Università di Genova | DIMES DIPARTIMENTO DI MEDICINA SPERIMENTALE

CRS -DI.ME.S. Contract Research Services

Experiments performed to evaluate the ability of Betterair's Enviro-Biotics® in inhibiting the infectivity of a SARS-CoV-2 pseudovirus.

Final report

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EXPREIMENTAL PROCEDURES

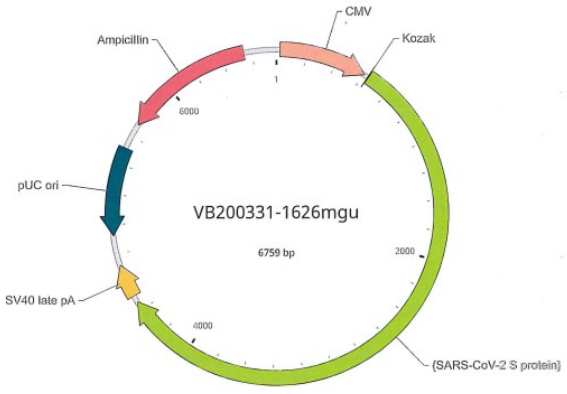
PseudoCovid-19 generation and characterization

Two different pseudoviruses with structural characteristics and infection mechanism equal to COVID-19 (Sars-CoV-2), due to the presence of Spike S protein on their surface, were generated. One pseudovirus had in its genome the genes for the expression of the EGFP fluorescence protein and puromycin resistance; the other one had the luciferase enzyme (LUC) and puromycin resistance genes. The vector sequence utilized for the generation of the pseudoviruses above was from VectorBuilder Inc (U.S.A.). In table 1 the Vector Summary and in Fig 1 the specific vector map.

Table 1

Vector ID	VB200331-1626mgu
Vector Name	pRP[Exp]-CMV>{SARS-CoV-2 S protein}
Vector Size	6759 bp
Vector Type	Mammalian Gene Expression Vector
Inserted Promoter	CMV
Inserted ORF	{SARS-CoV-2 S protein}
Plasmid Copy Number	High
Antibiotic Resistance	Ampicillin
Cloning Host	VB UltraStable (or alternative strain)

Fig. 1



The pseudoCovid-19 protocol has been standardized in order to obtain for each virus preparation an equal amount of viral particles in the same volume.
The expression of the Spike S in the generated pseudoCovid19, after HEK cells' transfection in specific condition, was evaluated by Western blot analysis.

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An aliquote of one of the bacth of the pseudoCovid-19 produced for the different tests was analyzed with transmission electron microscopy (TEM).

PseudoCovid-19 titration

A titration of the pseudoCovid-19 was performed in order to know the number of viral particles in the specific volume used in the whole experiments performed. The amount of transduction units per milliliter (TU/ml) present in pseudoCovid-19 preparation was calculated as the number of fluorescent cells in the wells of each dilution and detected by FACS analysis. The TU/ml was calculated in the wells where the amount of fluorescent cells was less than the 30% of the total.

PseudoCovid-19 inactivation on surfaces by Enviro-Biotics® cells

The interaction between the *Enviro-Biotics*®, containing *Bacillus* spores, and the pseudoCovid-19 was tested using a sterile plastic plate cover containing 96 wells. The experiments were carried out in a switched off, hermetically sealed biological hood with an inner volume of 0.42 m^3 at a temperature of 25 °C and at 80% RH.

Briefly, the *Bacillus* spores, contained in the commercial Betterair's spray can, were sprayed over the well covers, until reaching a volume of 30 µl/well that was followed by addition of 10 µl saline to each well. In parallel to the spore sprayed wells, 40 µl of saline was added to the negative control wells without any spores. The plates were pre-incubated in the hood for four hours. EGFP-pseudo Covid-19 was tested in the first viral deactivation experiment.

Following four hours preincubation, 30 µl of EGFP-pseudoCovid-19 were added to each well yielding a final volume of 70 µl/well. Five bacteria-virus incubation times were tested: 0 min, 15 min, 30 min, 1h, and 3h in triplicates.

At the end of each incubation, samples were collected from the plastic well surface, transferred to a 0.2 ml tube, centrifuged 3,500x*g* for 8 minutes and the recovered supernatant was filtered (0.2µm absolute filter) for eliminating any bacterial left-overs.

Immediately after the filtration and for each incubation time, the recovered supernatants were transferred to 96-well plates where Caco2 cells were seeded (15,000 cells/well, seeded 24 hours prior to the stimulation). The Caco2 cells were incubated with the recovered supernatant for 72 hours; at the end of 72 hours incubation, the Caco2 cells (for each different time point) were measured for their fluorescence both with a spectrofluorometer and with confocal microscopy acquisition to quantify the residual infectivity of the EGFP-pseudoCovid-19 (directly proportional to the measured fluorescence) after its incubation with pre-incubated Enviro-Biotics® cells.

After fluorimetric detection, puromycin was added to the same Caco2 cells in each well (in order to select only the infected cells with the EGFP-PseudoCovid-19) for the colony forming assay. The 12 days formed colonies were stained by crystal violet. The result obtained by the colony forming assay was acquired by camera snapshot and by spectrophotometric absorbance after dissolution of the crystal violet fixed in each well.

In the second deactivation test by *Bacillus* cells, LUC-pseudoCovid-19 was used instead of EGFP-pseudoCovid-19 for validating the *Bacillus* virucidal efficiency on a different system.

All the experimental conditions were identical to the EGFP-pseudoCovid-19 experiment except for the incubation time that lasted 120 hours and the evaluation of the infected Caco2 cells that was

quantified by chemiluminescence acquisition. The residual infectivity of the LUC-pseudoCovid-19 was directly proportional to the measured chemiluminescence that was detected as luciferase activity.

Bacillus cells viability

The *Bacillus* cells viability, after the four hours of preincubation on the plastic surface, was tested on agar plates containing 15 g/l agar, 2 g/l yeast extract, 2 g/l skim milk powder, 16 g/l glucose and 5 g/l sodium chloride. The Bacillus cells were suspended in saline buffer, diluted 1:10^6 and spread on agar plate. The agar plates were then incubated overnight a 37° C. The day after the bacterial colonies were counted.

RESULTS

PseudoCovid-19 characterization

The expression of the S-protein in the generated Pseudo Covid-19, after HEK cells' transfection, was detected by Western blot analysis (Fig1) in order to demonstrate the full homology of the pseudoCovid-19-protein with the SARS-CoV-2-protein.

Moreover, an aliquot of the Pseudo Covid-19 batches produced for the different tests in this study, was analyzed with transmission electron microscopy (TEM). A representative Pseudo Covid-19 image acquired with a TEM (50000x; 50kV) is shown in Fig. 2.

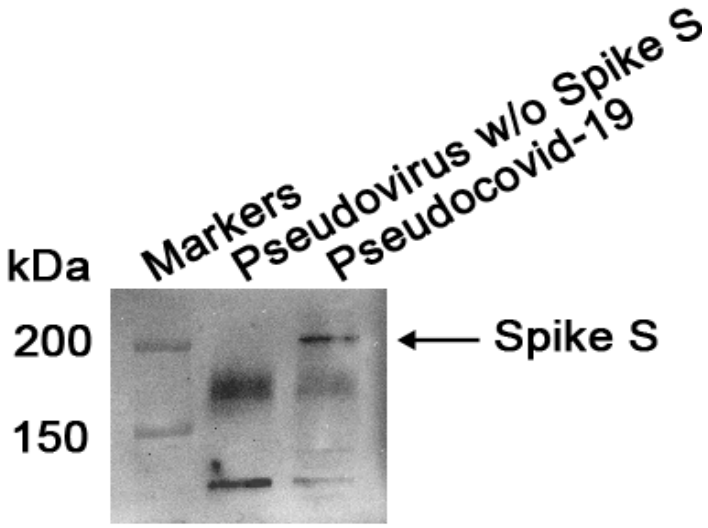

Fig. 1 Western blot performed on empty or over-expressing Spike S protein of the pseudoCovid19 used for all the tests performed. The identification of the Spike S was obtained using a specific mAb (Thermo Scientific, U.S.A) for such protein on lysate of the produced pseudovirus.

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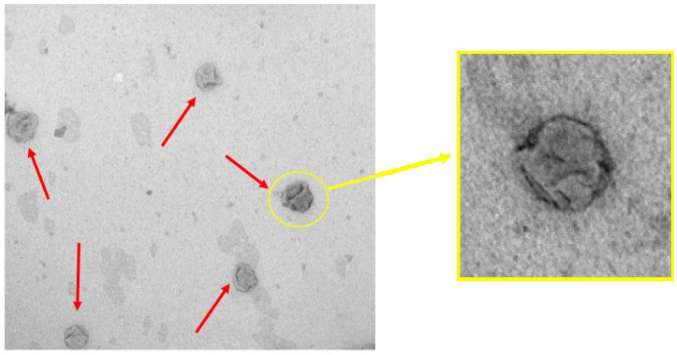

	diameter (nm)
Mean	131,9
Std. Deviation	33,51
Std. Error	4,693
Median	128,3

Fig. 3 A representative image of TEM acquisition of the produced pseudoCovid19. Red arrows indicate the viral particles in the field of acquisition; the yellow box is a zoom image of a single particle. The table on the right summarize the 2D dimension of the pseudoCovid-19.

Pseudo Covid-19 inactivation on surfaces by Enviro-Biotics® *Bacillus* cells

The tests with EGFP- and LUC-pseudoCovid-19 were perfomed according to the procedure reported in the "Experimental procedures" section.

The amount of pseudoCovid-19 pseudovirus particles used for each incubation point of the tests performed was 20,7 x 10^3, according to the results of the titration analysis which was 6,9 x 10^5 TU/ml of pseudoCovid-19 in each batch produced.

Results of spettrofluorimetric acquisition of the first inactivation test with EGFP-pseudoCovid-19, although the Enviro-Biotics® *Bacillus* spore suspension was applied to the tested surface only 4 hours prior to the viral inoculation, showed that *Bacillus* bacteria incubation with the viral particles were responsible for a rapid inactivation of the viruses. Within 15 minutes 67% of the viral particles (a Log reduction of 0.5) were neutralized and by 3 hours 97.7% of viral particles (a Log reduction of 1.6) (Fig. 4).

The *Bacillus* cell concentration at time 0 following 4 hours of pre-incubation, prior to the pseudo Covid-19 inoculation reached a CFU of 1.42 x 10^8 cell/ml indicating an initiation of spore germination. The original applied spore concentration by using the Betterair spray can was 8 x 10^7 spores/ml.

During this time, the viruses in the parallel untreated control surfaces were totally stable, indicating that there was no spontaneous reduction in the viral count. Confocal microscopy analysis of Caco2 infected cells confirmed the deactivation of the EGFP-pseudoCovid-19 particles (Fig. 5) demonstrating that Caco2 cells infected with the residual EGFP-pseudoCovid-19, previously incubated with active Enviro Biotics®, did not emit any fluorescence thus indicating the significant inactivation of the viral particles.

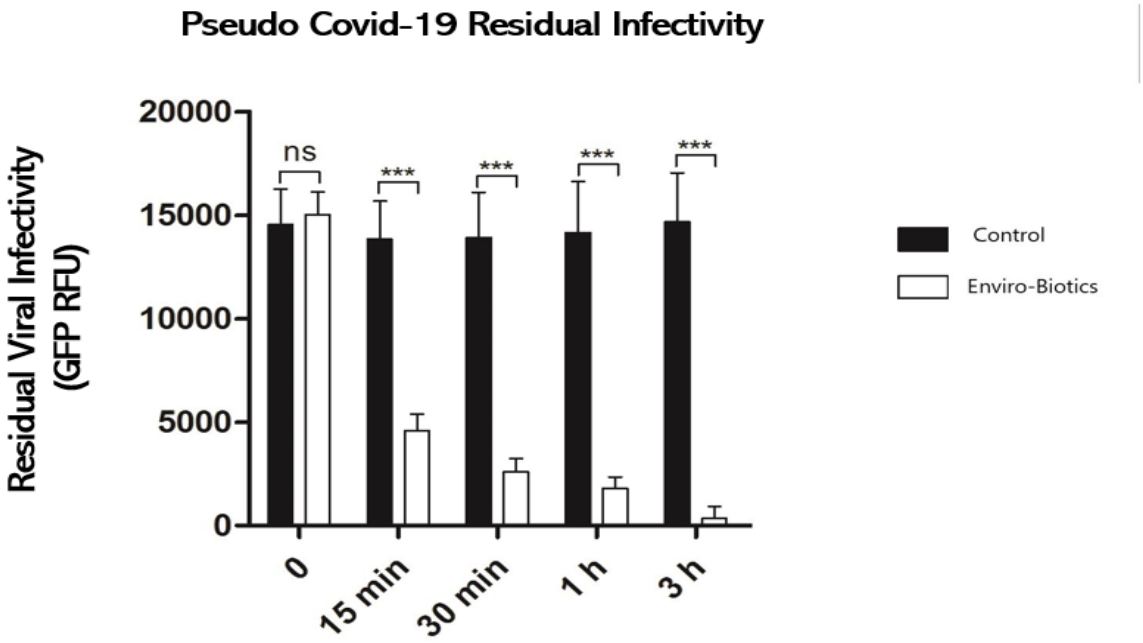

Fig. 4. Fluorimetric quantitation of EGFP-Pseudo Covid-19 residual infectivity detected as GFP fluorescence with a spectrofluorimeter, presented in Relative Fluorescence Units (RFU). Background auto-fluorescence deriving solely from cells was subtracted at each time point. Each bar is the mean±SD of a triplicate. P value was calculated by unpaired t-Test.

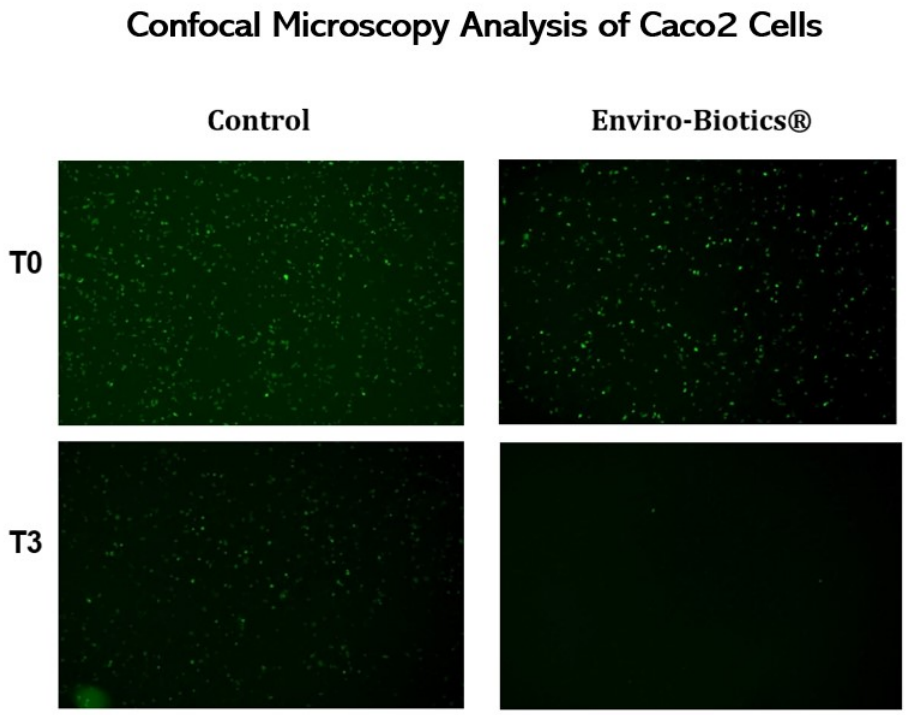

Fig. 5. Confocal microscopy analysis of Caco2 cells incubated with EGFP- Pseudo Covid-19 previously pre-incubated with and without active Enviro Biotic® Bacillus bacteria at T0 (Initiation of Bacillus – virus incubation) and following 3 incubation hours.

After confocal microscopy acquisition, puromycin was added in each well of the Caco2 host cells to enable another evaluation of the virucidal efficiency by the Bacillus cells. As depicted in Fig. 6, a colony forming assay revealed the significant deactivation effect of the Bacillus cells on the viral survivability. The puromycin resistance was also acquired by spectrophotometric absorbance after dissolution of the crystal violet fixed in each well (Fig.7).

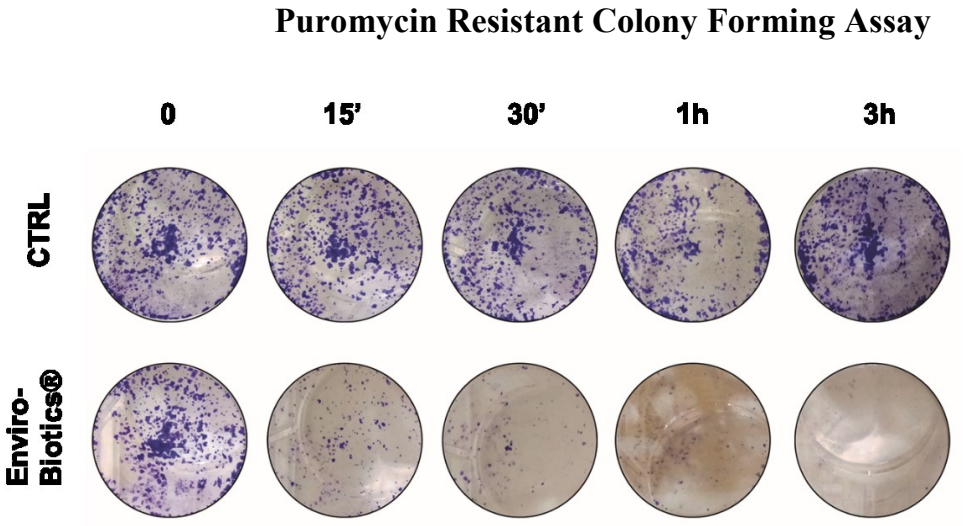

Fig. 6. A colony forming assay showing the plates with Coco2 cells that were treated with the sprayed *Bacillus* Enviro-Biotics® vs. the untreated control, following puromycin supplementation.

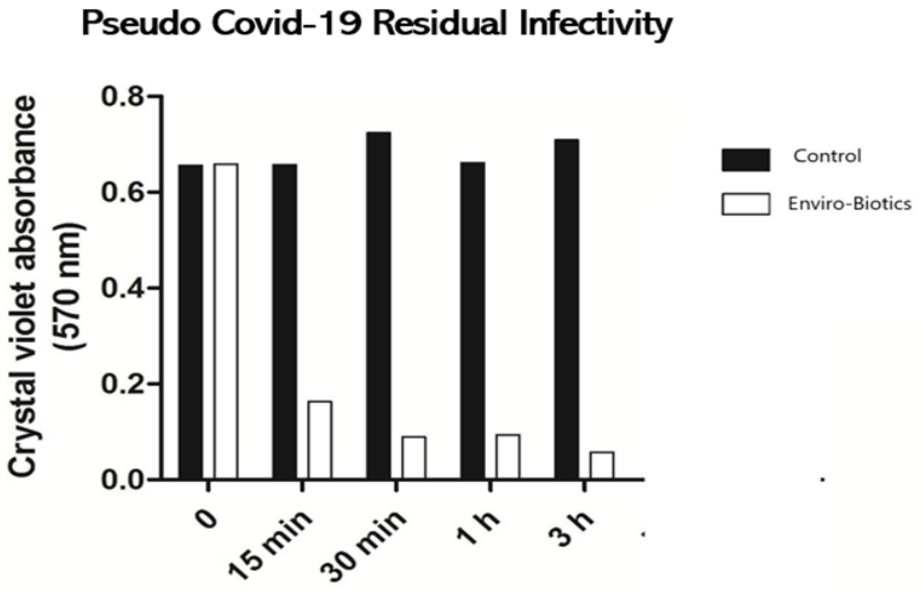

Fig. 7. Crystal violet quantitation by spectrophotometric absorbance at 570nm of the dissolved crystals corresponding to the colonies formed.

In order to validate the *Bacillus* Enviro-Biotics® antiviral efficiency, the bacterial cells were challenged by another pseudoCovid-19 construct. Similarly to the experiment that tested the inactivation of EGFP-pseudoCovid-19, the LUC-pseudoCovid-19 was found susceptible to the Enviro-Biotics® Bacillus cells. Following 15 minutes of incubation, the active viral load has decreased by 70%. Further incubation deactivated the viral particles and reached a reduction rate of 99% (a Log reduction of approx. 2) within 3 hours (Fig.8). This second test results confirmed the virucidal activity of the Enviro-Biotics® already within the first incubation hour, as previously observed by fluorescence quantitation performed in the test with EGFP-pseudoCovid-19.

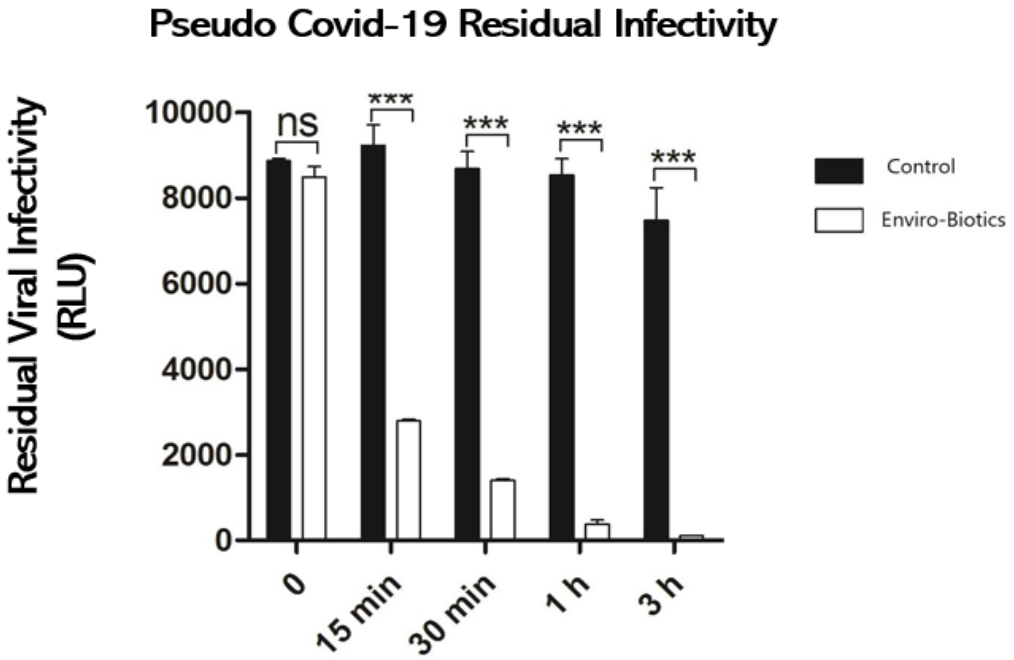

Fig. 8. Chemiluminescence quantitation of the LUC-Pseudo Covid-19 residual infectivity detected as luciferase activity, presented as Relative Luciferase Units (RLU). Each bar is the mean±SD of a triplicate. P value was calculated by unpaired t-Test.

CONCLUSION

The results herein reported indicate the significant anti-viral activity of Enviro-Biotics® Bacillus on a pseudoCovid-19, having full homology with the natural SARS-CoV-2 virus, and the potential use of such Bacillus cells in disinfecting indoor surfaces and objects.

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