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RESEARCH ARTICLE

Mechanism of Thrombosis During COVID-19 Infection Due to SARS-CoV-2 Virus and its Variants, and a Clinically Proven Strategy to Combat with Probiotics and their Immunomodulins.

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ABSTRACT

The US patent 11,077,052 B1, issued in August of the year 2021, has been granted relying on the research conducted until September 2020, using the multiple mixed strain probiotics and their immunomodulins to prevent or treat COVID-19 due to SARS-CoV-2 and its limited variants. Since then the SARS-CoV-2 virus continuously generated multiple variants with different genomic configurations, almost to the point of overriding the vaccines and medications. The objective of this research article is to evaluate the effectiveness of the invention outlined in the U.S. patent 11,077,052 B1 on the variants of SARS-CoV-2 evolved in the later years of 2020, 2021 and 2022. This research has great significance because vaccines are not totally effective because of the high rate of mutations of SARS-CoV-2 virus resulting in antigenically different variants, which are overriding the immunity conferred by vaccines. Thus, there is a definite need for a reliable alternative or adjuvant therapy, which can be used either by itself or used as adjuvant therapy along with vaccines to combat COVID-19 infection due to SAR-CoV-2 virus and its continuously mutating variants of different antigenicity. The results of this investigation proved that the use of multiple mixed strain probiotics along with their immunomodulins are effective not only on novel SARS-CoV-2 coronavirus evolved in late 2019 and mid 2020 but also the mutated variants in the later part of the years 2020, 2021, and 2022. The COVID-19 pandemic is not yet contained as of August 2022. The SARS-CoV-2 virus has been continuously mutating generating variants which are resisting vaccines and the treatments. The death's due to SARS -COV- 2 viral infections are attributed predominantly to cytokine storm and thrombosis resulting in ARDS (Acute Respiratory Disease Syndrome} and multi organ failure. The earlier published article thoroughly discussed about the genesis and control of cytokine storm to protect the victims. This article mainly emphasizes the Pathophysiology of thrombosis, due to SARS-CoV-2 virus and its recent multiple variants, and the ways and means of controlling it to prevent or cure COVID-19, using the multiple mixed strain probiotics and their Immunomodulins. The hormonal and enzymatic system variations causing the disturbance in homeostasis of the vascular system resulting in thrombosis, due to SARS-CoV-2 virus and its continuously evolving variants is thoroughly discussed. In addition, the infection pattern and progression of the SARS-CoV-2 virus causing endothelial cell lysis of the vascular system resulting in thrombosis has been elucidated with molecular details. The specific role of immunomodulins of the multiple mixed strain probiotics to prevent or treat COVID-19 disease induced thrombosis has also been presented. The physiological functions of individual components of the probiotics and the immunomodulins produced by the multiple mixed strain probiotics, is presented for the first time with explicitly proven molecular details, showing how they contribute individually and cumulatively to prevent or treat COVID-19 infection, due to SARS-CoV-2 virus and its multiple variants, generated during the years 2020 through 2022, to induce thrombosis.

Keywords: Multiple Mixed Strain Probiotics, Probiotic Immunomodulins, Immunomodulins, Postbiotics, Thrombosis, COVID -19, SARS-CoV-2, Corona Virus, ACE-2, ACE, US Patent # 11,077,052, Lysosomal Trafficking Pathway, Biosynthetic Secretory Pathway, Delta variant, Omicron Variant, Dysbiosis.

INTRODUCTION: The SARS-CoV-2 Coronavirus caused the worldwide pandemic, and the disease is named COVID-19 by the World Health Organization [1]. Starting from Nov. 2019 in Wuhan China, so far as of early August 2022, roughly 225 countries around the world contracted COVID-19 infections involving a total of roughly over 566 million people with a confirmed death of 6.39 million. The daily registered new cases are roughly 2.5 million, and according to some experts the total death rate may go up to 10 million, unless a new way of controlling this pandemic is discovered. The SARS-CoV-2 RNA virus is continuously mutating resulting in various variants with different rate of transmissibility and severity of infection. The variants are also attacking the younger population unlike its parent strain. Although several vaccines and therapeutic drugs have been developed and tried on people, the viral mutants are evading them through genetic variations. The WHO and CDC have categorized the SARS-CoV-2 viral variants into three classes, determined on the basis of their virulence, rate of transmission, and resistance to vaccines and treatments. They are: Variant of interest; Variant of concern; and Variant of high consequence.

A Variant of interest is the one which has an epidemiological impact, to suggest an emerging risk to global public health. Examples of variants of interest are Mu (B.1.621- origin Columbia – January 2021) and LAMBDA (C.37-origin Peru- December 2020), which are not highly transmissible.

A Variant of Concern is the one which can spread more easily and cause more serious disease or dent the effectiveness of vaccines and other fighting tools. The examples include ALPHA (B.1.1.7 – origin UK- September 2020), BETA (B.1.351 – origin ,South Africa – May 2020) , DELTA (B.1.617.2 – origin , INDIA – October 2020) , GAMMA (P.1 – origin, Brazil- November 2020), OMICRON (B.1.1.529 – origin , Multiple countries, but identified first in South Africa November 2021), and the BA.2, BA.2.12.1, BA.3, BA.4, BA.5, and the latest BA.2.75. The Delta variant which dominated in 2021 was more infectious than the earlier variants with an R-NAUGHT value of about seven, meaning each infected person could spread it to seven people. Whereas BA.5 could spread it to eighteen people. The researchers are predicting that in all probability soon BA.6 and BA.7 mutant variants may emerge in the forthcoming months.

A Variant of High Consequence has clear evidence that prevention measures or medical countermeasures have significantly reduced effectiveness relative to previously circulating variants. So far, according to WHO and CDC the variant of high consequence has not been encountered or identified yet. However, according to some medical experts, by looking into the world statistics, the Delta can be categorized under variant of high consequence. Although it has not been proven, perhaps some of the variants of omicron may also turn into variants of high consequence due to continuous mutations of omicron variant at a rapid pace.

The COVID-19 Deaths due to SARS-CoV-2 Virus and their mutant variants, are predominantly due to cytokine storm and thrombosis [2]. In this article the emphasis is on the genesis of thrombosis, which is causing death due the multiple organ dysfunction during COVID -19 disease, and measures to counteract it using the multiple mixed strain Probiotics and their Immunomodulins. In August 2021, the United States Patent and Trademark Office (USPTO) has issued Patent identified with US Patent # 11,077,052 B1 (patent application submitted in September 2020), with reference to treating SARS-CoV-2 coronavirus infection causing COVID-19 disease pandemic [3]. However, the research was conducted in the year 2020 and at that time although SARS-CoV-2 was mutating, the number of variants were significantly low compared to the years 2021 & 2022. Thus, we embarked on research to check the validity of the invention outlined in US patent 11,077,051 B1 to prevent or treat the COVID-19 due to the continuously evolving mutant variants of SARS-CoV-2, which were not present in the year 2020. It is highly publicized news regarding the U.S. president Joe Biden has been tested positive for COVID-19 on July 2022, despite the fact he was vaccinated and also double boosted indicating that the newly evolved variants are overriding the immunity conferred by vaccines. This research/review article is written to elucidate the possible molecular mechanism behind the patented invention to prevent or cure the thrombosis resulting from SARS-CoV-2 viral infection. Before proceeding with the section of materials and methods, the following details are presented regarding the general thrombosis and pathophysiology of specific thrombosis encountered during the COVID-19 infection, specifically considering the more talked about LONG – COVID complications, due to multiple variants of SARS-CoV-2 virus.

THROMBOSIS ENCOUNTERED UNDER THE NORMAL CIRCUMSTANCES IN THE ABSENCE OF SARS-CoV-2 INFECTION.

Thrombosis is formation of blood clots within the blood vessels, both in venous and arterial, limiting the blood flow, thus resulting in clinical complications. The complex homeostasis that exists between blood cells, coagulation factors, plasma

proteins, endothelial lining within the lumen of veins and arteries, inflammation factors and cytokines, keeps the ability of blood to flow freely in the blood vessels. When there is a severe imbalance to this physiological process, it may result in either thrombosis or coagulopathy (an increased risk of bleeding).

The cause of thrombosis is multifactorial. The common predisposing factors which cause thrombosis are: Damage to the endothelial lining of the blood vessel wall; Hypercoagulable state; Venous or Arterial blood stasis.

The vascular endothelial cells have abundant number of ACE-2 (Angiotensin Converting Enzyme - 2) receptors. The function of ACE-2 is to convert Angiotensin 2 to Angiotensin 1-7, since excess Angiotensin 2 increases the blood pressure through vasoconstriction.

On the other hand, "if Angiotensin 2 is converted to Angiotensin 1-7 by the ACE-2 enzyme, then the blood pressure is under control due to vasodilation and thus damage to the blood vessels will be significantly lower or even less. The next question is, what produces the substrate Angiotensin-2?

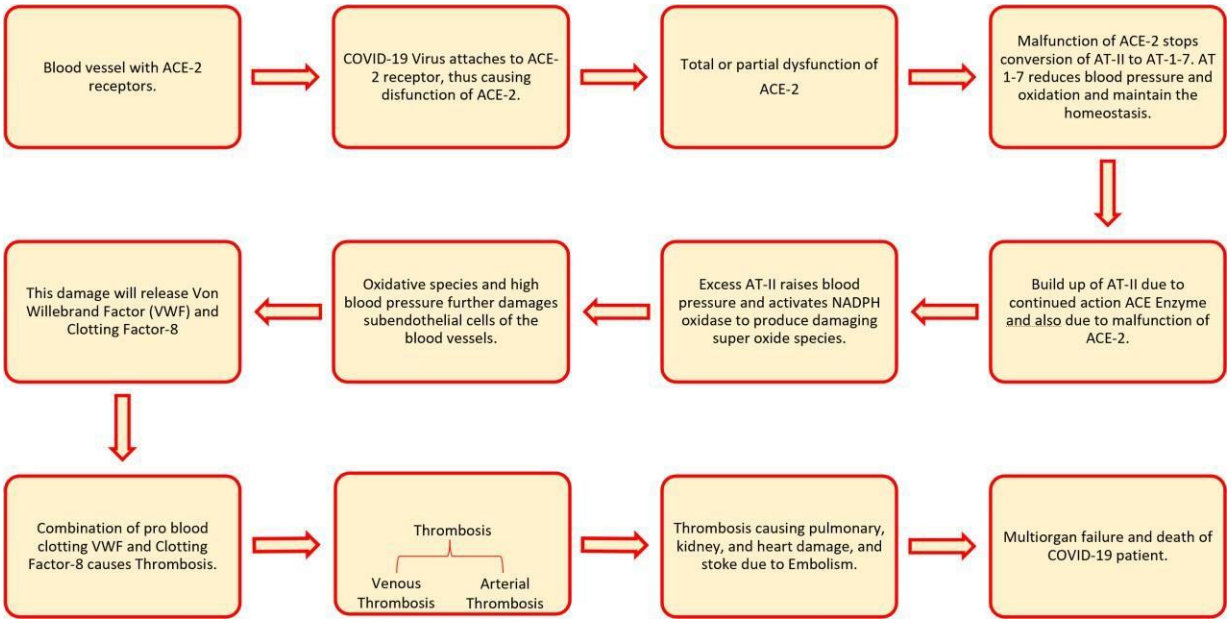
To start with the Renin -Angiotensin - Aldosterone system (RAAS) is an endocrine cascade. The Renin, the initial enzyme of the cascade, converts the pre-pro - hormone Angiotensinogen (which is produced by liver prominently) to pro-hormone Angiotensin-1. The ACE (Angiotensin Converting Enzyme) converts Angiotensin-1 hormone into Angiotensin-2 hormone. In this connection, as part of the homeostasis, Angiotensin-2 hormone gets converted to Angiotensin 1-7 by ACE-2 enzyme. The Angiotensin-2 causes constriction of blood vessels thus raises the blood pressure. If this is not controlled, the excess activity of Angiotensin-2 will do significant damage to the endothelial cells through activation NADPH oxidase to induce severe oxidation in the blood vessels. Due to the damage to blood vessels blood clots will form. Whereas, Angiotensin 1-7 has completely opposite effect compared to Angiotensin-2, in that it causes dilation of blood vessels to reduce the blood pressure to protect the capillary endothelial cells and thus to prevent blood clots. Thus, the homeostasis of the circulation system is maintained to protect the humans from the critical thrombus formation in the blood vessels.

How does the SARS-CoV-2 virus cause thrombosis through deactivation and/or activation of enzymes and hormones involved in the homeostasis of the circulatory system?

<u>During the SARS-CoV-2 infection, the following pathological changes take place in hormonal and enzymatic systems to induce thrombosis.</u>

The SAR-CoV-2 virus infects the ACE -2 receptor(s) on the endothelial cells of the vascular system. The SARS-CoV-2 virus spike protein down -regulates the ACE -2 enzyme thus preventing the conversion of AT -2 to AT 1-7. At the same time, it up regulates ACE (Angiotensin converting enzyme) to convert Angiotensin 1 to active Angiotensin 2. The excessive activity of Angiotensin 2 induces vasoconstriction and inflammation of the endothelial cells with activation of NADPH Oxidase to oxidize the endothelium of blood vessels. It further leads to excess production of Von Willebrand factor and clotting Factor-8 to start the process of forming blood clots to induce thrombosis. The entire mechanism of thrombogenesis due to SAR-CoV-2 infection disrupting the hormonal-enzyme dependent homeostasis has been schematically presented in Figure 1.

Figure 1: Pathophysiology of thrombosis in blood vessels due to COVID-19 systemic infection through the disruption of hormonal-enzyme dependent vascular homeostasis.



Unfortunately, due to the reduced and severe shortage of AT 1-7 during the COVID-19 infection, more Angiotensinogen gets converted to Angiotensin-1 and later to Angiotensin-2, thus aggravating the vasculitis and thrombus formation. The similar mechanism is operative in kidney, heart, gastrointestinal tract, and lungs vascular system causing the destruction of multiple organs due thrombosis, and also due to the resulting embolism. It has already been established that the preexisting hypertension, diabetes, and cardiac diseases exacerbate the thrombosis due to SARS-CoV-2 viral infection. The next question is what is the Pathophysiology of the thrombogenesis induced by corona virus? This question is answered using the sequential destructive pattern of SARS-CoV-2 viral infection of the endothelial cells, which is presented in a schematic format in figure 2. The details are as follows:

A Step wise progression of SARS-CoV-2 infection causing extensive lysis and damage to the vascular endothelial cells, resulting in thrombus formation, is as follows:

Binding: The SARS-CoV-2 virus primarily infects the respiratory and gastrointestinal tract cells, followed by kidney, endothelial cells of both venus and arterial circulatory systems. Even in the respiratory tract, viral preference is to attack the ciliated cells of trachea and the type-2 pneumocytes in the alveoli. The SARS-CoV-2 virus has greater affinity to the organs where there is predominant availability of Angiotensin – Converting- Enzyme - 2 (ACE-2), which is coded by ACE-2 gene in the cells.[4,5,6]

The SARS-CoV-2 virus binds to ACE-2 receptors of the host cell through its glycoprotein spike structure. This binding is further assisted and mediated by the host protease enzymes. This binding then facilitates the release of viral RNA into the cytoplasm of the host cell. When once it is in the host cell, since it is an RNA virus (unlike DNA viruses) transcription is not required and translation begins immediately utilizing the host ribosomes. As a result of the translation two large Polypeptide chains will be formed, they are: PP1a and PP1ab. [7,8,9]

Proteolysis: The polypeptides PP1a and PP1ab will be proteolyzed by the proteolytic enzymes 3 C like protease, 3CL protease and the papain like protease, which are virally encoded. In this connection it is to be noted that 3 CL protease cleaves or breaks the viral PP1ab Polyprotein at 11 sites to generate protein break down products required for viral multiplication. However, unlike 3 CL protease enzyme, the papain like protease enzyme cleaves at 3 sites, to generate specific protein break down products. A point of interest at this stage is, if the function of these proteases are blocked, corona virus cannot replicate to continue the infection. Since these proteases are potential targets for stopping viral replication, they are highly conserved in the SARS-CoV-2 virus. [10,11]

SARS-CoV-2 viral RNA Replication: The functional proteins or protein breakdown products forms the Replicase- Transcriptase complex to assist the replication of the virus, mediated by the RNA

dependent RNA Polymerase enzyme (Ra Rp). It is to be noted that Ra Rp is highly vulnerable to errors or error-prone and thus can lead to the emergence of viral mutations, which may lead to variants. We all know that RNA Polymerase, unlike DNA polymerase exhibits only polymerization activity but not proof-reading activity to minimize mutations. Various mutants of concern of the SARS- CoV-2 identified as variants such as ALPA, BETA, DELTA, GAMMA, and OMICRON, etc., are generated due to the lack of proofreading ability of RNA dependent RNA polymerase. The virulence and the infectivity rates of these mutants vary depending on their genetic alteration during mutation. For example, the novel coronavirus SAR-CoV-2, which emerged in November 2019 caused serve deaths due to thrombosis, yet it has a less infectivity rates. Whereas the Delta and Omicron mutants and others have high infectivity rates. Turn around, the mutants identified as variants MU and Lamda are categorized under variants of interest because of their less transmissibility. Now one can understand why so many variants of different pathogenicity are evolving as mutants from the novel SARS-CoV-2 coronavirus.

New SARS-CoV-2 Viral assembly: The viral particles are assembled at the mitochondria and golgi bodies of the host infected cell. The word assembly is used because all the pre-made viral components are assembled to form the new viral particles. After the assembly, the new virions of SARS-CoV-2 are released from the eukaryotic cell through exocytosis.[11]

Exit of new SAR-CoV-2 viral particles from the infected endothelial cells (exocytosis):

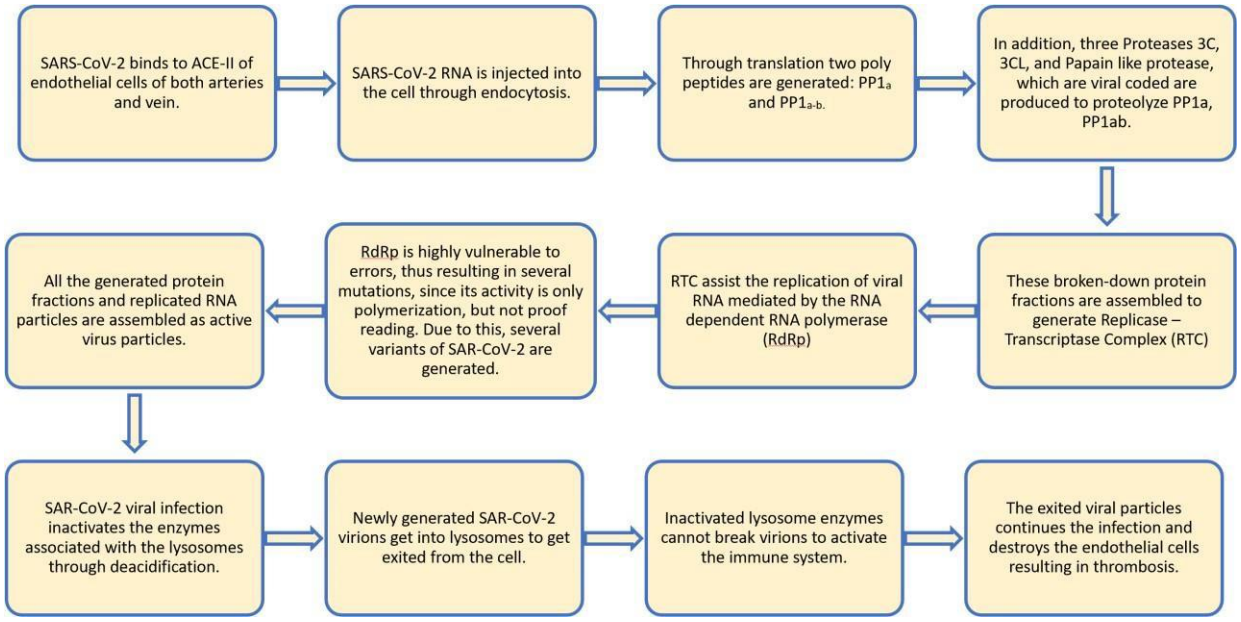
Although several investigators worked on the entry, replication and multiplication pathways, including life cycle of SARS-CoV-2 virus, yet not much information was available on the egress of the corona viral particles from the infected eukaryotic cells. Gosh et.al., for the first time has researched on the egress mechanism and came up with a breakthrough discovery.[12] Using the methodologies and virus-specific reporters they have demonstrated that β coronaviruses including SARS-CoV-2 utilized lysosomal trafficking for egress than the conventional biosynthetic secretory pathway, used by other viruses, such a Hepatitis C, Dengue and West Nile Virus, etc. The way the SARS-CoV-2 virus enters, replicates, and exits vary from individual to individual. Gosh et.al., have uncovered and established that this unconventional egress by the SARS-CoV-2 virus using lysosome pathway is by Arl8b-dependent lysosomal exocytosis.[12] According to the investigators, this non-lytic release of new formed corona viral particles in the infected human cells is associated with the lysosomal deacidification, Lysosome degradation enzyme deactivation, and severely impaired antigen presentation by the antigen presenting cells and major histocompatibility complex (MHC) , thus enabling the coronavirus to deceive the hosts immune system. Consequently SARS-CoV-2 virus was able to conceal its presence within the infected cell, to escape from the destructive effect of the adaptive immune system, specifically the T- killer cells. Additionally, in my opinion, even the production of Interferons (which can alert the neighboring cells regarding viral infections) may be blocked due to egress pathway utilized by the SARS-CoV-2 virus using the deacidified Lysosomes. Apparently acidified Lysosomes are important for protease enzymes for processing and presentation of antigen to antigen-presenting cells to elicit virus activated immune response. Since the corona viruses are deacidifying the lysosomes, the host immune system might not be operating efficiently to protect the host. Furthermore, on top of that the excess activation of non-antiviral immune cells can cause autoimmune neurogenerative diseases during COVID-19 infection.[13] This could be one of the reasons for the prolonged symptoms observed in LONG COVID.

According to some researchers, the Lysosomes deacidification during COVID-19 infection may be due to the action of specific corona virus proteins. Conversely other researchers postulated that Lysosomal deacidification can be indirectly due to too much cargo loading (too many viral particles in the infected cell) and/or the disturbed proton pump or ion channel trafficking.[14,15] The infected endothelial cells will eventually disintegrate and become non-functional leading to thrombosis. The length of time it takes from infection to the emergence of viral particles is called eclipse period. Whereas the number of viral particles generated from each infected eukaryotic cells is termed burst size. The eclipse period of SARS-CoV-2 virus is roughly 12-36 hours. Whereas, the burst size is approximately 700 virions. Apparently, the eclipse period and the burst size may vary from variant to variant and also depends on the health status of the human being. The progression of SARS-CoV-2 viral multiplication starting from entry through endocytosis, and to exit through exocytosis is presented schematically in figure 2.

The significant generation of variants during the progression of SARS-CoV-2 multiplication maybe one of the reasons why the Remdesivir, Hydro chloroquine and other targeted drugs, including vaccines are not fully effective to cure COVID-19 infection.[16,17,18,19] It is rather ironic to know that not all people exposed to SARS-CoV-2 were infected,

and not all infected people developed severe respiratory diseases.[20,21,22] It is only few people who have developed the acute symptoms and died due to COVID -19 infection. It clearly points out that the disease progression and pathogenesis is varying from individual to individual depends on their age, existing comorbid condition, and the composition of microbiota and microbiome, immunodeficiency, overall general health conditions, nutritional and food habits etc. etc. This has been thoroughly discussed by Reddy in his previous publication with regard to cytokine storm during COVID-19 infection.[2]

Figure 2: Schematic Molecular Mechanism of SARS-CoV-2 Viral Invasion and Multiplication in the Endothelial Cells Resulting In Vascular Damage and Thrombosis



As a separate note, the most significant feature outlined in the US patent #11,077,052 B1 was the serendipitous observation of how the multiple mixed strain probiotics not only prevented COVID-19 infection in elderly comorbid patients, but also significantly reduced the hypertension and diabetes. Furthermore, the patients were able to reduce the dosage of their specific medication (which has been proven to have severe side effects) while taking the multiple mixed strain probiotics.

The following probiotic cultures along with their immunomodulins and cell wall components belonging to various genera and species with proven therapeutic properties (with pertinent references) has been presented with explicit details to give a total and comprehensive understanding to the readers why different probiotic strains were included in this investigative research.

Lactobacillus plantarum: It is a lactic acid producing probiotic bacteria and produces lactic acid as an end product of growth. It has the following therapeutic properties: anticarcinogenic, anti-inflammatory, anti-diabetic, anti-obesity, reduces seasonal allergies and irritable bowel syndrome, significantly improves the function of immune system, reduces high blood pressure, reduces anxiety through production of anxiety and stress suppressing neurotransmitters, anti-viral effect on HINI virus and also on Coronavirus. It is a novel probiotic to prevent influenza viral infection, and it has an excellent ability to stick to the epithelial cells to replace or displace the pathogenic bacteria.[55]

Lactobacillus rhamnosus: It is an excellent naturally occurring probiotic with the following therapeutic properties: prevention and treatment of gastrointestinal linfections and diarrhea, stimulates the immune response, excellent adjuvant to enhance immune response following vaccination, reduces several viral infections through its anti-viral activity by shifting the T-helper cells to TH-I, has an excellent ability to stick to the epithelial cells thus exhibiting the wide spectrum inhibitory properties on both pathogenic bacteria and virus.[56]

Lactobacillus paracasei: It has significant therapeutic effect due to its immunomodulatory effect to suppress the inflammatory bowel diseases. In addition, even the heat killed bacterial cells of *Lactobacillus paracasei* have significant therapeutic effect. Thus, it can also serves as a para probiotic to stimulate the immune system. This probiotic has high adhesion capacity to stick to the epithelial cells to replace or displace the pathogenic bacteria.[57]

Lactobacillus casei: The following are the therapeutic benefits of *L. casei*: stimulates immune system, inhibits viral infections, decrease the incidence cold and influenza, reduces lower respiratory tract infections, inhibits pneumonia, stimulates immune system among the elderly population, decrease roto virus infection, inhibits respiratory tract infections, inhibits pneumonia due to bacterial infections such as *pseudomonas aeruginosa* etc., inhibits systemic inflammatory response syndrome.[58,59,60,61]

Lactobacillus helveticus: It prevents gastrointestinal infections, inhibits pathogens, exhibits immune modulation, and assists in maintaining the optimal composition of intestinal microbiota. It produces bacteriocin helvetcin, controls high blood pressure, alleviates symptoms of arthritis, help combat anxiety, improves sleep efficiency etc.[62,63,64,65,66]

Bifidobacterium bifidum: It has the following therapeutic benefits: reduces *H. pylori* infection, irritable bowel syndrome, lung infections, constipation, ulcerative colitis, and necrotizing enterocolitis. It produces a bacteriocin bifidocin-B, which is active against some food borne pathogens such as Listeria, Bacillus etc.[67]

Bifidobacterium longum: It has the following therapeutic functions: helps to reduce infections through stimulation of immune system, reduces production of chemicals that increase inflammation and oxidative stress. It produces bacteriocins bifilact Bg-12 and biflong Bg-46 which are inhibitory to pathogenic *Staph aureus* and *Salmonella typhimurium*.[57]

Streptococcus thermophilus: It has the following proven therapeutic benefits: it significantly improves skin health through hydration when applied as a cream, reduces lactose intolerance, mucositis, gastritis, ulcerative colitis and antibiotic associated diarrhea. It exerts anti-inflammatory effect by suppressing TH-I. It stimulates macrophages and T-cell regeneration and immunological defense mechanism in human stomach cells. It produces bacteriocin called thermophilin, which is a lantobiotic.[67,68,69]

Lactobacillus bulgaricus: It has the following therapeutic effects: decreases common colds due to viral infections, allergic rhinitis, periodontal diseases and other oral health problems, eczema, leaky gut, inflammation, triglycerides-LDL-and total cholesterol, significantly improves immunity and fights viruses, and improves longevity. It produces a bacteriocin called bulgaricin.[70]

Lactobacillus sporogenes: Also called *Bacillus coagulans*. It has the following therapeutic effects: it improves immune system and significantly decrease respiratory infections, acts as an adjuvant to improve vaccine efficiency, decreases roto viral diarrhea, traveler's diarrhea, and diarrhea caused by antibiotics, inhibit *Helicobacter pylori,* irritable bowel syndrome, (IBS), inflammatory bowel disease (IBD). It produces bacteriocin like substance called coagulin which exhibits inhibitory activity against pathogenic Listeria species.[71]

Lactobacillus acidophilus: It has the following therapeutic benefits: decreases depression, chronic fatigue syndrome, sleep problems, muscle or joint pain, extreme tiredness, lactose intolerance, irritable bowel syndrome, decreases cholesterol, over growth of candida which is responsible for itching and painful skin, and significantly boost the immune system. It produces a bacteriocin called acidophilin.[73,74,75]

Lactococcus lactis subsp. lactis: It has the following therapeutic benefits: improves the skin health, activates the plasmacytoid dendritic cells which improve both the innate and adaptive immune responses, activates the natural killer cells (NK cells) and enhances their cytotoxic activity, improves the resistance against the pneumococcal infections, reduces lung damage due to influenza virus and HINI infection, even the para probiotic fractions of this bacterium reduces allergic response, reduces bronchitis, alveola inflammation, reduces blood pressure, LDL cholesterol, triglycerides, reduces age related hearing loss, and induces cancer cell death. It produces bacteriocin called nisin, which is a class-I lantobiotic.[76]

Lactococcus lactis subsp. cremoris: It has the following therapeutic properties: antioxidant, improves the overall gut health, antidepressant, decreases anxiety, exhibits significant inhibitory effect on *Listeria monocytogenes*, up regulates the anti-oxidation metabolites such as folate and glutathione, it also down regulates the enzymes producing ROS (thus reducing oxidative stress) and has a significant effect on free radical scavenging to reduce oxidation of the tissues. It produces a bacteriocin called diplococcin, which is a class-I lantobiotic.[77]

Lactococcus lactis subsp. lactis var diacetylactis: It has the following know therapeutic properties: antifungal, and antimicrobial. It produces a bacteriocin NISIN-Z, which is class-I lantobiotic with inhibitory properties on gram negative pathogenic bacteria.[78]

Streptococcus faecium; It has the following therapeutic properties: produces broad spectrum of bacteriocins to prevent the infections due to *H. pylori*, *C. difficile*, *L. monocytogenes* and species of Salmonella, exhibits the immunomodulation property along with enhanced cytokine production and T- reg cells, has anti-inflammatory effect through production of butyrate to improve the intestinal epithelial cell integrity, several *S. faecium*

food strains are safe and significantly improves the gut health. This organism produces a class-III bacteriocin called enterolysin.[78]

Pediococcus acidolactici: It has the following therapeutic effects: reduces constipation, diarrhea, stress, significantly improves the immune response, suppresses the auto immune encephalomyelitis by inducing IL-10 and regulatory T-cells. These organisms produce class-II bacteriocin called pediocin.[78,48]

Leuconostoc mesenteroids subsp. cremoris: The following are their therapeutic properties: strong antimicrobial property to inhibit several pathogenic bacteria in the gastrointestinal tract, exhibits similar therapeutic properties as *Lactobacillus plantarum* in terms of adhesion, antimicrobial properties, and anti-inflammatory properties.[80]

Propionibacterium shermanii and Propionibacterium arabinosum: They have the following therapeutic properties: regulates intestinal microflora to arrive at the optimal concentration of microbiota and their microbiome, produces bacteriocins to inhibit the pathogenic bacteria, have an exceptional ability to scavenge mycotoxins to minimize mutagenesis of the epithelial cells, stimulates Bifidobacterium which in turn create homeostasis among other beneficial bacteria, and exhibits antifungal activity by producing propionic acid. These organisms produce bacteriocins named propionicin and *Propionibacterium jensenii* produces the bacteriocin jensenii.[81,82,83,84,85,86]

Brevibacterium linens: The following are the therapeutic properties: excellent immune stimulator, reduction of tumor incidence, improves digestion, reduction of serum cholesterol, it is highly proteolytic and lipolytic and thus can inactivate the Coronaviruses with lipid protective layer. It produces bacteriocin linocin which inhibits growth of Listeria, several Coryneforms and gram-positive bacteria, but will not inhibit gram negative bacteria.[87]

Penicillium roquefortii and Penicillium camembertii: These food grade molds have the following therapeutic properties: the metabolites of food grade *P. roquefortii* (andrastins A, B, C, and D) are potential inhibitors of cholesterol biosynthesis and thus contribute to cholesterol reduction, andrastin-A has strong antitumor properties, the metabolite roquefortine inhibits gram positive pathogenic bacteria and has anti-inflammatory and pro-regenerative properties. Food grade *Penicillium camembertii* also produces fat breaking and protein breaking enzymes and thus serves as a good digestive aid to curtail digestive disorders.[88]

Sacchromyces boulardii: This is food grade yeast and has the following therapeutic properties: prevents and treats intestinal diseases, has immunomodulatory effect, significantly improves the bioavailability of minerals, detoxifies mycotoxins and thus protect the epithelial cells, lowers serum cholesterol, antioxidant and anti-mutagenic and also has significant anti-tumor and anti-inflammatory properties, reduces the risk of cardiovascular diseases, cancer, and Alzheimer's disease.[78]

The following are the functions of the individual components of the immunomodulins produced by the multiple mixed strain probiotics, specifically pertaining to their role in preventing thrombosis. This will give a good understanding and appreciation to the reader regarding the role and functions of probiotic produced immunomodulIns to control the thrombosis during COVID-19 infection.

What are immunomodulins?

The immunomodulins are immunomodulatory molecules which helps the human body to defend against the pathogens by adjusting or altering the normal immune response to respond more effectively when a pathogen (pathogenic bacteria, virus, yeast and mold etc.) has been encountered or detected. In addition to other pharmacological immunomodulins, the soluble growth end products of probiotic bacteria such as Short Chain Fatty Acids (SCFA), organic acids (lactic, acetic, butyric, propionic etc.), salts of the organic acids (lactates, acetates, butyrates, propionates etc), bio-therapeutic peptides, and bacteriocins, etc., are considered as immunomodulins of microbiological origin. In addition to the by-products of probiotic growth, the following probiotic derived or associated compounds can also be categorized under immunomodulins. They are probiotic cell wall - peptidoglycan, DNA fragments, extra cellular lipopolysaccharides, polysaccharides, and all other minor cellular components. Let me delve into how probiotics and their growth end products specifically can retard COVID-19 infection to improve the health of host through immunomodulation, to eliminate skepticism on the part of medical community and the pharmaceutical industry. There are two aspects to consider on this subject of immunomodulins of the probiotics: First one is the functions or therapeutic effects of the probiotic cell components and the second one is the physiological properties of the growth metabolic end products of probiotics and their influence on preventing or treating COVID-19, to protect the host health. What are various probiotic cell wall components and how do they exert their therapeutic properties to control or reduce thrombosis associated with coronaviral infection? They are as

follows: Peptidoglycan, Teichoic acid, cell wall Polysaccharide, cell surface proteins, LPXTG proteins, S-layer proteins, pili proteins and moon lighting proteins etc.

Peptidoglycan: The cell walls of the probiotic bacteria contain thick peptidoglycan layer, which is a multilayer cross-linked glycan chain with repeating pentapeptide units of 1, 4 linked N-acetyl glucosamine and N-acetyl muramic disaccharide units.[25] The composition of glycans vary from probiotic strain to probiotic strain, depends on their genus and species.[26, 27] This is the main reason for using multiple mixed strain probiotics, rather than a single strain probiotic, to derive the maximum therapeutic effect, due to variance in the composition of the glycans of the cell wall peptidoglycans. The peptidoglycan of probiotic strains belonging to genus lactobacillus suppresses inflammation provoking interleukin-12 (IL-12), which are associated with autoimmune diseases (28). Peptidoglycan induces the inflammation reducing IL-10 and activation of T-regulatory cells, which simmer the inflammation (which is an etiological factor for thrombosis), through immunomodulation.[29]

Teichoic acid: It attenuates inflammation provoking interleukin-8 (IL-8).[30]

Cell wall polysaccharides: They modulate the systemic and mucosal immune responses.[31] They also exhibit immune suppressive effect on macrophages through induction of interleukin-10 and simultaneously suppressing the levels of inflammatory cytokines IL-6, IL-12, and IL-8.[32] They also exhibit antiviral effect by inducing production of IFN-Y (interferon gamma),[33] thus to prevent thrombosis during the COVID-19 infection.

Cell surface proteins: The Pili proteins decrease the production of proinflammatory cytokines, and also exhibit immunoregulatory function by interacting with monocytes and dendritic cells.[34,35] The activated dendritic cell functions is an essential requisite to stimulate T-cells to destroy the coronavirus, to protect the endothelial cell damage which results in thrombosis.

The probiotic growth end products or metabolites include the secreted proteins, peptides, organic acids, bacteriocins and other small molecules. The secreted proteins include proteins P40 and P-75, which exert immunomodulatory action to reduce inflammation. The P-40 and P-75 proteins protect the epithelial tight junctions and barrier functions to eliminate the entry of pathogens, including viruses.[36] The aggregation promoting factor (APF) is also a secreted protein of probiotics which helps to colonize the probiotic bacteria to inhibit the adhesion of pathogens by competitive exclusion or by co-aggregation with pathogens including bacteria and viruses. The bacteriocins produced by probiotic bacteria improve the host immunity through immunomodulation and through improvement of phagocytic activity of the macrophages.[37] All of the aforementioned properties of the secretary molecules are strain specific and thus selection of specific probiotic strains is important to maximize the therapeutic benefits of the multiple mixed strain probiotics to prevent or treat COVID-19.

The small molecules produced by probiotics are not strain specific yet helps to mediate the function of the probiotics to improve the host health. They include, short chain fatty acids (SCFA), conjugated linoleic acid (CLA), and various neurotransmitters. The SCFA's serve as energy source to epithelial cells[38] and also helps to regulate T-regulatory cells[39,40] and their functions to control anti-inflammatory effects,[41,42] to restore the immune tolerance in host.

The beneficial effects of the cell wall components of Probiotic bacteria and their secreted growth end products are mediated through an interaction between them and the host. They are recognized by the host through pattern recognition receptors (PRR's), which further induce downstream signaling cascades that confer the beneficial functions to the host specifically the immune enhancement, to prevent the thrombosis during the COVID-19 infection.

Now one can appreciate how multiple mixed strain probiotics and their soluble growth end products (immunomodulins) can prevent or cure COVID-19 infection and the resultant thrombosis, through reducing the inflammation causing pro-inflammatory cytokines such as IL-6, and simultaneously enhancing the inflammation reducing interleukins such as IL-10, and also the T-regulatory cells.[43]

MATERIALS AND METHODS

Although the method of preparation of the multiple mixed strain probiotic cultures along with their immunomodulins is presented with explicit details in the US patent # 11,077,052 B1, the combination of probiotic strains and their percentages varied in the preparations in accordance with the mode of application and tissue sensitivity, under this section. In August 2021, the United States Patent and Trademark Office has issued a new patent (US patent #11,077,052 B1) to prevent SARS-CoV-2 infection or COVID-19 disease, by using multiple mixed strain probiotics along with their immunomodulins, antioxidants, surfactants, and other microbial stimulants and protectants. It is to be noted that the application for patent was filed on

September 2020, at which time SARS-CoV-2 virus variants were significantly low. However, the patent clearly outlined the following multiple ways SARS-CoV-2 infection can be prevented or treated, as stated in the abstract of the Patent: A multi-phase treatment of a respiratory disease in which one treatment is a lysing defense by applying inhibitory agents to respiratory passages. Application may be oral gargling mouthwash, nasal irrigant or smelling salt. Another treatment is an immune system suppressing defense by providing a liposome-based countermeasure to excess activity of the host immune system during COVID-19 infection, when administered through oral route. The liposome is supposed to increases bioactivity of probiotics, probiotics-produced therapeutic peptides, bio-peptides, and antioxidant level in the blood with a sustained massive dose of antioxidants to counteract excess oxidation produced by excess activity of the host immune system. Thus, the following detailed experiments were conducted (under the super vision of physicians and medical professionals) to check the validity of the patented invention (U.S. #11,077,052 B1) to prevent or treat COVID-19 due to coronavirus multiple variants including the latest omicron and its subvariants evolved in late 2020, 2021, and 2022.

All the subjects participated in the clinical trials were checked prior to conducting the trials for the presence of COVID-19 infection using RT-PCR test. Similarly, they were checked for the COVID-19 infection during and at the end of the clinical trials using the RT-PCR test. Periodically, the suspected subjects were checked for the blood clots in the lungs using CT-Scans. In addition, all of the subjects were strictly monitored for any signs of COVID-19 infection using the symptoms such as, cough, fever, respiratory distress, and oxygen levels.

First Preparation as Mouthwash: The composition of mouthwash includes probiotics and their immunomodulins along with surfactant (polysorbate), pathogenic yeast and mold inhibitors (natamycin and sodium propionate), antioxidants (ascorbic acid/sodium ascorbate). The following probiotics along with their immunomodulins are included in the mouth wash: *Lactococcus lactis subsp. lactis; Lactococcus lactis subsp. lactis var diacetylactis; Streptococcus thermophilus; Lactobacilus bulgaricus;* and *Lactobacillus acidophilus*. These ingredients when used in mouthwash with gargle are aimed at lysing the SARS-CoV-2 virus and its multiple mutated variants in the buccal cavity by disrupting the lipid or fat molecular fractions of the membrane which support the spike proteins, and thus making the virus ineffective. The yeast and mold inhibitors natamycin and sodium propionate are intended to inhibit the pathogenic yeast and molds which assist the virus infection. The probiotic bacteria and their immunomodulins are included to stimulate the definisin (anit-microbial peptides) production by the oral epithelial cells to inactivate the SARS-CoV-2 virus and its variants along with other pathogenic bacteria. The probiotics are selected on the basis of their adhesion properties to adhere to the epithelial cells to exert their continuous therapeutic effects. The composition of the therapeutic mouth wash was prepared as outlined in the US patent #11,077,052 B1, including the probiotic strains and their relative percentages, along with other ingredients.

Dosage and Mode of application:

Roughly 10 to 20 ml of the mouth wash liquid was used to gargle for 30 to 55 seconds, touching the base of the tongue close to epiglottis. The volunteers were asked to gargle twice a day. It is important because SARS-CoV-2 virus, like any influenza virus, spreads through the mouth to gain entrance into the lungs. Twenty volunteers were included in this experiment. Fifty percent of the volunteers received vaccination and the other half were not vaccinated.

Second Preparation as Nasal Irrigant: The following probiotic strains are included in this preparation to administer through nostrils: *Lactococcus lactis subsp. lactis; Lactococcus lactis subsp. lactis var diacetylactis; Streptococcus thermophilus; Lactobacilus bulgaricus; Lactobacillus acidophilus; Lactococcus lactis subsp. cremoris;* and *Lactobacillus sporogenes*. Its composition is same as mouthwash but administered through nostrils. It is intended to exhibit similar functions as mouthwash to inhibit the SARS-CoV-2 virus, secondary pathogenic bacteria, black mold causing Mucormycosis, and pathogenic yeast such as *candida albicans* etc. in the nasopharyngeal orifice. In addition, the probiotic bacteria included are intended to adhere to the nasopharyngeal orifices and displace the viral particles and also function as antiviral microbiota. The composition of nasal irrigant was prepared according to the details presented in the US patent # 11,077,052 B1.

Dosage and mode of application:

Two and half grams of nasal irrigant powder was dissolved in 260 ml of Lukewarm water and the nostrils are purged with the application of a nasal device. The volunteers were asked to purge the nostrils once in a day or once in three days, depending on the severity of infection in that particular geographic area, during the pandemic period. The liquid should be purged alternatively through both the nostrils. Forty subjects were included in the study. In this group, only 18 people were vaccinated at the other twenty-two did not receive any vaccinations.

Third Preparation as Nasal Inhalation: The composition includes probiotics and their volatile immunomodulins and alcohol infused therapeutic herbal extracts imbibed on to magnesium sulphate and sodium chloride (sea salt), with inclusion of optional ingredients, ammonium carbonate, CBD (cannabidiol), sterilizing agent sodium hypochlorite, and parts per million or billion chloroform. The following probiotic cultures along with their immunomodulins are included in the sinus prep: *Propionibacterium sherimanii; Lactococcus lactis subsp. lactis; Lactobacillus sporogenes; Lactococcus lactis subsp. lactis var diacetylatis;* and *Pediococcus acidolactici*. SARS-CoV-2 virus and its multiple variants, which are lodged on trachea, bronchi, bronchioles, and alveoli, is supposed to be inactivated due to volatile vapors mainly through disruption of viral lipid fractions in the supporting membranes. In addition, the lung tissues are activated to ward off the infectious viral particles by stimulating ciliated cells. The composition of nasal inhalant used was prepared as per the specifications of the US patent #11,077,052 B1.

Dosage and mode of application:

The preparation was tried on patients of all age groups and genders ranging from 5 years to 90 years. They were asked to inhale once in the morning and once in the evening if they are at home or office. If they have to go to public places or a crowded place, they were asked to inhale before and after visiting the place. For example, in a restaurant or ballpark a person can inhale prior to going into the place and after leaving the place. Assuming the person stays at such place for 3 or 4 hours, inhaling at the beginning and ending of the 3 or 4 hours will do the job. Twenty people were included in this experiment. Out of the twenty, eight people were vaccinated at the remaining twelve did not receive any vaccinations. As a practical illustration, this preparation was also given to several patients who were tested positive for COVID-19. During a subsequent lockdown period due to the COVID-19 positive test, the patients were asked to smell twice in a day.

Fourth Preparation as Liposome Administered Orally: The multiple mixed strain probiotics along with their immunomodulins, and other antioxidants, surfactants, and yeast and mold inhibitors is prepared as a liposome using lecithin with sonication. Then the liposome is administered as tablet or capsule. This liposomal preparation is intended to get into the intestinal tract intact, and also into blood circulation, to inactivate the COVID-19 virus in the blood vessels, and in all other organs where ACE-2 receptors are high in numbers, such as heart, lungs, kidneys etc. The composition and method was similar to the details outlined in US patent # 11,077,052 B1. The following probiotic strains are included in this liposomal preparation: *Lactobacillus acidophilus*; *Lactobacillus sporogenes; Propionibacterium arabinosum; Propinibacterium shermanii; Streptococcus thermophilus; Lactobacillus casei; Saccharomyces boulardii;* and *Bifidobacterium bifidum*.

Dosage and mode of application:

This probiotic liposomal preparation was given to several test subjects of all ages who were monitored for coronavirus infection during the coronavirus pandemic period. They were asked to take orally an ounce of liquid preparation daily. If it is in the form of tablets or capsules, they were asked to take two capsules or tablets twice daily. Twenty subjects were included in the clinical trial. Ten individuals out of the twenty were vaccinated while the other half did not receive any vaccination.

The use of all four preparations (1-4) simultaneously administered in the sixty and above age comorbid patients with hypertension and diabetes to prevent COVID-19 infection due to variants evolved in late 2020, 2021, and 2022.

A total of ten male and female patients over the age of 60 with hypertension were administered all preparations (mouth wash, nasal purge, nasal inhalation, oral liposome preparation) over a period of six months, during 2021 and 2022. Six people had been vaccinated and the other four did not receive any COVID-19 vaccines. All of these patients were taking blood pressure medication. The blood pressure readings were monitored by their physicians. The volunteers were asked to gradually reduce the medication during the experimental trials, and yet monitor the blood pressure. The results were monitored on the basis of validated COVID-19 symptoms, oxygen readings, CT scans, and RT PCR Tests.

Similarly, ten patients with diabetes were selected and were administered all four preparations simultaneously using the composition and dosage, outlined earlier, for over a period of six months during the years 2021 and 2022. Five of the patients were vaccinated, whereas the rest of the five were not vaccinated. However, all volunteers were on medication. The subjects were monitored for the onset of COVID-19 infection using the methods outlined earlier.

Dosage and mode of application:

The dosages and application were the same as outlined earlier for each preparation.

It is to be noted that the sample size of the subjects participated are significantly low because of not having many volunteers during pandemic. With all this severe handicap we have managed to conduct the clinical trials at least to get trend analysis on

how well multiple mixed strains can prevent COVID-19 infection due to newly and continuously evolving viral variants.

Results

Clinical trials conducted on subjects of different age groups distinctly proved the following in terms of preventing or treating COVID-19 infection during the current pandemic period, employing the patented procedure outlined in the US Patent #11,077,052 B1.

Therapeutic mouth wash (first preparation): It was evident, proven through experimental methods outlined in the materials and methods section, that gargling with the experimental mouthwash composition outlined in preparation #1 was excellent in controlling or preventing the COVID-19 pathogenic viral infection, as well as the secondary bacterial, and yeast and mold infections in all the age groups tested, irrespective of their vaccination status. The results were evaluated on the basis of symptoms of COVID-19, oxygen readings using oxy meter, and RT-PCR test.

Nasal irrigant (second preparation): During COVID-19 pandemic, the experimental subjects who have used the liquid nasal irrigant did not contract any infection, as evidenced by the lack of clinical symptoms such as cough, high fever, sore throat, and signs of respiratory distress, as presented in the issued patent (US Patent # 11,077,052 B1), during the COVID-19 pandemic, irrespective of whether or not they are vaccinated. Conversely there were no side effects in the group who were vaccinated with the use of nasal irrigant.

As presented in the issued patent, during the COVID-19 pandemic, the people who have used this nasal irrigation did not contract any infection, as evidenced by lack of symptoms such as cough, high temperature, sore throat, fever, and respiratory distress. Furthermore, they have maintained good levels of oxygen in their blood (between 90 to 100 percent). The oxygen was measured using pulse oximeter. Oxygen levels below 90 (specifically between 85 and 90) were considered suspicious of having COVID-19, during the pandemic period. Several people in the age group of 60 and above also did not contract the infection. It distinctly proved that this nasal irrigation composition has significant inhibitory effect on the pathogenic nasal viruses, specially the COVID-19 causing coronavirus. This composition was tried on all age groups and genders ranging from 5 to 90 years. It is extremely surprising to note that the population of seventy and above, who are extremely susceptible to COVID-19 infections, did not pick up the infection during the corona pandemic period. The infection was evaluated on the basis of clinical symptoms. No side effects were observed in any age group indicating that the composition has been synergistically buffered. Some people observed that their nasal passages cleared so well, and they have never felt so good before.

Nasal inhalation (third preparation): All the subjects who have participated in the clinical experimental trials did not develop any typical COVID-19 symptoms or infection, irrespective whether or not they were vaccinated when used as directed, indicating that it is virucidal to the SARS-CoV-2 virus causing COVID-19 infection. In addition, the patients who were tested positive for COVID-19 (the subjects who did not use nasal inhalant prior to getting COVID-19 infection) also got cured while they were in quarantine, proven by the negative RT-PCR test. The respiratory distress symptoms were relieved significantly in less than three days.

The COVID-19 positive patients, who were asked to use nasal inhalation did not develop any COVID-19 distress symptoms during the 14 days quarantine. Furthermore, the test (RT PCR test) at the end of 14 days came out negative for COVID-19. Similar results were found with other subjects. The subjects who did not have COVID-19 symptoms did not develop COVID-19 disease at all when using this smelling salt, indicating that it is viricidal to the SARS-CoV-2 virus and their variants causing COVID-19 disease during the pandemic.

Liposomal preparation administered orally (fourth preparation): All of the people who have participated in the experimental trials did not develop COVID-19 infection. The oxygen levels were maintained above 95, without any signs of cough, fever, and respiratory distress. The CT-Scan also proved no signs of blood clots or any other obstructions in the capillaries of the lungs, indicating that the liposomal preparation given orally had a significant effect on preventing the COVID-19 infection, even due to SARS-CoV-2 variants that emerged in 2021-2022. Similar results were observed even in the patients who were tested positive for COVID-19 infection, indicating that liposomal preparation of the current invention can also be used successfully to treat SARS-CoV-2 viral infection.

The results of the trials using all four preparations on sixty and above age group with comorbid hypertension and diabetes:

None of the ten patients with comorbid hypertension (6 people who were vaccinated and four people who had never received vaccination) contracted COVID-19, proven by the lack of symptoms as well as the negative RT PCR test. The CT scans also revealed no signs of pulmonary thrombosis. The

volunteers also were able to reduce their dosage of medication by 50 percent, proving that multiple mixed strain probiotics along with their immunomodulins were able to prevent COVID-19 infection while the SARS-CoV-2 virus was mutating to generate antigenically different variants such as Delta and Omicron. Furthermore, the blood pressure readings were lower even if the prescription drug was used at one half dose, when the current invention was practiced. It is of great importance because even the vaccinated people (over 60 years of age with comorbid conditions with high blood pressure) did not contract fatal COVID-19 viral infection due to the continuously evolving SARS-CoV-2 viral variants.

Similar results (no COVID-19 infections) were obtained with the diabetic patients (Type-2), proving that the invention outlined in US Patent #11,077,052 B1 is still effective even with the continuously evolving antigenically different mutant variants of SARS-CoV-2 virus. Apparently, the multiple mixed strain probiotics along with their immunomodulins, were able to counteract the COVID-19 infection through immunomodulation, suggesting such a treatment modality can be used as a standalone or adjuvant therapy. Unlike vaccines, it is able to prevent COVID-19 infection, irrespective of the continuously evolving antigenically different mutants. Particular scientific interest in this study is the significant reduction of thrombosis, which is a major factor of multiorgan failure and death of the COVID-19 patients. It goes to prove that proper maintenance of the immune system through properly maintained microbiota and microbiome is a significant factor to prevent or treat COVID-19 infection, irrespective of the viral mutations and resulting variants and subvariants. These research results also proved that immunosenescence due to old age can also be controlled by administering proper multiple mixed strain probiotics, along with their immunomodulins, by preventing the dysbiosis, to protect the aged population due to any viral infections, specifically COVID-19. It is of immense interest and noteworthy that the proper microbiota and microbiome, with no dysbiosis dictates the immune response through proper immunomodulation to the point of preventing COVID-19 infection.

Since we could not get an accurate number of people infected without the use of multiple mixed strain probiotics along with their immunomodulins, a statistical analysis using a T-Test and statistical probability could not be determined. However, since all 120 participants who have been included in the clinical study did not contract COVID-19.While it is not statistically accurate, due to the limited number of test participants, one can interpret that the data is infact statistically significant in that one can interpret the probability as $P<0.05$, meaning the probability of the population who can avoid COVID-19 by using multiple mixed strain probiotics is around or greater than 95%.

If you take the overall world population and the total number of people who were infected with COVID-19, we can arrive at approximate percentage of infected people in the group. According to the WHO data, as of this writing 556 million people out of 7.5 billion people worldwide were infected with COVID-19. If you plot the data, 7-8 people in every 100 people were infected. However, this is not taking into account the people who were not tested since they were asymptomatic (although they might have been infected with COVID-19). If we assume that another 7-8% of asymptomatic people could have been positive for COVID-19, we can conclude that 15% of the people could have been infected with COVID-19. If we use this as a control figure and compare it to the limited clinical trials we have conducted using 120 people, the results are significant. Since we did not have enough data, in all fairness, a positive statistical data could not be presented. It is extremely difficult to conduct controlled experiments in large groups during the pandemic, considering the inherent risk and people unwilling to volunteer due to the scare of SARS-CoV-2 virus and its continuously mutating variants.

Discussion:

The figure 3 depicts the possible mechanism of how the multiple mixed strain probiotics and their immunomodulins prevent or cure the thrombosis induced by SARS-CoV-2 viral infection by maintaining the hormonal-enzyme dependent vascular homeostasis, in addition to reducing the cytokine storm. The multiple probiotic immunomodulins may accomplish this task through down- regulation of the production of ACE (Angiotensin Converting Enzyme) which converts Angiotensin to Angiotensin-2 (AT-2). When AT-2 production is reduced, the vascular endothelial cells will be protected due to reduced level of vasoconstriction induced by NADPH oxidase enzyme activity. It is also possible that the probiotic immunomodulins may simultaneously up-regulate ACE-2 production to covert AT-2 to AT 1-7 which can reduce the vascular endothelial cell damage (caused by SARS-CoV-2 coronavirus) by increasing vasodilatation with the aid of Nitric Oxide Synthase. Furthermore, the probiotic immunomodulins may further down-regulate the production and activity of Angiotensinogen, which is a substrate for ACE (Angiotensin Converting Enzyme) to produce AT-2. Even though SARS-CoV-

2 virus partly inactivated the ACE-2 enzyme, through either up-regulation and/or down-regulation of the appropriate hormone-enzymatic systems, multiple mixed strain probiotics and their immunomodulins can regulate and maintain the vascular homeostasis to prevent or reduce thrombosis during COVID-19 infection.

Figure 3: How does multiple mixed strain probiotics and their immunomodulins maintain the hormonal-enzyme dependent vascular homeostasis to prevent or cure the thrombosis induced by SARS-CoV-2 viral infection.

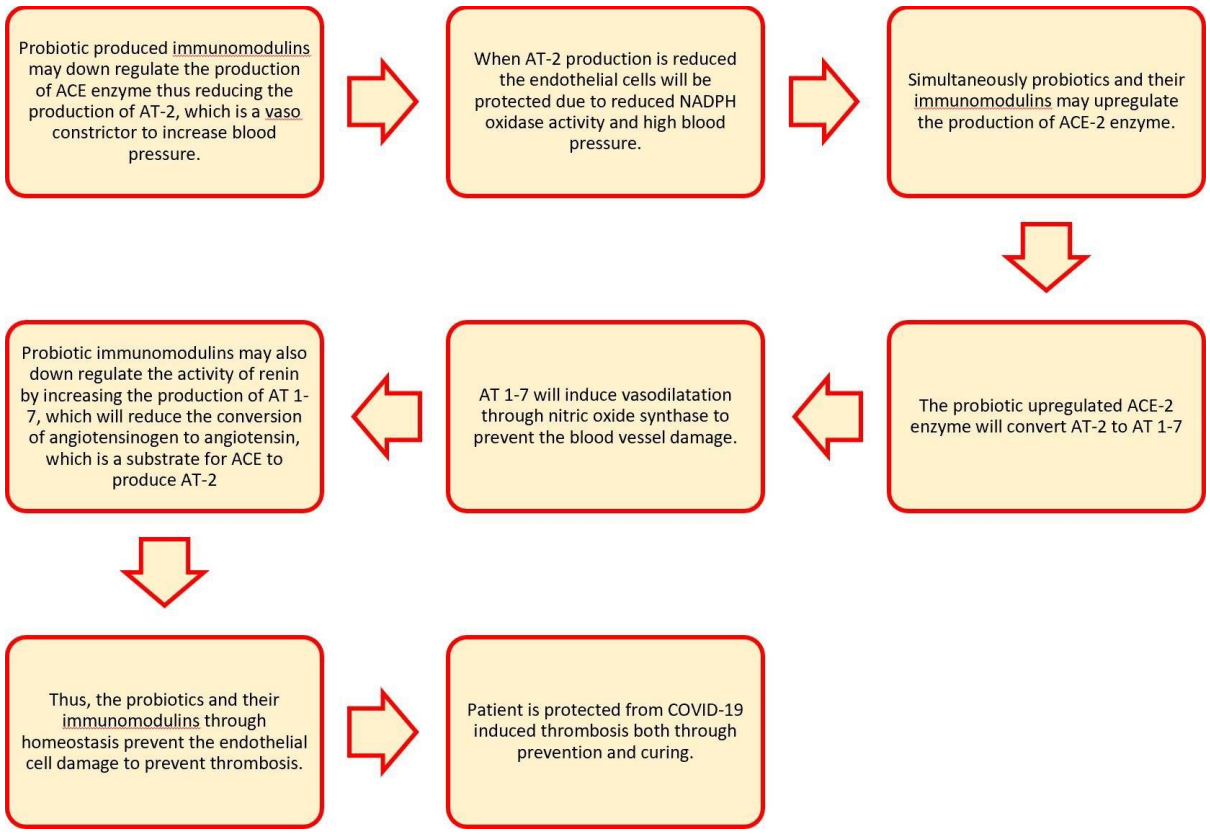
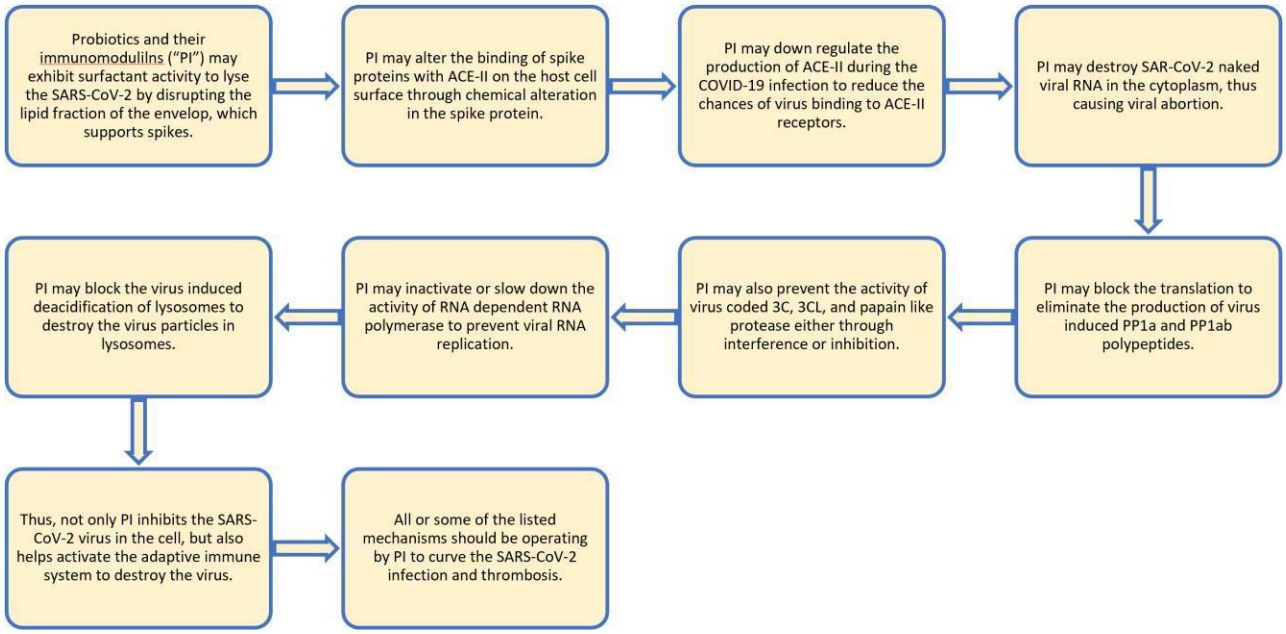


Figure 4, schematically presents the hypothetical molecular mechanism by which the multiple mixed strain probiotics and their immunomodulins can prevent the endothelial cell damage and thrombosis due toSARS-CoV-2 viral infection and multiplication in the eucaryotic cell(s). This can be attributed due to multifaceted mechanisms than a singular biochemical mechanism. Multifaceted mechanism exerted by probiotic immunomodulins include: The disruption of SARS-CoV-2 viral envelope through surface active mechanism; The alteration of viral spike protein configuration to eliminate spike protein- ACE-2 attachment; prevention of translation to produce viral encoded polypeptides PP1a, PP1ab, proteolytic enzymes 3c, 3cl, and RNA dependent RNA polymerase enzymes ; prevention of viral RNA replication ; prevention of virus induced deacidification of lysosomes to lyse the viral particles to assist the adaptive immune system etc.

Figure 4: Multifaceted Molecular Mechanism(s) of how Multiple Mixed Strain Probiotics and their Immunomodulins ("PI") can Protect the endothelial cell damage and thrombosis due to SAR-CoV-2 viral infection.



Since each probiotic strain produces strain specific bio-active immunomodulins, a combination of selective multiple mixed strain probiotics along with their diverse immunomodulins, biomolecules, in combination, may exert the preventive or cure of COVID-19 infection, as presented in US Patent# 11,077,052B1. Although exact mechanism cannot be elucidated, the patented discovery has validity in that it was very effective in treating COVID-19 infection due to SARS-CoV-2 virus and its multiple variants, confirmed by lack of thrombus formation with the aid of CT scan.

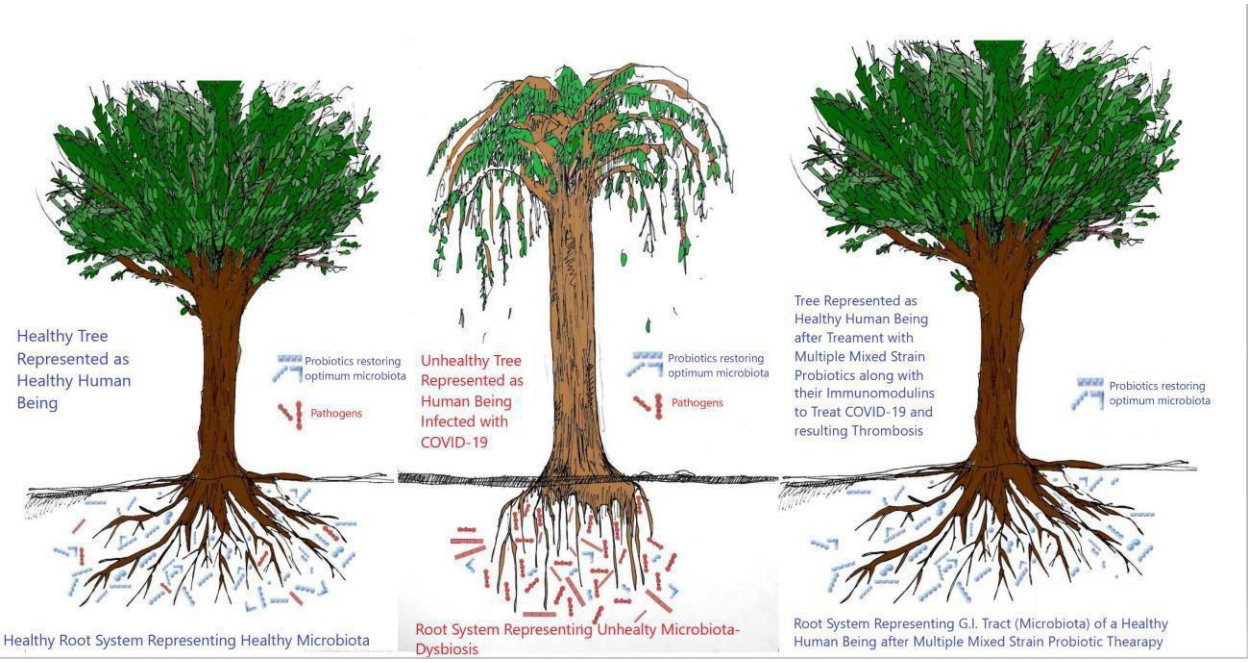
In addition, the multiple mixed strain probiotics along with their immunomodulins must have eliminated dysbiosis to protect the patient from thrombosis due to COVID-19 infection. It has been proven in this investigation, that administering multiple mixed strain probiotics along with their immunomodulins increased the fecal micobial contents by two logs, indicating increased growth and implantation of probiotics in the Gastrointestinal tract. Also, it has been confirmed that the increased microbial population were strains of probiotics administered. It is presented in a pictorial form for easy understanding using a non-traditional model of tree and root system, with the tree representing human body, and human intestinal tract represented as root system. It is presented in figure 5 (5a, 5b, 5c). In this connection, dysbiosis is the term used for the unfavorable negative variance in the composition of microbiota of the Gastro- Intestinal tract. Figure 5a represents a healthy human being with positively balanced microbiota in the gastrointestinal tract, with no dysbiosis, and consequently no COVID-19 infection. The dysbiosis due to dominant pathogenic flora with significant reduction of probiotics proved the individual picked up the COVID-19 infection due to depressed or negatively altered immune system. This is represented in figure 5b. The COVID-19 victim who has been treated with multiple mixed strain probiotics along with their immunomodulins recovered rather quickly with well-established probiotics in the GI tract, curing the dysbiosis. The pictorial details have been presented in figure 5c.

Figure 5: Pictorial demonstration of Probiotic Therapy on curing the COVID-19 Infection, due to dysbiosis resulting in thrombosis and cytokine storm, using tree as a model for an easy understanding (5a, 5b, 5c).

5a: Healthy tree, without COVID-19, represented as healthy human being, and healthy G.I. tract microbiota represented as root system.

5b: Unhealthy tree, with COVID-19, represented as unhealthy human being, and unhealthy G.I. tract microbiota (dysbiosis) represented as root system.

5c: Fully recovered COVID-19 patient, due to probiotic therapy, represented as healthy tree, and healthy G.I. tract microbiota as root system.



Taking into account the past and current research findings, mechanisms, and hypothesis put forward on this subject, I can safely hypothesize the following regarding the success behind the use of multiple mixed strain probiotics along with their immunomodulins to prevent or cure thrombosis due to COVID-19 viral infections, due to SARS-CoV-2 coronavirus and its multitude of variants, which is as follows:

The immunomodulins produced by the several probiotic strains, included in the multiple mixed strain probiotic blend, might have inactivated the SARS-CoV-2 viral envelop through the action of their surfactants; or their therapeutic peptides and bacteriocins might have physically or biochemically altered the spike proteins and/or configuration to eliminate binding to ACE-2 receptors; or the probiotic specific and nonspecific peptide fractions, entered into the cells through biosynthetic secretory pathways ,might have blocked the virus induced Proteolytic enzymes (3C, 3CL, and papain like protein enzyme) to cleve the translated poly proteins (PP1a and PP1ab); or they might have blocked or significantly reduced the activity of RNA dependent RNA Polymerase enzyme to stop the RNA viral replication; or it may also be possible that the Probiotic cell wall components and immunomodulins might have stopped or reduced or prevented the deactivation of Lysosomal enzymes during COVID-19 infection, thus to help adaptive immune system activation, to prevent the progression of COVID-19 infection.

In addition, the multiple immunomodulins produced by multiple Probiotic strains might have downregulated the production of Angiotensin 2, by inactivating or significantly reducing the ACE enzyme activity. Simultaneously they might have up regulated the ACE-2 enzymes to convert the AT -2 to AT 1-7, thus reducing the Vasculitis, Inflammation, and hypertension, through production of Nitric Oxide Synthase (NOS) and subsequent vasodilation, to prevent thrombosis.[49,50,51,52]

The use of multiple mixed strain Probiotics and their immunomodulins cannot be ignored to control or prevent thrombosis. The probiotics and their immunomodulins exhibit multitude of functions to inhibit SAR-CoV-2 and its mutants, and thus can be used as an adjuvant therapeutic agents, along with vaccines to control the COVID-19 infection. Thus, extensive research is required in this arena, not only to cure or prevent current COVID-19, but also any other unforeseen viral pandemics in the future, which can induce thrombosis and subsequent multiorgan failures resulting in death. Detailed, yet

limited clinical experiments conducted in this investigation proved that the composition and results presented in the US patent #11,077,052 B1 are highly reproducible to prevent or treat COVID-19 infection, not only due to SARS-CoV-2 virus, but also due to its multiple variants and subvariants.

CONCLUSION:

The results of this investigation proved that the multiple mixed strain probiotics along with their immunomodulins can prevent the COVID-19 infection irrespective of the number of variants evolved due to the continuous mutation of SARS-CoV-2 coronavirus, including but not limited to Delta, Omicron, and Omicron subvariants. The Pathogenesis of thrombosis causing multiple organ dysfunction has been systematically presented with minute molecular details. Mechanism of how SARS – CoV-2 variants, such as alpha, beta, gamma, delta, omicron, etc., are produced due to mutations involving lack of proof-reading ability of RNA dependent RNA Polymerase enzyme coded by SARS- CoV-2 viral genes has been presented in a schematic diagram. The hypothetical mechanism of retardation of thrombosis by the multiple mixed strain Probiotic produced immunomodulins has been presented. The dysfunction of ACE-2 due to SARS-CoV-2 infection and its subsequent inability to convert Angiotensin-2 to Angiotensin 1-7 resulting in hypertension and thrombosis during COVID-19 infection, has been clearly demonstrated. The importance of individual components of the Probiotic produced immunomodulins, to prevent or treat COVID-19, has been presented with specific emphasis on reducing the thrombosis. It has been well established that the multiple mixed strain probiotics can prevent COVID-19 infection, irrespective of multiple mutant variations of SARS-CoV-2 virus. A well-defined hypothesis has been presented in terms of the biochemical mechanisms exerted by the immunomodulins to retard the COVID-19 disease progression. The best alternatives to vaccines and medicaments is multiple mixed strain probiotic therapy to prevent or treat COVID-19 infection. A justifiable recommendation has also been made to use the multiple mixed strain Probiotics and their immunomodulins as an adjuvant therapeutic agent along with vaccines, to curb the COVID -19 pandemic.

Disclosure:

The author is a scientist heavily involved in probiotic research and holds over 150 US and International patents. His company (IMAC Inc.) manufactures food-grade microbial cultures and other essential high-tech enzyme fortified functional products that go into manufacturing cheese and other dairy products in the United States, Canada, Europe, Asia and South America.

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