*COVID*

MDPI

*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 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



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## 1. Correlation between COVID-19 Disease and GM

The diverse collection of bacteria known as the 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 greatest 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], and determining brain-gut interaction and consequently impacting psychological and neurological capacities [5]. In the last few years, there has been a huge increase in interest in studies on the effects of the 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 indicate that the gastrointestinal (GI) tract may also be negatively impacted, as the

ileum and colon have high expression of angiotensin-converting enzyme 2 (ACE2), an essential SARS-CoV-2 receptor [7]. Moreover, SARS-CoV-2 was identified in all GI tract tissues, and a significant proportion of patients continued 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 extrapulmonary 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-1β), interleukin 6 (IL-6), interferon-gamma (IFN-γ), monocyte chemoattractant protein 1 (MCP1), and interferon gamma-induced protein 10 (IP-10), and 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), interferon gamma-inducible protein (IP-10), monocyte chemoattractant protein-1 (MCP1), macrophage inflammatory protein 1α (MIP1α), 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 MIP1α, these cells trigger a 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 intensive 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 a population-based analysis of 2871 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 (MIS-C) [21] and in severe COVID-19 cases [11,12].

## 2. Correlation between 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 spike protein [22–24]. For a minimum of a decade, the scientific literature has documented and widely acknowledged the inflammatory toxicity of the spike protein [25–27]. The presence of 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 the 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 [37,38], lungs [37,39], kidneys [37,39], lymph nodes [40–42], spleen [37,39], heart [37,42], and brain [37,43]. 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 immunization and the detection of the vaccine-derived spike were 69 and 187 days, respectively [36]. Furthermore, it has been shown that both the whole spike protein and the S1 subunit, which includes the ACE2 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 [43,44]. The spike protein is harmful not only because it binds to ACE2 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 [45].

Research has demonstrated that the *Bifidobacterium* and *Faecalibacterium* genera are significantly reduced in the gut by both the SARS-CoV-2 [46] and mRNA vaccines [47]. To assess the relative abundance of *Bifidobacterium* in the gut, Hazan et al. [47] took stool samples from 34 people both before and one month after immunization. Their relative abundance dropped dramatically to about 50% of the initial level. The genus *Bifidobacterium* had median relative abundance values of 1.13% before and 0.64% after vaccination [47].

## 3. The Hypothesis

The present work proposes that the synthetic spike protein can enter 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 (Figure 1).

The spike protein of SARS-CoV-2 binds to the ACE2 receptor, which is also expressed on the surface of enterocytes in the gut [48]. This interaction could potentially disrupt the gut epithelial barrier, leading to increased intestinal permeability (leaky gut), as demonstrated in MIS-C, a rare but severe complication of SARS-CoV-2 infection [49]. The binding of the synthetic spike protein to the ACE2 receptors from enterocytes could trigger an inflammatory response, leading to the release of pro-inflammatory cytokines such as IL-6 and

TNF-α. Such disruptions could also alter the gut environment, making it less hospitable for beneficial bacteria and promoting the growth of pathogenic species.

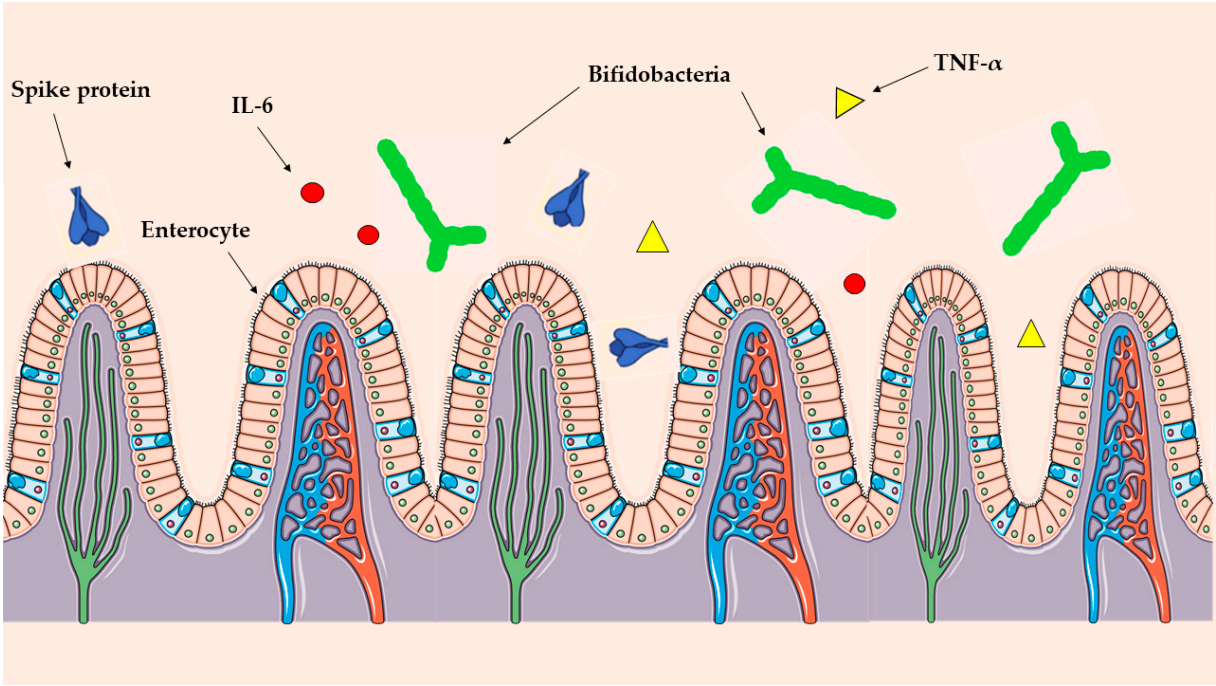


**Figure 1.** The synthetic spike protein could bind to ACE2 receptors on gut enterocytes, potentially triggering the release of pro-inflammatory cytokines like IL-6 and TNF-α. Such disruptions could modify the gut environment, negatively affecting beneficial bacteria and promoting the growth of pathogenic bacteria.

Another consequence associated with the binding of the SARS-CoV-2 spike protein to ACE2 receptors is tryptophan (Trp) depletion. ACE2 plays a significant role in Trp absorption by acting as a chaperone for the amino acid transporter BOAT1 [50,51]. Research revealed that ACE2-knockout animals had lower serum levels of amino acids, particularly Trp. Alongside this decline in Trp, there was a significant reduction in antimicrobial peptide (α-defensin) levels and intestinal dysbiosis. The negative effects caused by ACE2 deficiency were reversed by administering Trp directly in the diet, demonstrating that a severe disruption in local Trp homeostasis resulting from an ACE2 deficit increases the susceptibility to intestinal inflammation [52]. It is hypothesized that engineered spike can do the same.

The synthetic spike protein could also affect the gut-associated lymphoid tissue (GALT), a key component of the immune system in the gut [53]. Because factors such as age, diet, pre-existing health conditions, and genetic background can influence the GM and its response to vaccination, this work suggests testing the hypothesis in an animal model, in which those variables can be easily controlled.

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 (ABD) variants, 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 (PO) variants. 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 [54].

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.

## 4. Proposed In Vitro Analysis

### *4.1. 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% $CO_2$.

### *4.2. 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 h.

### *4.3. Cytokine Analysis*

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

### *4.4. 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.

### *4.5. Cell Viability Assay*

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

## 5. Potential In Vivo Study

### *5.1. Animal Model*

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

### *5.2. Administration of Synthetic Spike Protein*

- Administer synthetic spike protein via intramuscular injection.

### *5.3. Inflammatory Response*

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

### *5.4. Intestinal Tissue Analysis*

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

### *5.5. 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.

One can argue that it could be difficult to correlate the observed cytokine storm reproduced in Caco-2 cells with the in vivo serum cytokine levels, as any parameters here would not be specifically originating from the epithelium or intestine. However, our hypothesis, being based on a comprehensive analysis that includes examination of both the GM and cytokine dysregulation, integrates findings from in vitro and in vivo

analyses. This dual approach provides a deeper understanding of the complex interactions between the GM and the host immune response, particularly in how cytokines affect the GM in the context of SARS-CoV-2 infection. In 2021, Yonker et al. demonstrated that SARS-CoV-2 infection triggers excessive zonulin release that disrupts the integrity of the gut barrier, thus causing leakage of viral antigens to blood circulation and causing a cytokine storm-like syndrome called MIS-C [49]. That is why we are suggesting that serum cytokine measurement be conducted. The capability of the synthetic spike protein to cause damage to the gut epithelial barrier could be evidenced by the in vitro studies, while the cytokine measurement could confirm if the synthetic spike protein induces systemic pro-inflammatory responses.

## 6. Discussion

Although the mechanism by which SARS-CoV-2 infects these beneficial bacteria has been described [17–20,55], it is still unknown how the vaccine-derived spike protein caused such a reduction of helpful bacteria. In a later work, Hazan et al. [56] demonstrated that there was a persistent reduction in *Bifidobacterium* abundance following mRNA SARS-CoV-2 vaccination. They longitudinally recorded the relative abundance of the genus *Bifidobacterium* in four 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 had decreased to 15%, 0%, 35%, and 60% of pre-vaccine levels. Despite this significant reduction, no subjects in the study demonstrated significant clinical complications [56]. 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 spike 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 [57,58]. 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 [59]. 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 [60]; however, the spike protein may also interact with other receptors or parts of the cell, such as those found on the surface of bacteria [61]. 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 summary, while the mRNA vaccines played a crucial role in reducing disease severity and death, it is essential to investigate their long-term impact on gut health. The proposed hypothesis and experimental protocol provide a foundation for future studies to explore these effects. Understanding the long-term clinical implications will help in optimizing vaccine strategies and managing any potential adverse outcomes associated with changes in the GM. Variations in the composition of the microbiome have been linked to modifications in the effectiveness of certain immunological treatments, such as the COVID-19 vaccines [62–64]. The immunogenicity and effectiveness of vaccines can be enhanced by the GM [65–67]. Conversely, the opposite is also valid: the COVID-19 vaccines can have a major negative or positive impact on the GM, which could decrease or increase the overall number of organisms and species diversity [68].

A beneficial effect of an inactivated SARS-CoV-2 vaccine on GM was recently reported [64]. Researchers collected fecal samples from individuals who were injected with two doses of the BBIBP-CorV vaccine and from unvaccinated controls. The diversity and physiological functions of the microbiota of vaccinated and unvaccinated subjects were

compared. The phylum Firmicutes was discovered to be more abundant in vaccinated people in this study, whereas the unvaccinated group had more Bacteroidetes detected. As a result, the vaccinated subjects had an elevated Firmicutes/Bacteroidetes ratio [64]. Inflammatory bowel conditions such as ulcerative colitis and Crohn's disease have been associated with a lower F/B ratio [69].

Furthermore, the vaccinated participants showed a substantial increase in the butyrate-producing bacterium *Faecalibacterium prausnitzii* [64]. This is a relevant finding, since it was discovered that SARS-CoV-2 can infect and destroy this bacterium [17–20]. On the other hand, individuals who had received vaccinations did not exhibit a higher abundance of opportunistic pathogens like *Enterococcus* and *Prevotella* [64]. According to some studies, higher *Prevotella* abundance in HIV patients may be a contributing factor to the gut's chronic inflammation, which can cause mucosal dysfunction and systemic inflammation [70,71]. The study by Shen et al. [64] showed substantially enhanced profiles and physiological capabilities of the GM influenced by vaccination with the BBIBP-CorV vaccine, which may have profound impacts on the host's ability to build a strong immunological barrier.

On the contrary, a negative impact on certain bacterial species (*Bifidobacterium*) has been demonstrated for mRNA vaccines [47,56]. Unfortunately, those studies focused only on one species. It is suggested that the global diversity profile of the GM should be studied in future investigations. By measuring the relative abundance of the most beneficial and harmful species, more valid conclusions could be obtained about the effects of mRNA vaccines on GM. These different results suggest that inactivated COVID-19 vaccines induce a beneficial effect on GM, while mRNA vaccines do not. However, more studies are necessary to reach a definitive conclusion.

The reduction in *Bifidobacterium* abundance found by Hazan et al. [47,56] after the administration of mRNA vaccines should be further investigated, because there is a growing body of research suggesting a connection between reduced *Bifidobacterium* abundance and colorectal cancer [72–76]. Although Hazan et al. [47,56] did not report significant clinical complications in the mRNA-vaccinated individuals in the short term (1 year), continuous exposure to these vaccines could further reduce Bifidobacterium numbers, thus facilitating cancer growth. It is generally accepted that it takes approximately 10 to 15 years for benign polyps, specifically adenomas, to develop into colon and colorectal cancer [77–81].

*Bifidobacterium* can reduce inflammation by producing short-chain fatty acids (SCFAs) like butyrate, which have anti-inflammatory properties. SCFAs can inhibit the expression of pro-inflammatory cytokines and promote regulatory T cell differentiation, thereby reducing inflammation and potentially lowering cancer risk [82]. Animal studies have demonstrated that the administration of *Bifidobacterium* can reduce tumor growth, particularly by activating dendritic cells and enhancing T cell responses [83]. This immune modulation is also crucial in preventing cancer and improving the efficacy of immunotherapy [84].

Finally, for individuals who have received multiple mRNA vaccines and those who suffered severe COVID-19, it is suggested to consider consuming prebiotics and probiotic supplements containing *Bifidobacterium, Faecalibacterium prausnitzii*, and other beneficial species to recolonize their GM be considered, thus reestablishing the functionality of their gut immune system.

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