	1.12979702	12.39942714	2.63198463344399E-35	9.21194621705396E-34	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	plebeius
ASV_217	45.77577167	12.5267809	1.129873892	11.08688411	1.45259641590064E-28	3.38939163710149E-27	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Tannerellaceae	Parabacteroides	johnsonii
ASV_309	60.52674668	12.85841755	1.129833358	11.38080891	5.21140863375641E-30	1.45919441745179E-28	Bacteria	Desulfobacterota	Desulfovibrionia	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio	fairfieldensis
ASV_328	27.36761572	11.33415571	1.123464443	10.08857537	6.20636856209842E-24	9.65435109659754E-23	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	ovatus
ASV_384	36.43927284	12.11973529	1.129888243	10.72649031	7.64447009656451E-27	1.5288940193129E-25	Bacteria	Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	Eisenbergiella	tayi
ASV_635	21.05201209	11.23206477	1.138659715	9.864285715	5.9456105183706E-23	8.32385472571884E-22	Bacteria	Firmicutes	Clostridia	Oscillospirales	Ruminococcaceae	Negativibacillus	massiliensis

Supplementary Table 3 Equations used to calculate absolute (U/mL) and relative fractional avidity (%), and total absolute and relative fractional avidity indices

	Very low	Low	Medium	High	Very high
Absolute fractional avidity (U/mL)	$AFA_{vlo} = Y_0 - Y_{0.5}$	$AFA_{lo} = Y_{0.5} - Y_{0.75}$	$AFA_{med} = Y_{0.75} - Y_1$	$AFA_{hi} = Y_1 - Y_2$	$AFA_{vhi} = Y_2$
Total absolute fractional avidity index	$2 * AFA_{vhi} + 1 * AFA_{hi} + 0.25 * AFA_{med} + 0.25 * AFA_{lo} + 0.5 * AFA_{vlo}$				
Relative fractional avidity (%)	$RFA_{vlo} = \frac{AFA_{vlo}}{Y_0} * 100\%$	$RFA_{lo} = \frac{AFA_{lo}}{Y_0} * 100\%$	$RFA_{med} = \frac{AFA_{med}}{Y_0} * 100\%$	$RFA_{hi} = \frac{AFA_{hi}}{Y_0} * 100\%$	$RFA_{vhi} = \frac{AFA_{vhi}}{Y_0} * 100\%$
Total relative fractional avidity (TRFA)*	$TRFA = -0.533 * RFA_{vlo} + 0.0269 * RFA_{lo} + 0.477 * RFA_{med} + 0.492 * RFA_{hi} + 0.495 * RFA_{vhi}$				

AFA – absolute fractional avidity, RFA – relative fractional avidity

*Based on eigenvalue plot loadings of each avidity for PC1 of all avidity data