

Hypothesis

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Hypothesis

Could the Spike Protein Derived from mRNA Vaccines Negatively Impact Beneficial Bacteria in the Gut?

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Abstract: The emergence of mRNA vaccines for SARS-CoV-2 has opened a new page in vaccine development; nevertheless, concerns of the experts have been expressed about unintentional side effects on the gut microbiota (GM). Previous studies showed that this virus acts as a bacteriophage, which infects and destroys specific bacterial strains in the GM. The present manuscript hypothesizes that the synthetic spike protein could create changes in the composition and the functioning of the GM by entering the intestinal cells after vaccination and impairing the symbiotic relationship between intestinal cells and the GM. An experimental protocol to test the hypothesis is suggested.

Keywords: COVID-19; gastrointestinal (GI) tract; gut microbiota; microbiome; mRNA vaccine; SARS-CoV-2; spike protein

Correlation between COVID-19 Disease and GM

The diverse collection of bacteria known as GM inhabits the digestive systems of both humans and other animals. When compared to other areas of the body, the human GM contains the highest concentrations of bacteria and the most diversity of species [1]. The GM performs multiple important roles in the body, such as producing different antimicrobial compounds and inhabiting surfaces of the gut to protect the host from infections, thus boosting immunity [2], being essential to digestion and metabolism [3], controlling the growth and development of epithelial cells [4], determining brain-gut interaction and consequently impacting the psychological and neurological capacities [5]. In the last years, there has been a huge increase in interest in studies on the effects of GM on immunological homeostasis both within the gut and, crucially, at systemic locations. The GM is a vital part of an interesting ecosystem that develops a mutualistic relationship with its host by interacting and benefiting it on multiple intricate levels [6].

The COVID-19 pandemic produced by SARS-CoV-2 resulted in a broad spectrum of clinical manifestations, with pneumonia being particularly prevalent. Nevertheless, new data indicates that the gastrointestinal (GI) tract may also be negatively impacted, as the ileum and colon have high expression of angiotensin-converting enzyme 2, an essential SARS-CoV-2 receptor [7]. Moreover, SARS-CoV-2 was identified in all GI tract tissues, and a significant proportion of patients continue to excrete the virus in their feces even in situations where reverse transcription polymerase chain reaction results from respiratory samples were negative [8]. Consequently, the GI tract is immediately impacted by SARS-CoV-2 infection, which is thought to serve as an extra-pulmonary site for viral replication [9,10]. There is now a significant number of studies showing that the GI tract contributes to the etiology of the disease and how microbiota dysbiosis is directly linked to the clinical outcome [11], with the number of commensal bacteria decreasing in direct proportion to symptoms severity [11–16].

Some of the inflammation linked to COVID-19 is characterized by higher levels of interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), interferon-gamma (IFN- γ), monocyte chemoattractant protein-1 (MCP1), and interferon gamma-induced protein 10 (IP-10), may be explained by the decreased number of helpful commensals [13]. More serious cases of cytokine storm are linked to higher blood plasma levels of interleukin 2 (IL-2), interleukin 7 (IL-7), interleukin 10 (IL-10), IP-10, MCP1, macrophage inflammatory protein 1 α (MIP1 α), IL-6, and tumor necrosis factor-alpha (TNF- α). Fluid collected from lungs in patients with severe COVID-19 contained a population of monocyte-derived FCN1+ macrophages that had an inflammatory role. Additionally, peripheral blood from severe cases contains a higher proportion of CD14+ CD16+ inflammatory monocytes. By secreting inflammatory cytokines and chemokines such as MCP1, IP-10, and MIP1 α , these cells trigger the cytokine storm [13]. Increased levels of blood markers, such as aspartate aminotransferase, C-reactive protein, lactate dehydrogenase, and gamma-glutamyl transferase, as well as inflammatory cytokines, have all been associated with dysbiosis [12]. Interestingly, commensal bacteria have been shown to have immunomodulatory properties [12].

Yeoh et al. [12] performed a two-hospital cohort study aimed at improving comprehension of the function of the GI tract microbiota in COVID-19 patients and the effects of the disease. The goal of the research was to ascertain whether the degree of disease in COVID-19 patients was associated with their GM and whether this dysbiosis would improve if the virus was eliminated. For the investigation, blood and stool samples from 100 SARS-CoV-2 infected individuals were collected. Sequential stool samples were obtained from 27 of these patients up to 30 days after the virus had cleared. The GM was investigated using shotgun sequencing [12]. The concentrations of inflammatory cytokines and blood indicators were measured in plasma. The scientists found significant differences in the GM of patients and controls [12]. *Bifidobacterium*, *Eubacterium rectale*, and *Faecalibacterium prausnitzii* were found in lower concentrations in the patients, and these findings remained constant up to 30 days after the disease resolved [12,14]. According to other research, patients in critical condition had completely depleted *Bifidobacterium* and *Clostridium* genera. Furthermore, these individuals had a relative abundance of the *Pseudomonaceae* family, which has been connected to pathogenic illnesses such as severe acute respiratory syndromes [15].

Additional investigation showed that COVID-19 patients who needed to be admitted to the critical care unit (ICU) during their hospital stay had a lower baseline GM diversity (Shannon index) than patients treated in normal areas. A decrease in butyrate-producing bacteria and an increase in oral bacterial species were included in this index. The composition of the GM during hospitalization after severe COVID-19 was associated with 60-day mortality [16]. Comparably altered GM composition and functions (e.g., lower abundance of *Eubacterium rectale* and *Roseburia intestinalis* in the gut) are associated with COVID-19 mortality, according to the gut metagenomic data derived from the population-based analysis of 2,871 adult subjects from 16 countries [14]. It remained unclear how the virus caused the commensal bacteria to be so severely depleted. Nevertheless, Brogna et al. [17–20] revealed for the first time that certain bacteria, namely *Faecalibacterium prausnitzii* and *Dorea formicigenerans*, can be infected and destroyed by SARS-CoV-2 acting as a bacteriophage. It was

previously discovered that there was also a significant decrease in these species in children with multisystem inflammatory syndrome [21] and in severe COVID-19 cases [11,12].

Correlation between the Vaccination Status and GM

The vaccines' spike protein, especially in its free form, may be able to induce the same inflammatory cascades as the SARS-CoV-2's spike protein [22–24]. For a minimum of a decade, the scientific literature has documented and widely acknowledged the inflammatory toxicity of spike protein [25–27]. The presence of angiotensin-converting enzyme 2 (ACE2) receptors in nearly every part of the body, including the pharynx, trachea, lungs, blood, heart, vessels, intestines, brain, male genitalia, kidneys, and semen, as well as in bodily fluids like mucus, saliva, urine, cerebrospinal fluid, and breast milk, is the second factor that makes the spike/ACE2 interaction more toxic [28]. The spike protein can therefore cause inflammation in a variety of organs and systems. In fact, in addition to respiratory problems, the majority of COVID-19 patients also experience neurological, cardiovascular, intestinal, and renal dysfunctions [29–33]. Since spike protein is found in SARS-CoV-2 and also produced in response to mRNA vaccines, such toxicity consequently could be induced by both severe forms and long COVID-19, as well as all vaccines that are based on the unregulated synthesis of the spike protein by various cells, as opposed to vaccines that are made from inactivated whole virus or based on inactivated spike protein [22].

Following mRNA vaccine injection, the spike protein is known to be present on the cell surface as well as in considerable amounts in free form throughout the bloodstream, which travels to various organs such as the blood [34–36], liver [37–39], lungs [38,40], kidneys [38,40], lymph nodes [41–43], spleen [38,40], heart [38,43], and brain [38,44]. A recent study showed that 50% of the blood samples examined included the synthetic spike protein, which is difficult to break down. The time intervals between the immunization and the detection of the vaccine-derived spike were, respectively, 69 and 187 days [36]. Furthermore, it has been shown that both the whole spike protein and the S1 subunit, which includes the ACE-2 receptor-binding domain (RBD), can interact with the ACE2 receptors produced by different types of cells, such as endothelial cells and platelets, to induce an inflammatory response [44,45]. The spike protein is harmful not only because it binds to ACE-2 receptors but also because it interacts with the cancer suppressor genes P53 and BRCA, damages the mitochondria, causes coagulopathies by coming into direct contact with cellular proteins, accumulates and spreads prion proteins into their pathologic form, and is neurotoxic because spike accumulation inside cells may have also apoptotic effects [46].

Research has demonstrated that the *Bifidobacterium* and *Faecalibacterium* genera are significantly reduced in the gut by both the SARS-CoV-2 [47] and mRNA vaccines [48]. To assess the relative abundance of bifidobacterium in the gut, Hazan et al. [48] took stool samples from 34 people both before and one month after immunization. Their relative abundance dropped dramatically to about fifty percent of the initial level. The genus *Bifidobacterium* had median relative abundance values of 1.13% before and 0.64% after vaccination [48].

The Hypothesis

The present work proposes that the synthetic spike protein could enter into the intestinal cells and trigger an inflammatory response thus affecting the delicate balance between the GM and intestinal cells. Such dysbiosis could cause dysfunction or even death of these beneficial bacteria.

To prove or refute this hypothesis, we propose the following experimental protocol:

To synthesize the spike protein, it is recommended that researchers use the genomic sequence from the Alpha, Beta, or Delta variants (ABD) since a recent study in rhesus monkeys demonstrated that the gut bacteria in monkeys infected with these variants were found to be substantially different from those in monkeys infected with the Proto and Omicron variants (PO). In particular, compared to monkeys infected with PO variants, those infected with ABD variants had more pathogenic bacteria in their gut. In summary, the research showed that SARS-CoV-2 infection-related alterations in GM can increase inflammation and damage, especially in animals infected with the ABD strains [49].

We provide below a description of the steps that need to be taken to check the validity of this hypothesis *in vitro* and *in vivo*.

Proposed In Vitro Analysis

Cell Culture:

- Use human intestinal epithelial cells (e.g., Caco-2 cells).
- Grow cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics at 37°C with 5% CO₂.

Treatment with Synthetic Spike Protein:

- Divide cells into three groups: control (no treatment), low-dose spike protein (e.g., 10 ng/mL), and high-dose spike protein (e.g., 100 ng/mL).
- Treat cells for 24, 48, and 72 hours.

Cytokine Analysis:

- Collect cell culture supernatants at each time point.
- Measure cytokine levels (e.g., IL-6, IL-8, TNF- α) using ELISA kits.

Tight Junction Protein Expression:

- Harvest cells at each time point.
- Analyze the expression of tight junction proteins (e.g., occludin, claudin-1, ZO-1) by Western blotting and immunofluorescence.

Cell Viability Assay:

- Perform an MTT assay to assess cell viability after treatment.

Potential In Vivo Study

Animal Model:

- Use C57BL/6 mice, 8-10 weeks old.
- Divide mice into three groups: control (saline), low-dose spike protein (e.g., 10 μ g/kg), and high-dose spike protein (e.g., 100 μ g/kg).

Administration of Synthetic Spike Protein:

- Administer synthetic spike protein via intramuscular injection.

Inflammatory Response:

- Collect blood samples on days 0, 7, and 14.
- Measure serum cytokine levels (e.g., IL-6, IL-8, TNF- α) using ELISA kits.

Intestinal Tissue Analysis:

- Euthanize mice on day 14.
- Collect intestinal tissues for histological analysis (H&E staining) and immunohistochemistry for tight junction proteins.

Microbiome Analysis:

- Collect fecal samples on days 0, 7, and 14.
- Perform 16S rRNA sequencing to analyze microbiome composition.
- Compare the relative abundance of beneficial and harmful bacteria between groups.

Conclusions

Although the mechanism by which SARS-CoV-2 infects these beneficial bacteria has been described [17–20,50], it is still unknown how the vaccine-derived spike protein caused such a reduction of helpful bacteria. In a later work, Hazan et al. [51] demonstrated that there was a persistent reduction in bifidobacterium abundance following mRNA SARS-CoV-2 vaccination. They longitudinally recorded the relative abundance of genus Bifidobacterium in 4 subjects before receiving the mRNA vaccine (Pfizer or Moderna), approximately one month after the vaccine, and 6 to 9 months later. After that period, all Bifidobacterium relative abundance decreased to 15%, 0%, 35%, and 60% of pre-vaccine levels. Despite this significant reduction, no subjects in the study demonstrated significant clinical complications [51]. In our opinion, it is likely that the presence of

other beneficial bacteria, such as *Faecalibacterium prausnitzii* and *Dorea formicigenerans*, could dampen the damage caused by the synthetic pike protein.

Investigating whether the spike protein from vaccines can directly or indirectly interact with and potentially harm the GM is essential for several reasons:

1) The GM affects digestion, metabolism, immunological response, and even neurological functions. It is essential for preserving general health. This delicate ecosystem might be altered if the spike protein or its components were discovered to interact with and damage beneficial commensal bacteria in the gut.

2) Proper development and control of the immune system depend on gut microbes. Dysbiosis, or changes in the GM composition, has been connected to several immune-related diseases and disorders. Investigating the potential effects of the spike protein on the GM could provide insight into possible immune dysregulation mechanisms.

3) The ACE2 receptor is the primary route via which SARS-CoV-2 infects human cells; however, the spike protein may also interact with other receptors or parts of the cell, such as those found on the surface of bacteria. Studying these interactions is intriguing from a scientific standpoint because it may shed light on hitherto undiscovered aspects of viral biology and host-virus dynamics.

In conclusion, it is critical to investigate if vaccine-derived spike protein directly or indirectly impacts GM to comprehend potential impacts on the immune system, microbial homeostasis, and overall health. Because the new Omicron variants impact the GM and other organs with less severity than the original strains, the need to continue applying boosters should be re-evaluated.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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