





CLINICAL PRACTICE ARTICLE

REVISED The first report on detecting SARS-CoV-2 inside bacteria of the human gut microbiome: A case series on asymptomatic family members and a child with COVID-19 [version 2; peer review: 1 approved with reservations, 1 not approved]

Previously titled: The first report on detecting SARS-CoV-2 inside human fecal-oral bacteria: A case series on asymptomatic family members and a child with COVID-19

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Abstract

Many studies report the importance of using feces as source sample for detecting SARS-CoV-2 in patients with COVID-19 symptoms but who are negative to oropharyngeal/ nasopharyngeal tests. Here, we report the case of an asymptomatic child whose family members had negative results with the rapid antigen nasopharyngeal swab tests. The 21-month-old child presented with fever, diarrhea, bilateral conjunctivitis, and conspicuous lacrimation. In this study, analysis for the presence of SARS-CoV-2 in fecal samples by using Luminex technology allowed accurate detection of the presence of the viral RNA in the feces of the child and of all her relatives, which thus resulted to be positive but asymptomatic. It is the first time that SARS-CoV-2- is observed inside bacteria of the human gut microbiome and outside a matrix resembling extracellular bacterial lysates, in agreement with a bacteriophage mechanism with the images obtained by transmission electron microscopy (TEM), post-embedding immunogold, and by fluorescence microscope. In addition to the typical observations of respiratory symptoms, accurate evaluation of

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Approval Status ? X

1

2

version 2

(revision)

08 Aug 2022

version 1



02 Feb 2022

?

view

X

view

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Any reports and responses or comments on the article can be found at the end of the article.

clinical gastrointestinal and neurological symptoms, combined with efficient highly sensitive molecular testing on feces, represent an efficient approach for detecting SARS-CoV-2, and for providing the correct therapy in challenging COVID-19 cases, like the one here reported.

Keywords

SARS-CoV-2, gut microbiota, bacteriophage, feces, diarrhea, nasopharyngeal swab, fecal oral transmission, TEM image, case series



This article is included in the **Emerging Diseases and Outbreaks** gateway.

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Competing interests: Author Carlo Brogna, Domenico Rocco Bisaccia, and Francesco Lauritano are employed by Craniomed Group. Author Mauro Petrillo is employed by Seidor Italy Srl. Author Simone Cristoni is employed by ISB Srl. All authors of the manuscript declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVISED Amendments from Version 1

The second version of the manuscript has some changes.

The title has been changed to "The first report on detecting SARS-CoV-2 inside bacteria of the human gut microbiome: A case series on asymptomatic family members and a child with COVID-19". The words "bacteria of the human gut microbiome" had more appropriate to the written context. Having word limits for a case series, according to the guidelines, we added the supplementary materials (s.m.) to provide more details to the readers without requiring them to find references in our already published articles.

Supplementary materials can be viewed, in accordance with the journal guidelines, at the following DOI: [10.5281/zenodo.6974414](https://doi.org/10.5281/zenodo.6974414).

Few words or phrases have been improved for greater clarity.

Two new control panels have been added, Figure 3, panels F and G, in tiff format, 300 dpi, RGB, showing additional control in the feces of a healthy subject.

Table 1 has been expanded with a column showing the results of nasopharyngeal antigen swabs. References 9-11 and 16-20 have been added.

Reference 48 is now correct and had been described in the text (v1).

Dr. Vincenzo Costanzo, who acquired the images of the control samples in Figure 3, was added in the acknowledgments. All authors approved the changes and additions.

Any further responses from the reviewers can be found at the end of the article

Introduction

In the past two years, humanity has been combating the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 is a positive, single-stranded RNA virus of the *Coronaviridae* family, specifically of the subfamily *Orthocoronavirinae* (usually called "coronaviruses"). Its closest known relatives are those found in bat feces, like the coronavirus RaTG13.¹ Xu *et al.* (2020)² studied viral behavior in 10 children, ranging in age from two months to 15 years. Although all of them were positive to the initial nasopharyngeal test, for eight of them, the viral charge was also positive in the stool. Moreover, they continued to test positive in the stool even after the negative nasal swab for several days after hospital discharge. In another Chinese study, the researchers found viral positivity in the fecal samples of 205 patients.⁴ Many studies³⁻⁵ have observed that fecal-oral transmission of the virus is possible and that it is very common to detect this virus in feces. Nevertheless, in comparison to the closest SARS-like viruses, SARS-CoV-2 appears to diverge in the receptor-binding domain of the spike glycoprotein, which is considered a key player in the entrance of the virus in human eukaryotic cells throughout its interaction with the angiotensin-converting enzyme 2 receptor (ACE-2), which in turn is considered the entry point of the virus.⁶ ACE-2 receptors and host cell transmembrane serine protease 2 (TMPRSS2) are abundant throughout the intestinal tract^{7,8} and several studies have reported altered intestinal bacterial flora or intestinal bacterial co-infection in COVID-19 patients.⁹⁻¹¹ In terms of hosts, coronaviridae members are neither human-specific nor new in terms of discovery and treatments: a recent review describes the numerous zoonoses caused by the Coronaviridae family members,¹² and scientists searched for the pathogen in the stool,¹³ a method that was, and continues to be, very common in the veterinary field. Among the coronaviruses previously found and analyzed in feces, there are those responsible for animal diseases like the calves' enzootic pneumonia (caused by Bovine coronavirus, BCoV), or the porcine epidemic diarrhea (caused by the Porcine Epidemic Diarrhea Virus, PEDV). These diseases and other coronavirus-related ones very often show as initial clinical manifestation of violent diarrhea, and the affected animals have a significant alteration of the intestinal mucosa.^{12,14,15} Observations of possible links between the animal gut microbial environment and coronaviruses have been reported in some studies,¹⁶⁻²⁰ supported also by the use of transmission electron microscopy (TEM) image analysis which screens and looks for viruses-like particles.^{8,21} The observation of SARS-CoV-2 particles by TEM can complement the molecular traces of it.²² Finally, it is worth noting that almost all of the latest characterized SARS-like viruses have been found and sequenced in bat fecal samples.²³

Here, we report the case of a symptomatic child whose family members had negative results with rapid antigen nasopharyngeal swab test. Analyses of fecal samples detect the viral RNA presence in the feces of the child and of all her relatives, which thus resulted to be positive asymptomatic. Microscope image analyses confirm the presence of SARS-CoV-2-like particles on fecal samples of the family and suggest that bacteria, reservoirs of the virus, are the most critical factors of fecal-oral transmission in this pandemic. The present case report also emphasizes the importance of the rapid detection of SARS-CoV-2 in symptomatic and non-symptomatic subjects with negative results from nasal and oropharyngeal swabs by analyzing stool samples, and emphasizes the importance of the bacteriophagic mechanism of the virus and its fecal-oral transmission.

Case series description

A 21-month-old female, Caucasian child, presented to us with severe bilateral conjunctivitis, conspicuous lacrimation, diarrhea, malodorous stools, restlessness, and fever (38°C). The child's medical history was negative for any disease. Parents reported that about a year earlier, she had a period when she had a severe cold. They were alarmed by violent

diarrhea, which was preceded by 24 hours of constipation, as well as by the abnormal bilateral conjunctivitis with uncontrollable lacrimation. Rapid blood tests showed the following values (in bold are those out of normal range, NR): creatinine 0.18 mg/dL (NR: 0.40-1.10 mg/dL); glucose 97 mg/dL (NR: 60-110 mg/dL); aspartate transaminase 45 I.U. (NR: 10-50 I.U.); alanine transaminase 28 I.U. (NR: 10-35 I.U.); sodium 139 mEq/L (NR: 136-150 mEq/L); potassium 5.82 mEq/L (NR: 3.50-5.10 mEq/L); chloride 95 mEq/L (NR: 98-107 mEq/L); calcium 5.50 mEq/L (NR: 4.25-5.25 mEq/L); C-reactive protein 2.60 mg/L (NR: 0-5 mg/L); iron 28 mcg/dL (NR: 59-158 mcg/dL). Other complete blood count values were in the normal range.

The Caucasian family (six adults, three children) came to us, in the autumn of 2020, during one of the Italian regional lockdown periods. Some specific information on the family members were recorded, including age, sex, medical history, occupations, and relationships (see [Table 1](#)). They live in close proximity, divided among three apartments in one building ([Figure 1](#) panel A). The parents reported that the children never had a babysitter since this task was entrusted to their grandparents, who were in their building. Moreover, they reported that since the outbreak of the pandemic (March 2020), they had adopted a series of measures, probably excessive in their opinion, with the purpose of protecting the grandparents and children from sickness. Such measures included no contact with people outside the family context, disinfection of every product purchased, no summer holidays, no eating at restaurants or other public places, and limited outings for the four parents (am1, af1, am3, af3) for work reasons only. The grandfather (am2), grandmother (af2), and the three children (cf1, 2cf1, cm3) did not leave the building for the duration of the lockdown ([Figure 1A](#) and [Table 1](#)). All the parents (am1, af1, am3, af3) of the children working in the health care sub-area left home daily to work, and one of them worked in another geographical region. Considering their work position it is most likely that the family infection started with the contagiousness of one of the four parents (am1, af1, am3, af3) who were asymptomatic during working hours. Of interests is the medical history of one adult (am1), the father of child cf1 (our COVID-19 patient), that was hospitalized precisely one year prior (autumn 2019) with escalating symptoms of violent diarrhea, abdominal pain, fever (38°C), dyspnea, cough, headache, shortness of breath, and fainting. There was saturation of 91 SpO₂%, right bundle branch block, increased D-Dimer, increased liver values (GOT and GPT), and mild lymphopenia, treated with antibiotics.

We initially performed rapid antigen nasopharyngeal swab test (COVID-19 Ag Rapid Test Device, Abbott 41FK10) on the child (cf1), and it was negative. The same test was also performed on the parents (am1, af1) and the other six family members, and all results were negative. We had, in line with previous studies,^{24,25} experience of multiple negative results SARS-CoV-2 real-time reverse transcriptase polymerase chain reaction (RT-PCR) tests on oropharyngeal/nasopharyngeal (OP/NP) swab samples from individuals with a strong clinical suspicion of COVID-19.²⁶ Being in the presence of a very young patient, it was decided to adopt a fast high-throughput COVID-19 screening approach to detect the presence of SARS-CoV-2 directly from stool samples: in the following 24 hours, stool samples were collected from all nine family members, and molecular testing for SARS-CoV-2 was performed by using Luminex technology^{27,28} as described by us previously.²⁹ Negative and positive controls as bacterial cell cultures of stool samples were those used and described in this previous study.²⁹

A summary of the analyses is reported (all methods and materials are detailed in supplementary materials s.m.) in [Figure 1C-D](#) and [Table 1](#)³²: all family members had positive results to the Luminex molecular test, and the child with symptoms (cf1) showed the highest value of the Luminex assay. The other family members did not manifest any symptoms, despite being positive for the presence of viral RNA in their stools.

The child was treated for 48 hours only with rehydration and probiotics only; because of the absence of significant symptoms such as cough or dyspnea, no cortisone or antibiotics were administered. Conjunctivitis and lacrimation ceased about 72 hours later and the patient was discharged. The entire family, including the reported patient, were then instructed to take probiotics (*Lactobacillus reuteri*, 100 million units, one time per day, and *Bacillus clausii* 2 billion units, per day) in addition to bromelain, 300 mgr. per day, and colloidal copper, 20 ppm (parts per million) per day for 30 days, only as re-balancers of bacterial flora. After 60 days, both the rapid antigen nasopharyngeal swab test (COVID-19 Ag Rapid Test Device, Abbot 41FK10) and the Luminex test were repeated: all family members were negative to the rapid antigen tests, and only one family member ([Figure 1D](#) -am1) continued to have Luminex positive results. Patient am1, male, Caucasian and a healthcare employee, continued the treatment until he became negative at day 90 for the presence of SARS-CoV-2 in stools. The feces of this patient was cultured in bacterial culture media and after 30 days, the pellet of bacteria, have been analyzed by TEM, immune-EM, and by fluorescence microscopy, and a set of obtained images is shown in [Figure 2](#) (for more details see supplementary material-s.m.).⁵² At day 30 of bacterial culture of feces patient am1, the Luminex molecular test confirmed the presence of SARS-CoV-2 and the RNA viral concentration was increased from 24 arbitrary unit (AU) (initial) to 520 AU (Final) ([Figure 1B](#)) in accordance with our previous observations.²⁹ Transmission electron microscope images (panels A and B of [Figure 2](#)-Tecnai G2 Spirit BioTwin; FEI, equipped with a VELETTA CCD digital camera -Soft Imaging Systems GmbH) SARS-CoV-2 (black arrows) inside a bacterium (A) and outside a matrix

Table 1. Information about family members.

| Subject | Symptoms | Rapid antigen nasopharyngeal swab test (COVID-19 Ag Rapid Test Device, Abbott 41FK10) | Stools Luminex AU Test before therapy (Initial) | Stools Luminex AU Test 60 days after therapy (Final) | Medical history or comorbidities | Age | Degree of the kinship with the child (cf1) affected by Covid-19 | Occupation |
|---------|--|---|---|--|---|-----------|---|---|
| am1 | No | Negative | 24 | 16 | Hepatic Steatosis | 43 | Father | Worker in the health care sub-area |
| af1 | No | Negative | 23 | 0 | Congenital heart disease: patent foramen ovale (PFO) and atrial septal aneurysm (ASA) | 38 | Mother | Worker in the health care sub-area |
| cf1 | Yes: bilateral conjunctivitis, conspicuous lacrimation, diarrhea, restlessness, and fever (38°C) | Negative | 33 | 0 | Healthy | 21 months | the child affected by COVID-19 | - |
| 2cf1 | No | Negative | 20 | 0 | Healthy | 8 | Sister | Primary school student-lessons in "didactics distance" (DAD) for the lockdown period. |
| am2 | No | Negative | 20 | 0 | Benign prostatic hyperplasia (BPH) | 69 | maternal grandfather | Retired- full-time grandfather with grandson cf1, cf2 and cm3 |
| af2 | No | Negative | 13 | 0 | Diverticulosis; arterial hypertension | 67 | maternal grandmother | Retired- full-time grandfather with grandson cf1, cf2 and cm3 |
| am3 | No | Negative | 19 | 0 | Healthy | 42 | Uncle | Worker in the health care sub-area |
| af3 | No | Negative | 12 | 0 | Healthy | 39 | Maternal aunt | - |
| am3 | No | Negative | 20 | 0 | Healthy | 2 | Cousin | - |

Am1: adult male family 1; af1: adult female family 1; cf1: child female family 1; 2cf1: 2nd child female family 1; am2: grandfather family 2; af2: grandmother family 2; am3: adult male family 3; af3: adult female family 3; cm3: child male family 3.

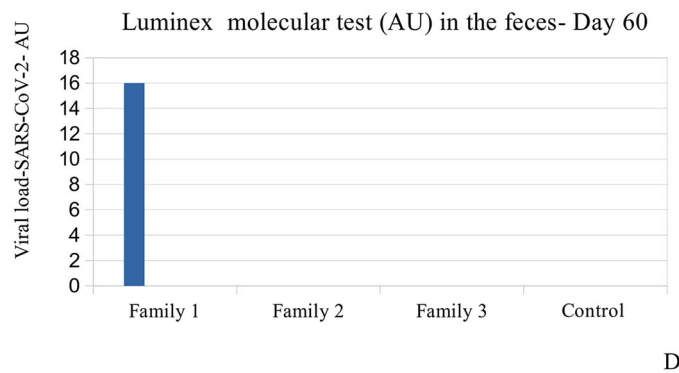
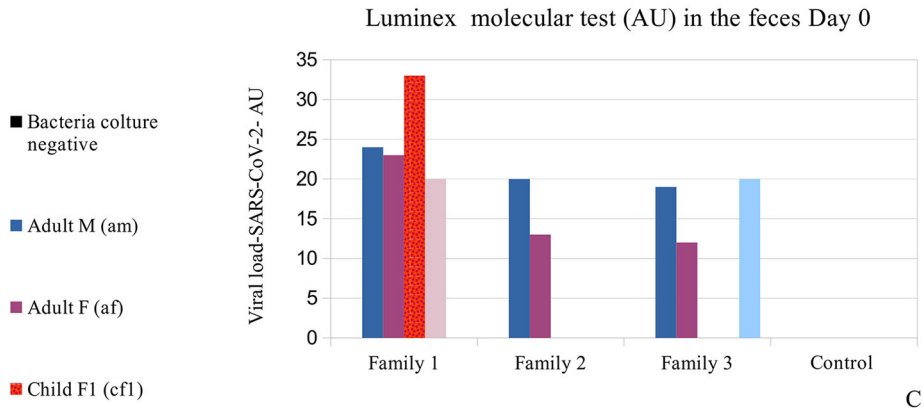
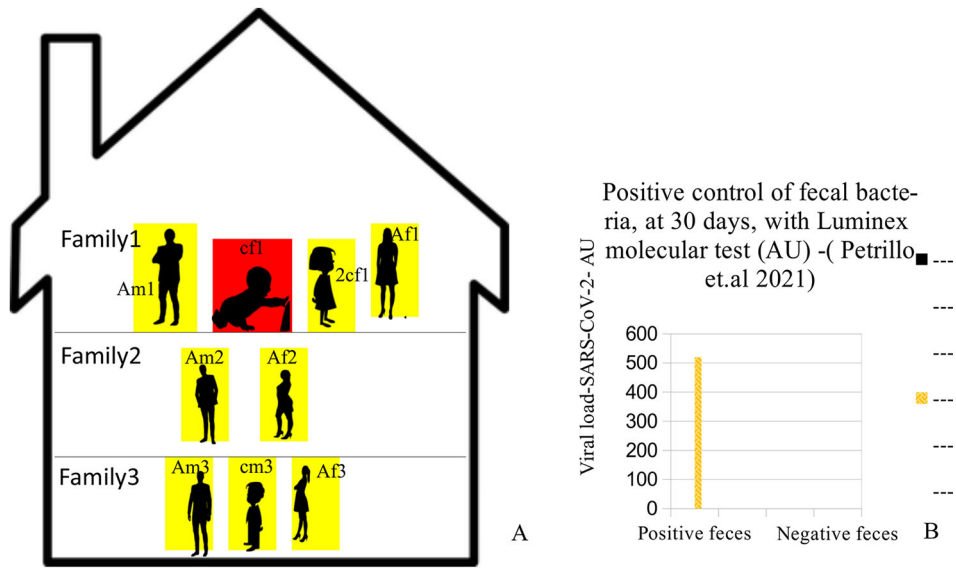


Figure 1. Case presentation and RNA viral concentration. (A) Distribution of the nine people analyzed in the family. Red (cf1: child female family 1) shows the child who was symptomatic and had positive results on the fecal molecular test. Yellow: the other family 1,2,3 members (Am1: adult male family 1; af1: adult female family 1; 2cf1: 2nd child female family 1; am2: grandfather family 2; af2: grandmother family 2; am3: adult male family 3; af3: adult female family 3; cm3: child male family 3) who had positive results on the Luminex molecular fecal test but negative results on the rapid antigen nasopharyngeal swab test. (B) This is the positive control of patient am1's bacteria, derived from a stool sample, after 30 days of bacterial culture using our previously published method,²⁹ performed with the Luminex molecular assay. The molecular assay reported a viral RNA concentration growth of up to 520 AU (arbitrary unit). (C-D) RNA Viral concentration initially and after 60 days. The family members hired supplemental therapy, only as re-balancers of bacterial flora, with colloidal copper and bromelain, as well as with probiotics therapy, only as re-balancers of bacterial flora, with *Lactobacillus reuteri* and *Bacillus clausii*.

resembling extracellular lysate of a bacterium (B). No eukaryotic cells have been ever observed after 30 days of bacterial culture. Post-embedding immunogold (Figure 2 Panel C, D): bacteria pellets were fixed with a mixture of 0.05% glutaraldehyde of 4% paraformaldehyde in 0.1M PBS (Phosphate-buffered saline) buffer, washed in PBS buffer, pelleted at 10000g and included in 3% agarose. The agarose block was cut into tissue-size pieces and the slices were post-fixed in 2% OsO₄, dehydrated in a series of ethanol solutions of increasing concentration and in propylene oxide and finally embedded in Epon 812. Thin sections were cut from embedded specimens using Reichert Jung Ultra microtome and are applied to Formvar/Carbon Supported nickel grids. Sections were blocked with normal goat serum for 1h at room temperature, incubated with rabbit monoclonal to SARS-CoV-2 nucleocapsid protein antibody (EPR24334-118, Abcam) and then with secondary anti-rabbit antibody 10nm gold-conjugated (Aurion). Electron microscopy images were acquired from thin sections under an electron microscope (Tecnai G2 Spirit BioTwin; FEI) equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH).

The immunofluorescence microscope (Figure 3, panels A; B, C, D - Zeiss Axioplan 2, AxioCam 305 color, magnification 100×) was performed in according to manufactures' protocol,^{30,31} using as primary antibodies versus SARS-CoV-2 Nucleocapsid protein ("Sars Nucleocapsid Protein Antibody [Rabbit Polyclonal] - 500 µg 200-401-A50 Rockland", and the "Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Cyanine3 #A10520" as secondary antibody). It was used also a primary antibody versus gram-positive bacteria ("Gram-Positive Bacteria Ab (BDI380), GTX42630 Gene Tex") and "Goat anti-Mouse IgG (H+L), Super-clonal™ Recombinant Secondary Antibody, Alexa Fluor 488" as secondary antibody. The images confirm the presence of SARS-CoV-2 particles (red light in the fluorescence images) in relationship with the bacteria (green light in the fluorescence images).

To our knowledge, this is the first time that a member of coronaviruses' family, the SARS-CoV-2, has been observed inside the bacteria of the human gut microbiome (Figure 2 panel A – Figure 2 panel C) and outside a matrix resembling extracellular bacterial lysates (Figure 2, panel B), in agreement with a phage-like behavior reported by us.²¹

Discussion

Zheng F. *et al.*^{32,33} observed that gastrointestinal symptoms are common in children with SARS-CoV-2 and are associated with fever, nausea, vomiting, and abdominal pain. However, their case series is probably not very large both because it is known that more than half of sick children have mild to moderate symptoms and because hospitalizations are not as common as for other respiratory viruses.³² A recent example of the possibility of fecal-oral transmission is well described in a short communication by Hansen *et al.*³⁴ These authors reported the case of an 86-year-old man who, despite of having been vaccinated (first dose of BNT162b2 mRNA COVID-19 vaccine), eighteen days after vaccination was admitted to the hospital for diarrhea, with no other symptoms of COVID-19, and had negative results on antigen and PCR testing until day 26, when he died of acute renal and respiratory failure. On day 24, the older man's roommate tested positive for SARS-CoV-2 RT-PCR on a nasal swab. Autopsy results of the 86-year-old decedent indicated the presence of the virus in the organs examined except for the liver and olfactory bulb.

In one of the first studies on SARS-CoV-2 in Wuhan, prominent symptoms of COVID-19 patients are described, including diarrhea³⁵ and in children, gastrointestinal disorders are the most prevalent.³² The persistence of coronaviruses in feces, for a long time, had already been observed many years ago. In one of the first case reports of 1982, Baker *et al.*³⁶ described the case of a 47-year-old Indian man who underwent surgery for a duodenal ulcer when he was 13 years old. The symptoms that forced hospitalization were diarrhea and steatorrhea. The man was monitored for eight months, and in 17 fecal samples, coronavirus-like particles were observed by electron microscopy. The images show two ovoid/geoid shaped coronavirus particles with the spike protein evident and one circular shaped coronavirus particle but without surface proteins, like those here reported in Figure 2.

Inclusion of symptoms other than respiratory, such as gastrointestinal symptoms, seems to be very important in the diagnostic process. Although diarrhea and conjunctivitis with lacrimation, as in our case, may be unlinked, they can be related to each other if the gut microbiota and the central, peripheral, and autonomous nervous systems are taken into account. The gut microbiota³⁷ seems to be extremely important and interconnected with the central, peripheral, autonomic, neuroimmune, and neuroendocrine nervous system axis. An altered gut microbiota or the total absence of bacteria, as in germ-free mice, can affect areas of the brain, including the hippocampus, the point of end of olfactory system.³⁸ Several studies have reported an impairment of intestinal gut microbiota³⁹ or respiratory and intestinal bacterial coinfection in COVID-19.⁴⁰

As shown in Figures 2-3, bacteria could play crucial role in the possibility of fecal-oral transmission. This news isn't so far away from the most recent studies²⁹ in which we described that RNA replication of the SARS-CoV-2 virus can take place in bacterial cultures. We also described that the use of certain drugs can decrease its replication *in vitro*.

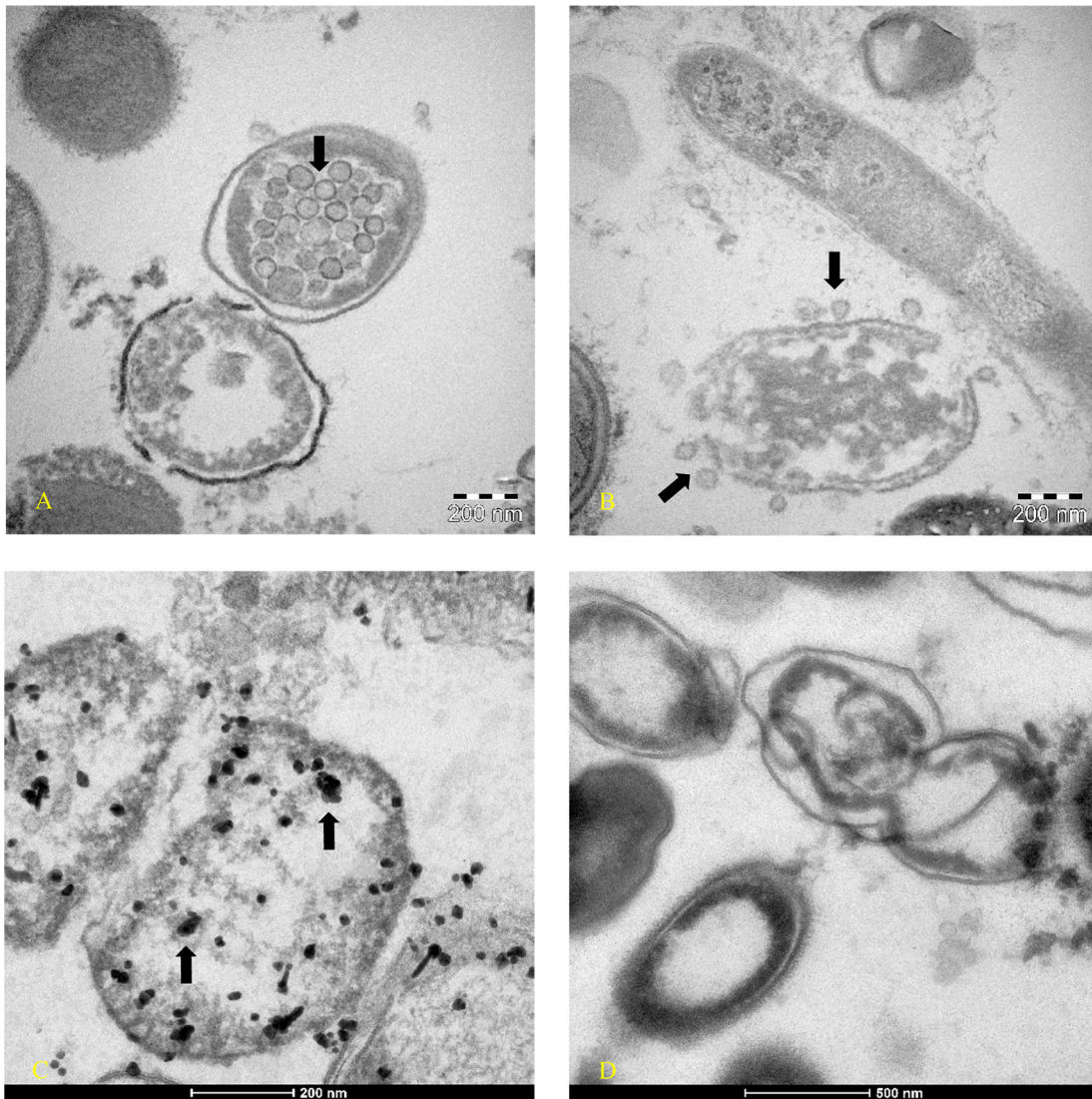


Figure 2. Transmission electron microscopy (post-embedded immunogold). Images were obtained at day 30 of bacterial culture of patient am1's feces, in which a molecular test with Luminex confirmed the presence of SARS-CoV-2 and an increase of RNA viral concentration from initial 24 arbitrary unit (AU) to 520 AU final. (A-B) Transmission electron microscope images (panels A and B-TEM FEI- Thermo Fisher Tecnai G2 operating at 120 kV) show SARS-CoV-2 (indicated by black arrows) inside a bacterium (A) and outside a matrix resembling extracellular lysate of a bacterium (B). (C-D) Post-embedding immunogold: rabbit monoclonal to SARS-CoV-2 Nucleocapsid protein antibodies ligating to the secondary anti-rabbit antibody 10nm gold-conjugated indicated the virus inside bacteria of gut microbioma (Tecnai G2 Spirit BioTwin; FEI equipped with a VELETTA CCD digital camera (Soft Imaging Systems GmbH)). (D) Negative control of bacterial stool culture of a healthy person after 30 days, without primary antibody with only the secondary antibody.

Moreover, in the same work, we observed, by mass spectrometry, the mutational phenomenon of viral proteins in bacterial cultures. Other authors have also noted the possibility that the spike protein of the SARS-CoV-2 may interact with the lipopolysaccharide of *Escherichia coli*⁴¹ or that the absence of proteobacteria could play a key role in the pathogenesis of respiratory viral diseases.³⁸ This is why early localization in the stool assumes considerable importance. Since the discovery of SARS-CoV-2, a plethora of commercial tests have become available, and, currently, more than 1,700 tests are commercialized in the European Union countries (source JRC COVID-19 *In Vitro* Diagnostic Devices and Test Methods Database⁴²). Rapid Antigen Tests (RATs) are recommended to be routinely used,^{43,44} especially on oropharyngeal/nasopharyngeal (OP/NP) swab samples. Researchers have had sometimes problems in terms of sensitivity and specificity with some of them.⁴⁵ Problems may arise because the tests were

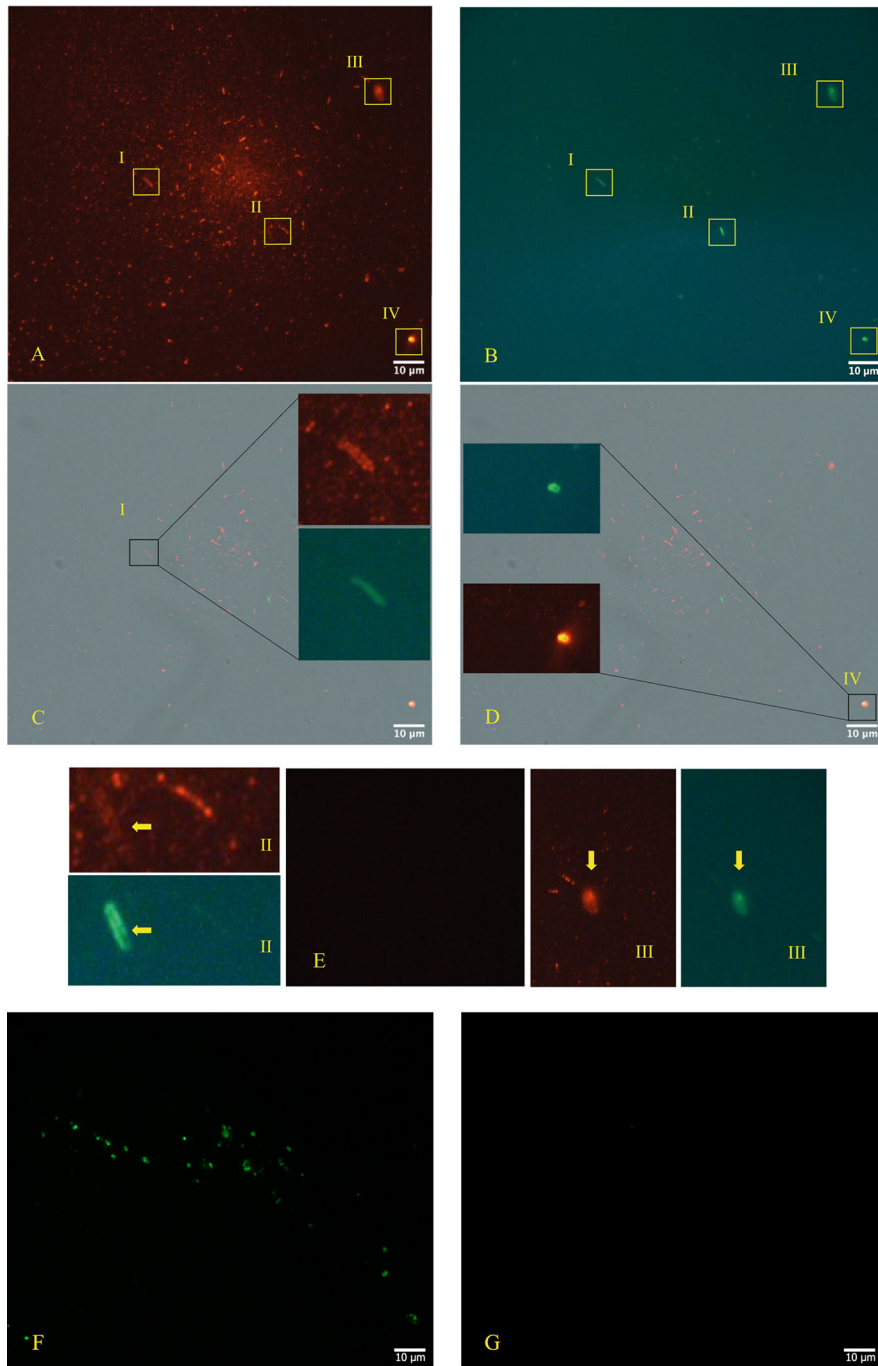


Figure 3. Fluorescence microscope images. Panels A, B, C, D (Zeiss Axioplan 2, Axiocam 305 color, magnification 100×) show immunofluorescence staining versus SARS-CoV-2 nucleocapsid protein (red light), gram positive bacteria (green light). Panel E is the negative control and panels F and G show a group of gram+ bacteria by fluorescence, derived from the stool bacteria culture of a healthy 18-month-old child (with healthy parents and never ill with SARS-CoV-2 at the time of collection and with their written consent) negative to molecular test to SARS-CoV-2, although the other primary antibody to the nucleocapsid protein is also included and does not show a red signa. The roman numerals I,II,III,IV and yellow rectangles indicate four gram-positive bacteria (green light) infected by SARS-CoV-2 (red light).

initially evaluated on samples from patients with severe COVID-19, who are suggested to develop a much higher immune response than those with mild or asymptomatic disease.⁴⁶

RT-PCR is considered the gold standard method for detection of SARS-CoV-2. However, we had previous experience of multiple negative results SARS-CoV-2 RT-PCR tests on OP/NP swab samples from individuals with a strong clinical suspicion of COVID-19.²⁶ Mardian *et al.* 2021 recommend fecal detection of viral RNA when nasopharyngeal swab data are questionable.⁴⁷ “In a Systematic Review and Meta-analysis, at the beginning of the pandemic, it was observed that viral RNA was present in the stool in 48.1% of patients during the disease and that 70.3% of patients had prolonged shedding that could extend beyond 33 days from the onset of the disease.”⁴⁸ Finally, in a recent study aimed to evaluate the role of fecal-oral transmission, unique RNA SARS-CoV-2 genomic sequence mutations have been observed by performing next-generation sequencing on the fecal samples.²⁵ In this case the Luminex technology as molecular testing tool was chosen because it is ideal for fast high-throughput COVID-19 screening and its clinical performance have been evaluated.⁴⁹

In consideration that SARS-CoV-2 was detected at low levels in fecal samples,⁵⁰ in addition to molecular test, was agreed to verify the presence of the virus by acquiring images of at least one sample. As proposed by Dittmayer and colleagues,²² in the case of COVID-19 diagnosis, the use of image analysis to confirm the presence of SARS-CoV-2 particles complements the detection of molecular traces of SARS-CoV-2 specific proteins or nucleic acids (and vice versa). In studies of infectious diseases, TEM is used very often to definitively prove the presence of an infectious unit. The images were obtained by TEM, immune-EM, and by fluorescence microscope. What we have noted is (in agreement with our first observations²⁹), that could be present an important role of bacteria in the fecal-oral transmission of SARS-CoV-2. The only limitations of such investigations are the high costs and long waiting times.

Conclusions

Here we report the case of a child symptomatic for COVID 19, transmitted by one of the parents, whose relatives had tested negative on the rapid antigenic nasopharyngeal swab test. Analyses of fecal samples by high-throughput COVID-19 screening (Luminex technology) allowed us to accurately detect the viral RNA presence in the faces of the child and of all her relatives, which thus resulted to be positive asymptomatic.

Microscopy images analysis was used as complementary approach to confirm the presence of SARS-CoV-2 in bacterial cultures obtained by fecal sample of an infected individual with the viral RNA load positive individual. The images obtained by TEM, immune-EM and by fluorescence microscope show SARS-CoV-2 inside human gut bacteria and outside a matrix resembling extracellular bacterial lysates, in agreement with a bacteriophage mechanism.²⁹ This first observation invites us to pay more attention to the fecal-oral transmission route of the virus and suggests as a further possible reservoir of the virus also the bacteria of the human gut microbiome.

We believe that accurate analysis of the human gut microbiome during viral infections, including SARS-CoV-2 infections, may be of great importance and may aid in diagnosis when other tests fail.²⁶ According to the other studies⁴⁷ faster and more versatile tests should be improved to decrease or cope with the contagiousness of the pathogens, especially to detect them in the stools. The observation of all clinical symptoms, typically respiratory, gastrointestinal, and neurological, combined with molecular testing (stool, sputum, tear, other fluids) and image analysis, represents the key for understanding the interaction of SARS-CoV-2 with the human gut microbiome and its product.⁵¹ Therefore, for the provision of the correct epidemiology, diagnosis and accurate therapeutic approach is important in the treatment of COVID-19, especially in challenging cases, such as the one reported here. This case also highlights the possibility of contagion from asymptomatic parents to their children.

Data availability

All data underlying the results are available as part of the article and are viewable at the following DOI <https://doi.org/10.5281/zenodo.6974414>, according to the journal guidelines; <https://zenodo.org/record/6974414#.YvFp6-xBxmA>.

Underlying data

All data underlying the results are available as part of the article and no additional source data are required.

Extended data

Zenodo: Supplementary materials (s.m.) of “The first report on detecting SARS-CoV-2 inside bacteria of the human gut microbiome: A case series on asymptomatic family members and a child with COVID-19” <https://doi.org/10.5281/zenodo.6974413>.

This project contains the following extended data:

- Supplementary material: Materials and methods of the tests described in the paper (detection of viral RNA by Luminex method, immunofluorescence at microscopy, electron microscopy, proteomics, and viral protein labeling by nitrogen radioisotope).

Data are available under the terms of the [Creative Commons Attribution 4.0 International](#) (CC BY Creative Commons 4.0 license).

Consent

Written informed consent for publication of their clinical details and clinical images was obtained from the parents of the child. Written informed consent for publication of their clinical details and clinical images was also obtained from all other patients involved in the study.

Acknowledgments

We thank Tigem Institute-Telethon Pozzuoli Naples for the microscope electron images preparations. We thank Biogem Institute and Dr. Costanzo Vincenzo of Ariano Irpino (Av), for fluorescence microscope images preparations. We are grateful to Electron microscopy facility of the department of chemical sciences- University of Study Federico II Naples (UniNa). We are also grateful to Marsan consulting and Dr Marino Giuliano for their full support. We thank the entire family and all the patients for allowing this work. This paper is a new gift for all the children of the world.

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Aravind Natarajan 

Department of Genetics, Stanford University, Stanford, CA, USA

Broгна C *et al.* The first report on detecting SARS-CoV-2 inside human fecal-oral bacteria: A case series on asymptomatic family members and a child with COVID-19

This manuscript by Broгна C *et al.* reports the first observation of SARS-CoV-2 “inside human fecal-oral bacteria” suggesting that SARS-CoV-2 can infect bacteria like a bacteriophage. This study is inspired by a symptomatic 21-month-old child whose nasopharyngeal sample tested negative on a rapid antigen test, but feces tested positive for SARS-CoV-2 viral RNA on a Luminex assay. This led the authors to carry out a contact tracing study, testing stool samples from family members that lived in the same building as the child for SARS-CoV-2 RNA; they found all the family members to be asymptomatic and positive for viral RNA in their stool. Notably, the child’s male parent (am1) continued to have extended shedding of viral RNA out to 90 days from the start of treatment. The authors collected stool samples from this parent to understand the pathobiology of SARS-CoV-2.

Specifically, they expand on their previous observation (featuring two shared authors with the current manuscript) that this virus could potentially display bacteriophage-like behavior and infect bacteria. In this previous work, they carried out *in vitro* culturing assays and reported that the concentration of viral RNA increased with time when incubated with fecal bacteria¹. In another publication (featuring seven shared authors with the current manuscript), the authors use TEM and immunofluorescence microscopy and report visualizing SARS-CoV-2 particles in gut bacteria². In the current work, they feature these same *in vitro* and microscopy experiments with a different stool sample and report the same conclusion - that bacteria in the gut are infected with SARS-CoV-2 viral particles.

I recommend this manuscript be rejected in its current form. It may be sent out for reviews again pending major revisions.

Major suggestions:

1. Throughout the manuscript, the authors suggest the fecal-oral transmission of SARS-CoV-2. However, whether there are infectious viral particles in stool capable of being transmitted is

at best still debated, with more evidence to the contrary³. Especially given the relevance of this matter to clinical decisions and public health, I encourage the authors to present a balanced view.

2. The authors report viral RNA concentration determined through Luminex in arbitrary units (AU). However, my understanding is that Luminex can be set up to contain standards that reveal an absolute concentration of viral RNA. Given how viral RNA concentrations are central data to the conclusions in this work, I believe that reporting the absolute concentration of viral RNA is important. Additionally, this will make the observations here more replicable across labs and viral RNA detection techniques.
3. Regarding the reported viral RNA concentrations, it is unclear what the specificity, sensitivity, and detection range of the Luminex assay are as carried out by the authors. Therefore, I recommend the authors include relevant controls to estimate these. Reporting this information along with their experiments will add important validity and context to the data.
4. Fig 3 is missing key controls. Recommend including a control of uninfected bacteria from a healthy donor stained with both α -SARS-CoV-2 nucleocapsid protein antibody and α -Gram-positive bacteria antibody.
5. The authors conclude from the microscopy data that fecal bacteria are infected by SARS-CoV-2 in samples collected on day 30 from an adult patient, am1. Notably, this patient along with the others in the study was prescribed probiotics with two Gram-positive bacteria (*Lactobacillus reuteri* and *Bacillus clausii*) in their diets for 30 days prior to sample collection. In the event that bacteria are in fact infected by SARS-CoV-2, it is unclear if this is an artefact of the probiotic supplements and treatment regime.
6. I encourage the authors to provide more methodological information in the manuscript. As a matter of principle, manuscripts should in and of themselves provide sufficient information for readers to repeat experiments without having to go down the rabbit hole of chasing down references to other works. The current manuscript exhibits this concern that is prevalent in scientific publications. Some instances of this in the current manuscript include -
 - "molecular testing for SARS-CoV-2 was performed by using Luminex technology as described by us previously" - It is unclear how the samples were prepared, if controls were included and what the parameters of this assay were.
 - Methodology used to isolate and culture bacteria from stool - It is unclear how bacteria were isolated from stool. This is important information to get a sense of how rigorous this method was, whether there are inherent biases regarding what bacteria are favored in the culturing process and if there are chances for other residual contaminants from the fecal sample.
 - Methodology used to collect and preserve stool samples - the majority of existing reports are unable to culture viable SARS-CoV-2 from fecal samples, even when the samples are preserved in Viral Transport Media (VTM). Therefore, clarity about how the current work collected and preserved samples are crucial to understanding how they have been successful at recovering viable viral particles.

Minor suggestions:

1. Request citation and clarity on the statement - "several studies have reported....intestinal bacterial co-infection in COVID-19 patients".
2. Request citation and clarity on the statement - "Observations of possible links between the animal gut microbial environment and coronaviruses have been reported over time". The current citations do not have evidence for a possible link to the gut microbial environment.
3. Fig 1 reports "Viral load" although what is actually reported is the viral RNA concentration, which can be different from viral load because not all viral particles are lysed and provide RNA for detection, and some viral RNA is reported to come from non-viral reservoirs⁴.
4. The manuscript calls for major editorial revisions given numerous improper usages of phrases. For instance - a) There are no "fecal-oral bacteria", which suggests specific bacteria in the feces are able to be transmitted orally. b) It is unclear what "Bacterial feces" means. c) "RNA viral load count" appears miswritten.

Additional notes:

1. I don't have experience with interpreting TEM images and therefore leave this comment here rather than as a cause for major revision. TEM images are complex requiring the appropriate collection and processing of samples, the inclusion of controls, and careful interpretation of observations. The improper use of EM to study SARS-CoV-2 from tissue samples has been a cause for concern^{5,6}. To my untrained eye, Fig 2 seems to be another example of misinterpreted EM data because -
 - If Fig 2a in fact displays SARS-CoV-2 viral particles inside a bacterium, it is surprising to me that the bacterial host appears to be entirely filled by viral particles with no room for its essential, native molecules. To me, this figure seems like that of the widely described multivesicular bodies that are unrelated to SARS-CoV-2 and yet regularly misinterpreted⁶.
 - The figures don't appear to have sufficient resolution to highlight the ultrastructures typical of SARS-CoV-2 that are required to unmistakably identify viral particles⁵.
 - If Fig 2b in fact presents the case of a bacteria with viral particles around it, it merely appears like viral particles proximal to the bacteria in this image, with no evidence that these viruses in fact infected the bacteria. The conclusion that viral particles are found in the extracellular matrix of gut bacteria is an over-reach without sufficient evidence.
 - The figure is missing controls such as stained and unstained images of comparable bacteria without the presence of the alleged SARS-CoV-2 viral particles. Given these observations and the importance of this figure to the central tenet of this manuscript, I recommend the editorial team seeks input from an expert in TEM.
2. The hypothesis that SARS-CoV-2 may infect bacteria is both biologically fascinating and clinically highly relevant. This is because there are no examples that I know of or can find of viruses that affect humans that can also retain the molecular machinery to affect bacterial cells. Therefore, the idea that this is possible is biologically fascinating and can pave the path to many follow-up studies. Further, SARS-CoV-2 continues to be a major threat, having claimed over 6 million people worldwide. Therefore, careful evaluation of the potential sources of future outbreaks is critical from a personal and public health standpoint. If in fact

bacteria in the gut can harbor infectious SARS-CoV-2 viruses, this adds to another dimension of the COVID-19 pandemic that calls for urgent precautionary measures. Unfortunately, the current manuscript is missing many controls and methodological details, and has insufficient data to make the case that bacteria are in fact infected by SARS-CoV-2. Given the hugely significant impact that concluding gut bacteria can be infected by SARS-CoV-2 through a bacteriophage-like property can have to humanity, I encourage the authors and editorial team to exercise caution and responsibility in promoting this conclusion with insufficient evidence.

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Is the background of the cases' history and progression described in sufficient detail?

Yes

Are enough details provided of any physical examination and diagnostic tests, treatment given and outcomes?

Yes

Is sufficient discussion included of the importance of the findings and their relevance to future understanding of disease processes, diagnosis or treatment?

Yes

Is the conclusion balanced and justified on the basis of the findings?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Fecal shedding of SARS-CoV-2 RNA

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 17 Jul 2022

Carlo Brogna, Craniomed group Srl, Montemiletto, Italy

Reviewer comments and Brogna et al. responses (R.)

Brogna C et al. The first report on detecting SARS-CoV-2 inside human fecal-oral bacteria: A case series on asymptomatic family members and a child with COVID-19

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R: First, we are honored by the reviewer's comments on the fecal-oral route of transmission of the virus SARS-CoV-2 because they were our doubts at the beginning of the pandemic and were the reason we wanted to do the check on prokaryotic cells.

In fact, we found it really embarrassing that no major research group in the world has documented a positive or also negative control on the interaction between Coronavirus, in general in the last 30 years, and SARS-CoV-2 in the last 2 years and prokaryotic unicellular cells.

Only the famous diatribes on the occurrence of bacterial cofactor in HIV (RNA virus) infection can be found in the literature. We respect the reviewer's opinion on the epidemiology and transmission route of the virus, but we believe that in a balanced context, the oro-fecal route of transmission should also, and not only be considered. Reference 3 that the reviewer cites is a letter, a commentary by Pedersen et al. to the work of Guo et al. (7), a manuscript that collected a review of other works emphasizing the fecal-oral transmission pathway. Guo et al. (8) responded to the letter from Pedersen et al., including more recent work and reiterating that the oro-fecal route of transmission must be considered. In addition, it should be considered that historically the coronaviruses family, which afflict animals in general, have long been listed in the literature as viruses with an oro-fecal route of transmission (many studies are present in the literature). Our third paper (2) supported the addition of the oro-fecal transmission route, including many new studies in this regard after the publication of the commentary by Pedersen et al. Since the beginning of the pandemic, several authors have pointed out that SARS-CoV-2 has a close relative: the coronavirus RATG13 (9). RATG13 was discovered in 2013, and picked up by researchers following the deaths of 3 out of 6 miners working in caves in Mojiang county in Yunnan, China (10), as currently recognized. The miners worked in caves where bat guano (stool) was present, and it is really hard to imagine that in contact with such material, the transmission route was respiratory.

However, the reviewer pays attention to this issue, and in line with his opinion, we have modified the text by adding the word "also" or "possible" next to "transmission route" where this was possible.

The authors report viral RNA concentration determined through Luminex in arbitrary units (AU). However, my understanding is that Luminex can be set up to contain standards that reveal an absolute concentration of viral RNA. Given how viral RNA concentrations are central data to the conclusions in this work, I believe that reporting the absolute concentration of viral RNA is important. Additionally, this will make the observations here more replicable across labs and viral RNA detection techniques.

R: We thank the reviewer for this observation and have included the conversion table with the corresponding formula in the supplementary materials (s.m.), table 1s of paragraph 4, in the second section, "Extra sample processing and extra data. » *The total turn around time was around 4 h. Luminex detection was reported in arbitrary units given in accordance with Florida et al. (4 in s.m. and 11 here) (Line 63-64 s.m. and 265-269 s.m.)*

Regarding the reported viral RNA concentrations, it is unclear what the specificity, sensitivity, and detection range of the Luminex assay are as carried out by the authors. Therefore, I recommend the authors include relevant controls to estimate these. Reporting this information along with their experiments will add important validity and context to the data.

R: More details have been included in the supplementary materials (s.m.), paragraph 6 of the first part of Materials and Methods: "*Luminex technology (Life Technology, USA) (ref. 3 in s.m. and 12 here,) was used to detect the viral RNA load in bacterial cultures. The detection was performed by using NxTAG® CoV Extended Panel, a real-time reverse transcriptase PCR assay detecting three SARS-CoV-2 genes on the NxTAG-enabled System MAGPIX® instrument, and the AccuPlex™ SARS-CoV-2 Reference Material Kit (SeraCare) as reference standard with sequences from the SARS-CoV-2 genome.*"

Fig 3 is missing key controls. Recommend including a control of uninfected bacteria from a healthy donor stained with both α -SARS-CoV-2 nucleocapsid protein antibody and α -Gram-positive bacteria antibody.

R: We agree with the reviewer that controls, in general, are essential. That's why it seemed, first of all, absurd to us that no researcher had designed tests to check for interactions between coronaviruses and prokaryotic cells. This is why we emphasized that it is important to do different tests with different methods so that each is a control of the other, and that is what we did in Brogna et. al (2) and repeat in the supplementary materials, now added. For the required control with fluorescence vision of gram+ bacteria and lack of fluorescence for viral proteins in question, a sample of stool from an healthy 18-month-old child, checked negative by molecular testing, with healthy parents and never ill with SARS-CoV-2 at the time of collection, was used. This choice, a child's faeces, was made because, after two years of the pandemic, it is very difficult to find uninfected or asymptomatic carriers. However, as usual, we had not put in the panels in Figure 3 such control though we had done so. We thought the evidence in panel E of figure 3 would be sufficient. However, the reviewer's suggestion is valuable, and we add the required control (figure 3 panels F and G) and comment on it below:

A panel (F, G) was added with the required control, a culture of only bacteria from the feces of a healthy individual, an 18-month-old child to be exact. The sample was obtained with the consent of both parents. As can be seen, only gram+ bacteria are highlighted by fluorescence, although both primary antibodies have been included, both to gram+ and to the nucleocapsid protein of SARS-CoV-2. The sentence "... and panels F and G show a group of gram+ bacteria by fluorescence, derived from the stool bacteria culture of a healthy 18-month-old child (with healthy parents and never ill with SARS-CoV-2 at the time of collection and with and with their written consent) negative to molecular test to SARS-CoV-2, although the other primary antibody to the nucleocapsid protein is also included and does not show a red signal". Line 193-199

The authors conclude from the microscopy data that fecal bacteria are infected by SARS-CoV-2 in samples collected on day 30 from an adult patient, am1. Notably, this patient, along with the others in the study was prescribed probiotics with two Gram-positive bacteria (*Lactobacillus reuteri* and *Bacillus clausii*) in their diets for 30 days prior to sample collection. In the event that bacteria are in fact infected by SARS-CoV-2, it is unclear if this is an artefact of the probiotic supplements and treatment regime.

R: We apologize to the reviewer if we are not clear. The Luminex data at day 60 refer to subsequent stool sampling from family members, whereas the microscope images are from bacteria culture of the am1 sample taken at time zero and cultured, as detailed in the supplementary materials, for 30 days in the laboratory. In addition, the probiotic legend was included because it is required by the case series checklist guidelines but should not be understood as an indication of possible therapy. The use of probiotics was used only as an aid in view of the fact that all the literature now agrees that SARS-CoV-2-induced alteration of bacterial flora occurs. The words « *only as re-balancers of bacterial flora...* » are added. Line101 e 127

I encourage the authors to provide more methodological information in the manuscript. As a matter of principle, manuscripts should in and of themselves provide sufficient information for readers to repeat experiments without having to go down the rabbit hole of chasing down references to other works. The current manuscript exhibits this concern that is prevalent in scientific publications. Some instances of this in the current manuscript include -

“molecular testing for SARS-CoV-2 was performed by using Luminex technology as described by us previously” - It is unclear how the samples were prepared, if controls were included and what the parameters of this assay were.

Methodology used to isolate and culture bacteria from stool - It is unclear how bacteria were isolated from stool. This is important information to get a sense of how rigorous this method was, whether there are inherent biases regarding what bacteria are favored in the culturing process and if there are chances for other residual contaminants from the fecal sample.

Methodology used to collect and preserve stool samples - the majority of existing reports are unable to culture viable SARS-CoV-2 from fecal samples, even when the samples are preserved in Viral Transport Media (VTM). Therefore, clarity about how the current work collected and preserved samples are crucial to understanding how they have been successful at recovering viable viral particles.

R: We thank the reviewer for this valuable advice and apologize because it was not our intention to direct him to read our other work, but unfortunately, as the manuscript is set up as a case series, we had a predetermined number of words and checklist guidelines to follow. To overcome this problem, we suggested that he read our other papers, but the reviewer rightly asked for more details, and we have summarized the

materials and methods of our other studies in the supplementary materials (s.m.) now attached. Of course, the words are in italics because we are reporting exactly what we have already published, with author's permission.

Please forgive the reviewer if we take the liberty to give some advice, since this is an open review, to anyone who would like to start repeating or improving the published experiments. Virus (SARS-CoV-2)-positive stool samples can be stored at 4°C in a previously sterile container as soon as they are collected from the patient. Culture in a multipotent medium, performed to grow only bacterial cells and exclude other eukaryotic cells, started after the homogenization and inorganic debris removal procedures, is best performed within 24 to 48 hours. Therefore, good timing between clinical collection and processing in the appropriate laboratory is important. In addition, freezing the samples, although it results in the preservation of proteins and nucleic acids, ruins the viability of bacteria.

Variations to the presented methodology can be made, or protein expression can be assessed by other methods and quantified over time. Our humble opinion suggests that the genetic part should always be verified with the actual protein expression and ultimately with the microscopic view of the viral particle.

Integration of data (genetic, proteomic, microscopy) is essential to observe typical or atypical viral behaviour.

Minor suggestions:

Request citation and clarity on the statement - "several studies have reported....intestinal bacterial co-infection in COVID-19 patients".

R : we have added the references 9-11 (line 20) :

- Yeoh YK, Zuo T, Lui GC, Zhang F, Liu Q, Li AY, Chung AC, Cheung CP, Tso EY, Fung KS, Chan V, Ling L, Joynt G, Hui DS, Chow KM, Ng SSS, Li TC, Ng RW, Yip TC, Wong GL, Chan FK, Wong CK, Chan PK, Ng SC. Gut microbiota composition reflects disease severity and dysfunctional immune responses in patients with COVID-19. *Gut*. 2021 Apr;70(4):698-706. doi: 10.1136/gutjnl-2020-323020. Epub 2021 Jan 11. PMID: 33431578; PMCID: PMC7804842;
- Yamamoto S, Saito M, Tamura A, Prawisuda D, Mizutani T, Yotsuyanagi H. The human microbiome and COVID-19: A systematic review. *PLoS One*. 2021 Jun 23;16(6):e0253293. doi: 10.1371/journal.pone.0253293. PMID: 34161373; PMCID: PMC8221462 ;
- Wang, B., Zhang, L., Wang, Y. et al. Alterations in microbiota of patients with COVID-19: potential mechanisms and therapeutic interventions. *Sig Transduct Target Ther* 7, 143 (2022). <https://doi.org/10.1038/s41392-022-00986-0>

Request citation and clarity on the statement - "Observations of possible links between the animal gut microbial environment and coronaviruses have been reported over time". The current citations do not have evidence for a possible link to the gut microbial environment.

R : We have refreshed the final part of the phrases in : « *in some studies,*» and we have

added the references 16-20 (Line 31):

- Li HY, Li BX, Liang QQ, Jin XH, Tang L, Ding QW, Wang ZX, Wei ZY (2020) Porcine deltacoronavirus infection alters bacterial communities in the colon and feces of neonatal piglets. *Microbiologyopen* 9:e1036 ;
- Felten S, Klein-Richers U, Unterer S, Bergmann M, Leutenegger CM, Pantchev N, Balzer J, Zablotski Y, Hofmann-Lehmann R, Hartmann K. Role of Feline Coronavirus as Contributor to Diarrhea in Cats from Breeding Catteries. *Viruses*. 2022 Apr 21;14(5):858. doi: 10.3390/v14050858. PMID: 35632600; PMCID: PMC9143444 ;
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- Saif LJ. Bovine respiratory coronavirus. *Vet Clin North Am Food Anim Pract*. 2010 Jul;26(2):349-64. doi: 10.1016/j.cvfa.2010.04.005. PMID: 20619189; PMCID: PMC4094360)

Fig 1 reports "Viral load" although what is actually reported is the viral RNA concentration, which can be different from viral load because not all viral particles are lysed and provide RNA for detection, and some viral RNA is reported to come from non-viral reservoirs⁴.

R : We have nothing to add to this reviewer's comment because we are in perfect agreement. The authors of reference 4 (Alexandersen et al.), clarify that nasopharyngeal samples contain subgenomic sequences of SARS-CoV-2. Textually they write: *'The results described here fully support that SARS-CoV-2 genomic and subgenomic RNAs are present in diagnostic samples even in late infection/after active infection'* and *'The detection of subgenomic RNA is therefore not direct evidence of active infection instead its presence at lower levels than virion genomic RNA results in detection for a shorter period of time unless using, e.g., highly sensitive NGS'*.

Similarly, in the tables presented in figure 1, we do not want to indicate who among the family members in the presented case series is infectious or not because the story in the paper describes well that they are all asymptomatic, except for the little girl. In the paper, we never speculate on the correlation between viral charge and high infectivity. The tables only show how with the passage of a fairly long time (figure 1 C and D), 60 days, the presence of traces of viral RNA is no longer detected, in agreement with many contemporary studies. It should also be pointed out that reference 4, Alexandersen et al., describes the nasopharyngeal sampling as a viral RNA collection event, and readers and scientists should reflect that the sampling is performed on mucous membranes that are overabundant with bacteria and that it

cannot be excluded that subgenomic sequences in non-SARS-CoV-2 sufferers could have bacteria, also, as a reservoir.

We do not associate the viral load to the disease state, nor do we speculate on high viral replication and high infectivity. Viral load is now a usual term used in scientific work, and the accuracy of the reviewer is relevant, but at this point, should specify the wording of many of the works currently published. However, we corrected everywhere the legend of figure 1 and other phrases in "RNA viral Concentration."

The manuscript calls for major editorial revisions given numerous improper usages of phrases. For instance - a) There are no "fecal-oral bacteria", which suggests specific bacteria in the feces are able to be transmitted orally. b) It is unclear what "Bacterial feces" means. c) "RNA viral load count" appears miswritten.

**R : a) we apologize to the reviewer. We want to say bacteria from the gastrointestinal tract, and we replaced the phrase with "bacteria of the human gut microbiome."
b) The sentence was rewritten: « *The feces of this patient was cultured in bacterial culture media and after 30 days, the pellet of bacteria, have been analyzed by TEM, immune-EM, and by fluorescence microscopy, and a set of obtained images is shown in Figure 2 (for more details see supplementary material-s.m.)*». Line 133-136
c) The word count is a typo, and we followed the reviewer's suggestion and pointed out that it is RNA concentration**

Additional notes:

I don't have experience with interpreting TEM images and therefore leave this comment here rather than as a cause for major revision. TEM images are complex requiring the appropriate collection and processing of samples, the inclusion of controls, and careful interpretation of observations. The improper use of EM to study SARS-CoV-2 from tissue samples has been a cause for concern^{5,6}. To my untrained eye, Fig 2 seems to be another example of misinterpreted EM data because -

If Fig 2a in fact displays SARS-CoV-2 viral particles inside a bacterium, it is surprising to me that the bacterial host appears to be entirely filled by viral particles with no room for its essential, native molecules. To me, this figure seems like that of the widely described multivesicular bodies that are unrelated to SARS-CoV-2 and yet regularly misinterpreted⁶. The figures don't appear to have sufficient resolution to highlight the ultrastructures typical of SARS-CoV-2 that are required to unmistakably identify viral particles⁵.

If Fig 2b in fact presents the case of a bacteria with viral particles around it, it merely appears like viral particles proximal to the bacteria in this image, with no evidence that these viruses in fact infected the bacteria. The conclusion that viral particles are found in the extracellular matrix of gut bacteria is an over-reach without sufficient evidence.

R: We thank the reviewer for finally raising the same issue as the public comment to

the present manuscript left by Dr. Michael Laue. We will disregard the fact that he states that he is not an expert in electron microscopy but we respectfully respond to his objections:

The article by Dittmayer and Laue et al. (5) that both we cite in the text of the manuscript and the reviewer cites in support of the misinterpretations that can be made during sample processing and during the interpretation of viral particles should be clarified that the authors emphasize the importance of matching, i.e., associating the EM image with genetics as well, attesting to the presence of the viral pathogen of interest or vice-versa. They are the ones who make it clear that imaging must be supported by genetic or protein testing, and in fact, they quote exactly that: "...*the presence of SARS-CoV-2 particles (figure A-D EM) complements the molecular traces of SARS-CoV-2s specific proteins or nucleic acids*". Our study demonstrated exactly that: EM images of virus-like particles combined with genetic confirmation of the presence of SARS-CoV-2, immunofluorescence microscopy with antibodies, and other tests, now described in the supplementary materials (s.m.). The addition of immunofluorescence images highlighting both GRAM+ bacteria and fluorescence of the nucleocapsid protein of SARS-CoV-2 is also important. However, as a final note, we have added in the supplementary materials (s.m.) many other evidences, including that of nitrogen isotope, added to the culture medium, which marks the proteins of SARS-CoV-2 (2) and lays a stable stone to our experiments.

With due respect to the reviewer, it should be noted that citation #6 (Calomeni E. et al.), taking as a reference to highlight how SARS-CoV-2 particles can be confused with multivesicular bodies, states the following: "*MVBs were always been identified in podocytes (1 to 4 podocytes per glomerulus), but we have not seen them in tubular epithelial cells. MVBs were occasionally observed in endothelial cells (mainly arterial or arteriolar) and in a parietal glomerular epithelial cell of biopsy.*"

MVBs theoretically may represent podocyte endocytosis with subsequent formation of intracytoplasmic microvesicles resembling viruses." " However, microvesicles are commonly "free-floating" in the cytoplasm of many cell types, including tubular epithelial cells."" caution is suggested when identifying a virus by EM in tissue sections."

In other words, MVBs are typical formations in animal tissues, not found in bacterial cultures. They cannot be confused with viral particles that we show in bacterial cultures even light-years away. They are by no definition at all associable with the viral particles we show in Figure 2A and 2B. They are completely different situations and two different substrates so there is really no basis for confusing the viral particles we show with the MVB.

It was made clear in the text that the images are from a 30-day culture of prokaryotic cells, bacteria, and that there are no mammalian cells; furthermore, Figure 2B, even for a non-expert in microscopy, cannot be misunderstood as it is an image showing a bacterial lysis phase with viral particles around it. In the work of Bullock et al., (13) cited in the comment by Dr. Laue just in Figures 1C and 2C, are clearly visible viral

particles indicated by the authors as coronavirus particles perfectly identical to those we show inside the bacterium in our Figure 2A and around the lysed bacterium in Figure 2B. Moreover, the authors cite the following sentence to demonstrate that *the protein spike, unless a special preparation with tannic acid is used, is almost never visible in microscopic images as they report:*" (ii) Coronaviruses do have projections on the surface; however, in thin sections, the "spikes" on the outside are not always (indeed, not usually) clearly visible, unless specially stained (e.g., with tannic acid). They may or may not appear as a very short 'fuzz.'" The ribonucleoprotein, with all due respect and contrary to Dr. Laue's comments, is clearly visible, just like that shown in the works of Dittmayer, Laue, et al. (5), and Bullock et al. (13).

The Aurion particles of 10 nm may overlap, but in the supplementary materials (s.m.), we show Figure 2s where the same test was repeated with the pre-ebbing technique in another facility and also satisfies this aspect challenged in the comments. (line 176- 194s.m.).

With due respect to the reviewer, all supporting data, including those added in the supplementary materials, dispel doubts shown, and it is still worth noting that the evidence of the nitrogen radioisotope found in the viral proteins after adding the element in the 30-day bacterial culture dispels all doubt. The data should be integrated and analyzed together, and not considered individually.

The figure is missing controls such as stained and unstained images of comparable bacteria without the presence of the alleged SARS-CoV-2 viral particles. Given these observations and the importance of this figure to the central tenet of this manuscript, I recommend the editorial team seeks input from an expert in TEM.

R : In Image 3, we added panels F and G with only controls on gram+ bacteria in the absence of SARS-CoV-2. The objections made by the reviewer seem identical to those in the commentary by Dr. Michael Laue, an imaging expert. Responding to the reviewer, we also questioned Dr. Michael Laue's objections.

We remind the reviewer that these are the first images of coronavirus in bacterial cultures. There are no other comparisons in the current literature, so any speculation on the present manuscript finds as debate the serious shortcoming of the failure to control infectivity first on prokaryotic cells, which are much more abundant above the epithelial layer where virus-receptor interaction takes place. What should be done before proclaiming for certain and establishing some possible pathogenetic mechanisms of infectivity, i.e., some possible viral protein and epithelial cell receptor binding interactions, is the control between coronaviruses in particular but also other RNA viruses in general and bacterial cells, much more abundant, it bears repeating, within the human microbiome. Moreover, even if an interaction does not take place, control also over bacterial metabolism and what they produce (2,14) in the presence of a novel viral pathogen should in no way be excluded. This, too, is a serious deficiency in possible controls.

The hypothesis that SARS-CoV-2 may infect bacteria is both biologically fascinating and clinically highly relevant.

R: We apologize to the reviewer: but with due respect: it is no longer a hypothesis but new observations (ref 2,14, 15).

We thank the reviewer for giving us an opportunity to engage in constructive scientific debate and to improve the present paper by highlighting what he thought was right and by improving what he thought was appropriate.

This is because there are no examples that I know of or can find of viruses that affect humans that can also retain the molecular machinery to affect bacterial cells. Therefore, the idea that this is possible is biologically fascinating and can pave the path to many follow-up studies. Further, SARS-CoV-2 continues to be a major threat, having claimed over 6 million people worldwide. Therefore, careful evaluation of the potential sources of future outbreaks is critical from a personal and public health standpoint. If in fact bacteria in the gut can harbor infectious SARS-CoV-2 viruses, this adds to another dimension of the COVID-19 pandemic that calls for urgent precautionary measures. Unfortunately, the current manuscript is missing many controls and methodological details, and has insufficient data to make the case that bacteria are in fact infected by SARS-CoV-2. Given the hugely significant impact that concluding gut bacteria can be infected by SARS-CoV-2 through a bacteriophage-like property can have to humanity, I encourage the authors and editorial team to exercise caution and responsibility in promoting this conclusion with insufficient evidence.

R : We believe that the reviewer now has much more clarification, but may notice more information about the concepts laid out and the importance of doing the checks between viruses and bacteria, to avoid epidemiologically and pathogenic misdiagnoses in the article under review also on F1000--Petrillo M, Querci M, Brogna C *et al.* Evidence of SARS-CoV-2 bacteriophage potential in human gut microbiota [version 1; peer review: awaiting peer review]. *F1000Research* 2022, 11:292 (<https://doi.org/10.12688/f1000research.109236.1>)

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2. Brogna C, Brogna B, Bisaccia DR, Lauritano F, et al.: Could SARS-CoV-2 Have Bacteriophage Behavior or Induce the Activity of Other Bacteriophages?. *Vaccines (Basel).* 2022; 10 (5). [PubMed Abstract](#) | [Publisher Full Text](#)
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 11. Florida, M. & Cristoni, S. PROSAD: A powerful platform for instrument calibration and quantification. *Rapid Commun. Mass Spectrom.* 2014. doi: <https://doi.org/10.1002/rcm.6808>.): ARB= $[\Sigma(SI*NF*COF) / \Sigma(NF*COF)]$ where SI: Signal intensity converted to Counts/s; NF: Noise Factor; COF: Correction Factor
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Competing Interests: No competing interests were disclosed.

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Reviewer comments -

1. The Figure 1B legend needs to be properly written or displayed.
2. Figure 1C, correct the spelling of culture as in Bacteria culture (legend).
3. In Table 1, data pertaining to the nasopharyngeal swab test need to be presented for all the subjects.
4. "At day 30 of bacterial culture of feces patient am1, the Luminex molecular test confirmed the presence of SARS-CoV-2 and the RNA viral load count was increased from 24 arbitrary unit (AU) (initial) to 520 AU (Final) (Figure 1B)..." – The figure description needs to be better (Figure 1B). The authors should better describe whose fecal sample was used in the culture in the figure description for 1B.
5. "Bacterial feces of this patient, after 30 days of bacterial culture, have been analyzed by TEM, immune-EM, and by fluorescence microscopy, and a set of obtained images is shown..." – Instead of bacterial feces, change to feces of this patient was cultured in bacterial culture media.
6. Figure 2D is not clear to this reviewer.
7. "In addition, the RNA virus could be present in the 48,1% of patients who were negative to OP/NP swab tests until 33 days." – Please correct the % depiction.
8. "Here, we report the case of a symptomatic child for COVID 19, brought to her by one of the parents, whose family members had negative results with rapid antigen nasopharyngeal swab test." – Please correct the grammatical error.
9. This reviewer has reservation on the "fecal-oral" transmission route being used by the authors. This paper and the earlier paper on this topic by the authors showed possible replication of the SARS-Cov2 virus like particles in bacterial culture. But neither observations prove the route of the viruses coming into the feces. As far as this reviewer is concerned, we still do not know how the virus gets to the gastrointestinal tract. So, instead of "fecal-oral" bacteria, gastrointestinal bacteria may be written.
10. "This surprising finding allows us to better clarify the first fecal-oral transmission of the virus and clearly shows that the reservoir of the virus is neither adults nor children but simply bacteria." – This reviewer does not agree with this statement especially the second half of it.

Overall, this is an interesting finding and corroborates the earlier report of the same author (F1000Res. 2021 May 11; 10:370) about the presence of the SARS-Cov2 like virus particles in bacterial culture. This report is an advancement as some evidence of the presence of the virus like particles have been shown using TEM, immune-fluorescence microscopy. The presence of SARS-Cov2 in feces has been documented before and as such this report does not add anything new to this however what is interesting is the replication potential of the virus in bacterial culture. The authors might want to provide more assays and evidence to showcase the “phage-like” activity of the SARS-Cov2 as they propose.

Finally, please crosscheck the text. Lots of grammatical and contextual errors are found, some of which have been highlighted above.

References

1. Petrillo M, Brogna C, Cristoni S, Querci M, et al.: Increase of SARS-CoV-2 RNA load in faecal samples prompts for rethinking of SARS-CoV-2 biology and COVID-19 epidemiology. *F1000Res.* 2021; **10**: 370 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the background of the cases' history and progression described in sufficient detail?

Partly

Are enough details provided of any physical examination and diagnostic tests, treatment given and outcomes?

Partly

Is sufficient discussion included of the importance of the findings and their relevance to future understanding of disease processes, diagnosis or treatment?

No

Is the conclusion balanced and justified on the basis of the findings?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: gut microbiome

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 10 Mar 2022

Carlo Brogna, Craniomed group Srl, Montemiletto, Italy

Dear Dr. Debojyoti Dhar,

Thanks a lot for your valuable comments and suggestions that you have provided in the report.

We will address all of them, together with those of other reviewers, in order to provide a

fully revised version of the manuscript.

Best regards,
Carlo Brogna, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Author Response 17 Jul 2022

Carlo Brogna, Craniomed group Srl, Montemiletto, Italy

Reviewer comments and Brogna et al. responses (R.)

First of all, we apologize to the reviewer for responding only now, but we also waited for the opinion of a second reviewer who arrived in early July so that we could review the manuscript only once.

1. The Figure 1B legend needs to be properly written or displayed.

R: the legend has been replaced in the following form: "This is the positive control of patient am1's bacteria derived from a stool sample, after 30 days of bacterial culture using our previously published method (21), performed with the Luminex molecular assay. The molecular assay reported a viral RNA concentration growth of up to 520 AU (arbitrary unit)." Line 94-99

1. Figure 1C, correct the spelling of culture as in Bacteria culture (legend).

R: Done. Line 97

1. In Table 1, data pertaining to the nasopharyngeal swab test need to be presented for all the subjects.

R: We have added again the corrected table 1, in which there is the column with the data request.

1. "At day 30 of bacterial culture of feces patient am1, the Luminex molecular test confirmed the presence of SARS-CoV-2 and the RNA viral load count was increased from 24 arbitrary unit (AU) (initial) to 520 AU (Final) (Figure 1B)..." – The figure description needs to be better (Figure 1B). The authors should better describe whose fecal sample was used in the culture in the figure description for 1B.

Done. Line 94-99

1. "Bacterial feces of this patient, after 30 days of bacterial culture, have been analyzed by TEM, immune-EM, and by fluorescence microscopy, and a set of obtained images is shown..." – Instead of bacterial feces, change to feces of this patient was cultured in bacterial culture media.

R: We have changed as suggested in: "The feces of this patient was cultured in bacterial culture and after 30 days, the pellet of bacteria....." Line 133-137

1. Figure 2D is not clear to this reviewer.

R: We apologize with the reviewer and have clarified our mistake in the legend, describing figure 2D: "(D) negative control of bacterial stool culture of a healthy person

after 30 days, without primary antibody with only the secondary antibody.” Line 174-176

1. “In addition, the RNA virus could be present in the 48,1% of patients who were negative to OP/NP swab tests until 33 days.” – Please correct the % depiction.

R: We apologize to the reviewer and thank him for pointing this out to us. There was an error in bibliography #48, which we have replaced correctly, and where the proposed data are now visible. For clarity, we rephrase the sentence in the text as follows:

“In a Systematic Review and Meta-analysis, at the beginning of the pandemic, it was observed that viral RNA was present in the stool in 48,1% of patients during the disease and that 70,3 % of patients had prolonged shedding that could extend beyond 33 days from the onset of the disease.” (2 here, 48in the manuscript). Line 264-267

8, “Here, we report the case of a symptomatic child for COVID 19, brought to her by one of the parents, whose family members had negative results with rapid antigen nasopharyngeal swab test.” – Please correct the grammatical error.

R: We replaced it with: “Here we report the case of a child symptomatic for COVID 19, transmitted by one of the parents, whose relatives had tested negative on the rapid antigenic nasopharyngeal swab test.” Line 2826-287

This reviewer has reservation on the “fecal-oral” transmission route being used by the authors. This paper and the earlier paper on this topic by the authors showed possible replication of the SARS-Cov2 virus like particles in bacterial culture. But neither observations prove the route of the viruses coming into the feces. As far as this reviewer is concerned, we still do not know how the virus gets to the gastrointestinal tract. So, instead of “fecal-oral” bacteria, gastrointestinal bacteria may be written.

R: We appreciate the reviewer’s critical viewpoint. In our recent paper published in ref. 3, we supported this possible additional transmission pathway. Still, the reviewer’s suggestion is very cautious, and in agreement with him, we have chosen to replace the term fecal-oral bacteria with “bacteria of the human gut microbiome”. We hope he agrees.

“This surprising finding allows us to better clarify the first fecal-oral transmission of the virus and clearly shows that the reservoir of the virus is neither adults nor children but simply bacteria.” – This reviewer does not agree with this statement especially the secondhalf of it.

R: With all due respect to the reviewer and his valuable help in improving this article, let us argue our opinion in a few lines. If we consider how many sites report the presence of viral RNA in wastewater (4-6), as described in the current literature, although everyone knows that RNA by definition is an easily degradable (7) molecule, and if we consider how the first six miners in the Yunnah caves in China, in close contact with bat guano (feces), became ill with CORONAVIRUS RATG13 pneumonia (8-9), and furthermore if we also consider our recent work Brogna et al. (3). where we performed many other tests, we can more confidently assert that bacteria *could*

represent a *potential reservoir* of the virus and, as in the work cited above, we argued that it could behave as both a lytic and lysogenic bacteriophage. However, with the caution suggested by the reviewer, we have changed the last sentence to: *"This first observation invites us to pay more attention to the fecal-oral transmission route of the virus and suggests as a further possible reservoir of the virus also the bacteria of the human gut microbiome."* Line 298-300

Overall, this is an interesting finding and corroborates the earlier report of the same author (F1000Res. 2021 May 11; 10:370) about the presence of the SARS-Cov2 like virus particles in bacterial culture. This report is an advancement as some evidence of the presence of the virus like particles have been shown using TEM, immune-fluorescence microscopy. The presence of SARS-Cov2 in feces has been documented before and as such this report does not add anything new to this however what is interesting is the replication potential of the virus in bacterial culture. The authors might want to provide more assays and evidence to showcase the "phage-like" activity of the SARS-Cov2 as they propose.

R: We can now reassure the reviewer on this point because the much-required evidence has been made public in the publications of Brogna et al. and Petrillo et al. (3,10), and we added some of the other results in the supplementary materials in which we have added evidence of nitrogen isotopic labeling also in viral particle proteins..

The reviewer's comments and corrections were invaluable in improving this work. We thank them for making the work solid.

Finally, please crosscheck the text. Lots of grammatical and contextual errors are found, some of which have been highlighted above.

The work was reviewed by a native speaker.

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 1

Author Response 10 Mar 2022

Carlo Brogna, Craniomed group Srl, Montemiletto, Italy

Dear Dr. Michael Laue,

Thanks a lot for your valuable comments and suggestions.

We will consider them with a revised version of the manuscript, which we will provide to address

the comments and suggestions of the reviewers.

Best regards,
Carlo Brogna, on behalf of the authors.

Competing Interests: No competing interests were disclosed.

Reader Comment 26 Feb 2022

Michael Laue, Robert Koch Institute, National Consultant Laboratory for Electron Microscopy of Infectious Pathogens (ZBS 4), Berlin, Germany

Dear colleagues,

I must comment on the ultrastructural data shown in Fig. 2 which should prove the presence of coronavirus particles. As for all objects identified by electron microscopy, images need to demonstrate sufficient specific structural detail to prove their identification. In Fig.2 A, B, the authors assigned vesicular structures as SARS-CoV-2 which do not show relevant structural detail of coronavirus particles, such as characteristic surface spikes and a granular interior representing the ribonucleoprotein (see e.g. doi 10.1016/j.kint.2021.01.004 or doi 10.1016/S01406736(20)320791 for reference). The immunogold data are also not convincing. Firstly, appropriate controls are not reported. Omission of the primary or secondary antibodies are not appropriate (see e.g. doi 10.1177/002215540004800201 or the book on Fine-structure immunocytochemistry by Gareth Griffiths, Springer 1993). The presumed gold particles shown in Fig. 2C are much larger than 10 nm, which should be the size of the gold colloid bound to the secondary antibody. Even if we consider clustering of the label, the distinct size of the gold particles usually provided by Aurion should be visible. Moreover, it is not clear to which structures the gold labels are associated. It is the strength of immunogold labelling to show both, the gold label and the structures of interest. In my opinion the data presented are not sufficient to prove the presence of SARS-CoV-2 particles in bacteria.

Competing Interests: I declare no competing interests

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