TITLE: **INCREASE OF SARS-COV-2 RNA LOAD IN FAECAL SAMPLES PROMPTS FOR RETHINKING OF SARS-COV-2 BIOLOGY AND COVID-19 EPIDEMIOLOGY**

AUTHORS

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SUMMARY

Scientific evidence for the involvement of human microbiota in the development of COVID-19 disease was reported recently. We elaborated these findings further and collected data on the relationship between faecal bacteria, isolated from stool from COVID-19 patients, and SARS-CoV-2. The preliminary results suggest that SARS-CoV-2 replicates in bacterial growth medium inoculated with a stool sample from an infected patient and that the replication follows bacterial growth.

These results are unexpected and when confirmed on large sample sizes hint towards novel hypotheses on the biology of SARS-CoV-2 and on the COVID-19 epidemiology.

The data reported here suggest a possible 'bacteriophage-like' behaviour of SARS-CoV-2, which to our knowledge was never observed or described before. The discovery of possible new modes of action of SARS-CoV-2 has far-reaching implications for the prevention and the treatment of the disease, necessitating quick sharing of even preliminary findings with the global scientific community.

INTRODUCTION

Recent papers and reviews [1–3] discuss the relationship between gastrointestinal microbiota and COVID-19 disease. In particular, the prolonged presence of SARS-CoV-2 viral RNA in human faecal samples from COVID-19 patients was recently reported[4] and the potential role of orofecal transmission of SARS-CoV-2 was recently examined in a systematic review[5]. SARS-CoV-2 faecal viral activity was depicted in association with gut microbiota composition in patients with COVID-19[6], and the live virus was detected in faeces[7]. At the same time, Woefel et al.[8] reported that 'infectious virus was readily isolated from throat- and lung-derived samples, but not from stool samples in spite of high virus RNA concentration', while Yao et al.[9] reported that 'SARS-CoV-2 is capable of replicating in stool samples', indicating that the detailed biology of SARS-CoV-2 in faeces is not fully elucidated. Our experiments further explored the relationship between COVID-19 disease and SARS-CoV-2 infected faeces to provide data relevant for pandemic understanding and disease management. The results however did not correspond with current thinking of the epidemiology of SARS-CoV-2 and therefore we believe a quick sharing with the scientific community of our findings is imperative.

ESPERIMENTAL DESIGN

We have first inoculated NutriSelect™ Plus nutrient broth, fit for the growth of more fastidious bacteria, with a faecal sample (stool) from one patient positive to SARS-CoV-2 and from one healthy individual (here called sample A and sample B, respectively).

After seven days, the presence of SARS-CoV-2 RNA in both samples was assessed by Luminex technology (NxTAG® CoV Extended Panel, a real-time reverse transcriptase PCR assay detecting three SARS-CoV-2 genes was used on the MAGPIX® NxTAG-enabled System MAGPIX instrument; signal acquisition was performed using the xPONENT and SYNCT software, Luminex Molecular Diagnostics), using a commercially available reference standard with sequences from the SARS-CoV-2 genome (AccuPlex™ SARS-CoV-2 Reference Material Kit, SeraCare). SARS-CoV-2 RNA was found in sample A but absent from sample B (see further).

Subsequently, an aliquot of sample A, containing a viral RNA load equivalent to 240 arbitrary units (ARB) determined by using the PROSAD methodology described by Floridia *et al.*[10] was centrifuged at 13,000 g. An amount of supernatant containing 180 ARB was inoculated in half of sample B (sample $B_{(A+)}$) while the pellet was re-suspended (sample C). All the samples (A, B, $B_{(A+)}$ and C) were incubated for 30 days under the same conditions in NutriSelect™ Plus nutrient broth, at 37°C, and the viral RNA load was measured for each sample at days 1, 2, 3, 7, 14, 21, and 30 following the date of inoculation (day 0).

On day 21, 18 aliquots from sample $B_{(A+)}$ were subject to further analysis. The SARS-CoV-2 RNA load was measured in each aliquot, and then a specific antibiotic (each of the following: metronidazole, clindamycin, lincomycin, piperacillin+tazobactam, vancomycin, amoxicillin, ampicillin, cefixime, ceftriaxone, meropenem, rifaximin, azithromycin, erythromycin, gentamicin, ciprofloxacin, colistin, levofloxacin, and teicoplanin) was independently added to each aliquot. After 3 days all aliquots were tested for SARS-CoV-2 RNA presence by Luminex technology.

In all samples and all aliquots of sample $B_{(A+)}$, bacterial growth and metabolic activity were analysed and monitored over time using SANIST Biotyper according to the method described by Cristoni *et al.*[11].
The details of the experimental design are reported as Materials and Methods in the Supplementary Materials file.

RESULTS

Extra-corporal SARS-CoV-2 RNA load *in vitro* highly increased over time in sample $B_{(A+)}$, slightly increased in sample A, decreased in sample C while, as expected, sample B was found constantly negative (Figure 1).
Samples A, $B_{(A+)}$ and C were found to contain some bacterial genera particularly abundant and metabolically active during the whole experiment as shown in Figure 2.
Aliquots of sample $B_{(A+)}$ tested after three days of culture in the presence of the single different antibiotics belonging to different classes (listed in Figure 3) were analysed and the SARS-CoV-2 RNA load measured in each of them.

Three days after the delivery of the different antibiotics, the SARS-CoV-2 RNA load was found to be influenced by their presence in different ways (Figure 3):

- SARS-CoV-2 RNA load was reduced to negligible levels in the four aliquots treated with metronidazole, vancomycin, amoxicillin and azithromycin, respectively;
- SARS-CoV-2 RNA load decreased by 20 % to 85 % in the aliquots treated with piperallicin+tazobactam, ampicillin, cefixime, ceftriaxone, meropenem, gentamicin, ciprofloxacin and teicoplanin. For example, cefixime induced a decrease of viral RNA load of 85%, ciprofloxacin of 61% and teicoplanin of 56%;
- SARS-CoV-2 RNA load did not substantially decrease in the aliquots treated with clindamycin, lincomycin, rifaximin, erythromycin, colistin and levofloxacin.

An evaluation on potential release of toxic metabolites in the cultures is ongoing. Preliminary evidence indicates their presence and that they are completely reduced to negligible levels in the aliquots treated with metronidazole and vancomycin administration (data not shown, *in preparation*).
These results need to be carefully interpreted, taking into account the different antimicrobials kinetics.

DISCUSSION

Although based on a single observation, our results suggest that the SARS-CoV-2 genome, or parts thereof, in addition to its known interactions with eukaryotic cells, is capable of replicating also outside the human body, insinuating a possible 'bacteriophage-like' mode of action. It is not clear whether the SARS-CoV-2 genome could just be replicated by its RNA polymerase (which would correspond to a bacteriophage pseudo-lysogenic mechanism), or if the production of full-blown SARS-CoV-2 viruses within the bacteria occur (which would correspond to the typical lytic cycle of bacteriophages). Anyhow, according to our knowledge, this is new and never described before for SARS-CoV-2.
Undoubtedly, results based on a single experiment have strong limits. Accordingly, additional experimental confirmation is ongoing. Preliminary results from three

independent replications of exactly the same experiment with the same samples A and B (day 10 at time of writing) show the same trend and lead to the same observations.
An even larger experiment using different samples in different combinations is also planned, aimed also at characterising further which bacterial species are candidate target(s) of the observed behaviour of SARS-CoV-2.
The experimental design followed and presented here was targeted to grow bacterial cells. However, the interaction between SARS-CoV-2 and other eukaryotic cells present in the stool samples, *in primis* human cells, could also be possible.
The possibility that our findings are in reality due to SARS-CoV-2 replicating in human cells present in the original faecal samples, was considered. The human cells most abundantly present in faecal samples are colonic epithelial cells (colonocytes). Loktionov in his review[12] reported that *'cell exfoliation from colonic epithelium appears to be a relatively rare event in normal conditions but its rate dramatically increases in neoplasia when cell removal by apoptosis in situ does not function properly'*. In addition, Iyengar et al.[13] reported that colonic epithelial cells terminally differentiated are devoid of proliferative activity. More recently, Nair et al.[14] and Chandel et al.[15] developed specific methodologies to recover viable colonocytes from stool. In our case, both sample A and B originated from adult individuals with no cancer and, in addition, it is unlikely that human cells potentially present in samples A and B are able to:

- grow in a culture medium typically formulated for bacteria and not containing growth factors, serum nor other important components for eukaryotic cell sustainment;
- survive in such a medium for 30 days, and in co-occurrence with an event of SARS-CoV-2 infection;
- multiply in the absence of specific CO_2 concentration conditions (5%).

Also the possibility of interaction between SARS-CoV-2 and other eukaryotic organisms present in the cultures, as e.g. parasitic nematodes has been considered. During the whole experiment, parasitic nematodes were not noted at visual inspections by eye. In addition, stool of sample B was certified to be 'parasite free'. Parasitic nematodes are usually not able to survive outside the host and many intestinal roundworms (like those of genus *Ascaris*) release antimicrobial factors that interfere with bacterial growth[16], in contrast with the found high increase of metabolic activities of some bacterial genera. Finally, in the used medium, chemical elements relevant for (parasitic and not) nematodes (e.g. cholesterol and traces of metals) are missing. If on the one hand, the possibility that a nematode or another unknown parasite is able grow in the medium cannot be excluded, the used conditions make this possibility very unlikely. Anyhow, the ability of SARS-CoV-2 to interact with nematodes has never been observed before and would be a novel observation as well.
Finally, the possibility of involvement of the mycobiome fraction present in the stool was considered. As reported by Chin et al. in [17], *'more multifaceted and multidisciplinary approaches have to be adopted to identify uncultivatable or low abundance fungi in the gut, to characterize the fungal species and strain diversity in the gut, and also to differentiate permanent and transient fungal species that reside in the gut'*, confirming that the human mycobiome is not yet fully characterised. Accordingly, while the ability of unknown fungi to grow in the used culture medium cannot be excluded, no significant metabolic activity of *Candida albicans,* most commonly found in the microbiome, was observed. Anyhow, like for nematodes, the ability of SARS-

CoV-2 to interact with fungal cells has never been observed before and would be surprising as well.

These results can potentially raise the epidemiology of SARS-CoV-2 to new insights with additional challenging aspects to be investigated and clarified, as e.g. the detailed understanding of such potential 'bacteriophage-like' behaviour and its relationship with SARS-CoV-2 mode of action on eukaryotic cells. Considering the possible impact and implications that such relationship has on the manifestation, therapy and control of COVID-19 disease, some questions immediately arise like e.g.:

- Can this 'bacteriophage-like' behaviour of SARS-CoV-2 explain the long-term presence of SARS-CoV-2 observed in some recovered patients[18]?
- Can antibiotics and/or bacteriophage-based therapies play a role in the treatment of COVID-19 affected patients[19]?
- How would the (antecedent) administration of antibiotics to patients, influencing the microbiota population, impact the clinical course of the disease[20]?
- Can the involvement of bacteria in COVID-19 epidemiology help to explain clinical observations, like the elevated serum C-reactive protein, procalcitonin, D-dimer, and ferritin associated with poor outcomes in COVID-19[21]?

These questions are only examples of the plethora of questions to be addressed. Our preliminary results support the way to tackle COVID-19 pandemic proposed by Mushi[22], i.e. by using the holistic One Health approach. If individuals are considered not only human bodies, but as 'holobionts', i.e. discrete ecological units that need to be studied and treated as such, a deeper understanding of the role of the microbial community living in the human body is fundamental to tackle COVID-19 disease, and not only.

DECLARATIONS

The scientific output expressed does not imply a policy position of the European Commission. Neither the European Commission nor any person acting on behalf of the Commission is responsible for the use that might be made of this publication.

INTELLECTUAL PROPERTY - PATENTS & COPYRIGHTS

The use of antibiotics has been deposited as pending patent (ITA 102020000022414) by S. Cristoni and C. Brogna.

The method to use faecal samples as source of viruses has been deposited as pending patent (ITA 102020000022519) by C. Brogna.

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FIGURES AND LEGENDS

Figure 1: SARS-CoV-2 RNA load variation over time.

SARS-CoV-2 RNA load measurements (reported as ARBs, see Supplementary Materials) of samples A (blue bars), B (orange bars), $B_{(A+)}$ (red bars), and C (azure bars) grown for thirty days, all under the same conditions, after inoculation of supernatant from sample A into sample B (day 0). SARS-CoV-2 RNA load in sample $B_{(A+)}$ showed a power increase trend over time (as shown in the small frame on top-left), slightly increased in sample A, and decreased in sample C. As expected, sample B was found constantly negative.

Figure 2: Bacteria genera

The presence of bacteria genera was monitored over time by looking at their metabolic activity as described by Cristoni et al.[11]. Measures on Y-axis are reported as "detection frequency" (range 0-10). The three charts report the most metabolically active genera identified together with the "generic bacterial gut flora" (representing other bacterial genera not classified by the instrument) at day 0, 1, 7, 14, 21, and 30 for samples A, $B_{(A+)}$ and C, respectively. Other microbial organisms were observed at low levels (2 or less after one week) and not reported in the figure: *Mycobacterium, Actinobacteria, Bacteroidetes, Blautia, Brevibacterium, Brevundimonas, Candida (C. albicans), Collinsella, Enterococcus, Eubacterium, Klebsiella, Lactonifactor, Microbacterium, Porphyromonas, Propionibacterium, Sphingomonas, Stenotrophomonas, Streptococcus gordonii, Xanthomonas*.

Figure 3: Effect of antibiotics on viral load.

SARS-CoV-2 RNA load measurements (reported as ARBs, see Supplementary Materials, *in preparation*) of eighteen aliquots pre- (red) and post- (three days, green) treatment with the following selection of antibiotics (ABX): Metronidazole (class: Azoles); Clindamycin, Lincomycin, Piperacillin+Tazobactam, Vancomycin (class: Carboxylic acids and derivatives); Amoxicillin, Ampicillin, Cefixime, Ceftriaxone, Meropenem (class: Lactams); Rifaximin (class: Macrolactams); Azithromycin, Erythromycin, Gentamicin (class: Organooxygen compounds); Ciprofloxacin, Colistin, Levofloxacin (class: Quinolines and derivatives); Teicoplanin (semisynthetic glycopeptide antibiotic). SARS-CoV-2 RNA load is reported as preABX-postABX variation in percentage.

Graphical abstract

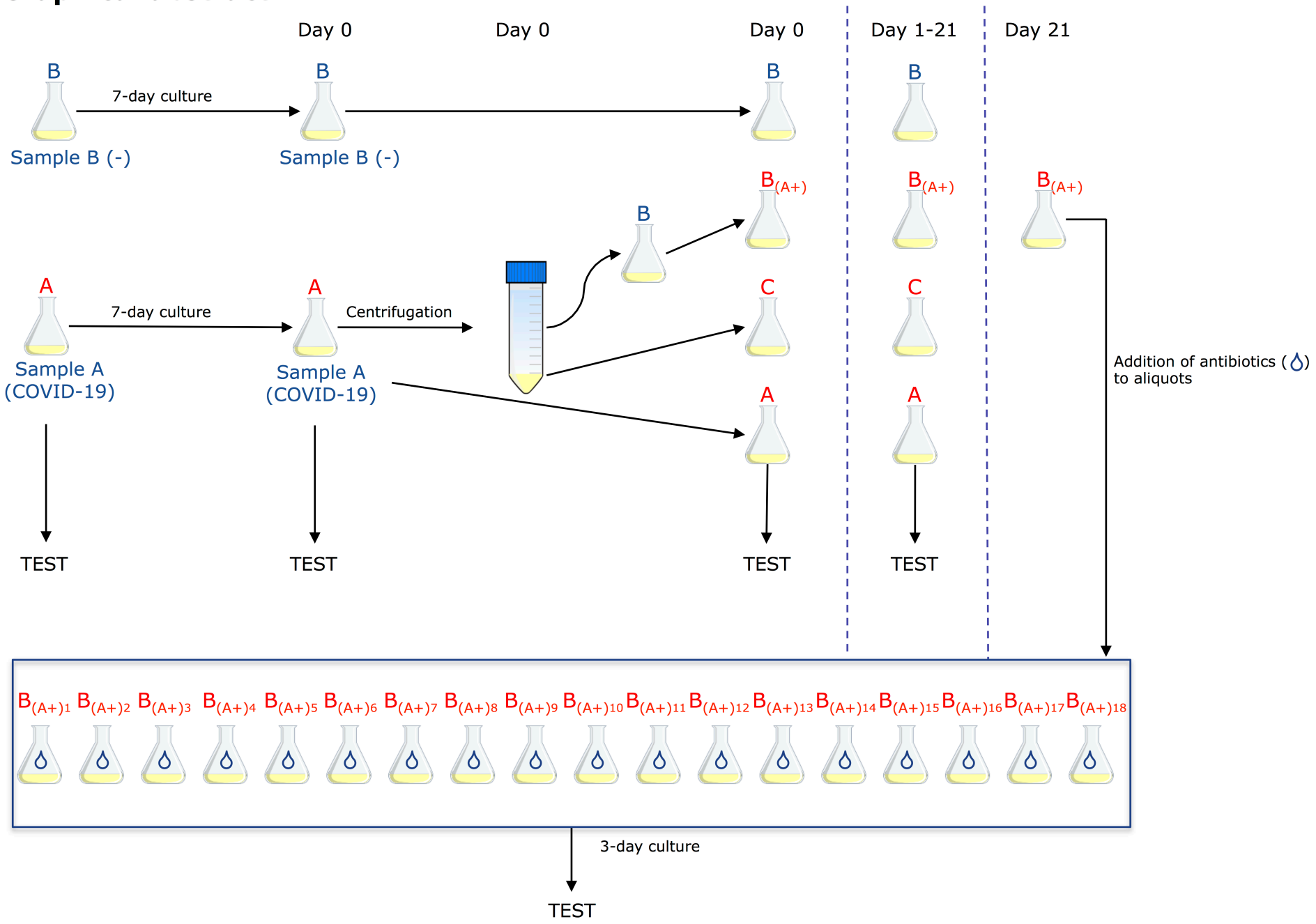


Figure 1

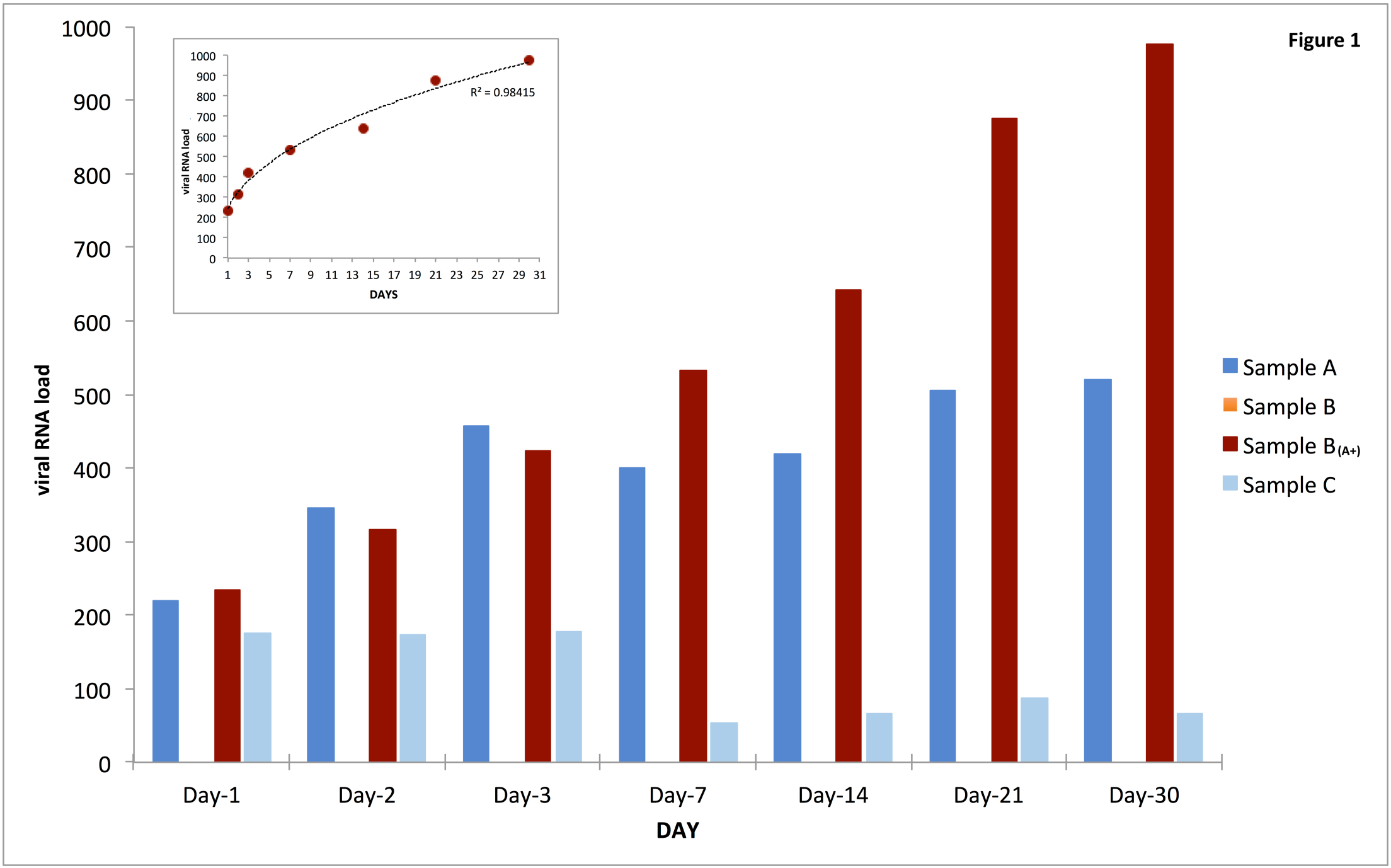
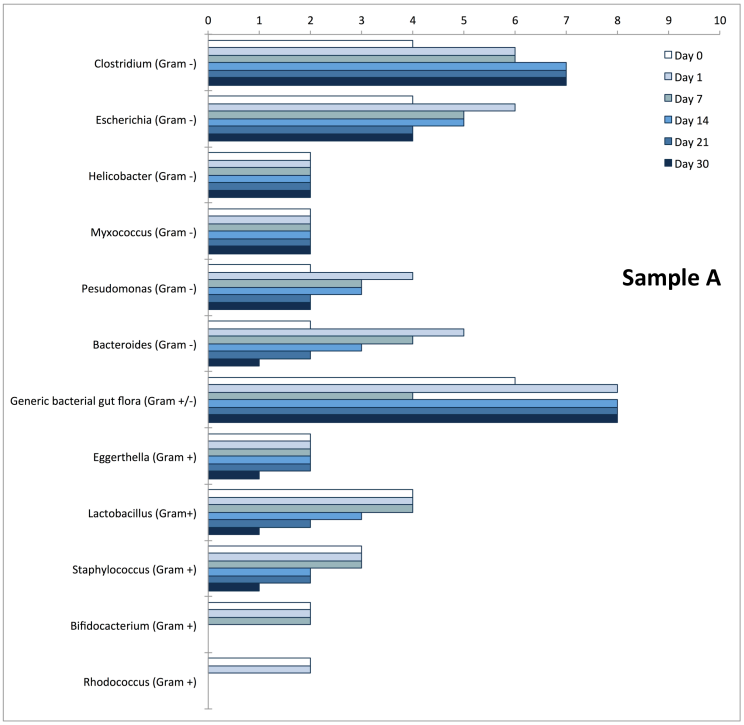

viral RNA load
1000
900
800
700
600
500
400
300
200
100
0
Day-1
Day-2
Day-3
Day-7
Day-14
Day-21
Day-30
DAY
Sample A
Sample B
Sample B(A+)
Sample C
viral RNA load
DAYS
R² = 0.98415

Figure 2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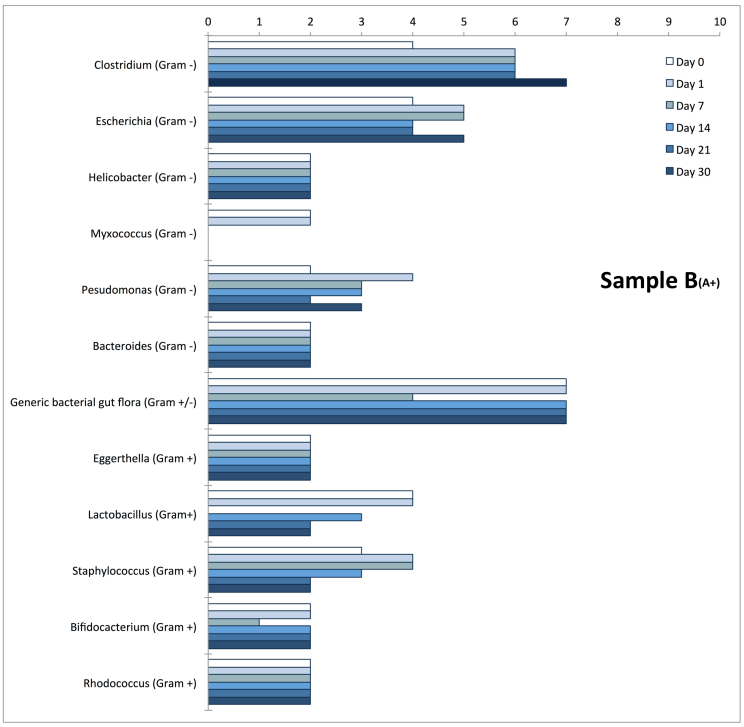
Clostridium (Gram -)
Escherichia (Gram -)
Helicobacter (Gram -)
Myxococcus (Gram -)
Pesudomonas (Gram -)
Bacteroides (Gram -)
Generic bacterial gut flora (Gram +/-)
Eggerthella (Gram +)
Lactobacillus (Gram+)
Staphylococcus (Gram +)
Bifidocacterium (Gram +)
Rhodococcus (Gram +)
Day 0
Day 1
Day 7
Day 14
Day 21
Day 30
Sample A


0 1 2 3 4 5 6 7 8 9 10
Clostridium (Gram -)
Escherichia (Gram -)
Helicobacter (Gram -)
Myxococcus (Gram -)
Pesudomonas (Gram -)
Bacteroides (Gram -)
Generic bacterial gut flora (Gram +/-)
Eggerthella (Gram +)
Lactobacillus (Gram+)
Staphylococcus (Gram +)
Bifidocacterium (Gram +)
Rhodococcus (Gram +)
Day 0
Day 1
Day 7
Day 14
Day 21
Day 30
Sample B(A+)

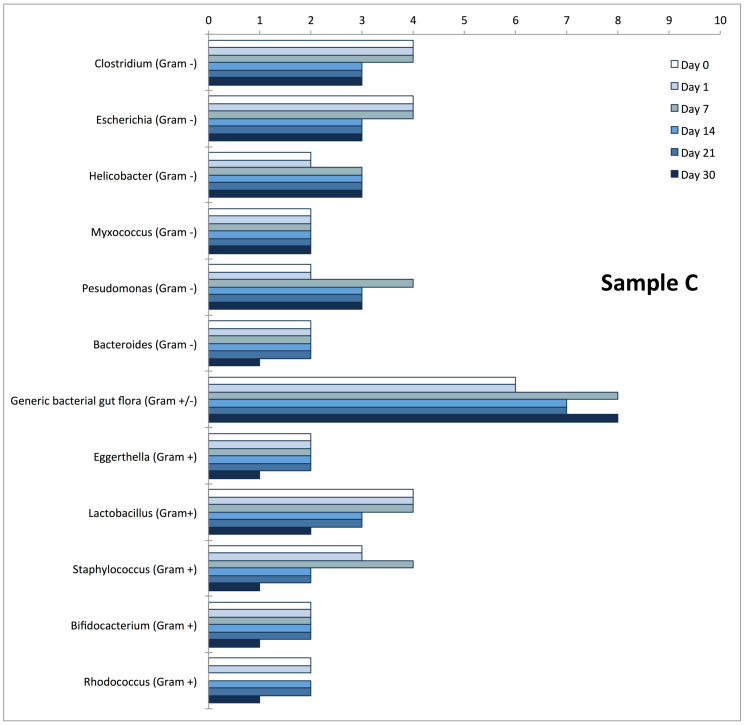
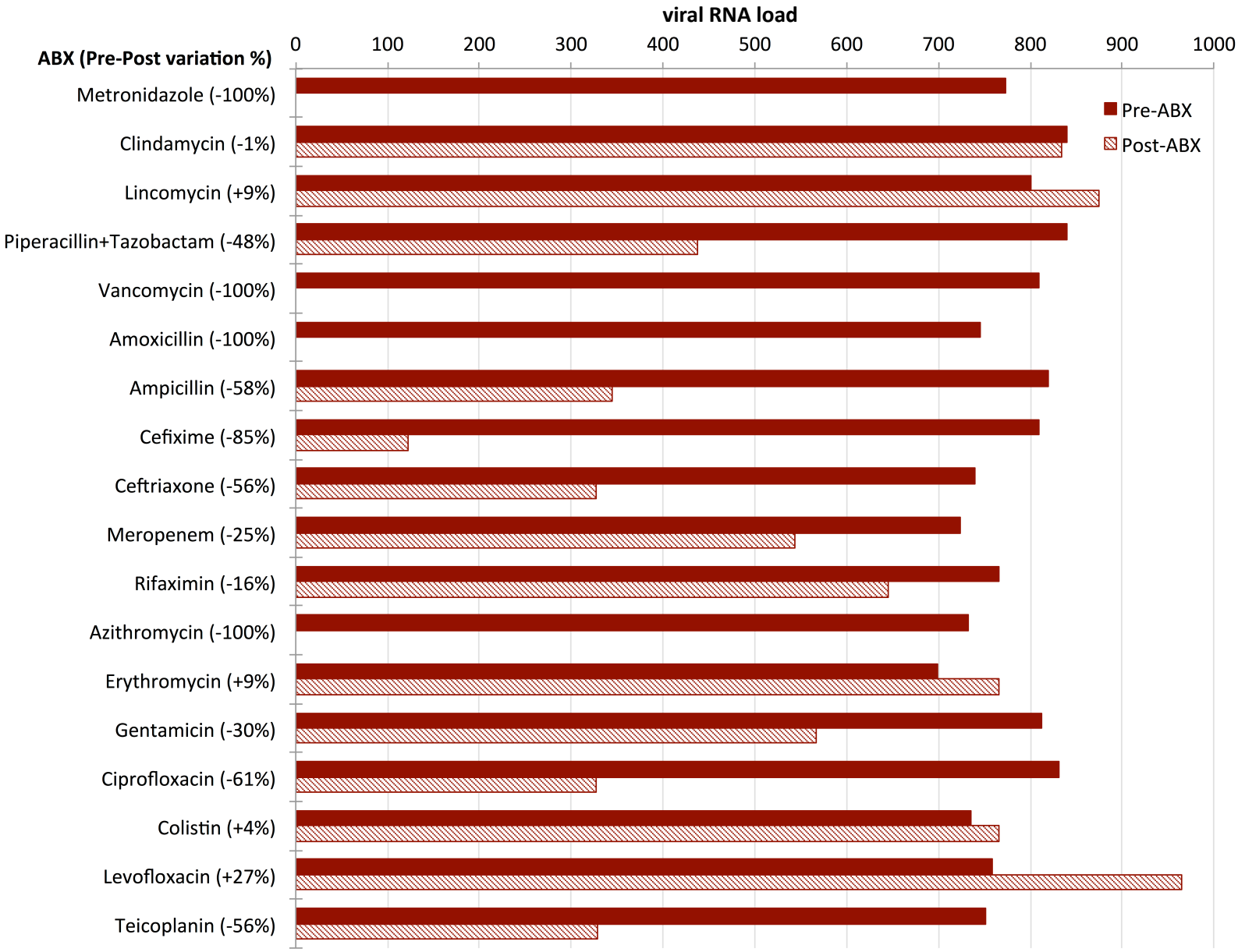

0 1 2 3 4 5 6 7 8 9 10
Clostridium (Gram -)
Escherichia (Gram -)
Helicobacter (Gram -)
Myxococcus (Gram -)
Pesudomonas (Gram -)
Bacteroides (Gram -)
Generic bacterial gut flora (Gram +/-)
Eggerthella (Gram +)
Lactobacillus (Gram+)
Staphylococcus (Gram +)
Bifidocacterium (Gram +)
Rhodococcus (Gram +)
Day 0
Day 1
Day 7
Day 14
Day 21
Day 30
Sample C

Figure 3


viral RNA load
0
100
200
300
400
500
600
700
800
900
1000
ABX (Pre-Post variation %)
Metronidazole (-100%)
Clindamycin (-1%)
Lincomycin (+9%)
Piperacillin+Tazobactam (-48%)
Vancomycin (-100%)
Amoxicillin (-100%)
Ampicillin (-58%)
Cefixime (-85%)
Ceftriaxone (-56%)
Meropenem (-25%)
Rifaximin (-16%)
Azithromycin (-100%)
Erythromycin (+9%)
Gentamicin (-30%)
Ciprofloxacin (-61%)
Colistin (+4%)
Levofloxacin (+27%)
Teicoplanin (-56%)
Pre-ABX
Post-ABX