

# Supportive therapy during COVID-19: the proposed mechanism of short-chain fatty acids to prevent cytokine storm and multi-organ failure

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1	Supportive therapy during COVID-19: the proposed mechanism of short-
2	chain fatty acids to prevent cytokine storm and multi-organ failure
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15	Abstract
16	The world is currently facing the COVID-19 pandemic that is taking a heavy toll on several
17	countries. While many infected patients have a good prognosis, in some cases the progression
18	can be serious and even lead to death. The commonly seen complications are a cytokine storm
19	and multi-organ failure that require intensive care. The mortality of critically ill patients
20	depends on age, sex, immune state or co-morbidities. There is an urgent need to discover a
21	biomarker to identify early on patients at risk of developing serious complications and to find
22	an effective treatment that could prevent disease progression and critical states. Recent
23	investigations have pointed to the possible contribution of intestinal dysbiosis to the
24	pathophysiology of COVID-19. Herein, we hypothesize that butyrate, a short-chain fatty acid

25	initially produced by the gut microbiota, could be administered as supportive therapy to prevent
26	immune system activation and disease progression.
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28	Keywords
29	COVID-19, cytokine storm, multi-organ failure, intestinal dysbiosis, short-chain fatty acids,
30	butyrate
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#### BACKGROUND

The coronavirus disease (COVID-19) pandemic for which the first case was reported in

December 2019 in Wuhan (China), is a highly contagious and life-threatening viral infection.

For the period from December 2019 to June 2021, it has already affected more than 176 million

people and caused more than 3.8 million deaths worldwide according to the World Health

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# 59

#### 60 Clinical presentation

Organization (1).

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which is responsible for COVID-19 is a new member of the *Coronaviridae* family. Transmission mostly occurs via respiratory droplets and aerosols from person-to-person. Once in the respiratory tract, the virus attaches to its specific receptor site, the angiotensin I converting enzyme 2 (ACE-2) and its entry into cells then requires transmembrane protease serine 2 (TMPRSS2) expressed by pulmonary epithelial cells. The virus penetrates host cells for replication followed by viral particles assembling and release of the new virions (2).

The clinical spectrum of COVID-19 ranges from asymptomatic, moderate, severe illnesses, up to critical states. The most frequent symptoms are fever, dry cough, dyspnea and fatigue that can be associated to myalgia, rhinorrhea, vomiting and cephalgia. Most of the infected patients have a good prognosis but in some cases, progression can become severe, possibly leading to death. The commonly seen complications in critically ill patients are acute respiratory distress syndrome (ARDS), sepsis, disseminated intravascular coagulation, acute liver or kidney injuries and pulmonary embolism. In rare cases, multisystem inflammatory syndrome, rhabdomyolysis, autoimmune hemolytic anemia and neurological complications have been observed (3,4). The mortality of critically ill patients depends on age (more than 65 years old), sex (male), co-morbidities (*e.g.*, metabolic syndrome, diabetes, cerebrovascular, cardiac or pulmonary diseases) and immune state (5,6). Furthermore, SARS-CoV-2 associated risk is increased with respect to seasonal influenza since its transmission rate is nearly three times higher (7,8). Fortunately, influenza vaccine is associated with a better clinical outcomes and presents no harmful effect on COVID-19 susceptibility (9).

82 Severe prognosis is correlated to the so-called "cytokine storm" which is frequently observed 83 in critically ill patients. Such an event is defined by a huge increase of pro-inflammatory 84 cytokines (namely interleukin (IL)-1 $\beta$ , IL-6, IL-12, interferon (IFN)- $\alpha/\beta$ , tumor necrosis factor 85 (TNF)- $\alpha$ ) and chemokines (namely C-C motif chemokine ligand (CCL)2, CCL3, CCL5). A 86 well-coordinated innate immune response is the first line of defense against pathogens and plays 87 a crucial role in the prevention of SARS-CoV-2 spreading (10). However, dysregulated and/or 88 excessive immune responses have been shown to fail at clearing the virus and in turn, can even 89 contribute to the pathogenesis of ARDS as well as multi-organ failure (3). The excessive 90 production of cytokines results in tissular infiltration of inflammatory cells (e.g., macrophages 91 and neutrophils); which favors (i) the alteration of the alveolar-capillary barrier, (ii) lung tissue 92 damage (vascular leakage, alveolar oedema) and (iii) hypoxia associated with ARDS (11). The 93 cytokine storm also plays a central role in extrapulmonary multi-organ failure (heart, kidney 94 and liver) (12). Moreover, decrease in natural killer (NK) and T cell populations ascribed to 95 lymphocytopenia have been also observed and correlated to disease severity (13,14). The 96 cytokine storm promotes tissue homing of immune cells and thrombosis. Moreover, a post-97 COVID syndrome leading to multi-organ sequelae is associated to immunologic aberrations 98 and inflammatory damages. This syndrome encompass a spectrum of pulmonary, hematologic, 99 cardiovascular, neuropsychiatric, renal, endocrine, dermatologic and gastrointestinal injuries.

# 101 Current pharmacological treatment of the cytokine storm

102 Since the beginning of the pandemic, many antiviral agents have been used or are still under 103 clinical evaluation for COVID-19 (e.g., lopinavir-ritonavir or darunavir-cobicistat 104 combinations, remdesivir, favipiravir, camostat mesylate-nafamostat) (15). However, once 105 immunologic complications occur, the use of standalone antiviral treatment is not sufficient. It 106 should be combined with anti-inflammatory treatments to manage the cytokine storm as well 107 as anti-coagulative drugs. Regarding the cytokine storm mechanism, the current therapeutic 108 approaches consist in: (i) supplementing with IFN- $\lambda$  to potentiate innate immunity, (ii) using 109 immunomodulators (e.g., corticosteroids, intravenous immunoglobulins) to restore immune 110 balance, (iii) inhibiting cytokine productions (e.g., IL-1 or IL-6 antagonists, TNF blockers, IFN-111  $\alpha/\beta$  inhibitors, ulinastatin, oxidase phospholipids, sphingosine-1-phosphate receptor 1 agonists 112 and stem cell therapy), (iv) scavenging cytokines (hemofiltration), (v) inhibiting mononuclear 113 macrophage recruitment and action (toll-like receptor (TLR) 7 antagonist and C-C motif 114 chemokine receptor type (CCR) 2 blockers), and/or (vi) strengthening the vascular barrier by 115 activating the endothelial Slit-Robot4 signal pathway (12,15,16).

116 The period of treatment administration for SARS-CoV-2 is crucial. Indeed, an early exposure 117 of the aforementioned strategies may inhibit the onset of the host's immune response, for 118 example if drugs are administered too early. However, the best strategy is to act before the 119 production of pro-inflammatory cytokines. For instance, montelukast commonly used in the 120 treatment of persistent asthma has recently been proposed for COVID-19 treatment in an 121 intensive care unit (17). Montelukast can inhibit cytokine production through the inhibition of 122 the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, 123 which down-regulates pro-inflammatory mediators. This approach seems promising since the

124 blockade of cytokine production might be efficient in preventing pro-inflammatory125 consequences.

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#### THE HYPOTHESIS

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In the present article, we aim to propose a new strategy dedicated to block the immune system overactivation. It is worth mentioning that after virus recognition, macrophages polarize towards the pro-inflammatory M1 phenotype. M1 macrophages actively produce proinflammatory cytokines which in turn drive viral replication and facilitate spreading of pathogens in the patient and tissue damage (18). Therefore, a very promising therapeutic alternative could be to modulate the differentiation of macrophages in order to orient them towards the anti-inflammatory M2 phenotype.

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#### 137 Indirect proof of macrophages M1/M2 ratio skewing in critically ill COVID-19 patients

138 Macrophages are major regulators of inflammatory response and also play a central role in host 139 defense (19). They are featured by an important cell heterogeneity and plasticity. Indeed, in 140 reaction to microenvironmental stimuli, macrophages can adopt different functional programs. 141 Macrophages can either differentiate through a pro-inflammatory (M1) or anti-inflammatory 142 (M2) phenotype (18). M1 macrophage polarization is mediated by IFN- $\gamma$ , TNF- $\alpha$  or 143 lipopolysaccharides stimulation. M1 transformation hallmarks are both the inducible nitric 144 oxide synthase (iNOS) expression and the pro-inflammatory cytokines IL-1β, IL-6, IL-12, IFN-145  $\gamma$ , TNF- $\alpha$  and chemokines CCL2, CCL3, CCL4, CCL5, CCL8 production. This leads to 146 pathogens destruction for which the time-window of inflammatory processes usually ranges 147 from hours to days, but it has been shown that it can last for months or years, resulting in non-148 resolving inflammation. On the other hand, M2 macrophage polarization involves other interleukins stimulation (namely IL-4 and IL-13) and promotes up-regulation of genes associated to tissue repair and remodeling (e.g., *arginase l (arg1)*) (18). M2 macrophages exert their anti-inflammatory response *via* some specific cytokines like IL-1, IL-13, tumor growth factor (TGF)- $\beta$  and chemokines CCL1, CCL2, CCL13 (18). Therefore, M1 and M2 macrophages can easily be distinguished by their cytokine profiles as well as the expression of cell surface markers.

155 M1/M2 macrophages balance is finely regulated by specific signaling pathways. NF- $\kappa$ B and 156 signal transducer and activator of transcription (STAT) 1 signaling pathways lead to M1 157 phenotype, while STAT3, STAT6 and PPAR $\gamma$  signaling pathways favor the M2 phenotype (19). 158 An imbalance between these two phenotypes has been already observed in chronic 159 inflammatory diseases such as asthma, chronic obstructive pulmonary disease or 160 atherosclerosis (20,21).

161 According to cytokines and chemokines profiles at the systemic level of critically ill patients 162 (*e.g.*, IL-1 $\beta$ , IL-6, IL-12, TNF- $\alpha$  and CCL5), it has been suggested that macrophages involved 163 in the cytokine storm have the pro-inflammatory M1 phenotype (22). However, such an 164 observation still needs to be validated in clinical samples.

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# 166 Intestinal dysbiosis, macrophages polarization and arguments for supportive therapy with 167 short-chain fatty acids

168 Clinical characteristics of patients who develop severe forms of COVID-19 consistently suggest 169 the contribution of intestinal dysbiosis. These patients are those with specific risk factors in 170 relation to unhealthy gut microbiome status (23). Moreover, COVID-19 infection itself is 171 associated to the alteration of the gut microbiome characterized by an enrichment of 172 opportunistic organisms and depletion of beneficial commensals (24,25). Therefore, there is a 173 link between gut microbiota alterations and the pathophysiology of COVID-19. Maintaining a

174 healthy microbiome status is a possible strategy to tame COVID-19 severity. Gut microbiota is 175 known as a key factor of host immune system homeostasis and its composition has been found 176 depleted with bacteria associated to immunomodulatory effects in some COVID-19 patients 177 (24). Furthermore, intestinal dysbiosis has been linked to the decrease of main pro-resolving 178 Faecalibacterium prausnitzii, a butyrate-producing bacteria, has been found mediators. 179 depleted in critically ill patients (26). This has also been associated to a pro-inflammatory state 180 featured by (i) a decrease of regulatory T cells and (ii) polarization of macrophages into the M1 181 phenotype (27).

182 Short-chain fatty acids (SCFAs) belong to the pro-resolving mediators that participate in the 183 dynamic host-microbiome network to regulate immune response. SCFAs are produced by the 184 gut microbiota from the anaerobic fermentation of indigestible polysaccharides such as dietary 185 fibers and resistant starches. Acetate, propionate and butyrate are key since they ensure the 186 communication between the host and the microbiome, playing an important role in host 187 homeostasis (28). SCFAs can act on various immune cells in the gut to inhibit inflammation 188 through multiple mechanisms. For instance, they modulate the differentiation of regulatory T 189 cells or inhibit histone deacetylase (HDAC). They can also specifically activate some G protein-190 coupled receptors (GPCRs, e.g., FFAR2, FFAR3) in order to enhance the intestinal barrier 191 function and to modulate the immune system. Moreover, butyrate mainly triggers the metabolic 192 shift in macrophages towards anti-inflammatory M2 phenotype via the inhibition of HDAC3 193 (28). In other words, SCFAs may provide a better control of the inflammatory response, 194 suggesting that their therapeutic properties are worth exploring.

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## 196 Focus on the systemic anti-inflammatory effects of butyrate

SCFAs are first absorbed by intestinal epithelial cells and are then metabolized in the liver.Only a small fraction reaches the systemic circulation. However, it has been suggested that

199 systemic SCFAs are protective against allergic asthma (29). Butyrate can inhibit adhesion of 200 eosinophils to the endothelium and is known to improve allergen-induced inflammation in mice 201 (29). Moreover, HDAC inhibitors are protective against vascular inflammation and 202 atherosclerosis by modulating the endothelial function and production of pro-inflammatory 203 cytokines, particularly with butyrate. SCFAs also improve kidney function after acute kidney 204 injury induced by ischemia-reperfusion (28). Butyrate has been thoroughly evaluated as HDAC 205 inhibitor and pointed as the most pharmacologically active of SCFA (30). HDAC inhibition by 206 butyrate promotes the inhibition of lipopolysaccharide-induced production of nitric oxide (via 207 iNOS) and pro-inflammatory cytokines (IL-6, IL-12) release (30). More interestingly, butyrate 208 has shown inhibition of NF-kB signaling pathway and facilitates the expression of anti-209 inflammatory cytokines such as IL-10 in mononuclear cells and neutrophils (31). Recently, it 210 has been shown in gut epithelial organoids and in some gastric cells lines that butyrate can 211 downregulate genes essential for SARS-CoV-2 infection (Ace2, Tmprss2) and can also 212 upregulated other antiviral pathways (32,33). Altogether, all these studies provide strong 213 evidence that SCFA and especially butyrate exhibit a worth-investigating anti-inflammatory 214 action that might be used to prevent or alter the course of cytokine storm in critically ill COVID-215 19 patients.

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# 217 Butyrate supplementation for preventing the cytokine storm

Similar to the observations made on experimental models of viral infection (e.g., Influenza A), we propose that the inflammatory response during COVID-19 involves an increased M1/M2 macrophage phenotype ratio (34). The M1 phenotype is likely to be associated to the activation of the NF- $\kappa$ B pathway and the pro-inflammatory cytokine production (e.g., IL-6, IL-12, TNF- $\alpha$ ). This could at least partially explain the observed excessive cytokine release. Therefore, a relevant alternative is to restore the M1/M2 balance ratio by favoring M2 macrophage 224 differentiation. Butyrate supplementation is expected to help polarize macrophages into M2 225 phenotype, which in turn might decrease pro-inflammatory cytokine production. Butyrate is 226 proposed to inhibit the NF-kB pathway by preventing proteasomal degradation of the NF-kB 227 inhibitor, namely IkB. Ji et al. elegantly demonstrated that butyrate facilitates M2 macrophage 228 polarization by HDAC inhibition and STAT6 signaling pathway activation. Furthermore, they 229 observed an upregulation of M2 markers such as *arg1* and *found in inflammatory zone 1 (fizz1)* 230 (35). Furthermore, butyrate is known to prevent excessive neutrophil recruitment into the 231 airways via a GPCR-dependent receptor and by an alteration of the CXCL1 synthesis, which is 232 a neutrophil chemoattractant produced by macrophages (34). In other words, butyrate 233 supplementation could also limit inflammatory cell infiltration in the airways, which in turn 234 might reduce tissue and vascular disruption. In addition, butyrate also stimulates hematopoiesis 235 by favoring the development of macrophage progenitors with the M2 phenotype (36). Through 236 the expression of the transcription factor forkhead box protein P3 (foxp3) butyrate also 237 promotes regulatory T cells, which modulate the immune system and secrete anti-inflammatory 238 cytokines (IL-10). Butyrate is also known to limit the thrombo-inflammation phenomenon by 239 activating the tissue-plasminogen activator (t-PA), thus likely to play a role in anti-coagulation 240 COVID-19 strategies (Figure 1).

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#### **EVALUATION OF THE HYPOTHESIS**

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# 244 Supportive therapeutic strategy with SCFA

The cytokine storm plays a critical role in COVID-19 pathophysiology depending on macrophage activation. Supplementation with SCFAs and especially butyrate could help to modulate the immune system activation early on. Our idea is to systematically associate SCFAs serum profiling, cytokine dosage and/or M1/M2 macrophages ratio determination in each 249 patient positive for COVID-19 at the early stages of the disease. In face of the absence of validated borderlines of these tests in specific cohort of COVID-19 patients, investigations will 250 251 be performed according to limits routinely applied for other pathologies. This might help to 252 detect intestinal dysbiosis and identify patients at risk of developing severe immune 253 complications that could benefit from this supportive therapy. In addition to clinical 254 characteristics, these parameters could also serve to elaborate a strong predictive score of 255 COVID-19 progression and to initiate early butyrate supplementation as a supportive treatment 256 in such detected vulnerable patients (Figure 2).

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## 258 Preclinical proof-of-concept of butyrate as supportive therapy

259 Recently, butyrate treatment shows beneficial effects in influenza-induced mice by preventing 260 excessive neutrophil recruitment into the airways (34). These results have driven the hypothesis 261 that butyrate could serve as a supportive therapy during COVID-19 infection. Preclinical 262 investigations performed with gut epithelial organoids or gastric cell lines have shown down-263 regulation of SARS-CoV-2 entry genes under butyrate treatment (32,33). Another level of 264 proof-of-concept could be reached by investigating the effects of butyrate supplementation in 265 a relevant mouse model of SARS-CoV-2 infection. This could be achieved with mice 266 expressing human ACE2 enzyme, inoculated with SARS-CoV-2 that develop ARDS within 267 five days (37).

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# 269 Clinical trial design

Our hypothesis could be tested through a randomized double-blind prospective trial. This trial would enroll patients tested positive for SARS-CoV-2 by reverse transcription polymerase chain reaction (RT-PCR) and who are at risk of progressing to severe COVID-19 (older patients with confirmed co-morbidities like heart disease, hypertension, chronic respiratory disease,

obesity, diabetes). After signing the informed consent, patients would be randomized to either 274 275 "butyrate" or "placebo" groups. Patients would be proposed either butyrate capsules or placebo 276 on a daily basis and for a fortnight. A remote telehealth monitoring service for clinical events 277 would be performed from diagnosis to the final outcome. This would include the use of 278 validated specific and general health questionnaires to document disease progression. Blood 279 samples would be collected before treatment initiation and at 7 and 14 days after, for SCFA 280 profiles and cytokine dosage. The primary endpoint could be a combined score aggregating 281 clinical adverse events, severity markers, sequential organ failure assessment, the need and 282 duration of hospitalization, time until virus negativation or death due to SARS-CoV-2 infection 283 (Figure 3).

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- **CONCLUSION**
- 287 The cytokine storm acts as a critical factor in the occurrence of severe-to-death patients with 288 COVID-19. This event is mostly defined by the unbalanced increase of circulating pro-289 inflammatory cytokines, which in turn may lead to multi-organ failure. To counterpoise this 290 phenomenon, we propose an early identification of patients at risk by investigating 291 macrophages polarization. The decision to supplement with butyrate will be made on the basis 292 of SCFA profiles, cytokine dosage and M1/M2 macrophage ratio. SCFAs and mainly butyrate 293 can be considered as pro-resolving mediators due to their already known role in the immune 294 response modulation. It is important to note that pro-resolving mediators have the major 295 advantage of not blocking the initial immune response but rather downregulating the process to 296 resolve inflammation, which is crucial during COVID-19.
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307	Consent statement/Ethical approval
308	Not required
309	
310	Conflict of interest
311	There are no competing interests to declare.
312	
313	

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	<ul><li>34.</li><li>35.</li><li>36.</li><li>37.</li></ul>

#### 437 FIGURES LEGENDS



# 438

439 *Figure 1:* The proposed mechanism of short-chain fatty acids to prevent cytokine storm
440 and multi-organ failure

441 (1) SARS-CoV-2 enters and colonizes the host organism mainly through the airways. (2) 442 Intestinal dysbiosis is found in patients with severe forms of COVID-19. Lymphocytopenia is 443 also observed and SARS-CoV-2 inhibits regulatory T cells and leads to activation of the NF-444  $\kappa$ B signalling pathway which promotes the cytokine storm. (3) Supplementation with butyrate 445 could help to limit the cytokine storm through the inhibition of the NF- $\kappa$ B pathway and 446 promotion of anti-inflammatory M2 macrophages. Moreover, butyrate could restrain the 447 thrombo-inflammation phenomenon by activating the t-PA. Created with Biorender.com



# 449 *Figure 2:* Path for the decision of supportive therapy with SCFA

450 From the blood sample of a person tested positive for COVID-19, we propose to perform

451 plasma SCFA profiling, relevant cytokine dosage and M1/M2 macrophages ratio at early stages

452 of the disease. These data will serve as inputs to build a predictive score of disease severity or

- 453 progression and to decide SCFAs supplementation.
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### 458 *Figure 3:* Flowchart of the proposed clinical trial

This clinical trial will enrol patients positive for COVID-19 (RT-PCR test) at mild to moderate stage who have co-morbidities. After randomization, blood samples will be collected at different endpoints for SCFA profile and cytokine dosage. The primary endpoint could combine clinical adverse events, disease severity markers, sequential organ failure assessment, the need and duration of hospitalization, time until virus negativation or death due to SARS-CoV-2 infection.