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ORIGINAL RESEARCH ARTICLE

# Modulation of host immune response by carotene supplementation in a COVID-19 vaccination mouse model

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## Abstract

Carotenoids, known for their immunomodulatory and gut microbiota-modulating effects, have drawn attention as potential dietary adjuvants to enhance vaccine efficacy and maintain gut health. This study aimed to evaluate the effect of carotene supplementation on immune response with insight into gut microbiome using an *in vivo* animal model. The BALB/c mice were fed daily with CaroGaia (50 mg/kg of body weight), a carotene supplement that contained 33.3% α-carotene and 66.6% β-carotene, by oral gavage for 70 days. Mice fed with the vehicle served as controls. The mice in the vaccinated groups received two doses of the CoronaVac inactivated virus vaccine on days 14 and 42. Flow cytometry revealed no significant modulation of lymphocyte subsets (total T lymphocytes, T-helper cells, cytotoxic T lymphocytes, and B cells) with carotene supplementation. In addition, there were no significant differences in the levels of SARS-CoV-2 immunoglobulin G and interferon-gamma in plasma between treatment and control groups. In contrast, the vaccinated carotene group showed an increased SARS-CoV-2 antigen-specific splenocyte proliferation. In the gut microbiome, carotene supplementation appeared to alter the gut microbiota composition. However, no significant changes were observed in the short-chain fatty acids (SCFA) levels, such as acetic acid, butyric acid, and propionic acid. Furthermore, the differential abundance analysis showed that carotene supplementation reduced the levels of SCFA producers (*Odoribacter* and *Monoglobus* genera) in unvaccinated mice compared to the control group, while it enriched the level of SCFA producers (Ruminococcaceae family) and reduced pathobiont levels, commensal bacteria that have pathogenic potential (*Mucispirillum* genus), in the vaccinated group.

***Keywords:*** COVID-19; SARS-CoV-2; Vaccination; Carotenoid; Carotene; Immune-modulation; Gut microbiota; Short-chain fatty acid

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## 1. Introduction

The coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in China in late 2019.[1] By March 2020, the World Health Organization had declared it a pandemic. The virus rapidly spread worldwide, causing severe disruptions to societies, economies, and politics. The pandemic had led to millions of infections and deaths without signs of abating. Given

the ability of vaccines to expose the immune system to antigens and activate targeted immune responses, there was an urgent need to roll out vaccination programs to reduce the morbidity and mortality associated with COVID-19. Therefore, mass vaccination against SARS-CoV-2 has been widely implemented to curb the spread of the disease.

Vaccination offers substantial protection by generating immunological memory against the virus, enabling the rapid production of neutralizing antibodies upon subsequent exposure. Among the COVID-19 vaccines, the mRNA platform has garnered significant attention as it represents the first widespread application of mRNA vaccination in humans. Despite this advancement, the seemingly traditional inactivated virus vaccines continue to be administered due to factors including public hesitancy toward mRNA vaccines and the logistical challenges posed by the stringent storage and delivery requirements.[2] However, inactivated virus vaccines have generally demonstrated lower efficacy against COVID-19 than their mRNA counterparts.[3] Two to three doses of inactivated COVID-19 vaccines were reported to provide 90.15% protection against severe and critical COVID-19 cases, even in the most vulnerable elderly population (≥60 years old).[4] However, the immune response specific to SARS-CoV-2 elicited by two doses of CoronaVac, an inactivated virus vaccine, was reported to wane significantly after 6 months.[5] Consequently, a booster or third dose of the vaccine was highly recommended.[5] Hence, there is a need to improve and maintain the immune response to the vaccine. Various factors affect the normal functioning of the immune system, including nutrition, lifestyle habits, stress levels, and sleep quality.

The immune system comprises a repertoire of cells, tissues, and molecules that work together to mediate resistance against infections.[6] It is well established that B cells, cluster of differentiation 4-positive ($CD4^+$) regulatory T (Treg) cells, and $CD8^+$ cytotoxic T lymphocytes (CTLs) are the three essential components of the adaptive immune system responsible for controlling SARS-CoV-2 infection.[7] Clinical trials reported that inactivated SARS-CoV-2 vaccination activates both innate and adaptive immunity, resulting in the upregulation of immune cells, such as $CD16^+$ monocytes, $CD4^+$ Treg cells, and $CD8^+$ CTLs, along with increased levels of neutralizing antibodies.[8] B cells play a pivotal role in antibody-mediated (humoral) immunity.[9] Upon activation, the activated antigen-specific B cells differentiate into plasma cells, which produce antigen-specific antibodies, and memory B cells, which enable a swift response upon reinfection.[9] The T-helper (Th) cells secrete cytokines that can regulate B cell activation and CTLs,[7] while the CTLs kill virus-infected or abnormal cells.[10] A well-balanced diet is widely recognized as crucial for optimal cellular functioning, including those in the immune system.[11] When the immune system is activated during an infection, it requires abundant energy for clonal expansion and the production of new proteins and structures. Therefore, optimal nutrition is essential for immune cells to respond to pathogens rapidly and effectively. Micronutrients, such as vitamins and trace elements, are essential to support both the innate and adaptive immune systems.[11] Research suggests that deficiencies or low levels of these micronutrients can weaken immune function and reduce resistance to infections.[11]

Nutritional interventions have potent effects on the activation of host immune systems. Carotenoid supplements are nutritional interventions that exhibit diverse bioactivities, including antioxidant, anti-inflammatory, and anticancer properties.[12] The main carotenoids in crude palm oil are α- and β-carotenes, which comprise 41.3% and 41.0% of the total carotenoids in commercial red palm oil, respectively.[13] Carotenes are fat-soluble Vitamin A pre-cursors metabolized in the gut or liver to produce retinol, commonly known as Vitamin A. Vitamin A deficiency is the most common micronutrient deficiency globally, especially in developing countries with low meat and protein intake.[14] In addition, Vitamin A deficiency in infants and children has been associated with reduced immune response and is a risk factor for several diseases, particularly vision impairment.[15] Besides animal products, Vitamin A can be obtained from Vitamin A pre-cursors, such as α- and β-carotenes.[14] Carotenoids have been reported to have immunomodulatory effects by increasing the activity of natural killer (NK) cells and plasma interferon-gamma (IFN-γ) levels in a xenograft model of human breast cancer.[16] In this model, carotene supplementation upregulated anti-inflammatory markers (interleukin 4 [IL-4] and IL-13) while downregulating pro-inflammatory markers (IL-β, IL-6, and tumor necrosis factor-α) in nude mice.[17] In addition, carotene supplementation enhanced the production of peripheral blood NK cells and B cells, thus supporting the immunomodulatory effects of carotenes.[17] Numerous cellular models concluded that β-carotene and its metabolites display antioxidant activity through the inhibition of reactive nitrogen ($ONOO^-$) and oxygen species ($H_2O_2$), respectively, reducing lipid peroxidation activity.[15] In addition, Vitamin A supplementation in porcine circovirus type 2-vaccinated pre-pubertal gilts has been reported to significantly increase circovirus antibody titers compared to the control group.[18] Although numerous studies have demonstrated the immunomodulatory potential of Vitamin A and its pre-cursors as dietary supplements, their mechanism of action remains poorly understood.

The gastrointestinal tract (GIT) is the home to the gut-associated lymphoid tissue (GALT), the largest immune organ in the human body.[19] GALT forms part of the mucosa-associated lymphoid tissue and comprises many immune cells. Hence, GIT is a primary site for immune activities. At the same time, the GIT, particularly the colon, serves as an invaluable repository for gut microbiota, such as bacteria, viruses, and fungi.[19] The comprehensive collection of gut microbiomes can contribute to a vast palette of metabolic enzymes and pose a greater metabolic potential than their host.[19] Due to the proximity between gut microbiota and GALT, they inevitably affect one another as these gastrointestinal inhabitants can interact with nutrition and selectively promote or inhibit specific bacteria, thereby modulating gut microbiota populations.[20] In addition, the gut microbiome plays crucial roles in nutrition metabolism, energy metabolism, and immunomodulation.[20] Studies reported that short-chain fatty acids (SCFAs), metabolites produced by the gut microbiota, can enhance B-cell metabolism to support the production of the energy required to generate antibodies and cytokines.[21] These metabolites also upregulate genes responsible for plasma cell differentiation and class switching, vital for immune responses to pathogens or vaccines. The present literature suggests that carotene supplementation can modulate host gut microbiota and SCFA production.

The SCFAs are key metabolites produced by gut microbiota, which can modulate immune response and regulate the activity of innate cells, such as macrophages, neutrophils, and dendritic cells.[22] For example, SCFAs were reported to limit SARS-CoV-2 infection by downregulating the level of angiotensin-converting enzyme 2, the main receptor responsible for viral infections, while improving antiviral immunity.[23] In addition, SCFAs can also control SARS-CoV-2 infection by downregulating transcription of genes responsible for modulation of viral entry and replication, such as IFN, transmembrane protease serine 2, and retinoic acid-inducible gene 1 receptors, while maintaining the permeability of intestinal cells.[19] Moreover, SCFAs can modulate the differentiation of T lymphocytes and B cells, which are responsible for the adaptive immune response.[22]

This study used a mouse model to investigate how daily carotene supplementation modulates the immune response to COVID-19 vaccination. This research aimed to unveil the mechanism of action of carotene supplementation in immune response post-vaccination and provide evidence of its health benefits. In addition, this study also explored how carotene influences gut microbiota and SCFA production, offering insights into the overall action of carotene supplementation.

## 2. Materials and methods

### 2.1. Materials

CaroGaia (30% oil suspension) used for dietary intervention was a gift from PhytoGaia Sdn Bhd, Malaysia. The vehicle used was palm oil (Buruh Cooking Oil, Lam Soon, Malaysia). The inactivated SARS-CoV-2 vaccine (CoronaVac, Sinovac Biotech Ltd, China) was a kind gift from Pharmaniaga Lifescience Sdn Bhd, Malaysia.

### 2.2. Ethical approval

The ethical approval for this study was obtained from the Monash University Animal Ethics Committee (AEC Project ID: 29899).

### 2.3. Animal study design

Ten-week-old male BALB/c mice were obtained and housed individually at the animal holding facility of Monash University Malaysia. A total of 32 mice were used in this study. The mice were acclimated to the housing conditions for 1 week before the study began. During the study, the mice were subjected to a 12-h light/12-h dark cycle with *ad libitum* access to food and water. A standard mouse pellet chow was provided throughout the study. The experimental design is illustrated in Figure 1. Briefly, 32 male BALB/c mice were evenly divided into two dietary intervention groups, that is, (i) control (fed with the vehicle – palm oil) and (ii) carotene-fed group. The mice were fed daily with 100 µL of carotene (50 mg/kg body weight) or palm oil (vehicle) through gavage. On day 14, the mice in each dietary group were split into two sub-groups, that is, (i) non-vaccinated (phosphate-buffered saline [PBS]) and (ii) vaccinated (CoronaVac) (Table 1). Vaccination was administered by an intramuscular injection into the hind leg of the mice using a 30G needle, with 0.1 mL of CoronaVac (0.6 µg) as vaccine intervention for the vaccinated subgroup and 0.1 mL of PBS as control for the non-vaccinated subgroup. On day 42, plasma samples were collected from the mice and were given a second dose of CoronaVac or PBS. At the study's endpoint (day 70), the mice were humanely euthanized, and samples of plasma, feces, and spleens were collected for analysis.

### 2.4. Mice euthanasia

On day 70, the mice were weighed to calculate and prepare the dosage for a ketamine and xylazine cocktail, at concentrations of 100 mg/kg and 10 mg/kg, respectively. The concoction was administered through intraperitoneal injection. Once the mice were determined to be fully anesthetized, euthanasia was performed by cardiac puncture.

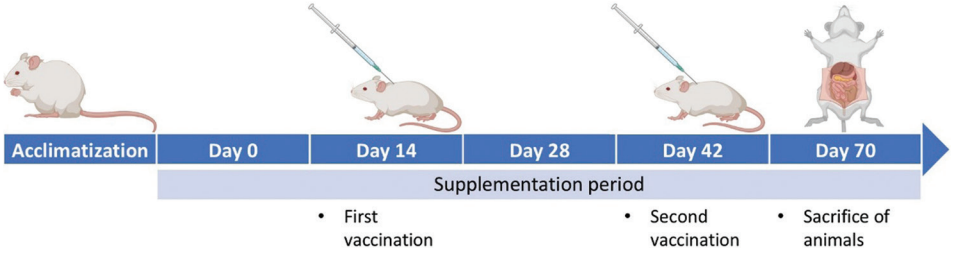


**Figure 1.** The animal study design. In the supplementation period, half the mice were fed daily with 100 µL vehicle (palm oil), while the other half were fed with 100 µL carotene (50 mg/kg body weight) by oral gavage for 70 days. For vaccination, half the mice in each supplementation arm received intramuscular injections of 100 µL (0.6 µg) of the CoronaVac or 100 µL PBS (control) on days 14 and 42.

**Table 1. Animal grouping**

| Dietary intervention | Vaccine intervention | Number of mice |
|---|---|---|
| Vehicle (palm oil) | PBS | 8 |
| | C19V | 8 |
| Carotene (50 mg/kg body weight) | PBS | 8 |
| | C19V | 8 |
| Total | | 32 |

Abbreviations: C19V: CoronaVac vaccine; PBS: Phosphate-buffered saline.

### 2.5. Isolation of splenocyte

During the autopsy, the spleen was aseptically collected and transferred to a petri dish containing 6 mL of RPMI 1640 culture medium (11875-093, Gibco, USA) supplemented with 5% fetal bovine serum (FBS; A5256701, Gibco, USA), 1% penicillin-streptomycin (15140122, Gibco, USA), and 1% glutamine. The splenic capsules were carefully snipped to release the splenocytes into the medium. The resulting splenocyte suspension was transferred into 15 mL Falcon tubes and kept on ice. Splenocytes were isolated through centrifugation (1,200 rpm for 10 min at 4°C).

### 2.6. Splenocyte proliferation assay

This assay was carried out to determine the proliferation of splenocytes in response to stimulation with CoronaVac. The supernatant was discarded, and the splenocytes were resuspended in 1 mL of complete culture medium (RPMI 1640 medium supplemented with 10% FBS and penicillin-streptomycin). The cell density was adjusted to $5 \times 10^4$ splenocytes/mL in complete medium containing 10 µg/mL of COVID-19 vaccine. The cells were seeded into a 96-well culture plate at 200 µL per well ($5 \times 10^3$ cells/well), and the plate was incubated for 72 h at 37°C in a humidified incubator with 5% $CO_2$. Splenocyte proliferation was assessed using the Cell Counting Kit-8 (CK04-01, Dojindo, Japan). The culture supernatants were harvested and stored at -80°C for subsequent cytokine analysis.

### 2.7. Immunophenotyping using flow cytometry

The peripheral blood lymphocytes collected in heparinized tubes were analyzed using fluorochrome-labeled antibodies to $CD3^+$ T lymphocytes, $CD4^+$ Th cells, $CD8^+$ CTLs, and B cells, and a flow cytometer (FACSVerse cell analyzer, BD, USA). The immunostaining was carried out according to the manufacturer's protocol.

### 2.8. IFN-γ production

The amount of IFN-γ, a cytokine crucial for macrophage activation and antigen presentation, in the supernatant of splenocyte cultures, was quantified using a commercial mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (#88-7314-88, Invitrogen, USA) using the manufacturer-recommended protocol.

### 2.9. Plasma SARS-CoV-2 recombinant receptor-binding-domain antibody levels

The plasma SARS-CoV-2 recombinant receptor-binding-domain (RBD) antibody level was quantified using the SARS-CoV-2 RBD mouse immunoglobulin G (IgG) ELISA kit (CoV2-RBD mIgG, Novatein Biosciences, USA) as per the manufacturer-recommended protocol. A mouse SARS-CoV-2 spike-neutralizing antibody (#40592-MM57, Sino Biological, China) was used as a positive control. The assay was carried out according to the recommended protocol.

### 2.10. Characterization of gut microbiota

#### *2.10.1. DNA purification and sequencing*

To evaluate the impact of carotene supplementation on gut microbiota, 16S amplicon sequencing was performed on fecal samples collected at the endpoint, day 70. The bacterial genomic DNA (gDNA) was extracted using the QIAamp® PowerFecal® Pro kit (QIAGEN, Germany) according to the manufacturer's instructions. The quality and concentration of the gDNA were determined using a nano-spectrophotometer. The V3–V4 region was targeted for sequencing, using forward primer

5'-CCTAYGGGRBGCASCAG and reverse primer 5'-GGACTACNNGGGTATCTAAT. The DNA samples were sent to an accredited laboratory (NovogeneAIT Genomics, Singapore) for library preparation, sequencing, and bioinformatics analysis.

#### *2.10.2. Bioinformatics analysis pipeline*

The raw FASTQ files from sequencing were analyzed using the bioinformatics methods outlined in Table 2.

### 2.11. Determination of SCFA levels in fecal samples

The levels of SCFAs in fecal samples were identified and determined using gas chromatography-mass spectrometry (GC-MS) (Agilent 7890A Gas Chromatograph, Agilent, USA). The water-methanol (80:20, v/v, pH 1.5 – 2.5) diluent, standard stock, and internal standards were prepared according to Gray *et al.*[24] with slight modifications. Stock solutions of acetic acid (30.0 µL), propionic acid (30.3 µL), isobutyric acid (39.0 µL), and butyric acid (32.7 µL) were prepared in acidified diluent to achieve the final concentrations of 52.60 mM, 39.43 mM, 42.05 mM, and 36.80 mM, respectively. The internal standard (4-methyl valeric acid) was spiked into all calibration standards at a concentration of 7.9 mM. The calibration curves of all SCFA standards were then established to allow quantification of SCFA levels in fecal samples.

The fecal samples were withdrawn from −80°C storage, thawed, and homogenized in 1,993 µL of the water-methanol diluent with 7 µL of phosphoric acid (85% w/w) and mixed thoroughly for 10 min using a vortex (Vortex-Genie 2, Scientific Industries, USA). The fecal suspensions were centrifuged (12,000 rpm for 10 min), and 1,800 µL of the supernatant was transferred to 2 mL GC vials. The 46.7 µL of internal standard (7.9 mM) was added to each vial before analysis.

SCFAs were quantified using an Agilent 8890 gas chromatograph (G3542A, Agilent Technologies, USA) paired with a 5977C mass selective detector (G7077C, Agilent Technologies, USA), and a 7693A automatic liquid sampler (G4513A, Agilent Technologies, USA). Hydroguard FS water-resistant guard column (5 m length, 0.25 mm ID, 10079, Restek, USA) was used in combination with a high polarity, DB-FATWAX Ultra Inert PEG column (30 m length, 0.25 mm ID, 0.25 film thickness, G3903-63008, Agilent Technologies, USA). Data were analyzed using the Enhanced ChemStation software (version E.02.02.1431, Agilent, USA).

### 2.12. Statistical analysis

All statistical analyses were performed using Prism 10.3.1 (GraphPad, USA). Comparisons between two groups were evaluated with a two-tailed unpaired *t*-test with Welch's correction, while multiple group comparisons were conducted using analysis of variance (ANOVA) with Tukey *post hoc* test. A $p<0.05$ was considered statistically significant.

## 3. Results

This section outlines the results of the study.

### 3.1. Modulation of immune responses by carotene supplementation

The effect of carotene supplementation under various conditions is outlined in the following subsections.

#### *3.1.1. Effect of carotene supplementation on lymphocyte subsets*

The data from the flow cytometry analysis revealed no significant differences in $CD3^+$ T lymphocytes, $CD4^+$ Th cells, $CD8^+$ CTLs, and B cells between the baseline groups and carotene supplementation groups (Figure 2 and Table A1).

#### *3.1.2. Effect of carotene supplementation on SARS-CoV-2-specific antibody production*

The plasma samples collected on days 42 and 70 of the intervention were used to determine the SARS-CoV-2-specific

**Table 2. Methods used for bioinformatics analysis**

| Data processing | Description | Methods/packages |
|---|---|---|
| Sequence assembly | Merge paired-end reads | BBMap |
| Data split | Trimming primer sequences | QIIME |
| Amplicon sequence variant (ASV) denoise | Reconstruct ASVs from noisy amplicon sequencing reads | DADA2 in QIIME |
| Taxonomy classification | Classify pre-processed reads to the respective taxonomy | QIIME using silva138 AB V3–V4 classifier |
| Generation of phyloseq object | Export QIIME artifact into phyloseq | QIIME2R |
| Heatmap | Visualization of microbiome compositions | pheatmap in R |
| Alpha diversity | Estimation of alpha diversity | Tidyverse in R |
| Beta diversity | Estimation of beta diversity | mia and miaViZ in R |
| Differential abundance analysis | Identify differentially abundant microbes | LEfSe, DESeq2, and corncob in R |

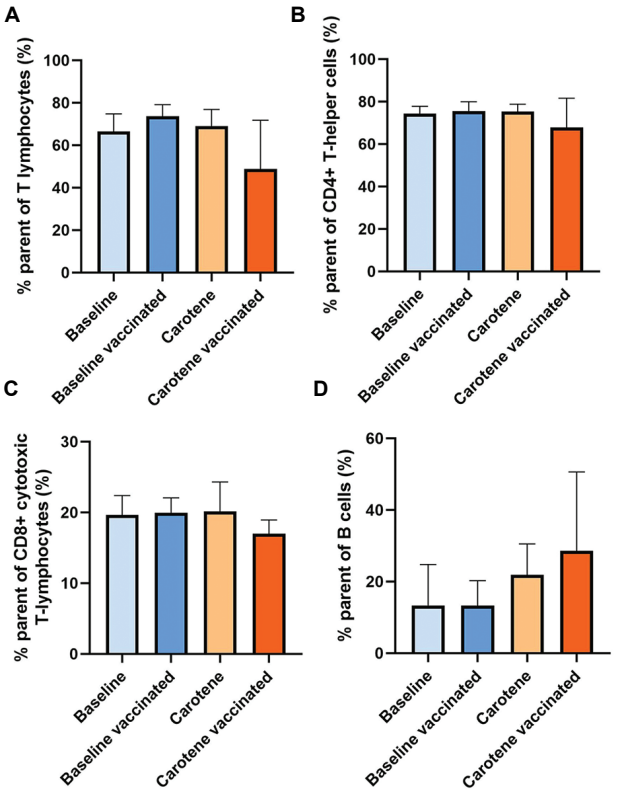


**Figure 2.** Quantification of (A) cluster of differentiation 3-positive ($CD3^+$) T lymphocytes, (B) $CD4^+$ T-helper cells, (C) $CD8^+$ cytotoxic T lymphocytes, and (D) B cells in the peripheral blood of mice from the four study groups: (i) baseline (control mice fed daily with the vehicle palm oil), (ii) baseline vaccinated (control mice fed daily with the vehicle and injected with CoronaVac), (iii) carotene (mice fed daily with carotene), and (iv) carotene vaccinated (mice fed daily with the carotene and injected with CoronaVac). Multiple comparisons using ordinary one-way ANOVA revealed no significant differences in the percentage of lymphocytes between these groups ($p>0.05$). Data are presented as mean ± SD, derived from three mice ($n=3$) per group.

IgG antibody level (Figure 3). There were no significant differences in the plasma SARS-CoV-2 levels between the carotene supplementation group and the vehicle group.

#### *3.1.3. Effect of carotene supplementation on SARS-CoV-2 antigen-induced splenocyte proliferation*

Splenocytes from carotene-fed mice cultured in the presence of the SARS-CoV-2 antigen showed significantly higher proliferation than splenocytes from the vehicle-vaccinated animals ($p<0.05$, Figure 4 and Table A2).

#### *3.1.4. Effect of carotene supplementation on IFN-γ production*

The supernatant of splenocyte cultures from the carotene-vaccinated group showed higher levels of IFN-γ production (Figure 5). However, the difference was not statistically significant ($p>0.05$).

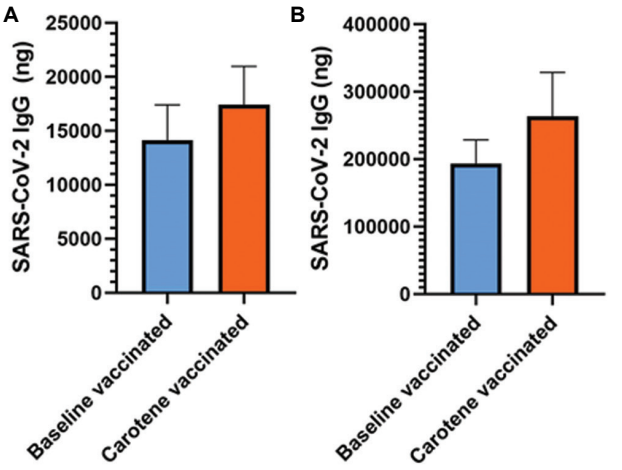


**Figure 3.** Quantification of plasma SARS-CoV-2 immunoglobulin G (IgG) levels from mice in the baseline vaccinated (control mice fed daily with the vehicle and injected with CoronaVac) and carotene vaccinated (mice fed daily with the carotene and injected with CoronaVac) groups on days (A) 42 and (B) 70. Data are presented as mean ± SD, derived from three mice ($n=3$) per group.

### 3.2. Modulation of gut microbiota by carotene supplementation

#### *3.2.1. Alpha diversity*

Alpha diversity determines the structure of single microbial communities based on richness and evenness.[25] Richness represents the total number of species present in a community, whereas evenness represents the uniformity of relative species abundances within a community.[25] Several alpha diversity indices, such as Chao1, Inverse Simpson, Pielou, and Shannon, are applied to determine alpha diversity. The Chao1 index is a diversity estimator of species richness, highlighting rare species. The inverse Simpson index measures both richness and evenness.[25] Pielou's index indicates the evenness of species distribution. The Shannon index also measures species richness and evenness, emphasizing rare species more than the Simpson index.[25] Carotene supplementation did not significantly affect the alpha diversity in vaccinated and unvaccinated subjects compared across all groups (Figure 6), suggesting that carotene supplementation did not substantially impact the overall microbial diversity in terms of richness and evenness.

#### *3.2.2. Beta diversity*

Beta diversity is a comparative analysis of the composition of different microbial communities. It measures how distinct one microbial community is from another, illustrating the variability of microbial composition between samples.[25] No significant differences were observed across the groups in the principal coordinate analysis scatter plot based on multidimensional scaling of Aitchison distance (Figure 7).

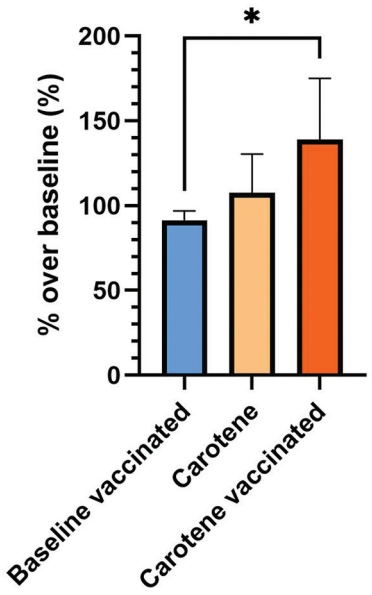


**Figure 4.** Splenocyte proliferation rate across baseline (fed daily with the vehicle), baseline vaccinated (fed daily with the vehicle and injected with CoronaVac), carotene (fed daily with carotene), and carotene vaccinated (fed daily with carotene and injected with CoronaVac) groups at day 70. The splenocytes were cultured in the presence of CoronaVac (10 μg/mL) for 72 h. Splenocyte proliferation was determined using the Cell Counting Kit-8. Data are presented as the percentage of cell culture compared to proliferation observed in the splenocyte cultured from mice in the baseline group, expressed as mean ± SD, derived from six mice ($n$=6) per group.
Note: *$p$<0.05.

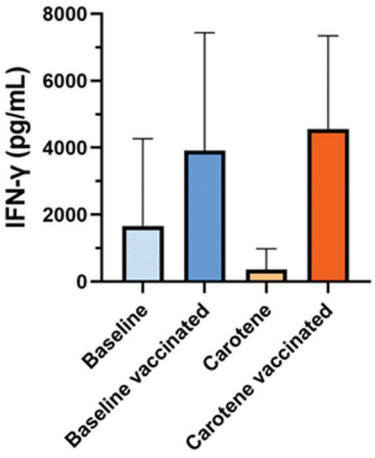


**Figure 5.** Quantification of interferon-gamma (IFN-γ) across baseline (fed daily with the vehicle), baseline vaccinated (fed daily with the vehicle and injected with CoronaVac), carotene (fed daily with carotene), and carotene vaccinated (fed daily with the carotene and injected with CoronaVac) groups at day 70. The splenocytes were cultured in the presence of CoronaVac (10 μg/mL) for 72 h. The IFN-γ concentration in each group's culture supernatant was determined using a commercial IFN-γ enzyme-linked immunosorbent assay kit. Data are presented as mean ± SD, derived from three mice ($n$=3) per group.

#### *3.2.3. Taxonomic abundance cluster heatmap*

The composition of the microbial community was visualized with a composition heatmap (Figure 8). The Y-axis represents the bacterial taxa, and the X-axis represents samples. The colour of each intersection depicts the taxon abundance in the sample.

#### *3.2.4. Differential abundance analysis*

Differential abundance analysis is carried out to determine the differences between microbial communities. In this project, multiple differential abundance analysis methods (e.g., DESeq2, LEfSe, and Corncob) were utilized to cross-validate results as different methods varied in statistical assumptions regarding data distribution, sparsity, and the handling of zero inflation.[26] Applying multiple methods yielded overlapping and distinct sets of differentially abundant taxa. The differences can be attributed to the varying statistical assumptions. Overlapping taxa suggest a high level of confidence in the biological relevance of these taxa, as they are robustly detected across methods that handle data differently. This combined approach allows for a comprehensive assessment of microbial changes, and taxa that were consistently identified across methods were prioritized for interpretation in this study.

(a) Modulation of gut microbiome by carotene in unvaccinated groups

Carotene supplementation in the unvaccinated group displayed a reduction in *Odoribacter*, as identified by both LEfSe and DESeq2 analyses, and a reduction of *Monoglobus*, as indicated by DESeq2 and corncob methods (Figure 9).

(b) Modulation of gut microbiome by carotene in vaccinated groups

The DESeq2 and corncob analyses reported enrichment of the Ruminococcaceae family and reduction of the *Mucispirillum* genus in the vaccinated carotene supplementation group (Figure 10).

### 3.3. Effect of carotene supplementation on SCFA production

SCFAs are crucial metabolites produced by intestinal microbiota that contribute to intestinal and immune homeostasis. Targeted metabolomics was carried out to quantify the levels of SCFAs in fecal samples using GC-MS (Figure 11). There were no significant differences observed in the fecal acetic acid, butyric acid, or propionic acid across the four groups.

## 4. Discussion

In this study, the effects of carotene supplementation from palm oil as an adjuvant to enhance or modulate the

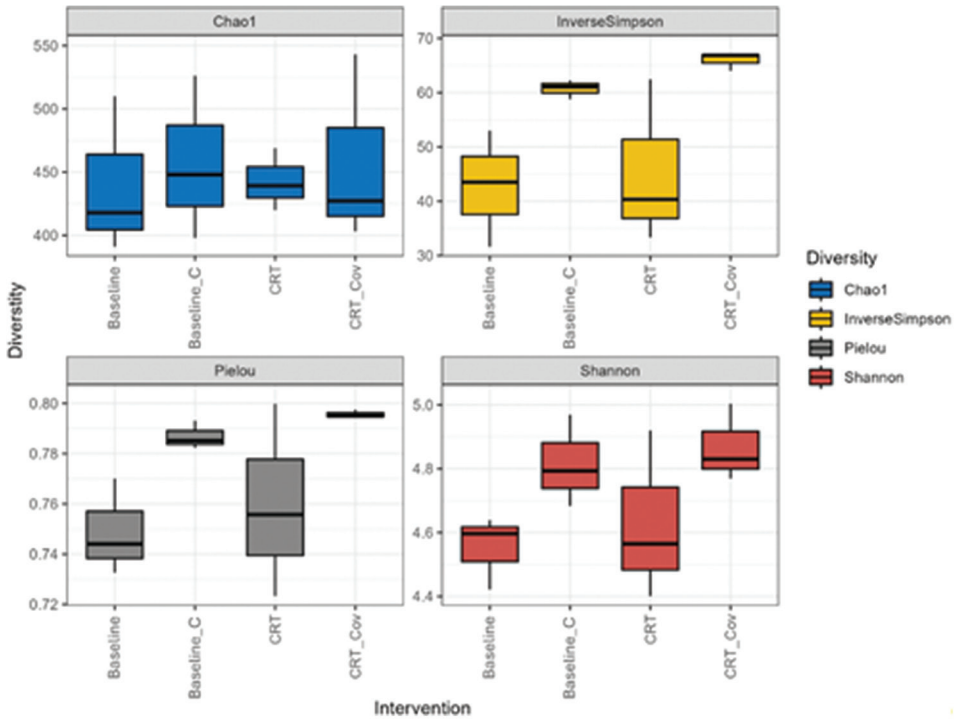


**Figure 6.** Box plots of alpha diversity indices (Chao1, Inverse Simpson, Pielou, and Shannon) of microbial diversity in feces of Baseline (fed with the vehicle), Baseline_C (fed with the vehicle and vaccinated with CoronaVac), CRT (fed with carotene), and CRT_Cov (fed with carotene and vaccinated with CoronaVac) groups. No significant differences were observed across the four groups in all indices. Data are presented as mean ± SD, derived from three mice ($n$ = 3) per group.

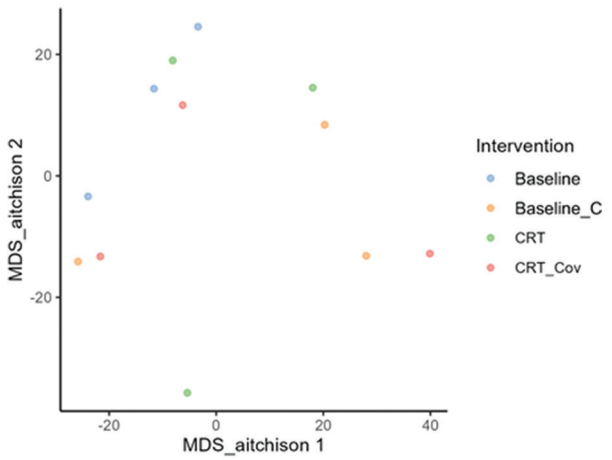


**Figure 7.** Scatter plot of principal coordinates analysis illustrating the beta diversity of microbial communities in feces from Baseline (fed with the vehicle), Baseline_C (fed with the vehicle and vaccinated with CoronaVac), CRT (fed with carotene), and CRT_Cov (fed with carotene and vaccinated with CoronaVac) groups. No significant differences were observed across the four groups. Data are derived from three mice ($n$=3) per group.
Abbreviation: MDS: Multidimensional scaling.

immune response to an inactivated COVID-19 vaccine were explored in a mouse model. The results from the flow-cytometry analysis showed no significant difference in the percentages of adaptive immune cells, such as B cells, $CD4^+$ Th cells, and $CD8^+$ CTLs, across the study groups. The daily supplementation of carotene did not significantly modulate lymphocyte counts in vaccinated and unvaccinated mice. The result aligns with an earlier random clinical trial, where the effect of daily β-carotene supplementation on immunity was investigated.[27] The β-carotene supplementation reported no significant changes in *in vitro* lymphocyte proliferation and production of IL-2. In addition, β-carotene supplementation resulted in no significant difference in profiles of leukocyte subsets ($CD3^+$ total T lymphocytes, $CD4^+$ Th cells, $CD8^+$ CTLs, and B cells). In contrast, the results from another random clinical trial demonstrated that moderate daily supplementation of β-carotene (15 mg/day for 26 days) enhanced cell-mediated immune responses in non-smoking males through upregulating the expression of major histocompatibility class II proteins, for example, the human leukocyte antigen-DR – a functional surface protein on monocytes associated with improved host immune response to antigenic stimulation.[28]

Humoral immunity was evaluated by quantification of antibody levels. In this study, daily carotene

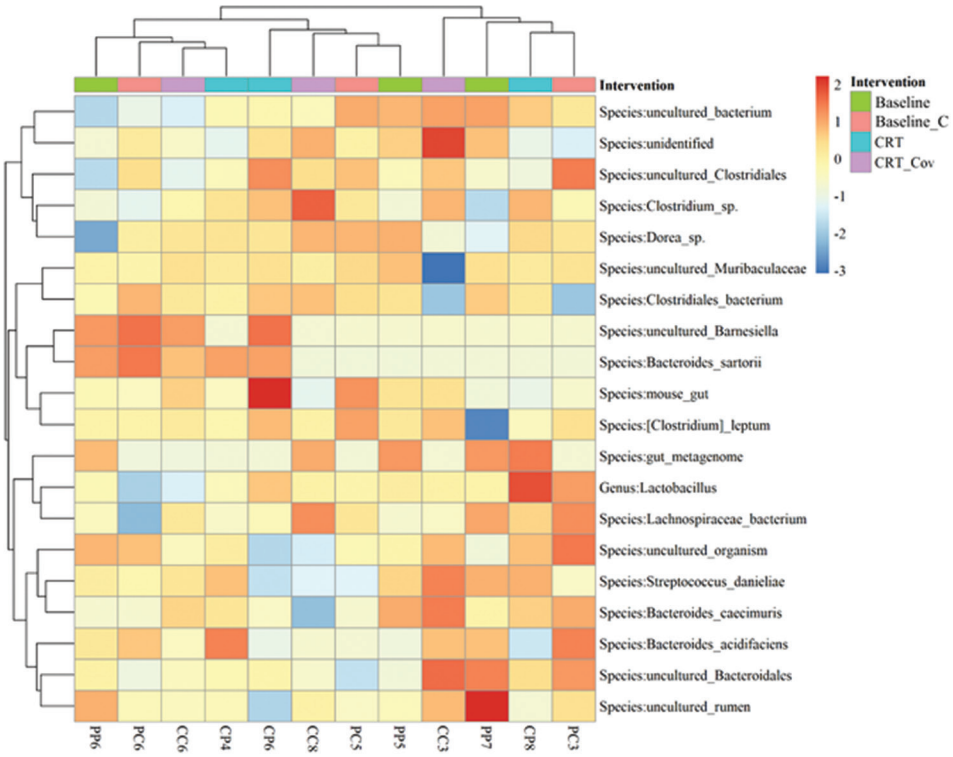


**Figure 8.** Taxonomic abundance cluster heatmap illustrating the abundance information of the top 20 phyla of all samples from Baseline (fed with the vehicle), Baseline_C (fed with the vehicle and vaccinated with CoronaVac), CRT (fed with carotene), and CRT_Cov (fed with carotene and vaccinated with CoronaVac) groups. The scale and legend in the upper right corner of the figure depict the colors in the heatmap associated with the relative abundance of operational taxonomic unit (y-axis) within each sample (x-axis).
Abbreviations: CC: Carotene vaccinated; CP: Carotene; PC: Baseline vaccinated; PP: Baseline.

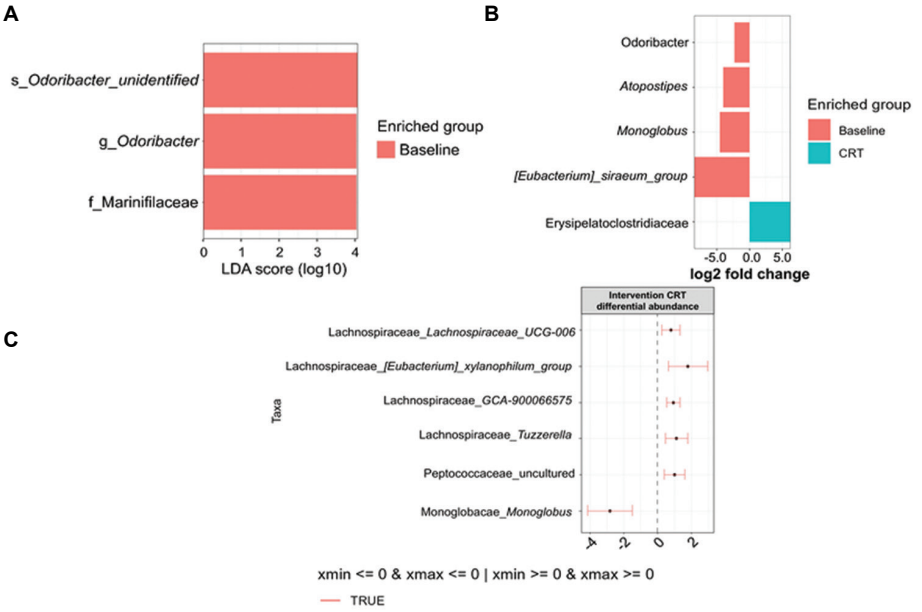


**Figure 9.** Comparison of differentially abundant bacterial taxa between unvaccinated groups receiving carotene supplementation (CRT) and vehicle palm oil (Baseline). (A) LEfSe histogram of linear discriminant analysis (LDA) scores for significant abundance differential in bacterial taxa. An LDA >4 represents species whose abundance shows differences between groups. (B) DESeq2 analysis graph showing log2 fold change to measure the significant abundance of the bacterial taxa ($p$<0.05) between groups. (C) Corncob plot using 5% false discovery rate-adjusted $p$-value ($q$-value) cutoff to measure the significant abundance of the bacterial taxa between groups. Data are derived from three mice ($n$=3) per group.

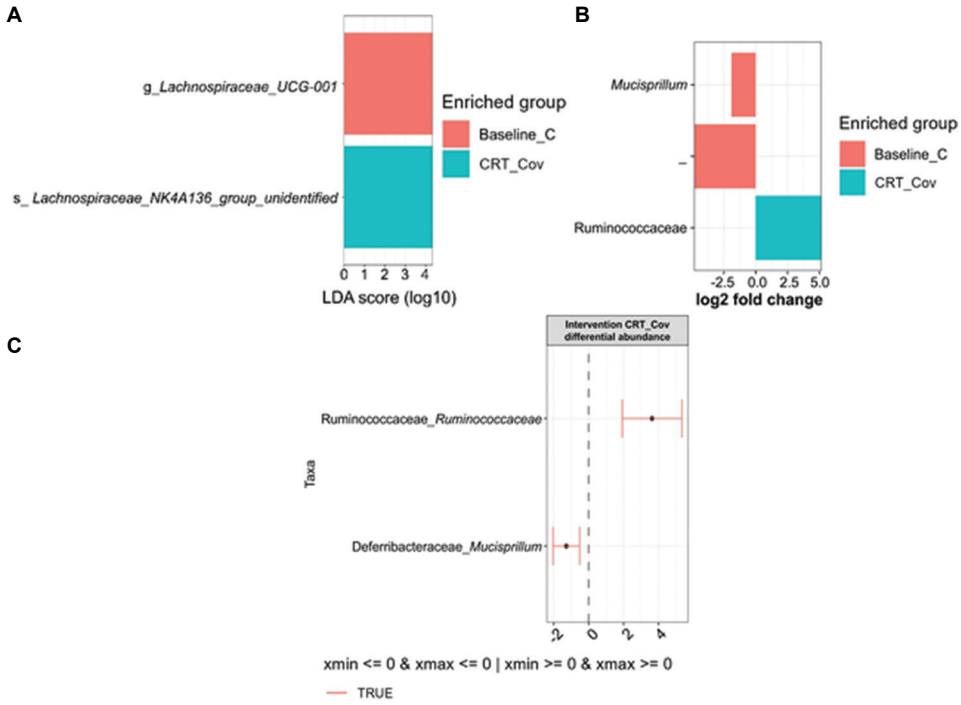


**Figure 10.** Comparison of differentially abundant bacterial taxa between vaccinated groups receiving carotene supplementation (CRT_Cov) and vehicle palm oil (Baseline_C). (A) LEfSe histogram of linear discriminant analysis (LDA) scores for significant abundance differential in bacterial taxa. An LDA >4 represents species whose abundance shows differences between groups. (B) DESeq2 analysis graph showing log2 fold change to measure the significant abundance of the bacterial taxa ($p< 0.05$) between groups. (C) Corncob plot using 5% false discovery rate-adjusted $p$-value ($q$-value) cutoff to measure the significant abundance of the bacterial taxa between groups. Data are derived from three mice ($n$=3) per group.

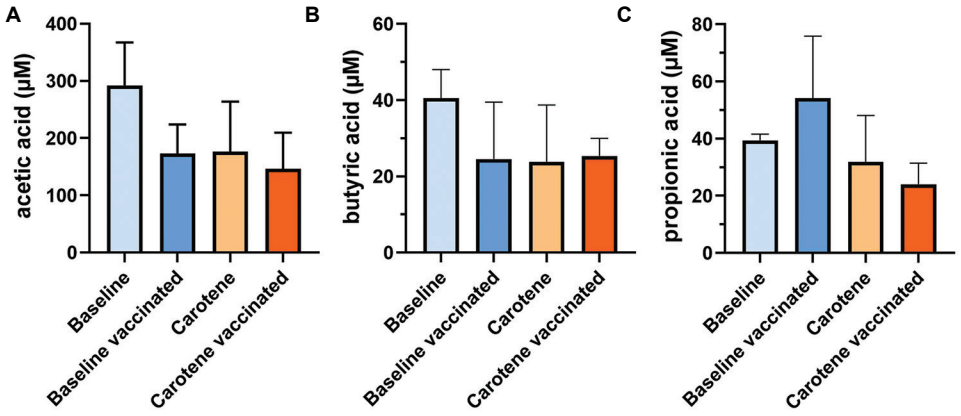


**Figure 11.** Quantification of short-chain fatty acids in mouse fecal samples after 70 days of nutritional interventions (carotene or vehicle) using gas chromatography-mass spectrometry. (A) Acetic acid. (B) Butyric acid. (C) Propionic acid. Data are presented as mean ± SD, derived from three mice ($n$=3) per group, and were analyzed using one-way ANOVA with Tukey *post hoc* test.
Notes: Baseline: mice fed with vehicle (palm oil); Baseline vaccinated: Mice fed with vehicle and vaccinated with CoronaVac; Carotene: mice fed with carotene; Carotene vaccinated: mice fed with carotene and vaccinated with CoronaVac.

supplementation reported no significant effect on SARS-CoV-2 IgG levels compared to the baseline group. A similar result was observed in Vitamin A dietary intervention in calves inoculated with an inactivated bovine coronavirus (BCoV) vaccine, where there were no significant differences in antibody responses (serum $IgG_2$, IgM, IgA and fecal IgA) between groups fed with high Vitamin A (3,300 U/kg) and low Vitamin A (1,100 U/kg).[29] However, the high Vitamin A-supplemented group displayed significantly higher serum $IgG_1$ titers than the low Vitamin A-supplemented group after two doses of the inactivated BCoV vaccine. Similar findings were also reported in influenza A-infected BALB/c mice supplemented with Vitamin A, where the control group (4,000 IU/kg diet)

reported a significantly higher IgG response than the high Vitamin A-supplemented group (250,000 IU/kg diet).[30]

The proliferation rate of splenocytes in response to an antigen represents the spleen's multiplication rate in response to the antigenic stimulus. This serves as an indicator of vaccine response, immune activation, and T-cell and B-cell responses. In this study, the splenocytes from vaccinated mice fed with carotene showed a higher proliferation rate ($p<0.05$) than those from vaccinated mice fed with the vehicle, suggesting that carotene supplementation can modulate the host immune system. A similar result was reported in a study on astaxanthin, a type of carotenoid, in BALB/c mice, where lipopolysaccharide (LPS)-induced lymphocyte proliferation was significantly increased by astaxanthin administration.[31]

Interferon-gamma is a pro-inflammatory cytokine produced by activated Th1 cells, CTLs, and NK cells.[32] This cytokine is pivotal in stimulating and modulating cell-mediated immune responses and class-switching antibodies to the IgG class.[7,31] Therefore, IFN-γ is a key biomarker to evaluate cellular immune response, where it is primarily produced by Th1 cells.[33] In this study, the differences in the levels of IFN-γ produced by antigen-stimulated splenocytes across all groups were not statistically significant ($p>0.05$), indicating that the supplementation of carotene did not affect the production of IFN-γ regardless of the status of vaccination. However, both the vaccinated control and carotene groups displayed elevated levels of IFN-γ compared to their unvaccinated counterparts, indicating the activation of T lymphocytes by the inactivated virus vaccine. In contrast to the present result, cultured splenocytes from BALB/c mice fed with β-carotene and immunized with ovalbumin were reported to produce higher IFN-γ and show increased IFN-γ mRNA expression compared to the control group.[34] In another study, supplementation of astaxanthin, a carotenoid, to BALB/c mice showed significantly higher IFN-γ production in response to LPS or concanavalin A.[31] The results of the present study suggest that daily supplementation of carotene after vaccination can increase SARS-CoV-2-specific humoral responses by promoting the proliferation of lymphocytes.

In the present study, daily carotene supplementation in unvaccinated BALB/c mice was observed to have reduced *Odoribacter* and *Monoglobus* in the gut microbiome compared to unvaccinated controls. *Odoribacter* is a member of the SCFA producers in a healthy, balanced gut microbiota.[35] *Monoglobus* is primarily represented by *Monoglobus pectinilyticus,* a gut bacterium that breaks down pectin.[36] On the other hand, carotene supplementation in vaccinated mice caused the enrichment of the Ruminococcaceae family and the reduction of the *Mucispirillum* genus compared to the vaccinated control group. The Ruminococcaceae family is a prominent butyrate producer.[37] *The Mucispirillum* genus, primarily represented by *Mucispirillum schaedleri*, is a mucus-resident intestinal bacterium in rodents and is considered a pathobiont, a commensal organism that may contribute to disease.[38] This indicates the potential of carotene supplementation to inhibit harmful bacteria in the gut. The findings from this study revealed that carotene supplements significantly decreased the abundance of SCFA producers (e.g., *Odoribacter*) and pectin-degrading bacteria in the feces of unvaccinated mice. In contrast, in the carotene-supplemented vaccinated group, there was an increase in the abundance of butyrate producers (e.g., Ruminococcaceae) and a decrease in the abundance of potential pathobionts (e.g., *Mucispirillum*) in the host gut microbiome. The Ruminococcaceae and *Odoribacter* enriched by carotene supplementation were reported to have a strong association with the metabolism and absorption of β-carotene.[39]

In this study, the GC-MS analysis revealed that carotene supplementation did not significantly modulate SCFA (e.g., acetic acid, butyric acid, and propionic acid) levels in the fecal samples. This result is in contrast to recent research that deployed an *in vitro* anaerobic fermentation model to study gut microbiome interaction with β-carotene, where β-carotene significantly increased the production of acetic acid and propionic acid compared to the control group.[10] The discrepancy in findings can be due to the difference in the research models, where quantification of SCFAs was affected by absorption of SCFAs in the colon but not in the *in vitro* model. Besides, this animal study was challenged by vaccination. The immunological challenge can cause an inflammatory reaction associated with gut dysbiosis, potentially inhibiting SCFA producers. This finding was supported by a recent study where COVID-19 patients reported significantly reduced SCFA levels.[19]

## 5. Conclusion

This study used the BALB/c mice model to explore the interaction of carotene supplementation with an inactivated SARS-CoV-2 virus vaccine. To evaluate the association between carotene and vaccine response, this study investigated the effect of carotene on immune parameters, gut microbiome, and SCFA levels. Carotene supplementation did not significantly affect the antibody levels related to the inactivated SARS-CoV-2 vaccine, IFN-γ levels, and fecal SCFA levels. However, it increases the proliferation rate of splenocytes in vaccinated mice.

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## Conflict of interest

The authors declare that they have no competing interests.

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## Ethics approval and consent to participate

Ethics approval to carry out this study was obtained from the Animal Ethics Committee (AEC) of Monash University (AEC Project ID: 29899).

## Consent for publication

Not applicable.

## Availability of data

Data used in this work are available from the corresponding author upon reasonable request.

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## Appendices

**Table A1. Raw data for $CD3^+$ T lymphocytes from peripheral blood samples**

| Sample | % parent |
|---|---|
| Baseline 1 | 57.8 |
| Baseline 2 | 67.8 |
| Baseline 3 | 74.1 |
| Baseline vaccinated 1 | 71.5 |
| Baseline vaccinated 2 | 79.9 |
| Baseline vaccinated 3 | 69.4 |
| Carotene 1 | 74.1 |
| Carotene 2 | 72.9 |
| Carotene 3 | 59.9 |
| Carotene vaccinated 1 | 24.0 |
| Carotene vaccinated 2 | 69.0 |
| Carotene vaccinated 3 | 53.7 |

**Table A2. Raw data for splenocyte proliferation**

| Sample | % over baseline |
|---|---|
| Baseline vaccinated 1 | 91.2 |
| Baseline vaccinated 2 | 82.8 |
| Baseline vaccinated 3 | 97.2 |
| Baseline vaccinated 4 | 94.2 |
| Baseline vaccinated 5 | 87.3 |
| Baseline vaccinated 6 | 96.0 |
| Carotene 1 | 90.9 |
| Carotene 2 | 128.3 |
| Carotene 3 | 96.4 |
| Carotene 4 | 143.5 |
| Carotene 5 | 99.4 |
| Carotene 6 | 87.1 |
| Carotene vaccinated 1 | 139.9 |
| Carotene vaccinated 2 | 163.3 |
| Carotene vaccinated 3 | 90.4 |
| Carotene vaccinated 4 | 121.9 |
| Carotene vaccinated 5 | 194.2 |
| Carotene vaccinated 6 | 124.6 |