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PhD Thesis:

Impact of gut microbiota on clinical management of critical patients

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Dedicated to my aunt, Giuliana,

Even though you're not here with us physically,

your spirit will always be in our hearts

"Research is creating
new knowledge"

Neil Armstrong

Summary

Abstract

Background. Severe COVID-19 and MIS-C are rare but serious outcomes associated to the SARS-CoV-2 infection. The MIS-C onset, 2-6 weeks after infection, advises an immune-mediated mechanism involving gastrointestinal system suggesting a gut microbiota connection. This study aims to compare the gut microbiota between severe COVID-19 children or MIS-C using different biomolecular approach, outlining opportunities involving the gut microbiome monitoring and treatment to avoid the most severe outcomes.

Methods. Gut microbiota and specific microbial modulations were analysed starting from faecal samples of children at the admission in the hospital. The study included patients hospitalised during 2020-21 (age 6±5 years) affected by severe COVID-19 (37 patients) or MIS-C (37 patients). Microbial differences were assessed by both NGS and qRT-PCR approach, including specific target such as *Akkermansia muciniphila*, *Bacteroides spp.* and *Bifidobacterium spp.*

Results. Gastrointestinal symptoms were reported in 29% of COVID-19 patients and 81% of MIS-C patients. In the 75% of the cases the pharmacological treatment included antibiotics and cortisone that showed an influence on microbiota composition. The early age (<6 months) has the greatest influence on the microbiota biodiversity while, only considering low-abundance species present in the gut, a significant difference in alfa and beta diversities were reported between COVID-19 or MIS-C patients. The Firmicutes/Bacteroidetes ratio was 0,82±0,17. A significant decrease of Firmicutes (- 20%) was observed in patients treated with antibiotics. The levels of *Bacteroides spp.*, *Bifidobacterium spp.* and *Akkermansia muciniphila* were substantially homogeneous, whereas an increased activity of *Bifidobacterium spp.* was observed in children with positive faecal samples (p=0,019).

Conclusions. Not so marked differences were reported into the gut microbiota profiles, at the time of hospitalization, between children with such different clinical presentation. An in-depth evaluation of the less known species present and active in the gut could be the key for the formulation of treatments able to reduce the risk of severe outcome and also to develop microbiota-based biomarkers for MIS-C early diagnosis.

1. Introduction

The COVID-19 (Coronavirus Disease 2019) pandemic, which emerged in late 2019, was responsible for a critical impact on global health, with unique challenges in understanding its diverse clinical presentations across different age groups and populations. COVID-19 is primarily caused by the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), which infects the respiratory tract and can lead to a wide range of signs/symptoms from mild respiratory distress to severe complications like Acute Respiratory Distress Syndrome (ARDS) and multi-organ failure [1, 2]. In adult population, severe cases are commonly characterized by a hyperinflammatory response, commonly known as "cytokine storm" (Figure 1). This severe inflammation involves the excessive release of cytokines such as IL-6, IL-1β and TNF-α, which leads to widespread tissue damage, compromised respiratory function and significant systemic complications, including cardiovascular, renal and neurological dysfunctions. Among adults, those 50-64 years experienced the most severe disease [3, 4]. ARDS, driven by this exaggerated immune response, showed the highest mortality rates among adult subsets, especially in those with pre-existing conditions such as cardiovascular disease, obesity, diabetes or chronic respiratory illnesses [5, 6].

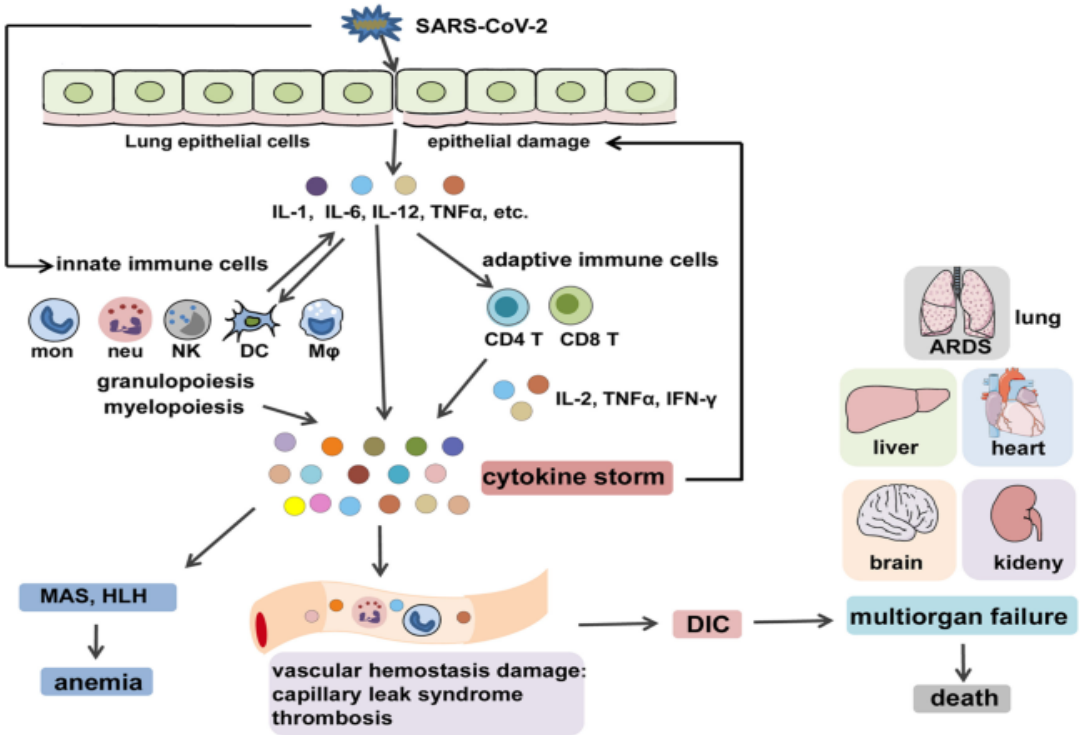


Figure 1. Cytokine storm pathway in SARS-CoV-2 infection (https://www.nature.com/articles/s41392-021-00679-0).

On the other hand, children with COVID-19 often were admitted to clinical departments with milder symptoms than adults, with significantly lower rates of severe respiratory distress and death. This has led to a hypothesis that age-related differences in immune system function and receptor expression may account for the variance in disease severity. In children, the expression of Angiotensin-Converting Enzyme 2 (ACE2) - the primary receptor SARS-CoV-2 uses for cellular entry - is lower in lung tissues compared to adults, potentially reducing the viral load in the respiratory system and the likelihood of severe respiratory complications [7, 8]. Additionally, children's immune systems rely more on innate immune responses, which are more effective in controlling viral infections at an early stage compared to the adaptive immune responses that are predominant in adults [9]. Despite these protective factors, COVID-19 impact on children is still of significant concern due to the emergence of Multisystem Inflammatory Syndrome in Children (MIS-C), a rare but severe post-infectious complication associated with SARS-CoV-2 infection [10]. MIS-C typically develops few weeks after the initial infection, showing itself with persistent fever, gastrointestinal symptoms and multi-organ involvement. The syndrome resembles Kawasaki disease in some features but is distinct in its higher frequency of cardiac complications, including myocarditis and shock, which require intensive care management [11,12]. The exact mechanisms underlying MIS-C remain unclear, however it was proposed that the syndrome results from a delayed immune response, possibly triggered by molecular mimicry or autoimmunity [13, 14]. Thus, the critical need for understanding MIS-C's pathogenesis has led researchers to investigate various contributing factors, including genetic predispositions, immune system maturation and potential environmental triggers. Moreover, recent findings suggest that the gut microbiota - the extensive community of microorganisms relying within the gastrointestinal tract - may play a key role in the modulation of immune responses against SARS-CoV-2 infection, particularly in MIS-C patients. The gut microbiome is well known to interact with the systemic inflammation, the immune regulation, and even mental health through the production of short-chain fatty acids (SCFAs), vitamins and other metabolites essential for maintaining gut barrier integrity [15-17]. The so-called "Dysbiosis", an imbalance in gut microbial communities, has been implicated in various inflammatory and autoimmune diseases and it is increasingly recognized as a player to severe COVID-19 outcomes. Dysbiosis can lead to increased gut permeability, or "leaky gut," which allows bacterial toxins and antigens to translocate into the bloodstream, triggering systemic inflammation

and exacerbating immune responses [18]. This intersection of the gut microbiota and immune system dysregulation highlights the need for further exploration of microbial changes in pediatric COVID-19 patients in order to provide an in-depth understand of their role in disease progression and severity.

1.1. SARS-CoV-2 and the gut microbiota

SARS-CoV-2 enters host cells via the ACE2 receptor, a protein highly expressed across multiple organs, including the lungs, heart, kidneys, and gastrointestinal (GI) tract. Within the GI tract, ACE2 is particularly abundant in the epithelial cells lining the intestines, where it plays a key role in the regulation of nutrient absorption, immune function and maintaining of gut barrier integrity [19, 20]. This dual functionality of ACE2 - both as an entry receptor for SARS-CoV-2 and a critical component in gut homeostasis – could be the proof of COVID-19 role on effects not only of respiratory systems but also on gastrointestinal health. When SARS-CoV-2 binds to ACE2 in the bowel, it can disrupt the receptor's function, impairing the regulation of amino acid absorption and impairing local immune responses. This disruption in gut homeostasis may contribute to common GI symptoms observed in COVID-19 patients, including diarrhea, nausea and abdominal discomfort, which have been reported across various age groups and severity levels. Moreover, several studies have detected SARS-CoV-2 RNA in the feces of COVID-19 patients, including asymptomatic carriers, thus highlighting that the virus can actively replicate in the GI tract and may persist long after respiratory symptoms have resolved [21, 22]. The persistence of viral RNA in the gut suggests that the GI tract could act as a secondary reservoir for the virus, extending the period during which an individual might potentially spread the virus through fecal-oral transmission. This mode of transmission, however not yet fully understood, could have critical implications for public health, particularly in highly populated or resource-limited settings where sanitation could represent a challenge. In addition to being a district for viral replication, the continuous persistence of SARS-CoV-2 in the gut may contribute to chronic immune activation and to the prolonged symptoms experienced in some COVID-19 patients, commonly referred to as "long COVID". COVID-19-induced dysbiosis, or disruption of the gut microbiota, has emerged as a critical area of research, given its implications for immune health. Dysbiosis

in COVID-19 patients is characterized by a marked reduction in beneficial bacterial diversity, including Bacteroidetes and Firmicutes, which are essential for producing short-chain fatty acids (SCFAs) like butyrate [23, 24]. SCFAs play a vital role in supporting immune health, strengthening the gut barrier and reducing inflammation. The depletion of SCFA-producing bacteria reduces the gut's natural anti-inflammatory defenses, creating an environment more susceptible to inflammation and immune dysregulation. Simultaneously, studies show an increase in pathogenic bacteria, such as *Enterococcus* spp. and *Streptococcus* spp., which can exacerbate local inflammation and weaken the gut's immune resilience [25]. This bacterial imbalance can have significant consequences not only for gut health but also for systemic immunity, as the altered gut microbiota composition can contribute to broader immune activation throughout the body. The connection between the gut microbiome and respiratory health, often described as the "gut-lung axis," could explain light on how gut dysbiosis can exacerbate respiratory symptoms in COVID-19. The gut-lung axis is a bidirectional pathway that allows the gut microbiome to influence immune responses in the lungs and vice versa. For example, SCFAs produced in the gut can circulate to the lungs, where they play an important role in modulating local immune responses, reducing inflammation, and protecting lung tissue from pathogen invasion [26, 27]. In COVID-19, the depletion of SCFA-producing bacteria disrupts this protective mechanism, leaving the lungs more vulnerable to inflammation and infection. This dysregulation within the gut-lung axis highlights the importance of maintaining gut microbiota health, as disruptions in gut microbiota can influence respiratory outcomes, potentially worsening symptoms and contributing to the cytokine storm observed in severe COVID-19 cases. The effects of SARS-CoV-2 on the gut are not only local but also systemic, as gut dysbiosis compromises the integrity of the intestinal barrier. The epithelial cells that line the gut are normally tightly joined, creating a barrier that prevents pathogens, toxins, and other antigens from crossing into the bloodstream. However, SARS-CoV-2-induced dysbiosis disrupts this barrier, leading to what is often described as "leaky gut" syndrome [28, 29]. This condition allows microbial products such as lipopolysaccharides (LPS) to translocate into the bloodstream, where they can activate immune cells and contribute to systemic inflammation. The release of these microbial components into the circulatory system can act as a continuous immune stimulus, potentially exacerbating the inflammatory response in severe COVID-19 cases. The systemic inflammation stemming from gut permeability may further aggravate respiratory symptoms, indicating that

preserving gut barrier integrity could be a critical component of managing COVID-19 respiratory complications (Figure 2).

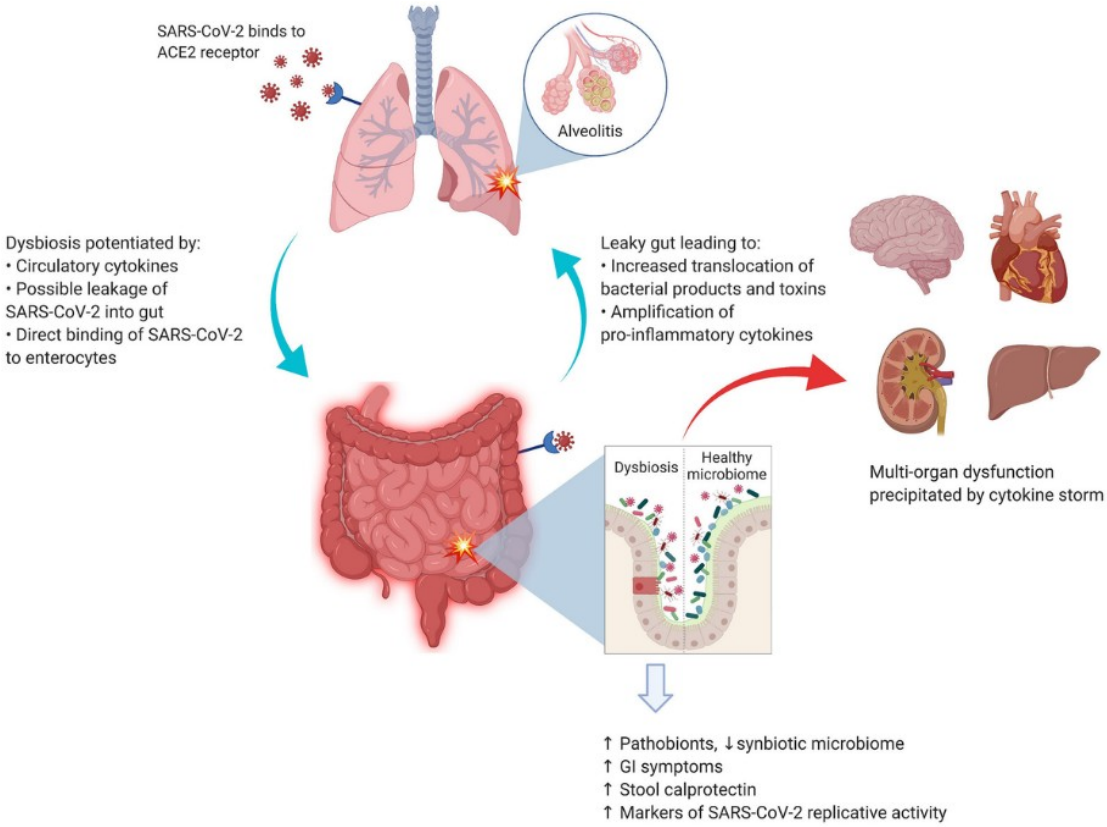


Figure 2. Gut-Lung Axis in SARS-CoV-2 Infection (https://www.frontiersin.org/journals/immunology/articles/10.3389/fimmu.2021.765965/full).

1.2. Gut microbiota in pediatric patients

The gut microbiota in children differs fundamentally from that of adult age and it showed rapid and dynamic development, particularly within the first three years of life. Key factors, such as maternal features, the method of birth (vaginal delivery or cesarean section), early feeding practices (breastfeeding, formula feeding), antibiotic exposure and interactions with environmental microorganisms could significantly influence the pediatric microbiome, providing the pillars for the child's immune system and metabolic health [30, 31]. During this critical period, a balanced and homeostatic gut microbiome is pivotal since it trains the immunological system to a wide variety of external antigens in a controlled

manner, allowing it to recognize non-threatening antigens and to respond effectively to those pathogens (Figure 3).

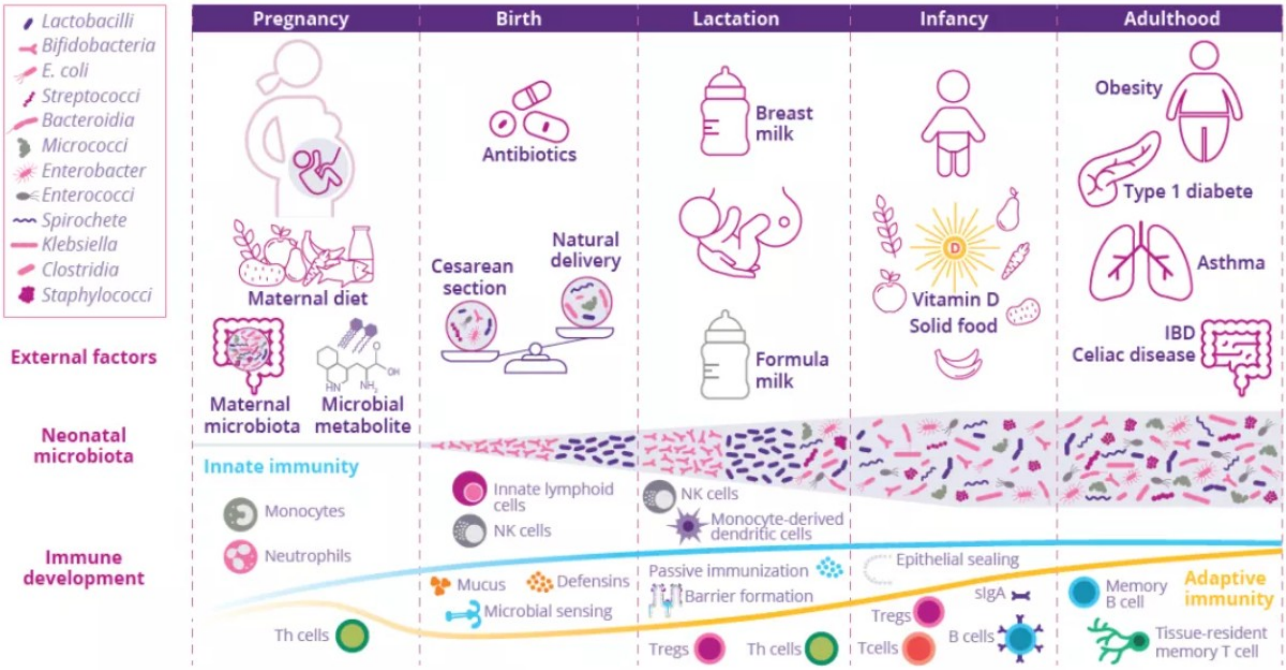


Figure 3. Interactions between pediatric gut microbiota and immune responses (https://www.biocodexmicrobiotainstitute.com/en/pro/factors-influencing-microbiota-development-and-maturation-immune-system-early-life).

This early-life exposure to microbial diversity is very useful, like suggested by studies that showed that children who develop a well-balanced microbiome are less susceptible to allergies, asthma and autoimmune diseases later in life. Otherwise, disruptions in the pediatric microbiome during this growing period may have long-term impacts on health, potentially increasing the risk of chronic diseases characterized by immune dysregulation. Children infected with SARS-CoV-2 frequently present with gastrointestinal symptoms, such as abdominal pain, vomiting and diarrhea, which are less common in adult COVID-19 cases. These symptoms are believed to be associated with a higher expression of ACE2 receptors in the pediatric gut, as compared to respiratory tissues [32, 33]. This high ACE2 expression in the GI tract makes the pediatric gut a primary site for SARS-CoV-2 entry and interaction, allowing the virus to firstly influence gut health and potentially amplify localized immune responses. The frequent GI symptoms in pediatric COVID-19 patients highlights the impact of SARS-CoV-2 on the developing microbiome and strengths the

importance of understanding these effects within the broader context of immune and gastrointestinal health.

Studies have also shown that SARS-CoV-2 RNA is often detectable in stool samples from pediatric COVID-19 patients, thus suggesting that virus can persist in the GI tract for a long time after the resolution of respiratory symptoms [34, 35]. This prolonged viral presence can produce severe effects on the pediatric microbiome, disrupting its balance and leading towards a condition of dysbiosis. Dysbiosis in children involves an imbalance in gut bacterial populations, characterized by a reduction in beneficial commensals like Bacteroides and Firmicutes, bacteria known for their roles in producing short-chain fatty acids (SCFAs), maintaining gut barrier integrity and regulating immune function [36]. The reduction of these protective bacteria weakens the gut's immune defense, making it more susceptible to inflammation and pathogenic colonization. Otherwise, there may be an overgrowth of pathogenic bacteria that further exacerbate immune dysregulation, promoting an inflammatory environment that could have systemic effects.

In addition to the direct impact of SARS-CoV-2, treatment protocols for pediatric COVID-19 patients, including antibiotics and corticosteroids, can contribute to a higher disruption of the microbiome balance. Although these medications are essential in managing bacterial infections and controlling inflammation, they also reduce populations of beneficial bacteria and allow for the proliferation of pathogenic species. This microbial imbalance can produce a feedback loop that worsens dysbiosis and potentially leads to immune dysregulation [37]. As a result, these microbial imbalances in childhood can affect the development of the immune system, increase susceptibility to future infections and potentially predispose children to chronic diseases in the adult stage. As researchers investigate the specific impacts of SARS-CoV-2 on the pediatric microbiome, it is becoming increasingly evident that the long-term consequences of COVID-19-induced dysbiosis in children require careful consideration and, potentially, could open the way to microbiome-targeted interventions.

Early-life dysbiosis not only impacts immune responses but also has long-term implications for neurological and cognitive development. The gut microbiome is key player of the gut-brain axis, a complex communication network between the gut and brain that influences neurological health and behavior. In children, disruptions of the microbiome can interfere with normal brain development, potentially leading to an

increased risk of anxiety, depression and other neurodevelopmental disorders. This concern is particularly relevant in the context of SARS-CoV-2, since viral-induced dysbiosis may affect the production of neurotransmitters and immune signals that influence neuronal health. Understanding the impact of COVID-19 on the pediatric microbiome and its interactions with the developing immune system and brain is critical, as this knowledge could guide interventions to support healthy development and long-term resilience [38, 39].

Different studies suggest that specific beneficial bacterial strains, such as *Bifidobacterium* spp. and *Lactobacillus* spp., may offer protective effects against severe inflammatory responses, particularly in pediatric COVID-19 and MIS-C cases. These bacteria, often administered as probiotics, are well known to enforce the gut barrier function, to support immune tolerance and to contribute in the reduction of inflammation, making them promising candidates for microbiome-targeted therapies. By promoting the growth of beneficial bacteria, these therapies may help to contrast the dysbiotic effects of COVID-19, supporting the recovery of gut health and reducing the risk of complications in infant age [40]. Microbiome-focused therapies could provide a safer and non-invasive approach to managing inflammatory responses in pediatric COVID-19, offering a relevant added value to conventional treatments. The potential for using probiotics or dietary modifications to restore microbial balance in children with COVID-19 could represent a significant step forward in pediatric infectious disease management, supporting not only immediate recovery but also long-term immune health.

1.3. Multisystem Inflammatory Syndrome in Children (MIS-C)

Multisystem Inflammatory Syndrome in Children (MIS-C) is a severe, hyperinflammatory syndrome that typically arises 2-6 weeks after SARS-CoV-2 infection in children. MIS-C is characterized by high fever, gastrointestinal symptoms (e.g. abdominal pain, vomiting, diarrhea) and multiorgan involvement, particularly affecting the cardiovascular and gastrointestinal systems. Although it shares some clinical similarities with Kawasaki disease - an inflammatory condition that affects blood vessels in children – the MIS-C is distinct in its severity and its tendency to present with cardiac complications, such as myocarditis, coronary artery dilation and even shock [40, 41]. The differences between

Kawasaki disease and MIS-C are resumed in Table 1. In some cases, the cardiovascular involvement is so severe that children require intensive care and advanced life support, underscoring the urgency of identifying and managing MIS-C in COVID-19-positive pediatric populations.

Table 1. Comparison of Kawasaki disease and MIS-C.

	Kawasaki disease	MIS-C
Demographics		
Age	6 months to 5 years	6–11 years
Sex	Male predominance (~1.5:1)	No apparent predominance
Race or ethnicity	Highest incidence in Japan, China, South Korea and Taiwan	Highest incidence in children of African and Hispanic heritage
Pathogenesis		
Trigger	Unknown but some data suggest possible preceding viral or bacterial infection	Onset ~3–6 weeks after SARS-CoV-2 exposure
Immunological characteristics		
Similarities	Enhancement of IL-1β^+ neutrophils and immature neutrophils	
	T cell activation by a conventional antigen	SARS-CoV-2 viral spike (S) protein acts like a superantigen, triggering a cytokine storm
	High levels of IL-17	High levels of IL-15, IFNγ in severe cases
Differences	Relatively less frequent MAS-like cytokine profile	>50% of patients with MIS-C have a MAS-like cytokine phenotype
	Rare lymphopenia	Frequent lymphopenia
	Anti-SARS-CoV-2 IgG not reported	Anti-SARS-CoV-2 IgG reported
Clinical features		
Similarities	Similar associations with fever, rash, cervical lymphadenopathy, neurological symptoms, extremity changes	
Differences	Relatively high incidence of conjunctival injection and oral mucous membrane changes	Relatively high incidence of gastrointestinal symptoms, myocarditis and shock, and coagulopathy
Management		
Common	IVIG, glucocorticoids, acetylsalicylic acid	IVIG, glucocorticoids, acetylsalicylic acid
Rare	Infliximab, ciclosporin and anakinra	Anakinra, tocilizumab

Elevated levels of inflammatory biomarkers, including interleukin-6 (IL-6), C-reactive protein (CRP), ferritin and D-dimer are frequently observed in MIS-C, thus highlighting a hyperactive immune response. This immune overactivation, often described as a cytokine storm, leads to widespread tissue inflammation and damage, potentially resulting in multiorgan failure if not adequately managed [42]. Some researchers suggest that MIS-C

may result from molecular mimicry, wherein SARS-CoV-2 antigens resemble host proteins, leading to an immune response that mistakenly targets the body's own tissues. Another hypothesis suggests that the lingering viral antigens may continuously stimulate the immune system, causing sustained inflammation and contributing to MIS-C's severe manifestations [43, 44]. This autoimmune-like response poses significant clinical challenges, since it not only causes widespread inflammation but can also lead to irreversible damage to several organs, highlighting the need for early recognition and intervention in order to prevent severe complications (Figure 4).

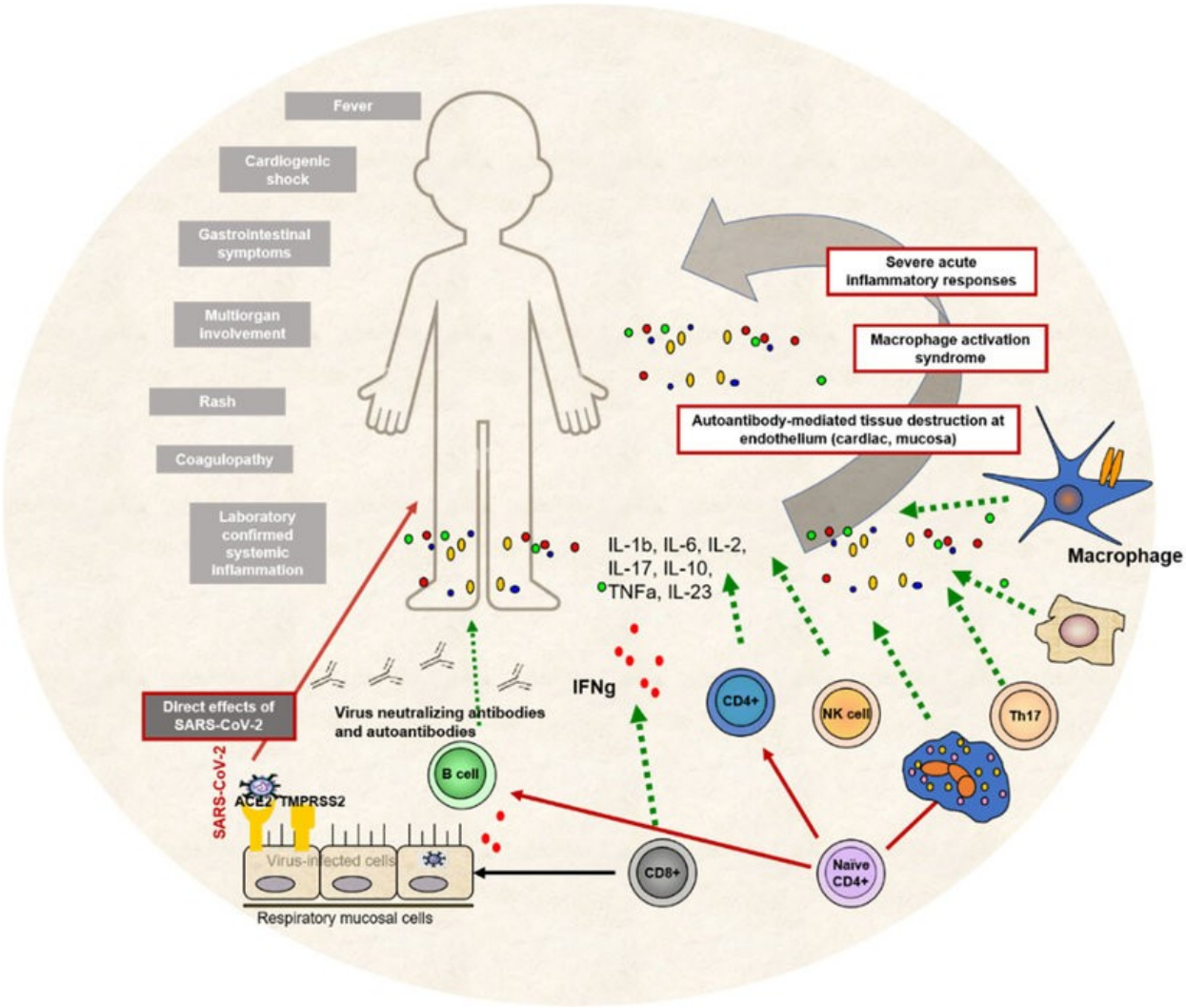


Figure 4. MIS-C immunological pathogenesis (https://www.mdpi.com/2073-4468/11/2/25).

Gastrointestinal symptoms are prevalent in MIS-C, with many children experiencing severe abdominal pain, vomiting and diarrhea. This frequent manifestation of GI signs and suggested that SARS-CoV-2 may weaken gut barrier integrity, resulting in a condition

known as “leaky gut”. Under normal circumstances, the epithelial cells lining the GI tract form a barrier able to prevent the entry of bacterial endotoxins and viral antigens in the bloodstream. However, SARS-CoV-2 infection appears to disrupt this barrier, allowing these pathogen components to translocate into the bloodstream, where they can trigger systemic inflammation and exacerbate immune responses. This hypothesis is further supported by observations that children with MIS-C often exhibit significant dysbiosis, characterized by reduced diversity of beneficial bacteria and an overgrowth of pathogenic species, which can intensify the immune response and contribute to systemic inflammation [45, 46].

The potential role of dysbiosis in MIS-C suggests that microbiome-based therapies could be beneficial in managing the syndrome’s severe symptoms. By restoring microbial balance in the gut, it may be possible to reduce systemic inflammation and improve outcomes in children affected by MIS-C. For instance, probiotics and dietary interventions that increase the presence of anti-inflammatory bacterial strains, such as *Bifidobacterium* spp. and *Lactobacillus* spp., could help strengthen gut barrier integrity, reduce endotoxin translocation and sustain immune modulation. The identification of specific microbial signatures associated with MIS-C risk could also lead to the development of predictive biomarkers, enabling clinicians to proactively identify and to monitor children at high risk, providing an additional instrument of preventive care [47, 48].

1.4. Microbiome Biomarkers and Predictive Models

Given the microbiome’s critical role in immune function, it has become a field study for the search of biomarkers that could be useful to predict severe COVID-19 complications, such as the MIS-C. Specific bacterial taxa, including Bacteroidetes, Firmicutes, *Bacteroides* spp., *Bifidobacterium* spp., and *Akkermansia muciniphila* are known to support anti-inflammatory pathways and maintain the GI barriers’ integrity. These protective bacteria produce SCFAs which play a pivotal role in modulating immune responses and preventing systemic inflammation. Butyrate, in particular, has been shown to enhance mucus production in the gut lining, reduce permeability and directly inhibit inflammatory processes within immune cells. These characteristics suggest that the relative

abundance of these bacteria could serve as indicators of immune resilience or susceptibility to inflammatory disease [14, 49].

Efforts are currently underway to develop predictive models that integrate gut microbiota profiles with traditional clinical biomarkers - such as CRP, IL-6 and ferritin levels - to help identify pediatric patients at high risk for MIS-C. These models could allow for early and targeted interventions, enabling healthcare providers to monitor high-risk children and to modify treatment plans. For example, a combination of microbial markers indicating low diversity in SCFA-producing bacteria, paired with elevated inflammatory biomarkers, may indicate a heightened risk for severe COVID-19 outcomes or MIS-C. This early-warning capability could enable proactive treatment measures, such as administering anti-inflammatory medications or microbiome-targeted therapies, potentially reducing the severity of symptoms and the need for intensive care [14, 50].

In addition to diagnostics, microbiome-targeted therapies are emerging as promising strategies for preventing or managing severe COVID-19 complications. Probiotics, prebiotics and dietary modifications that increase the presence of beneficial bacteria have shown a potential role in supporting immune health, modulating inflammation and restoring gut barrier integrity. Probiotic strains such as *Bifidobacterium* spp. and *Lactobacillus* spp. have demonstrated anti-inflammatory effects by promoting SCFA production, which enhances gut barrier integrity and prevents the translocation of bacterial endotoxins. Instead, prebiotics, such as inulin and fructooligosaccharides, also serve as food for beneficial bacteria, stimulating their growth and activity. These microbiome-based interventions could serve as complementary therapies, especially for pediatric COVID-19 patients with MIS-C or severe gastrointestinal symptoms, helping to create a more resilient immune environment and supporting overall health [14, 49].

Beyond preventive and therapeutic applications, microbiome data may also inform long-term monitoring of COVID-19 and post-COVID syndromes. For example, dysbiosis appears to be a recurrent factor in long-COVID patients, with affected individuals exhibiting lower levels of anti-inflammatory bacteria and higher levels of pro-inflammatory species compared to those who fully recover from the disease. By tracking microbiome changes over time, clinicians may be able to monitor the recovery direction of COVID-19 patients and could identify those at risk of developing long COVID. This approach not only aids in assessing a patient’s current health status but may also help

prevent long-term complications by guiding individualized post-recovery interventions [51].

Moreover, the integration of microbiome profiles with conventional clinical biomarkers represents an innovative approach to precision medicine in infectious disease care. This approach could be a promising tool in pediatric populations, where microbiome-targeted interventions may offer a safer, non-invasive alternative to traditional pharmaceuticals, which can have side effects that are particularly challenging for young patients. Instead, these data-driven models and therapies hold significant potential for advancing patient-centered care, with the ultimate goal of reducing the incidence of severe complications such as MIS-C and providing a holistic approach to managing COVID-19 and other infectious diseases in children. This capability is especially valuable in identifying children who may be predisposed to immune dysregulation and severe inflammatory responses. With continued research and advancements in microbiome science, the use of microbiome-based diagnostics and therapeutics may become an added tool to pediatric infectious disease management, potentially transforming the approach towards clinical conditions like MIS-C, long COVID and even broader immune-related disorders [39, 52].

2. Aim of work

Pediatric patients represent a particular cohort in the research fields linked to COVID-19 due to distinctive immune responses, especially in MIS-C individuals where a multiorgan inflammation follows the SARS-CoV-2 infection. The reported involvement of the gastrointestinal system in this patient subset's, alongside with the relatively high prevalence of gastrointestinal symptoms in severe COVID-19 cases among children, suggests that gut microbiome alterations could play a role in both immune dysregulation and inflammatory responses.

The aim of the present study is to undertake a comparative analysis of the gut microbiota in pediatric patients diagnosed with severe COVID-19 and MIS-C, evaluating microbial diversity, composition and the abundance of specific bacterial taxa. Using a combination of molecular techniques, including Next-Generation Sequencing (NGS) and quantitative real-time PCR (qRT-PCR), this study will profile the gut microbiome of these patients to identify unique microbial signatures associated with severe disease. In this way, it could be possible to reveal variations that correlate with immune dysregulation and inflammation, with an in-depth evaluation of gut dysbiosis influence the disease progression. Additionally, specific microbial biomarkers will be identified and assessed for their potential to predict the onset of MIS-C, with an emphasis on taxa known for their role in immune modulation, such as the Firmicutes/Bacteroidetes ratio, which has been suggested as a potential marker in other inflammatory conditions.

These data could contribute to the field of microbiome-based precision medicine by potentially providing novel non-invasive biomarkers for identifying and managing severe outcomes in pediatric COVID-19 cases. The study's findings may also offer a starting point for microbiome-targeted therapeutic approaches, which could act as an added value to traditional treatments, reducing adverse effects and promoting a more comprehensive management of pediatric infectious diseases. Finally, this research aims to advance the understanding of SARS-CoV-2 interaction with the pediatric gut microbiome and poses the way for innovative strategies in the diagnosis and prevention of severe complications in children with COVID-19 and MIS-C.

3. Materials and methods

3.1. Clinical Setting

This study was conducted during the period April 2020 to February 2022 in the Regina Margherita Children's Hospital (Torino, Italy), the main tertiary care center specializing in the treatment of pediatric patients.

Overall, 74 individuals were enrolled and divided in the following subsets: COVID-19 group (n =37) and MIS-C group (n =37). Criteria of inclusion for the first group was the positivity to SARS-CoV-2 infection (throughout a molecular swab) without suspect or confirmation of MIS-C disease, while for the second subset MIS-C cases were defined according to CDC guidelines, characterized by persistent fever, systemic inflammation and multi-organ dysfunction. All enrolled patients in the MIS-C subset resulted positive to SARS-CoV-2 infection by serological test (ELISA assay) and/or molecular test. Exclusion criteria comprised patients with pre-existing gastrointestinal disorders or immunosuppressive conditions unrelated to COVID-19. Moreover, at time of the admission, data only about demographic features and clinical presentation were collected. No data about ethnicity, socioeconomical conditions and eating habits were available. Meanwhile, a written informed consent from patients' parents was obtained. The official approval for this study was obtained from the Institutional Ethical Committee (Città della Salute e della Scienza di Torino, protocol number: 00564/2020).

At the admission, for each patient, stool samples were collected for SARS-CoV-2 testing and microbiome analysis at the first evacuation after the hospital's admission, immediately stored at -80°C and delivered to the Hygiene laboratory of the Department of Public Health and Paediatrics Sciences (Torino, Italy).

3.2. Nucleic acids extraction and quantification

Both DNA and RNA were extracted from fecal samples. Total nucleic acid extraction was performed using PowerFecal DNA and PowerMicrobiome RNA isolation kits (Qiagen, Germany). The extracted nucleic acids were quantified using spectrophotometer Tecan Infinite® 200 PRO, using a Nano Quant Plate (Tecan Trading AG, Switzerland) and the software iControlTM (version 1.11.10), which allows a 260 nm spectrophotometric

reading. Moreover, the DIN of the extracted genomic DNA was evaluated by Tape Station 4150 (Agilent technologies). DNA samples were stored at -20°C for the NGS step while RNA samples were stored at -80°C until molecular analysis was performed for the detection of SARS-CoV-2 infection.

3.3. SARS-CoV-2 detection in stool samples

RNA extracts were tested for SARS-Cov2 positivity using the Novel Coronavirus (2019-nCoV) Real Time Multiplex RT-PCR Kit (LifeRiver Ltd, USA) onto the CFX Instruments (Bio-Rad, USA), following the suggested thermal protocol included in the kit instructions and as recommended in the literature [53]. Briefly, a multiplex qPCR was performed on three different target viral genes: gene E, gene N e gene RdRp. The gene E encode for the Envelope small membrane protein, which has an ion channel activity and it is involved in the formation of the envelope membrane of the virion inducing cell membrane curvature and scission. The gene N encode the Nucleoprotein, which packages the positive strand viral genome into a helical ribonucleocapside (RNP) and connects viral genome to the membrane trough the interaction with M protein. Finally, the gene RdRp encode the RNA-dependent RNA-polymerase, which is pivotal for viral genome synthesis. A sample could be considered positive when 2 out of 3 genes exit before the 41st qPCR cycle threshold.

3.4. Metagenomics analysis

3.4.1. NGS workflow

Metagenomics analysis was performed starting from the extracted bacterial DNA previously obtained from stool samples for gut microbiota profiling. DNA extracts were prepared for NGS analysis of 16s rRNA through the set-up of metagenomic libraries, each one marked with a unique barcode. For each sample, analysis of microbial composition was performed with the Ion 16S™ Metagenomics Kit (Thermofisher Scientific, USA) according to manufacturers' instructions. Briefly, an initial multiplex PCR assays was done for amplification of 16S rRNA hypervariable regions 2, 4, 8 and 3, 6–7, 9 in two tubes, the first yielding amplicon fragments of ~250 bp, ~288 bp, and ~295 bp while the second yielding amplicon fragments of ~215 bp, ~260 bp and ~209 bp respectively using primers

and reagents provided with the kit. Subsequently, equimolar quantities of amplified products were pooled and were purified with Agencourt Ampure reagent (Life technologies, USA). DNA amount was estimated as per manufactures' instructions with the Qubit 4 Fluorometer (Thermofisher Scientific, USA). After end repair of the fragments, adapter/barcodes were ligated to the amplified fragments as per the instructions using the Ion Xpress™ Barcode Adapters 1-16 Kit (Thermofisher Scientific, USA). A further PCR amplification of this library of amplicons was done using primers and reagents provided with the Ion Plus Fragment Library Kit (Thermofisher Scientific, USA). The samples were quantified in qPCR with the Ion Universal Library Quantitation kit (Thermofisher Scientific, USA) and diluted to obtain equimolar quantities and collected in a metagenomic pool. Finally, the next generation sequencing of the amplified library was performed after emulsion PCR and chip preparation onto the Ion chef platform (Thermofisher Scientific, USA) and sequencing was done using Ion 530™ Chip micro-chip onto Ion S5 instrument (Thermofisher Scientific, USA) according to manufacturers' instructions. The output reads were aligned and mapped using Ion Reporter™ software with default parameters (Thermofisher Scientific, USA) for metagenome analysis including read mapping, annotation and reporting. The workflow was resumed in figure 5.

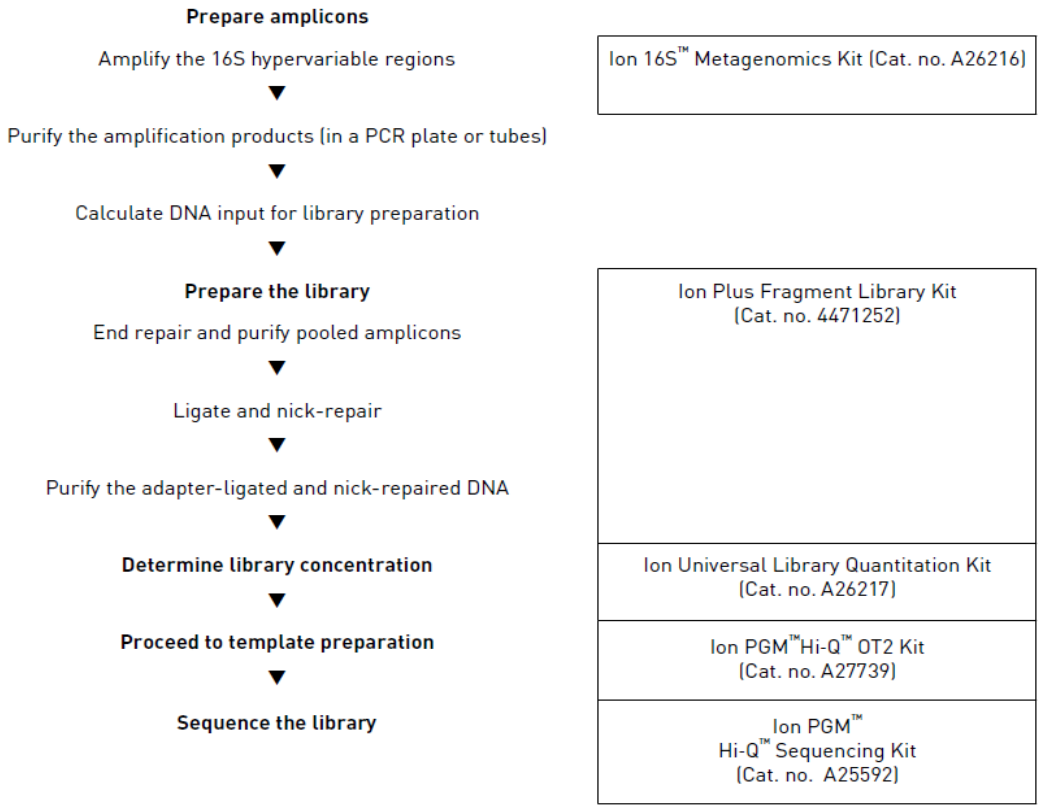


Figure 5. Overview of NGS protocol.

3.4.2. Thermofisher Ion Chef™ System

The Ion Chef Instrument is a fully automation platform designed for the first step of sequencing setup workflow: (I) templates preparation and (II) chips loading for Ion Torrent systems. The instrument reduces hands-on time while ensuring high reproducibility in genomic studies. It is designed to execute key preparatory steps such as emulsion-PCR (ePCR), which is essential for the amplification of DNA libraries by attaching DNA templates to ion sphere particles (ISPs) (Figure 6).

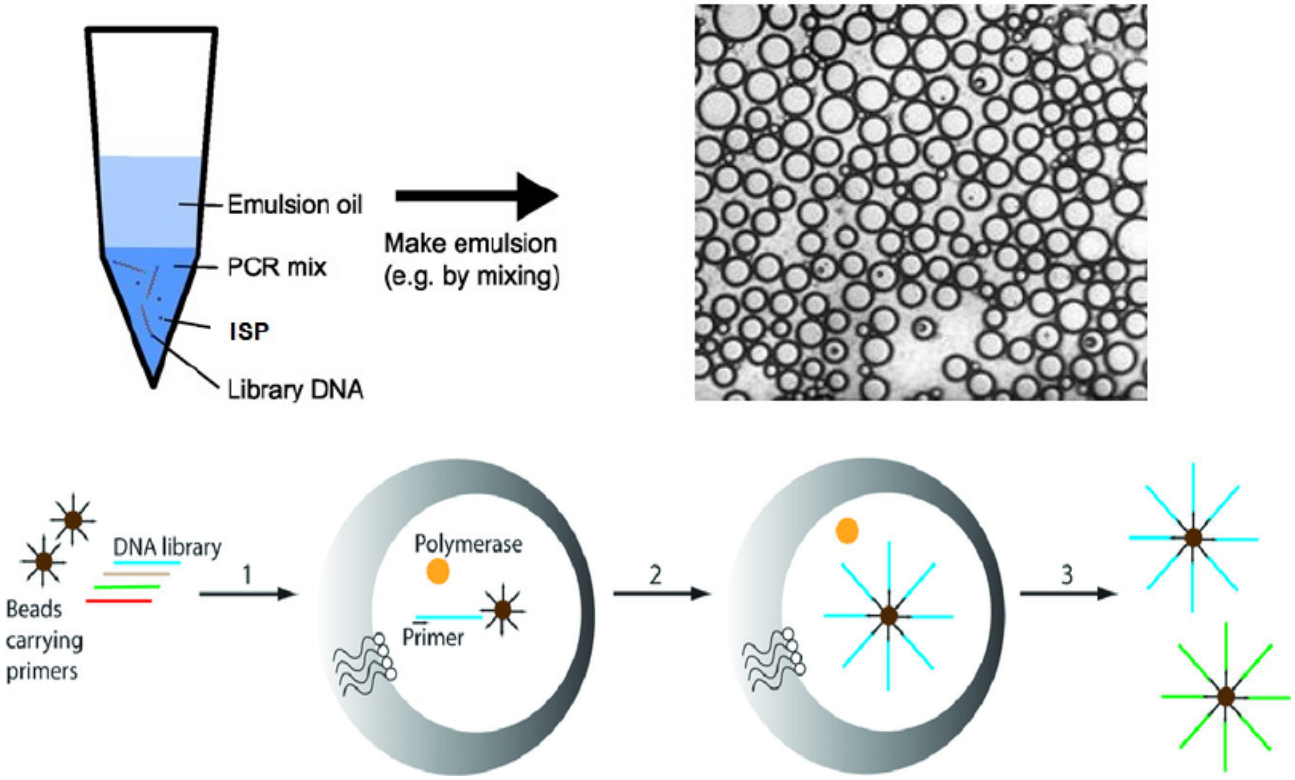


Figure 6. Overview of emulsion-PCR.

The Ion Chef is also responsible for enriching functional ISPs, separating them from non-functional particles to guarantee the accuracy of downstream sequencing processes. The instrument is set to load these ISPs onto sequencing chips, ensuring uniform distribution across wells and sealing the chips to prevent contamination during sequencing. This integrated process is based on linkage between a magnetic support and streptavidin beads: only ISPs that performed a successful amplification step are covered by several amplicons to which biotin and streptavidin beads can bind. Subsequently, the combination ISP-biotin-

streptavidin can be preserved in the washing steps while those ISPs without any amplified read are removed during the enrichment phase (Figure 7).

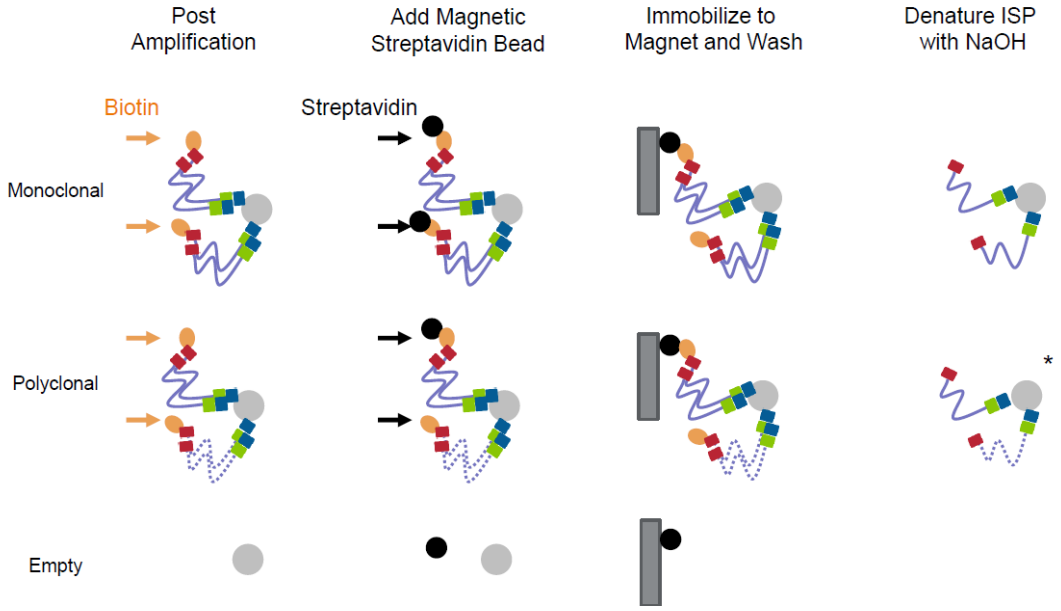


Figure 7. Overview of enrichment step.

The system is able to prepare templates for two sequencing chips in one run, accommodating Ion 520, 530 and 540 chips for a wide range of throughput requirements. Its touchscreen interface and pre-programmed protocols provide a seamless workflow that reduces errors and ensures optimal sequencing outcomes. In figure 8 is reported the Ion Chef work desk set-up.

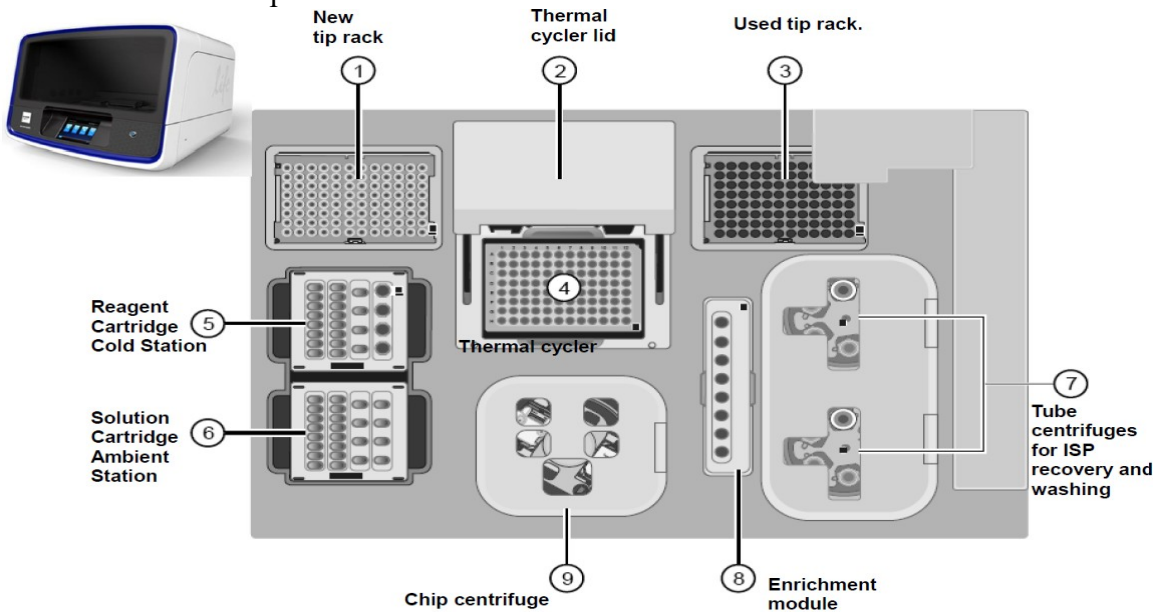


Figure 8. Ion Chef: structure.

3.4.3. Thermofisher Ion GeneStudio S5 System

The Ion S5 Sequencer is a platform designed for fast and cost-effective next-generation sequencing. It employs semiconductor sequencing technology, which eliminates the need for optical systems or fluorescent dyes. This innovation simplifies the sequencing process, translating biochemical reactions directly into digital data. The heart of the Ion S5 system is its semiconductor chip, which contains millions of microwells where sequencing reactions occur. When nucleotides are incorporated by DNA polymerase, hydrogen ions are released. These ions create voltage changes that are measured in real time, enabling nucleotide detection (Figure 9). This mechanism allows for rapid sequencing with minimal complexity. The Ion S5 platform is compatible with interchangeable chips (Ion 520, 530, 540 chips) that provide scalability for various experimental needs.

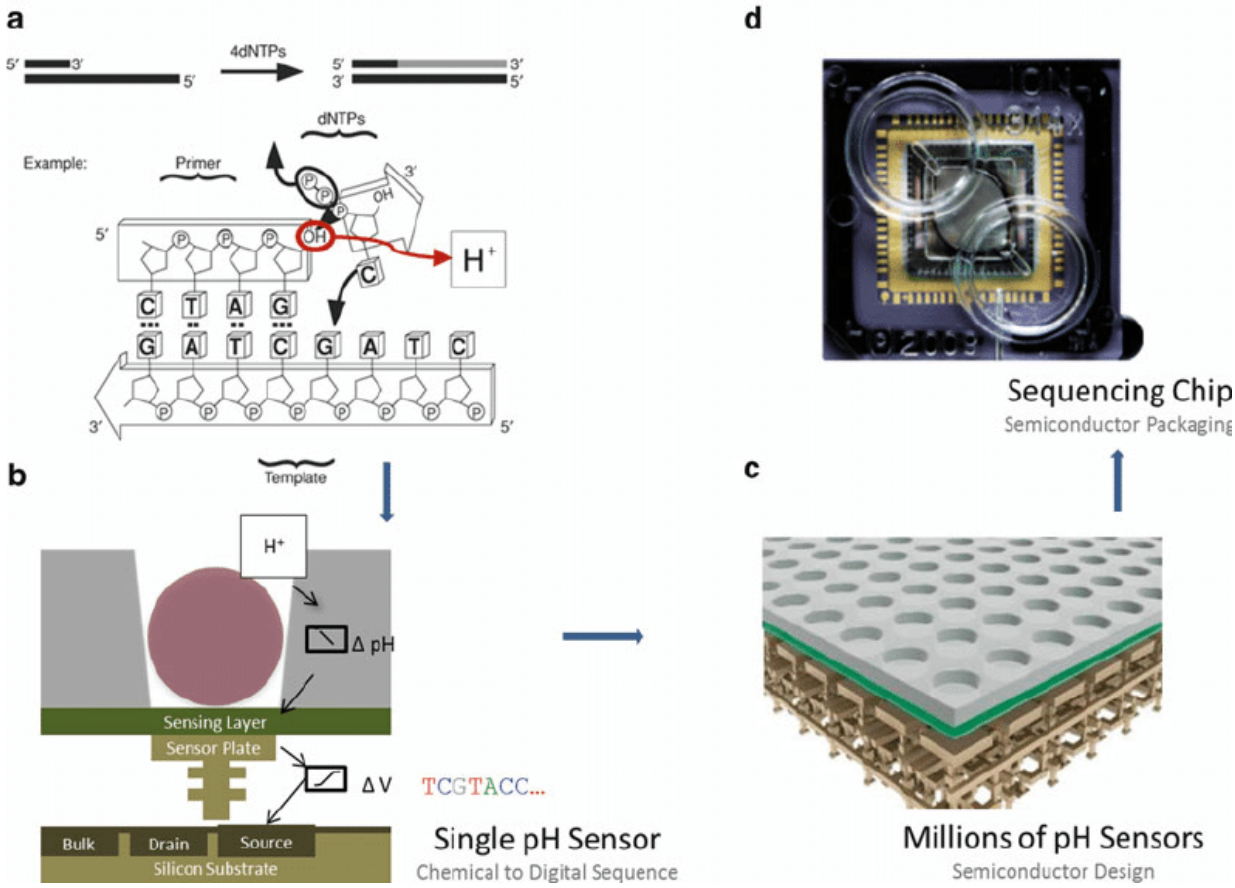


Figure 9. Ion S5 system: principle of semiconductor sequencing.

Together, the Ion Chef and Ion S5 represent a streamlined and integrated sequencing solution. Indeed, the Ion Chef automates the preparation of high-quality sequencing templates, while the Ion S5 ensures the sequencing step. This integration reduces manual

workload, enhances accuracy and facilitates high-throughput data generation in genomic studies.

3.5. Microbiota biomarkers

RNA extracts obtained from previous step were converted in cDNA using iScript cDNA Synthesis kit (Bio-Rad, USA). Briefly, 2 µl of the extract were taken and processed according to manufacturer's instructions. 20 µl obtained were immediately diluted to reach the final volume of 60 µl and the cDNA was stored at -20°C. Subsequently, qPCR assay was performed with a CFX Touch System both for DNA control genomic and cDNA samples (Bio-Rad, USA) to quantify the following targets: *Bacteroidetes, Firmicutes, Bacteroides spp., Bifidobacterium spp., Akkermansia muciniphila*. Standard curves were obtained through six serial dilutions of the certified genomic DNA. Primers and genomic DNA used are reported in table 2.

Table 2. Oligonucleotide primers, probes and genomic standards used for Microbiota biomarkers analysis.

Microbial Target	**Sequences**		**Standard Genomic DNA**
Total Bacteria	F	5′ACTCCTACGGGAGGCAGCAG3'	*Desulfovibrio vulgaris*
16 s rDNA	R	5′ATTACCGCGGCTGCTGG3'	ATCC 29579D
Total Bacteria	F	5′AGAGTTTGATCMTGGCTCAG3'	*Desulfovibrio vulgaris*
16 s rDNA	R	5′TTACCGCGGCKGCTGGCAC3'	ATCC 29579D
	Probe	5′CCAKACTCCTACGGGAGGCAGCAG3'	
Bacteroidetes	F	5′CATGTGGTTTAATTCGATGAT3'	*Bacteroides fragilis*
16 s rDNA	R	5′AGCTGACGACAACCATGCAG3'	ATCC 25285D
Bacteroides	F	5′GAGAGGAAGGTCCCCCAC3'	*Bacteroides fragilis*
16 s rDNA	R	5′CGCTACTTGGCTGGTTCAG3'	ATCC 25285D-5
Firmicutes	F	5′ATGTGGTTTAATTCGAAGCA3'	*Clostridium acetobutylicum*
16 s rDNA	R	5′AGCTGACGACAACCATGCAC3'	ATCC 824D
Bifidobacteria	F	5′CTCCTGGAAACGGGTGG3'	*Bifdobacterium longum infantis*
16 s rDNA	R	5′GGTGTTCTTCCCGATATCTACA3'	ATCC 15697D
Akkermansia muciniphila	F	5′CAGCACGTGAAGGTGGGGAC3'	*Akkermansia municiphila*
16 s rDNA	R	5′CCTTGCGGTTGGCTTCAGAT3'	ATCC-BAA835D

Negative controls (without DNA) were performed using ultrapure water. Each test was done in triplicate. For *Bacteroidetes, Firmicutes, Bacteroides spp., Bifidobacterium spp.* and *Akkermansia muciniphila*, 2 µl of extracted DNA or RNA pure or diluted was added to a reaction mix consisting of 10 µl SsoAdvancedTM Universal SYBR Green Supermix

(Bio-Rad, USA), 0.5 µl of the forward and 0.5 µl reverse primers (10 µM final concentration) and 7 µl of ultrapure water for a 20 µl final reaction volume. The reaction conditions for all targets except *Firmicutes* were set as follow: 95°C for 3 mins (1x), 95°C for 10 secs, and 59°C for 15 decs, 72°C for 10 secs (39x), 65°C for 31 secs, 65°C for 5 secs + 0.5°C/cycle, ramp 0.5°C/sec (60x). The thermal protocol followed for Firmicutes, instead, were set as follow: 95°C for 2:30 mins (1x), 95°C for 10 secs, and 60°C for 20 secs, 72°C for 15 secs (39x), 65°C for 31 secs, 65°C for 5 secs + 0.5°C/cycle, ramp 0.5°C/sec (60x). The reaction efficiency ranged from 90 to 110%.

3.6. Data analysis and statistics

Statistical analyses were performed using the SPSS Package, version 27.0 (IBM Corp.). A descriptive analysis of the variables was conducted. The data were reported as absolute numbers and percentages for categorical variables while as mean and standard deviation for continuous variables. Moreover, the subjects were divided into two groups based on the diagnosis: COVID-19 or MIS-C. Differences between COVID-19 and MIS-C children were assessed using the χ^2 test with Fisher's correction for categorical variables and MannWhitney test for independent samples for continuous variables. A p-value of $p<0.05$ was considered significant for all analyses. For biomolecular data we applied (1) a log transformation to non-normally distributed data, (2) the Spearman's correlation to assess relationships between variables, (3) T-test and paired T-test (where appropriate) to compare means, (4) ANOVA for multivariate analysis followed by a Tukey post-hoc test for multiple comparisons. The mean differences and correlations were considered significant for $p<0.05$ and highly significant for $p<0.01$.

For the microbiota, we elaborate the data using the microbiome R package software. For alfa diversity the follow indexes were calculated: Shannon, Simpson, Pielou and Lladsser indexes. For the beta-diversity evaluation different comparison model were applied: Bray-Curtis, Jaccard, Camberra/Mahnattan.

4. Results

4.1. Cohort Demographics and Clinical Data

The study cohort included 74 pediatric patients hospitalized during the COVID-19 pandemic. Patients were divided into two groups: 37 diagnosed with severe COVID-19 and 37 with Multisystem Inflammatory Syndrome in Children (MIS-C). The MIS-C patients were significantly older than those with COVID-19, with a mean age of 8.1 ± 4.0 years compared to 5.1 ± 4.9 years ($p = 0.004$). No significant differences were observed in sex distribution between the two groups, with females comprising 56.8% of the COVID-19 group and 59.5% of the MIS-C group ($p = 0.814$).

Gastrointestinal symptoms were significantly more frequent in MIS-C patients, reported in 80.6% of cases, compared to 32.4% in COVID-19 patients ($p < 0.001$). This highlights the greater involvement of the gut in MIS-C. Pharmacological treatments also varied between groups; while 97.2% of MIS-C patients received antibiotics, only 54.1% of COVID-19 patients underwent similar treatment ($p < 0.001$). Corticosteroid administration was more common in MIS-C patients as well, at 30.6% versus 5.4% in COVID-19 cases ($p = 0.005$). Moreover, hospitalization duration was longer for MIS-C patients (12.2 ± 3.8 days) compared to COVID-19 patients (8.7 ± 7.1 days, $p < 0.001$).

The table 3 summarizes the cohort demographics, clinical characteristics, and pharmacological treatments, highlighting the significant differences between the two groups. The continuous variables are expressed as means and standard deviations, while the categorical variables are expressed as percentages. p-values for MIS-C vs COVID-19 comparisons are included.

Table 3. Cohort characteristics: number of involved patients, sex, age and clinical data.

		COVID-19 (Mean ± SD or percentage)	MIS-C (Mean ± SD or percentage)	p value (test χ^2 or Mann-Whitney)
Age (years)		5.1±4.9	8.1±4.0	0.004
Sex	F	56,8%	59,5%	=0.814
	M	43,2%	40,5%	
Gastrointestinal symptoms	No	67.6%	19.4%	<0.001
	Yes	32,4%	80.6%	
Antibiotics	No	45.9%	2.8%	<0.001
	Yes	54.1%	97.2%	
Cortisone	No	94.6%	69.4%	0.005
	Yes	5.4%	30.6%	
Immunoglobulins	No	100.0%	91.7%	0.073
	Yes	0.0%	8.3%	
Colchicine	No	100.0%	97.2%	0.307
	Yes	0.0%	2.8%	
Antimycotics	No	100,0%	97.2%	0.307
	Yes	0.0%	2.8%	
Hospitalization days		8.7±7.1	12.2±3.8	<0.001
Days since nasopharyngeal swab		8.9±15.2	13.3±16.1	0.125
PCR		85.7±226.2	201.0±99.6	<0.001
DNA ng/µl		22.64±18.48	34.31±31.68	0.155
RNA ng/µl		104.45±152.29	54.86±74.82	0.118
SARS-CoV-2 RNA in stool	No	64.9%	91.9%	0.005
	Yes	35.1%	8.1%	
Nasopharyngeal swab positivity	No	0%	62%	<0.001
	Yes	100%	38%	

4.2. Alpha-Diversity

The alpha-diversity of the gut microbiota was evaluated using Shannon, Simpson, and Pielou indexes. Overall, no statistically significant differences were observed between MIS-C and COVID-19 groups in these metrics, indicating similar levels of microbial richness and evenness at the phylum and genus levels. However, age-specific analysis revealed significant differences within the COVID-19 group.

Among younger COVID-19 patients (<6 months), the Shannon diversity index was significantly lower (2.06 ± 0.77) compared to older children (2.83 ± 0.73, p = 0.00873).

This trend reflects the natural developmental trajectory of the pediatric microbiota, where diversity increases with age.

The Pielou evenness index showed no significant differences across age groups or between MIS-C and COVID-19 groups, indicating a consistent distribution of microbial taxa. Table 4 presents the detailed alpha-diversity metrics for both groups.

Table 4. Alfa-diversity patients expressed as mean and standard deviation: of the following indexes: Shannon, Simpson, Pielou, Lladser.

(A) COVID-19 and MIS-C and p-value of the Wilcoxon's test

Alfa-diversity index	COVID19 (mean ± s.d.)	MIS-C (mean ± s.d.)	p value
Shannon	2.641±0.809	2.889±0.770	0.2285
Simpson	0.705±0.166	0.754±0.133	0.2789
Pielou	0.606±0.152	0.618±0.111	0.9606
Lladser	0.003±0.008	0.016±0.003	0.0248

(B) Only COVID-19 children comparing by age and p-value of the Wilcoxon's test

Alfa-diversity index	Age ≥ 0.5 year (n=27) (mean ± s.d.)	Age < 0.5year (n=9) (mean ± s.d.)	p value
Shannon	2.8342±0.7382	2.062±0.768	0.00873
Simpson	0.7387±0.1551	0.60548±0.1674	0.03919
Pielou	0.622±0.147	0.557±0.164	0.2626
Lladser	0.0013±0.0008	0.0087±0.0159	0.00873

4.4. Beta-Diversity

A marked difference could be observed using different approaches for the beta diversity evaluation considering both age class and conditions. Considering all the patients independently by the two age-class: nurslings (age ≤6 months) or older children (age > 6 months), the Adonis test using Jaccard, Bray-Curtis and Manhattan index is always significant (p=0.001). The same Adonis test remain significative only for the Manhattan model when the nurslings are excluded (p=0.021). Of course, the hospitalised COVID19 patients were younger children respect the MIS-C, as already showed, and such diversity in the age is linked to a wide diversity of both the feeding and the gut microbiota. On the other hand, the Manhattan metric is a dissimilarity measure that involves not only the abundance (as Bray-Curtis) but also it adds the absolute differences among the coordinates. This produces a greater contribution to the dissimilarity of the differences observed among the low abundant or marginal microbial genera. Figure 10 shows the PCoA plots illustrating the clustering of microbial communities for COVID-19 and MIS-C groups.

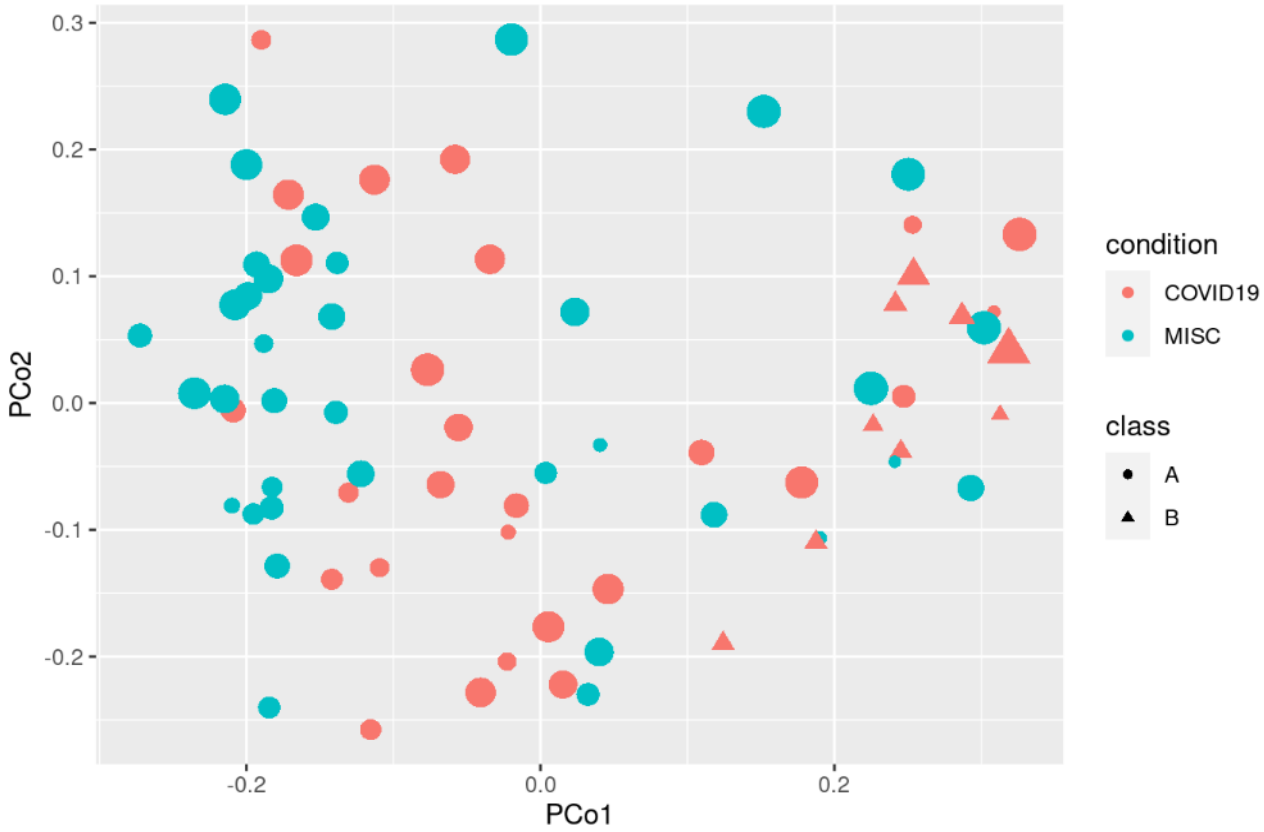


Figure 10. Principal Coordinator analysis by Manhattan Distance metric in relation to the patient condition (COVID19 or MIS-C, red or blue colour respectively) and age class (cut off at 0.5 years, circle A - older / or triangle B - younger).

4.5. Analysis of microbial profiles

The microbial profiles of COVID-19 patients displayed greater variability, particularly among younger children (<0.5 years), where the relative abundances of Firmicutes and Bacteroidetes were distinct compared to older children. These age-related differences align with the developmental trajectory of the gut microbiota, which undergoes rapid changes during infancy. In MIS-C patients, the microbiota appeared more uniform, with a relative increase in *Streptococcus* species, often associated with opportunistic infections, suggesting a potential role in the heightened inflammatory state observed in these patients. Antibiotic treatment had a pronounced effect, correlating with a ~20% reduction in Firmicutes and a significant decrease in beneficial taxa such as *Bifidobacterium* spp. and *Akkermansia muciniphila*. Corticosteroid use further suppressed activity in certain low-abundance species, reinforcing the influence of pharmacological interventions on gut microbiota composition. Despite these alterations, age and treatment effects were more apparent in younger patients, where reduced diversity and the dominance of specific taxa were most pronounced.

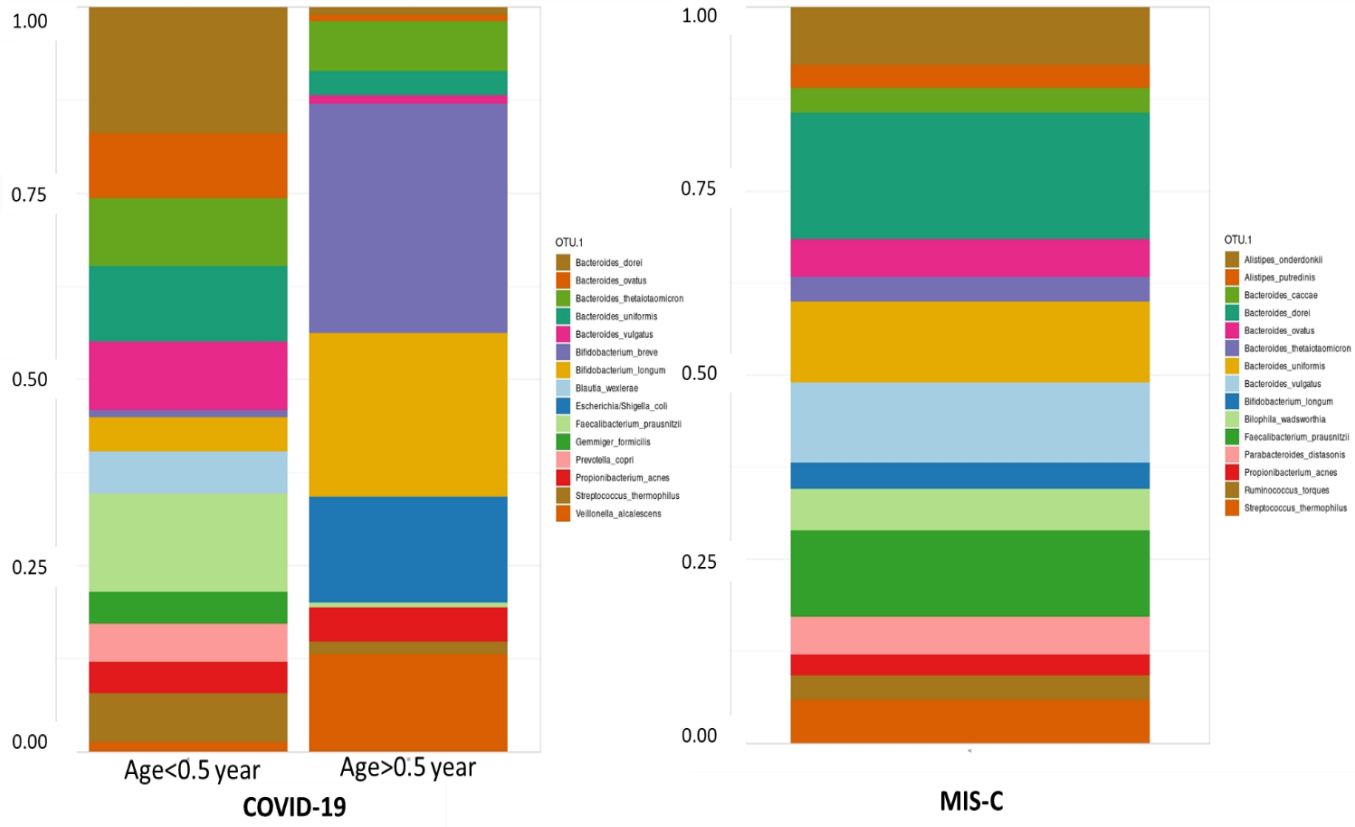


Figure 11. Relative abundance of main microbial species observed in COVID-19 (subdivided by age, cut off 0.5 years) and MIS-C.

4.6. qRT-PCR analysis

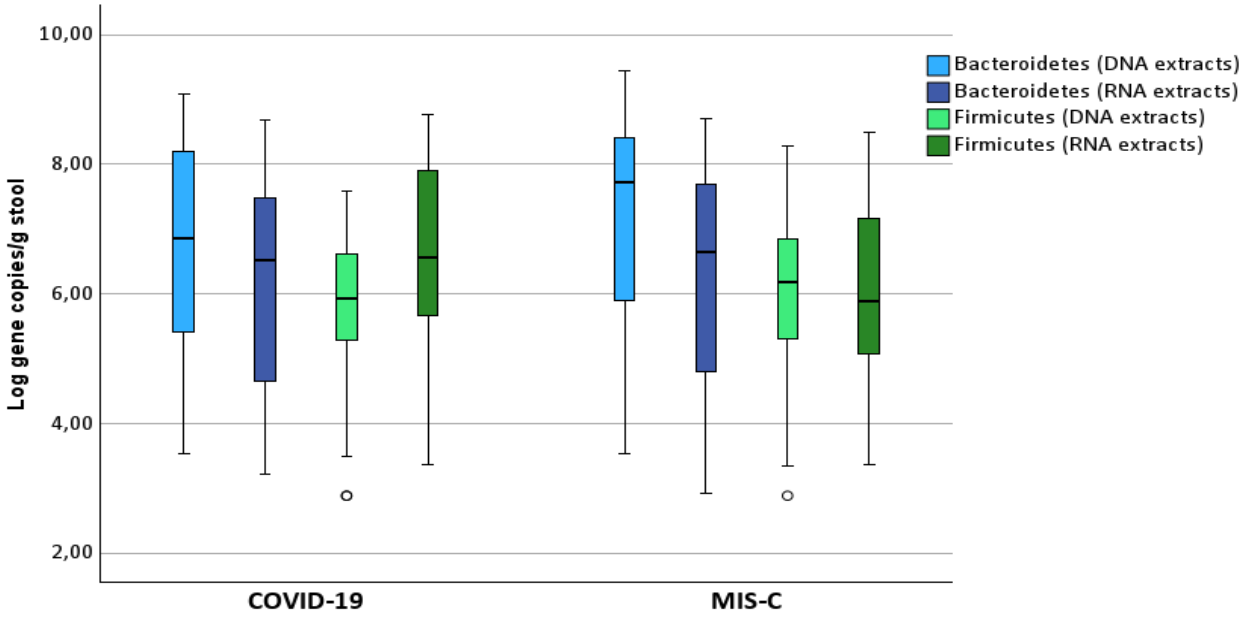
The quantifications of specific microbial starting from the DNA are significantly and strictly correlate to the same evaluation stating form the mRNA (Table 5). This shows as the presence of a microbial group (phylum, genus or specie assessed starting form DNA), is also matched with an active form of such microbials (starting from mRNA, under a transcriptomic approach). On the other hand, *Bacteroides* spp. is strictly correlated with its phylum, Bacteroidetes both considering the presence but also its activity. Respect the Firmicutes a decrease of the correlation rho observed between present and active form can be linked to the presence of silent microbes. The presence of *Bifidobacterium* spp. belonging to Actinobacteria is significantly correlated to the presence of *Akkermansia muciniphila* belonging to the Verrucomicrobia phylum. However, the activity of such two microbials has not correlated each other or to other quantified microbial groups. This could be a confirm of the relevance of low abundant species in the gut when a microbiota signature of disease is researched.

Table 5. Correlation among the concentrations of the microbials detected by qRT-PCR starting from DNA (yellow) and from mRNA (Blue). Spearman's rho and its significant (* or **) is included for each couple following by the p value for each line.

	Bacteroidetes	*Firmicutes*	*Bacteroides*	*Bifidobacterium*	*Akkermansia*	*Bacteroidetes*	*Firmicutes*	*Bacteroides*	*Bifidobacterium*	*Akkermansia*
Bacteroidetes	1,000	,369**	,900**	0,083	0,087	,657**	0,195	,513**	0,162	-0,067
		0,001	0,000	0,487	0,470	0,000	0,099	0,000	0,171	0,571
Firmicutes		1,000	,332**	0,226	,252*	,275*	,302**	,275*	0,018	0,157
			0,004	0,054	0,033	0,019	0,009	0,019	0,880	0,185
Bacteroides			1,000	-0,006	0,142	,699**	,245*	,480**	0,117	-0,145
				0,957	0,235	0,000	0,036	0,000	0,323	0,221
Bifidobacterium				1,000	,281*	-0,035	-0,010	-0,057	,332**	0,037
					0,017	0,770	0,936	0,633	0,004	0,758
Akkermansia					1,000	,298*	0,142	0,052	0,145	,496**
						0,011	0,236	0,666	0,225	0,000
Bacteroidetes						1,000	,597**	,768**	,417**	0,180
							0,000	0,000	0,000	0,124
Firmicutes							1,000	,686**	,454**	0,188
								0,000	0,000	0,109
Bacteroides								1,000	,442**	0,107
									0,000	0,368
Bifidobacterium									1,000	0,155
										0,188

The composition in terms of phyla and more specific microbial target are showed on (figure 12A and 12B respectively). The quite similar abundance, observed by NGS method, between COVID-19 and MIS-C, both starting from DNA and mRNA was confirmed also using qRT-PCR method and degenerate primers.

A)



B)

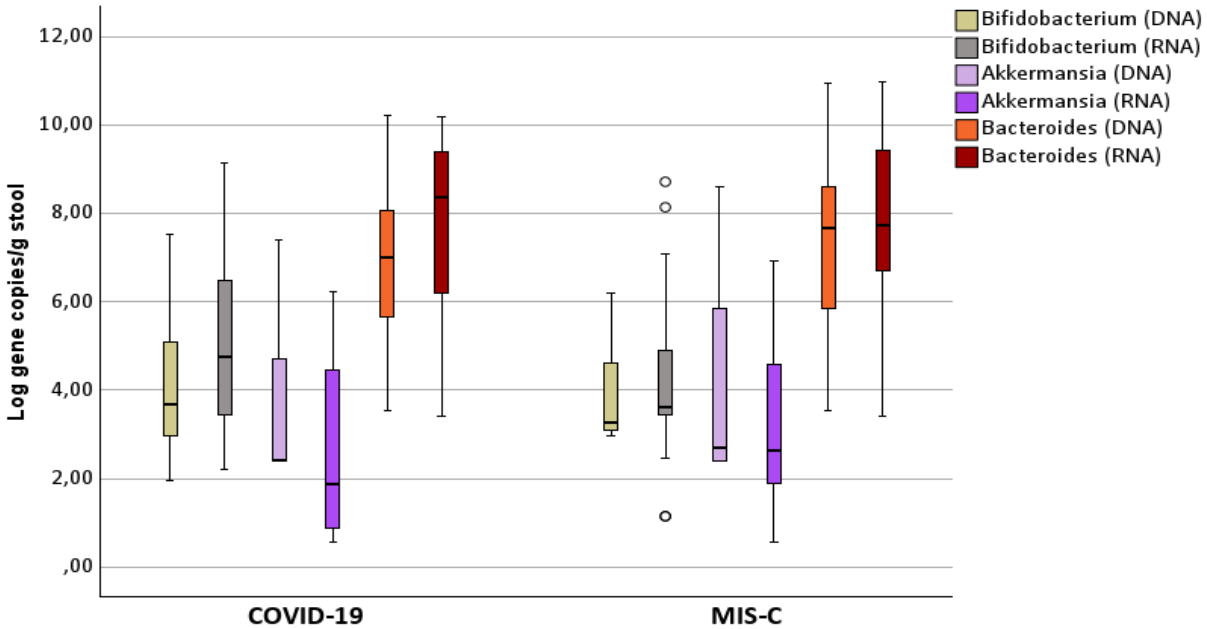


Figure 12. Box plot of the qRT-PCR results subdivided by disease, starting both from DNA and mRNA extract. For Phyla (A) and for detected genera and species (B).

Among the specific genera, *Bifidobacterium* is one of the most variable microbial targets. It is different between COVID-19 and MIS-C both when RNA was analysed (5.14 vs 4.21 Log gene copies/mg in faeces, $p<0.05$) but also between positive and negative stool to SARS-CoV-2 both when DNA and RNA were analysed (2 Log higher concentration in the positive stools, $p<0.05$). Such data is affected by the higher proportion of very young patients with COVID-19 and SARS-CoV-2 positive stool. Moreover, *Bifidobacterium* was affected by the antibiotic treatment (-29%, $p=0.002$).

The C reactive protein level increase with the increase of the *Akkermansia muciniphila* levels (Spearmans'rho =0,265, $p =0.043$), even if such bacteria is generally quite low (30% samples <LOQ). An impact of the sporadic pharmaceutical treatment was observed. Cortisone inhibited both the presence and the expression of *Bacteroidetes* and more in detail, of *Bacteroides spp.*, *Bifidobacterium spp.* and *Akkermansia muciniphila* ($p<0.05$).

5. Discussion

The relationship among SARS-CoV-2 infection, gut microbiota alterations and immune responses represents a novel area of investigation, particularly in pediatric populations. This study contributes to this emerging field by providing a comprehensive analysis of gut microbiota dysbiosis in children diagnosed with COVID-19 and MIS-C. While several studies were focused on adult age, the pediatric immune system, characterized by its developing and adaptive nature, and its interaction with the SARS-CoV-2 infection, represents a study field yet to evaluate. This study advances the understanding of gut microbiota alterations in pediatric SARS-CoV-2 infections, providing more details on their implications for disease severity and immune modulation. Throughout the use of advanced molecular techniques, we have delineated microbial signatures associated with severe outcomes such as MIS-C. The findings align with and build upon previous studies, enabling a broader contextualization of the gut microbiota's role in SARS-CoV-2 infections while identifying similarities, differences and gaps in the current literature. Moreover, the integration of next-generation sequencing (NGS) and comprehensive molecular analyses, allowed to provide an in-depth evaluation of our results with existing studies, offering both confirmation of known patterns and novel perspectives on pediatric cohorts.

The reduced microbial diversity observed in pediatric patients in this study is consistent with similar findings in adults. For instance, a reduced microbial diversity in severe COVID-19 adult patients, with an enrichment of potentially pathogenic taxa as observed [54]. However, the taxonomic composition in our pediatric cohort exhibited notable differences, suggesting that age-specific factors play a significant role in shaping the microbiota response. Similarly, Liu et al. [49] reported microbiota dysbiosis in post-acute COVID-19 syndrome, with a shift toward a pro-inflammatory microbial environment. These studies underscore the importance of age-stratified microbiome research to better understand the interplay between SARS-CoV-2 infection and host-microbiota dynamics.

Interestingly, unlike studies such as Xiang et al. [55], which highlighted significant increases in taxa associated with inflammation in adults, our pediatric cohort exhibited a more nuanced microbial shift, with specific taxa demonstrating subtle changes rather than outright dominance. This suggests that the developing immune system in children may

modulate microbial responses differently, potentially offering resilience against severe microbial disruptions seen in adults.

Comparing methodological approaches, in our study we used the Ion Torrent sequencing platform allowed for high-throughput, cost-effective profiling of microbial communities. This contrasts with studies using Illumina platforms, such as Zuo et al. [33], which provide comparable resolution but require more complex sample preparation. The Ion Torrent platform's user-friendly workflow and reduced error rates in homopolymeric regions make it particularly well-suited for clinical research settings, especially when working with limited pediatric sample volumes. These technical advantages support the robustness and reproducibility of our findings while highlighting the importance of selecting sequencing technologies tailored to specific research needs.

Beyond microbiota diversity, the functional analysis in our study suggests potential disruptions in metabolic pathways critical to maintaining gut and systemic health. These disruptions align with finding that linked altered microbial functions to heightened inflammatory responses in COVID-19 patients [56]. The observed changes in metabolic pathways, such as those involved in short-chain fatty acid production, may have downstream effects on immune regulation, as suggested by D'Amico et al. [57]. However, the pediatric microbiota's developmental stage may result in distinct functional profiles compared to adult studies, underscoring the importance of age-specific investigations.

When comparing studies among themselves, significant overlaps and distinctions emerge. Enaud et al. [58] highlighted the role of the gut-lung axis in mediating respiratory infections, emphasizing microbial metabolites that influence immune signaling. This aligns with findings by Bacorn et al. [59], who identified persistent microbiota alterations in pediatric long-COVID cases, suggesting that microbiota recovery may lag behind clinical symptom resolution. These studies collectively emphasize the microbiota's central role in shaping COVID-19 outcomes across age groups, though they also highlight the need for longitudinal designs to capture the temporal dynamics of microbial recovery, an area not addressed in our cross-sectional analysis.

The potential clinical implications of these findings are significant. Probiotic and prebiotic interventions, as explored by Wais et al. [56], represent promising strategies to mitigate microbial dysbiosis. However, their effectiveness in pediatric populations requires further

investigation, as children's microbiota exhibit higher plasticity and developmental variation compared to adults. Studies like Giovanetti et al. [60] underline the importance of tailored interventions targeting specific microbial deficiencies, a consideration particularly relevant for pediatric patients.

This study's primary strengths include its focus on a pediatric cohort, the use of advanced sequencing technologies and rigorous analytical methods. However, several limitations warrant discussion. The cross-sectional design limits our ability to infer causal relationships between microbiota alterations and disease severity. Longitudinal studies, such as those by Mehta et al. [61] have showed dynamic changes in microbiota composition over time, providing critical insights into recovery trajectories. Future research should adopt similar approaches to better elucidate the microbiota's role in disease progression and resolution.

In conclusion, this study highlights significant microbial alterations in pediatric SARS-CoV-2 patients, underscoring the gut microbiota's role in disease progression and recovery. The findings emphasize the need for age-specific research and tailored therapeutic strategies to address the unique microbiota signatures observed in children. By comparing our results with existing studies, this discussion illustrates the complexities and nuances of microbiota-host interactions in COVID-19, offering valuable insights for future research and clinical applications

6. Conclusion

By focusing on children, this study has highlighted unique microbial and immune signatures distinguishing pediatric cases of severe COVID-19 and MIS-C from those seen in adults. The reduced microbial diversity observed in the pediatric cohort, particularly among those with MIS-C, correlates with disease severity and emphasizes the importance of age-specific microbiota research. The advanced molecular approaches employed, including next-generation sequencing, provided robust insights into microbial disruptions and their potential role in mediating immune dysregulation and inflammation, thus adding a valuable dimension to the broader understanding of pediatric infectious diseases. One of the study's significant contributions lies in its exploration of gut microbiota's role in immune modulation. The differences in microbial composition between MIS-C and COVID-19 patients reflect the varying immune challenges posed by these conditions. The enrichment of opportunistic pathogens and the depletion of beneficial taxa, particularly short-chain fatty acid-producing bacteria, provide a mechanistic basis for the observed systemic inflammation and gastrointestinal symptoms. Furthermore, the demonstrated correlations between microbial profiles and clinical biomarkers affirm the potential utility of microbiota-based diagnostics in stratifying disease severity and predicting outcomes in pediatric populations. The implications of this research extend beyond diagnostics to potential therapeutic applications. Microbiota-targeted therapies, including probiotics and prebiotics, could emerge as promising avenues to mitigate dysbiosis and restore immune homeostasis. However, their application in pediatric populations requires careful consideration of age-specific microbiota dynamics and developmental factors. The study also highlights the need for longitudinal research to capture the evolving interplay between the gut microbiome, immune responses, and disease outcomes over time, paving the way for personalized and predictive approaches to pediatric care. The findings hrein reported underscore the need for age-specific research, the integration of microbiota data into clinical workflows and the exploration of innovative, non-invasive therapies tailored to pediatric patients. By contextualizing these results within the broader landscape of microbiota research, the study not only advances the field but also sets the stage for future investigations that could revolutionize pediatric infectious disease management and improve outcomes for vulnerable populations.

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