SARS-CoV-2 Elimination from Surfaces by Applying Bio-Control *Bacillus* Agents

ABSTRACT

The highly contagious SARS-CoV-2 is transmitted by droplets, fomites and aerosols. Whereas aerosols float in the air for a while, droplets and fomites emitted by SARS-CoV-2 infected patients, settle rapidly on indoor surfaces and objects, and persist there for days and even weeks. The acceptable way to treat and disinfect contaminated areas is by using hazardous chemicals. That said, chemical treatment has a major shortfall as it is harmful therefore can't be applied when humans are present, and its efficiency is lost shortly after its application. Another method is air filtration that may assist in cleaning the air but doesn't affect surface contamination. In this study we have elucidated the microbial bio-control approach that aims to deactivate the Corona virus on indoor surfaces and objects. We have exhibited a significant reduction of 98% in Pseudo Covid-19 survivability within only three hours after applying Enviro-biotics® *Bacillus* cells. This Bio-control preventative approach may mitigate the fomite spread, enable safer, sustainable and long-lasting protection against the SARS-CoV-2 threat.

INTRODUCTION

The unwavering spread of the Covid-19 pandemic that has begun on December 2019 in Wuhan China (1) and has swept the world by storm, taking (so far) the lives of about 1.5 Million people according to WHO, and still continues to endanger the human society. Preventive measures such as social distancing, mask usage and endless surface disinfection have been applied all around the globe (2). As the pandemic is still not showing signs of abatement, and considering the precarious increasing infection rate, there is an urgent need for a worldwide combined endeavor in finding solutions to the Covid-19 pandemic.

The need to protect surfaces and objects has become apparent with the discovery that the Covid-19 persists on surfaces and objects for long durations, far exceeding any prior estimates (3,4,5). In addition, the Covid-19 is transmitted when we sneeze, speak, cough or breath out, scattering droplets and fomites that descend rapidly within a 2 meters radius, on surfaces and objects (6,7,8). Consequently, the duration Covid-19 stays airborne may be 1% of the total time of potency where the remaining 99% of the time it persists and survives on surfaces and objects. The search for a better and long-lasting surfaces and objects protection, evolved the need for a more advanced technology. Since the reliance on chemical disinfectants is highly problematic for many reasons – unsafe, harmful for humans and environments, deploy in a manner most areas are not reachable or not practical (example, a contaminated keyboard cannot be cleansed by washing it with aggressive chemicals), may induce viral resistance due to continuous selective pressure and have a short duration of

efficacy. Therefore, the protection against Covid-19 by using chemical disinfectants is limited and inferior (9,10,11). These circumstances have created new challenges and opportunities for creative and innovative solutions. As part of the search for innovations, we have encountered a new technology, a biological procedure that protects surfaces and objects by using environmental probiotics (Enviro-Biotics ®).

Enviro-Biotics® is a microbial suspension consisting of multiple strains of *Bacillus* species which are the main natural producers of extra-cellular proteolytic enzymes (12) that can break down protein molecules. SARS-CoV-2 possesses the typical coronavirus structure with the “spike protein” on the membrane envelope (13); the spike protein is a crucial recognition factor for virus attachment and entry to the host cells (14,15), therefore, proteolytic cleavage of the spike protein, eliminates its human cell receptor recognition, prevents the virus-cell attachment and blocks the viral infection.

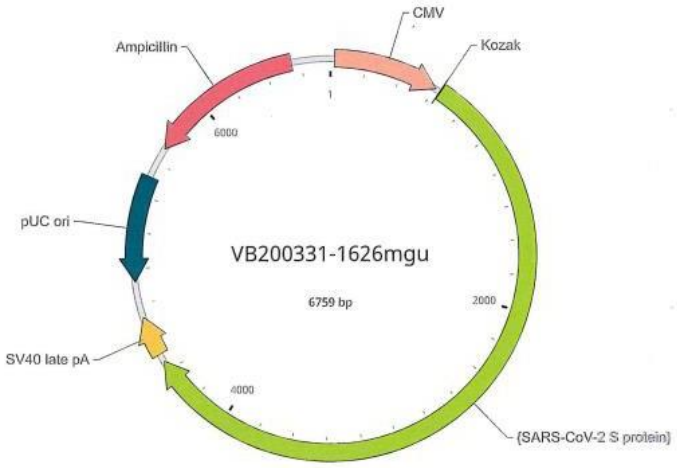
The aim of this research was to examine the virucidal efficiency of the Enviro-Biotics® *Bacillus* cells against corona viruses on surfaces and to validate its capacity to eliminate viral infectivity.

MATERIALS AND METHODS

Features of the Pseudo Covid-19 produced

We have generated two different Pseudo Covid-19 viruses with structural characteristics and infection mechanism identical to COVID-19 (Sars-CoV-2). They are both covered with Sars-CoV-2 Spike S proteins on their surface. One Pseudo Covid-19 virus had in its genome both EGFP fluorescence expression protein and puromycin resistance genes. The other Pseudo Covid-19 virus had the luciferase enzyme (LUC) and puromycin resistance genes.
The vector sequence utilized for the above Pseudo Covid-19 viruses’ generation was obtained from Vector-Builder Inc (U.S.A.). The table below presets the vector summary and the specific vector map is depicted in the following figure.

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 UtraStable (or alternative strain)



The Pseudo Covid-19 preparation protocol has been standardized in order to obtain an equal number of viral particles for each virus preparation in the same volume.

Pseudo Covid-19 titration

In order to determine the viral particles concentration in the specific volume used in the performed experiments, a titration of the Pseudo Covid-19 was performed. The amount of transduction units per milliliter (TU/ml) present in the Pseudo Covid-19 preparations was calculated as follows:

- The number of fluorescent cells in each dilution well was calculated by FACS analysis.
- The TU/ml was calculated in the wells where the fluorescent cells amount was less than 30% of the total.
- The TU/ml values of each dilution were averaged to obtain the final Pseudo Covid-19 concentration which was 6.9×10^5 TU/ml.
- In each experimental test point the amount of Pseudo Covid-19 particles was 2.10×10^4.

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

The interaction between the *Bacillus* cells and the Pseudo Covid-19 was tested using a sterile plastic plate cover containing 96 wells.
The experiments were caried 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.

The *Bacillus* spores 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. The *Bacillus* viability after four hours of preincubation 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.

EGFP- Pseudo Covid-19 was tested in the first viral deactivation experiment. Following four preincubation hours, 30 µl of EGFP- Pseudo Covid-19 were added to each well yielding a final volume of 70 µl/well. Five bacteria-virus duration incubation times were tested: 0 min, 15 min, 30 min, 1h, and 3h in triplicates. Following the completion 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 that were inoculated beforehand by Caco2 cells (15,000 cells/well, seeded 24 hours prior to the stimulation). The Caco2 cells were incubated with the recovered supernatant for 72 hours.

The Caco2 cells (for each different time point) were then measured for their fluorescence with a spectrofluorometer to quantify the residual infectivity of the EGFP-Pseudo Covid-19 (directly proportional to the measured fluorescence) after its incubation with pre-incubated Enviro-Biotics® cells.

Background auto-fluorescence deriving solely from cells was subtracted at each time point. P value was calculated by unpaired t-Test.

Puromycin was added to the same Caco2 cells, after their fluorescence spectrofluorimetric acquisition, in each well (in order to select only the infected cells with the EGFP- Pseudo Covid-19) for the colony forming assay performance. The 12 days formed colonies were stained by crystal violet. The result obtained by the colony forming assay was acquired by photo and by spectrophotometric absorbance after dissolution of the crystal violet fixed in each well.

A confocal microscopy acquisition of Caco2 cells infected with the EGFP Pseudo Covid-19 was recovered from each point of the experiment, with and without *Bacillus* spores.

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

All the experimental conditions were identical to the EGFP- Pseudo Covid-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- Pseudo Covid-19 was directly proportional to the measured chemiluminescence that was detected as luciferase activity.

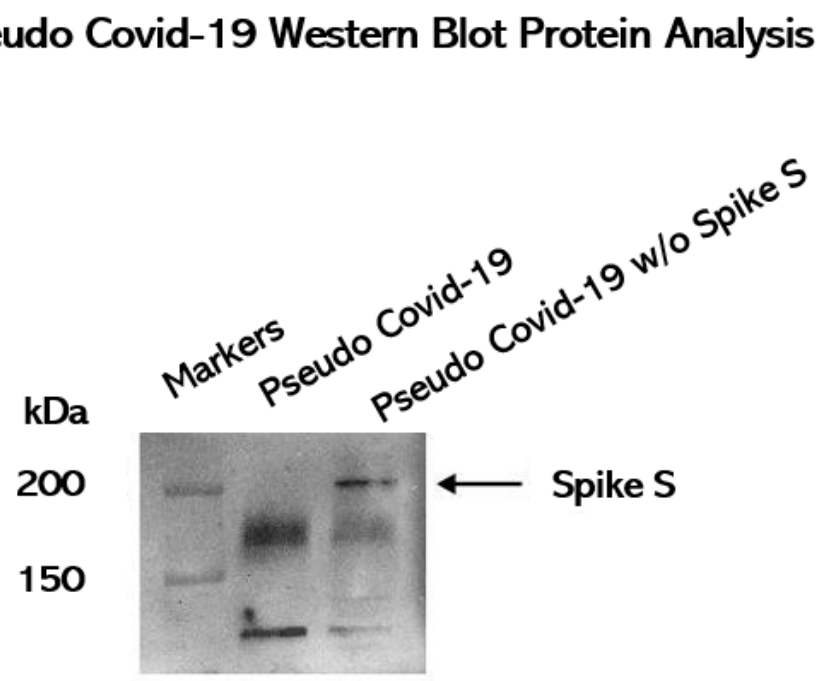
RESULTS

Pseudo Covid-19 preparation

The SARS-Cov-2 virus binds to the host receptor by using its spike (S)-protein, that facilitates cell entry. The S-protein plays a crucial role in the virus attachment, membrane fusion and subsequent infection (16). The S-proteins of other Corona viruses such as 229E and OC43 show a significant lower affinity to the ACE2 human cell receptor than the S-protein of SARS-Cov-2. Therefore, we have selected an enveloped pseudoCovid19 virus that fully expresses the SARS-Cov-2 S-protein. Western blot analysis of the Pseudo Covid-19 virus was caried out for demonstrating the 100% tested pseudoCovid19 S-protein homology with the SARS-CoV-2 S-protein.

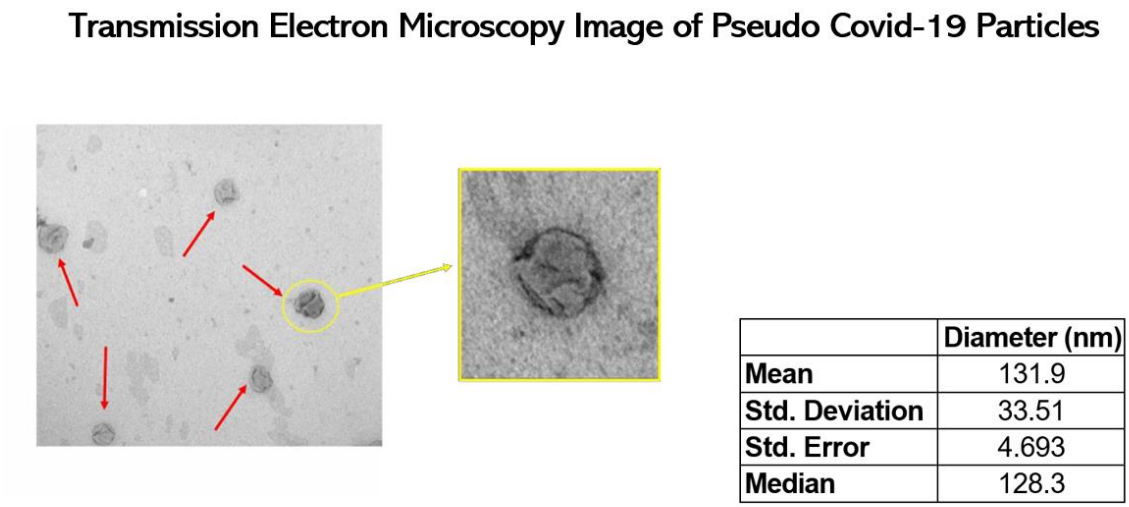
The expression of the S-protein in the generated Pseudo Covid-19, after HEK cells' transfection, that was demonstrated by Western blot analysis is presented in Fig1.

Fig.1: Western blot performed on spike-less and over-expressing Pseudo



Covid-19 Spike S-protein used for all the tests performed in this study. The identification of the Spike S-protein was obtained by using a specific mAb (Thermo Scientific, U.S) for this protein in a Pseudo Covid-19 lysate.

Following the empirical irrefutable proof for the SARS-CoV-2 S-protein presence in the Pseudo Covid-19, an aliquot of one 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



	Diameter (nm)
Mean	131.9
Std. Deviation	33.51
Std. Error	4.693
Median	128.3

Fig. 2: A representative image of the produced Pseudo Covid-19 by TEM acquisition.

The arrows indicate the viral particles in the field of acquisition; the enlarged picture is a zoom image of a single particle. The table on the right summarizes the dimensions of the Pseudo Covid-19.

The mean diameter of this virus is 1 magnitude of order smaller than the *Bacillus* cell diameter. In some general anti-viral modes of action, the diameter ratio between the controlling cell and the viral particle may be crucial for facilitating the elimination or inactivation of the virus.

Since the presumed mode of action is based on secretion of virucidal metabolites, rather than some kind of phagocytosis, the specific dimensions seem less relevant to the specific mode of virucidal action. Nevertheless, the Pseudo Covid-19 was designed also for ensuring full identity to the SARS-CoV-2 particle dimensions.

Proteolytic activity of the Enviro-Biotics® *Bacillus* cells

The presumed anti-viral mode of action of Enviro-Biotics® *Bacillus* cells is the extracellular proteolytic cleavage of the Coronavirus S-protein. As Proteases enzymatically hydrolyze peptide bonds in substrate proteins, we have tested the *Bacillus* cells degradative mechanism capacity. Enviro-Biotics® *Bacillus* cells were inoculated on an agar plate consisting skim milk as the nitrogen source.

As presented in fig.3, the *Bacillus* cells have degraded and consumed the proteinaceous skim milk substrate and subsequently clarified the zone circulating the colony. This clear metabolic physiological activity indicates to the *Bacillus* cells potential capacity to deactivate adjacent proteins.

Fig. 3: Skim milk degradation by metabolites secreted from an Enviro- Biotics® *Bacillus* colony.

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

Plastic is one of the main inanimate surfaces that are potential routes of SARS-CoV-2 transmission. The duration persistence of SARS-CoV-2 on plastic surfaces was reported to reach 4 days (17), 9 days (18) and 14 days (19). Therefore, we selected plastic wells as the tested surface in this study.

Although the Enviro-Biotics® *Bacillus* spore suspension was applied to the tested surface only 4 hours prior to the viral inoculation, *Bacillus* bacteria incubation with the viral particles resulted in a rapid inactivation of the viruses. Within 15 minutes 67% of the virus particles were neutralized and by 3 hours 97.7% of virus particles were inactivated as shown in 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 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 was performed as another procedure to examine the deactivation of the EGFP-Pseudo Covid-19 particles. As presented in fig.5, cells incubated with EGFP-pseudocovid-19 that were previously incubated with active Enviro Biotics® do not emit any fluorescence indicating the significant inactivation of the viral particles.

In this experiment, Puromycin resistant EGFP-Pseudo Covid-19 were used as the tested viral particles. The antibiotic Puromycin addition to the Caco2 host cells enabled another evaluation of the virucidal efficiency of 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).

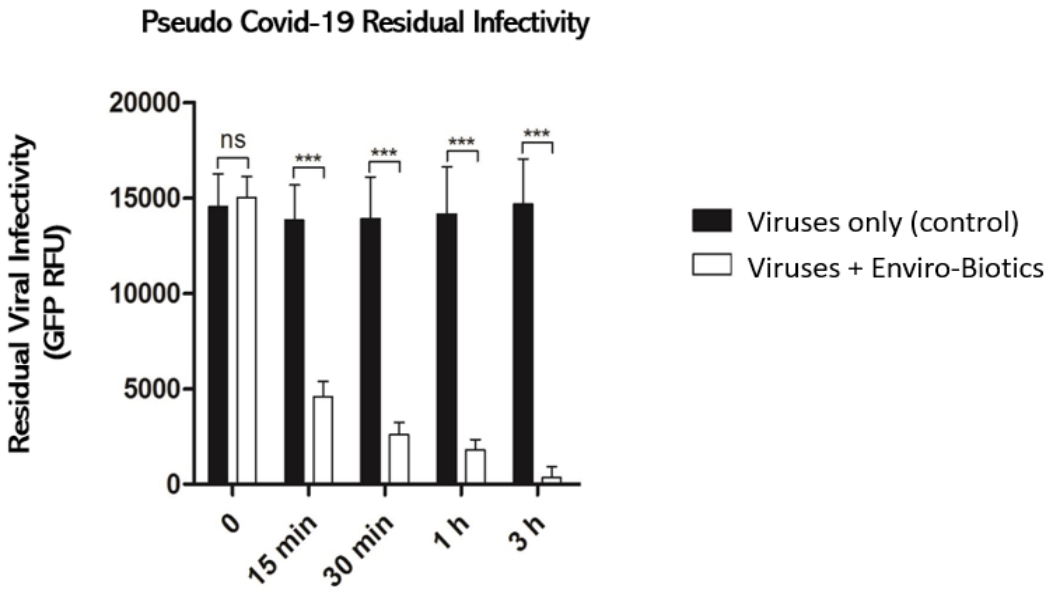


Fig.4: Fluorometric quantitation of EGFP-Pseudo Covid-19 residual infectivity detected as GFP fluorescence with a spectrofluorometer, presented in Relative Florescence Units (RFU). Each bar is the mean±SD of a triplicate. P value was calculated by unpaired t-Test.

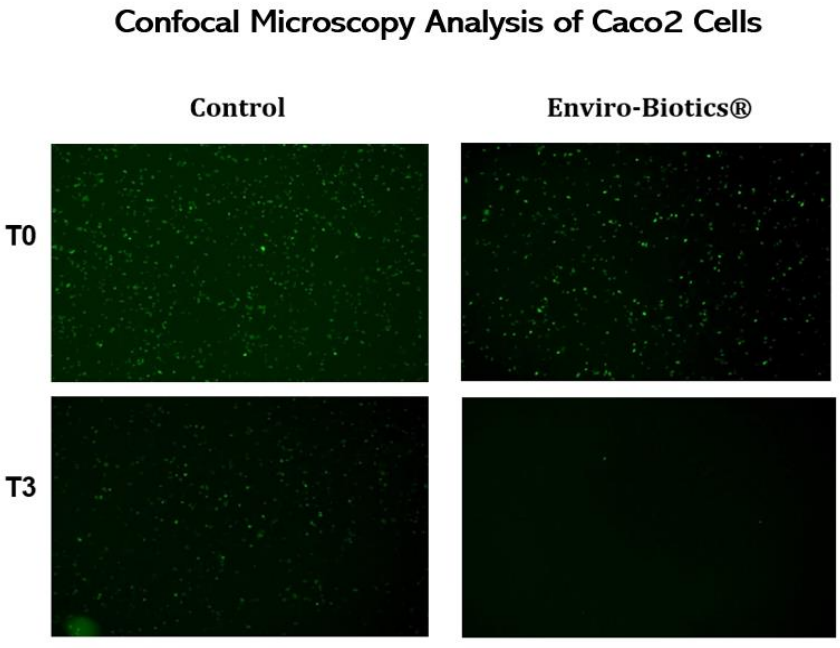
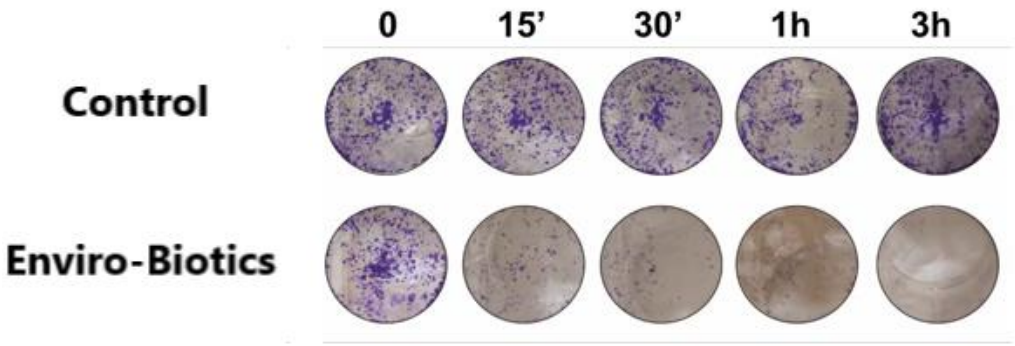


Fig. 5: Confocal microscopy analysis of Caco2 cells incubated with EGFP- Pseudo Covid-19 previously pre-incubated with and without active Enviro Biotic® *Bacillus* bacteria at T0 (Initiation of *Bacillus* – virus incubation) and following 3 incubation hours.



Puromycin Resistant Colony Forming Assay

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.

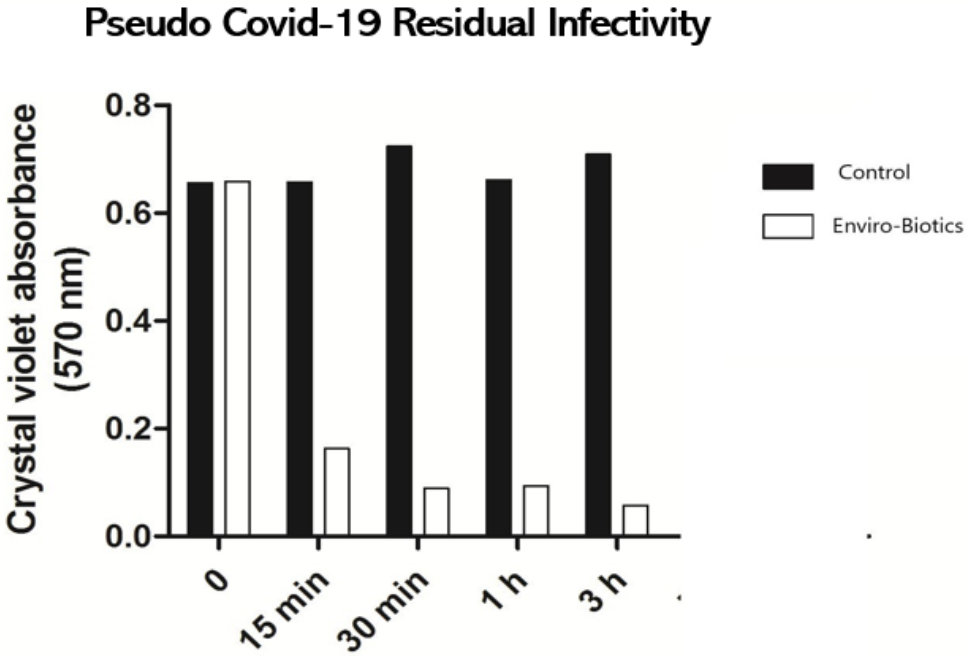


Fig. 7: Crystal violet quantitation by spectrophotometric absorbance at 570nm of the dissolved crystals corresponding to the colonies formed.

In order to validate the *Bacillus* Enviro-Biotics® antiviral efficiency, the bacterial cells were challenged by another Pseudo Covid-19 construct.
Similarly to the experiment that tested the inactivation of EGFP-Pseudo Covid-19, the LUC-Pseudo Covid-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 virus particles and reached a reduction rate of 98.6% within 3 hours as presented in fig.8.

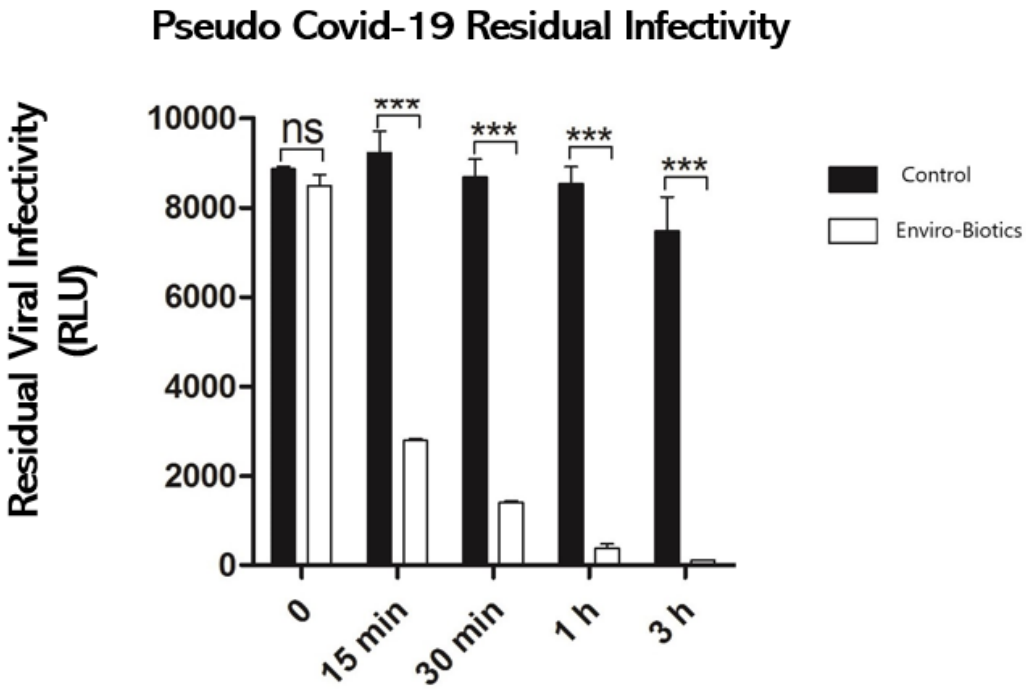


Fig. 8: Chemiluminescence quantitation of the LUC-Pseudo Covid-19 residual infectivity detected as luciferase activity, presented as Relative Luciferase Units (RLU). Each bar is the mean±SD of a triplicate. P value was calculated by unpaired t-Test.

The second test results exhibited above 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-Pseudo Covid-19. These findings indicate the significant anti-viral capacity of Enviro-Biotics® *Bacillus* and their potential use in disinfecting indoor surfaces and objects.

DISCUSSION

Microbial Bio-control of bacterial and fungal pathogens is a well-established discipline that was investigated and reported in innumerable scientific papers (20,21). The modes of action are diverse but the principal theme is that applying beneficial microorganisms reduces the presence of pathogens (22,23,24).
The Sars-Cov-2 pandemic, and the high viral survivability on surfaces and objects caused an urgent need for sustainable and effective disinfecting technology.

Testing the effect of the Enviro-Biotics® *Bacillus* on pseudo-viruses showed a rapid and significant reduction of the viral survivability (Fig. 4, 8).
Virucidal biocontrol has been barely studied and as far as we know, this is the first trial of its kind. This trial not only tested beneficial microorganisms spraying against viruses, but also exhibited a clear viral inactivation.
In order to back up the evaluation of the viral inactivation by spectrofluorimetric acquisition of fluorescence, we performed a colony forming assay (fig.6), that demonstrated once again the clear virucidal effect of the Enviro-Biotics® *Bacillus*. Another pseudo-virus- LUC pseudoCovid19, was constructed with the Sars-Cov-2, S-protein for proving the efficiency in a different system. The viral inactivation process was repeated in this system too (fig.8). The virucidal rates were consistent with those accepted for the EGFP-Pseudo Covid-19, thus, validating the effectiveness of the microbial based, viral-control technology.
One proposed viral inactivation mechanism by bacteria is the secretion of metabolites which adversely affect viral particles (25). The scarce scientific studies that investigated the role of microorganisms as potential virucidal agents, focused on viruses other than Corona. Laboratory studies have indicated that some bacteria are microbial predators of viruses which use the virus' capsid as a nutrient source (26,27). Ward et al. (28) indicated that proteolytic bacterial enzymes inactivate echovirus particles by cleavage of virus proteins thus exposing the viral RNA to nuclease digestion. Deng and Caliver (29) demonstrated the unique role of proteases in viral inactivation processes in the presence of bacteria, by using protease inhibitors. Bacteria may also inactivate viruses by other substances such as small molecules. These are referred to as variolitic substances (29).
Due to the significantly fast viral inactivation that was mostly completed within 15 minutes as exhibited in this study, we propose that the Enviro-Biotics® *Bacillus* viricidal mode of action is based on proteases, as proteases are primary metabolites.
This assumption is also based on one of the characteristics of the *Bacillus* genus. The *Bacillus* cells – which are the active ingredients of the Enviro-Biotics® are known as efficient proteolytic enzyme producers. Moreover, we have demonstrated the proteolytic capacity of the *Bacillus* strains that compose the Enviro-Biotics® formulation (fig.3). In this study, the *Bacillus* spores were applied only four hours prior to the incubation with the viruses, enabling only a

short interval for spore germination, propagation and entering full metabolic activity. The prior sterilization of the tested surface may have also hindered the bacterial activity. Herrman et al. (30) reported that viral inactivation by bacteria is more rapid in contaminated environments as opposed to sterile environments.

Continuous application of Enviro-Biotics® *Bacillus* cells on actual indoor environment consequents in increased virucidal efficacy due to surface colonization and to the nutritive nature of surfaces and objects that induce microbial metabolic activity such as secretion of anti-viral proteases and small molecules.

Most SARS-CoV-2 particles were found in droplets and fomite that are larger than 5μm, thus they are not suspended in the air, but settle on indoor inanimate surfaces and objects. Most coronaviruses, can persist on surfaces for days while some survive for up to a month (31). The SARS-CoV-2 are transmitted by self-inoculation from surfaces to nose, mouth, or eyes after touching contaminated objects (32).

Significant environmental contamination by the SARS-CoV-2 patients through respiratory droplets and fecal shedding (33,34), suggests that the surfaces are a potential transmission medium and supports the need for strict adherence to environmental hygiene indoors.

This study aims to offer a continuous and sustainable preventive solution to the SARS-CoV-2 transmission and infection that stems from contaminated inanimate surfaces and objects.

CONCLUSIONS

The data presented in this study demonstrates that the microbial bio-control approach is applicable and efficient in preventing covid-19 infections originated from surfaces and objects. Applying Enviro-Biotics *Bacillus* cells in indoor environments induces biologic deactivation of viral particles. The drastic viral survivability reduction demonstrated in this study is pertinent to the indoor, public health and transport sectors. The Enviro-Biotics solution should be considered when implementing strategies designed to mitigate fomite transmission for the current Covid-19 pandemic.

REFERENCES

1. Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., ... & Niu, P. (2020). A novel coronavirus from patients with pneumonia in China, 2019. *New England Journal of Medicine*.
2. Wang, C., Horby, P. W., Hayden, F. G., & Gao, G. F. (2020). A novel coronavirus outbreak of global health concern. The Lancet, 395(10223), 470-473.
3. Kampf, G., Todt, D., Pfaender, S., & Steinmann, E. (2020). Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents. *Journal of Hospital Infection*, *104*(3), 246-251.
4. Otter, J. A., Donskey, C., Yezli, S., Douthwaite, S., Goldenberg, S., & Weber, D. J. (2016). Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. *Journal of Hospital Infection*, *92*(3), 235-250.
5. Riddell, S., Goldie, S., Hill, A., Eagles, D., & Drew, T. W. (2020). The effect of temperature on persistence of SARS-CoV-2 on common surfaces. *Virology Journal*, *17*(1), 1-7.
6. Hui, D. S., Chow, B. K., Chu, L., Ng, S. S., Lee, N., Gin, T., & Chan, M. T. (2012). Exhaled air dispersion during coughing with and without wearing a surgical or N95 mask. Plos one, 7(12), e50845.
7. Sun, C., & Zhai, Z. (2020). The efficacy of social distance and ventilation effectiveness in preventing COVID-19 transmission. Sustainable cities and society, 62, 102390.
8. Shafaghi, A. H., Rokhsar Talabazar, F., Koşar, A., & Ghorbani, M. (2020). On the Effect of the Respiratory Droplet Generation Condition on COVID-19 Transmission. Fluids, 5(3), 113.
9. Casey, M. L., Hawley, B., Edwards, N., Cox-Ganser, J. M., & Cummings, K. J. (2017). Health problems and disinfectant product exposure among staff at a large multispecialty hospital. American journal of infection control, 45(10), 1133-1138.
10. Dumas, O., Varraso, R., Boggs, K. M., Quinot, C., Zock, J. P., Henneberger, P. K., ... & Camargo, C. A. (2019). Association of occupational exposure to disinfectants with incidence of chronic obstructive pulmonary disease among US female nurses. JAMA network open, 2(10), e1913563-e1913563.
11. Weinmann, T., Forster, F., Von Mutius, E., Vogelberg, C., Genuneit, J., Windstetter, D., ... & Gerlich, J. (2019). Association Between Occupational Exposure to Disinfectants and Asthma in Young Adults Working in Cleaning or Health Services: Results From a Cross-Sectional Analysis in Germany. Journal of occupational and environmental medicine, 61(9), 754-759.

12. Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology, 59(1), 15-32.
13. Li, F. (2016). Structure, function, and evolution of coronavirus spike proteins. Annual review of virology, 3, 237-261.
14. Belouzard, S., Millet, J. K., Licitra, B. N., & Whittaker, G. R. (2012). Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses, 4(6), 1011-1033.
15. Hantak, M. P., Qing, E., Earnest, J. T., & Gallagher, T. (2019). Tetraspanins: architects of viral entry and exit platforms. Journal of virology, 93(6).
16. Padhi, A. K., & Tripathi, T. (2020). Can SARS-CoV-2 Accumulate Mutations in the S-Protein to Increase Pathogenicity?. ACS Pharmacology & Translational Science, 3(5), 1023-1026.
17. Rabenau, H. F., Cinatl, J., Morgenstern, B., Bauer, G., Preiser, W., & Doerr, H. W. (2005). Stability and inactivation of SARS coronavirus. Medical microbiology and immunology, 194(1-2), 1-6
18. Dong, J. H. X. P. (2003). Stability of SARS coronavirus in human specimens and environment and its sensitivity to heating and UV irradiation.
19. Chan, K. H., Peiris, J. M., Lam, S. Y., Poon, L. L. M., Yuen, K. Y., & Seto, W. H. (2011). The effects of temperature and relative humidity on the viability of the SARS coronavirus. Advances in virology, 2011.
20. Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. Journal of experimental Botany, 52(suppl_1), 487-511.
21. Fravel, D. R. (2005). Commercialization and implementation of biocontrol. Annu. Rev. Phytopathol., 43, 337-359.
22. Gardener, B. B. M., & Fravel, D. R. (2002). Biological control of plant pathogens: research, commercialization, and application in the USA. Plant Health Progress, 3(1), 17.
23. Jones, R. J., Hussein, H. M., Zagorec, M., Brightwell, G., & Tagg, J. R. (2008). Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiology, 25(2), 228-234.
24. Lalloo, R., Ramchuran, S., Ramduth, D., Görgens, J., & Gardiner, N. (2007). Isolation and selection of Bacillus spp. as potential biological agents for enhancement of water quality in culture of ornamental fish. Journal of Applied Microbiology, 103(5), 1471-1479.
25. Vasickova, P., Pavlik, I., Verani, M., & Carducci, A. (2010). Issues concerning survival of viruses on surfaces. Food and Environmental Virology, 2(1), 24-34.
26. Cliver, D. O., & Herrmann, J. E. (1972). Proteolytic and microbial inactivation of enteroviruses. Water Research, 6(7), 797-805.
27. Herrmann, J. E., & Cliver, D. O. (1973). Degradation of coxsackievirus type A9 by proteolytic enzymes. Infection and Immunity, 7(4), 513-517.
28. Ward, R. L., Knowlton, D. R., & Winston, P. E. (1986). Mechanism of inactivation of enteric viruses in fresh water. Applied and environmental

microbiology, 52(3), 450-459.
29. Deng, M. Y., & Cliver, D. O. (1992). Inactivation of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manure. Applied and Environmental Microbiology, 58(6), 2016-2021.
30. Herrmann, J. E., Kostenbader Jr, K. D., & Cliver, D. O. (1974). Persistence of enteroviruses in lake water. Applied microbiology, 28(5), 895.
31. Ren, S. Y., Wang, W. B., Hao, Y. G., Zhang, H. R., Wang, Z. C., Chen, Y. L., & Gao, R. D. (2020). Stability and infectivity of coronaviruses in inanimate environments. World Journal of Clinical Cases, 8(8), 1391.
32. National Health Commission. Guideline of Diagnosis and Treatment for COVID-19 (7th Edition) Available from: http://www.gov.cn/zhengce/zhengceku/2020-03/04/5486705/files/ae61004f930d47598711a0d4cbf874a9.pdf.
33. Ong, S. W. X., Tan, Y. K., Chia, P. Y., Lee, T. H., Ng, O. T., Wong, M. S. Y., & Marimuthu, K. (2020). Air, surface environmental, and personal protective equipment contamination by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) from a symptomatic patient. Jama, 323(16), 1610-1612.
34. Young, B. E., Ong, S. W. X., Kalimuddin, S., Low, J. G., Tan, S. Y., Loh, J., ... & Lau, S. K. (2020). Epidemiologic features and clinical course of patients infected with SARS-CoV-2 in Singapore. Jama, 323(15), 1488-1494.