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Possible correlation between the probiotic activity of bacterial honey isolates and the *in vitro* inhibition of coronavirus 2 replication responsible for acute respiratory syndromes

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Abstract

Various strategies, like those using vaccines and antibiotics, have been examined for the prevention and treatment of virus's diseases, but until this moment infection control is not at sufficient level. Exopolysaccharides, especially from probiotics, became one of the most innovative approaches for antiviral agents. This research tried to highlight the effect of a probiotic polysaccharide, such as levan, in COVID-19 prevention. Accordingly, 5 levans types previously obtained from bacterial honey isolates were tested against COVID-19. The most promising result was recorded with levans from *Pseudomonas aeruginosa* HI1 (levAE) and *Bacillus subtilis* 9A (lev9A). The lowest cytotoxicity was obtained from lev9A (CC_{50}=5.567e+006 mg/ml) and the most promising IC_{50} was obtained by levAE (10.75 mg/ml) followed by lev13M (142.5 mg/ml) then lev9A (1299 mg/ml). The dialysis process of levAE greatly affected the virus inhibition activity (IC_{50} of levAE/D =7.773e+006 mg/ml). *Pseudomonas aeruginosa* HI1 and *Bacillus subtilis* 9A were highly tolerant to the acidic (pHs 2, 3) and alkaline conditions (pHs 9, 11). Moreover, when incubated with 0.3 bile salt for 24h, their surviving rates recorded 94% and 100% respectively. H_2O_2 tolerance showed 77% surviving of *Pseudomonas aeruginosa* HI1 and 100% surviving of *Bacillus subtilis* 9A. The blood hemolysis and the antibiotics sensitivity tests confirmed the isolate's safety. The hypothesis that the isolates adhere to the lung cells, could explain the ability of the isolates and their levans to inhibit covid-19 replication.

Key words: Levan-Probiotic-Covid 19.

1. Introduction

One of the biggest disasters that faced all the world between 2020-2021 and caused the death of many people is the emergence of severe acute respiratory syndrome coronavirus 2 "SARS-CoV-2". Despite the development of many vaccines, its danger still exists because of the rapid mutation rate of the virus and the emergence of immuno-escape variants [1]. The symptoms demonstrated by individuals infected with the novel coronavirus fall on a wide spectrum of severity ranging between mild infections showing symptoms of fever, dry cough, fatigue, and diarrhea, while on the other end of the observed spectrum symptoms included shortness of breath, difficulty breathing, chest pain and loss of speech or movement. There is no conclusive explanation for this variation in the symptoms of infection, but scientists attributed it to the strength of the immune system, which differs from one person to another. The different severity of the virus is probably due to the multiplicity of its mutations [2]. Therefore, we urgently need to find a drug that can protect the body's immune system and act as an antivirus.

The levansucrases [sucrose: 2.6-β-D-fructan-2.6-β-D-fructosyltransferase, E.C.2.4.1.10] form the β-[2→6] levan through the transfructosylation reaction. Levan [β-[2,6]-linked fructose polymer] has received great attention in recent years due to its ability to perform multiple functions. Its high molecular weight and water solubility make it attractive for various industrial applications, including cosmetics, pharmaceutical coatings, and adhesives [3]. Levan was detected as a carrier for drug delivery systems [4], an anti-inflammatory and antioxidant compound [5], anti-tumor [6], and film agent. Sezera *et al.* reported levan as nanoparticles in drug delivery systems [4]. In the last few years, bacterial honey iso-

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lates were identified as a new source for low molecular weight levan with tailored properties. For instance, it was mentioned as an antivirus to many types of viruses that cause problems for birds and humans, such as the New castle disease virus [NDV] which causes a huge economic loss in the poultry birds' section [7]. Also, it was mentioned as an anti-virus against avian influenza HPAI, H5N1, and adenovirus type 40 [8]. The levan acting mechanism is suggested to begin when the levan incubates with the virus for appropriate time since the levan could aggregate the virus using the hydroxyl groups which acquired the levan its adhesion property and could inhibit the fusion of the protein active site of the virus. This step causes feebleness in the virus's capability to penetrate the host [7].

In this study, the antivirus activity of 5 types of levan produced from different bacterial honey isolates was evaluated against COVID-19. The most promising result was obtained by lev9A and levAE. The adhesion property of the two types was estimated. Also, the probiotic activity of the two bacterial honey isolates was studied.

2. Material and methods

Levans' precipitation

The levan yielding organisms, *Bacillus paranthracis* (lev13M) [result under publication], *Bacillus subtilis* MT453867 (H) [9], *Enterococcus faecalis* Esawy (levG) [10], *Pseudomonas aeruginosa* HI1 from which the crude (levAE) and the dialyzed (levAE/D) levans were obtained [11] and *Bacillus subtilis* (9A) (lev9A) [result under publication] were cultivated on the medium containing (g/l): 80 sucrose, $MgSO_4$ 0.2, KH_2PO_4 1 and yeast extract 1, under shaking flask cultivation technique [12]. After the early stage of the stationary phase, the culture filtrate [CF] was centrifuged at 5000 ×g to get rid of bacterial cells. The levAE/D was obtained by the dialysis of levan against deionized water for 24 h by using a dialysis membrane [MWCO 14,000 Da, diameter 60 mm] to remove the unfermented sucrose, and any fermentation products had low molecular weights [MW]. All levans were precipitated with three volumes of ice-cold ethanol [99%] except levAE.

Cytotoxicity [CC_{50}] determination

Levans were obtained and dissolved in distilled H_2O and stored at -80°C. To assess the half-maximal cytotoxic concentration [CC_{50}], the stock solutions of levans were diluted further to the working solutions with DMEM. The cytotoxic activity was tested in VERO-E6 cells by using crystal violet assay as previously described [13] with minor modifications. Briefly, the cells were seeded in 96 well-plates [100 µl/well at a density of 3×10^5 cells/ml] and incubated for 24 h at 37 °C in 5% CO2 incubator. After 24 h, cells were treated with various concentrations of levans in triplicates. At 72 h post-treatment, the supernatant was discarded, and cell monolayers were fixed with 10% formaldehyde for 1 h at room temperature (RT). The fixed monolayers were then dried well and stained with 50 µl of 0.1% crystal violet for 20 min on a bench rocker at RT. The monolayers were then washed, dried overnight and the crystal violet dye in each well was then dissolved with 200 µl methanol for 20 min on a bench rocker at RT. The absorbance of crystal violet solutions was measured at λmax 570 nm as a reference wavelength using a multi-well plate reader. The cytotoxic concentration 50% (CC50) value was calculated using nonlinear regression analysis of GraphPad Prism software (version 5.01) by plotting log concentrations of levans versus normalized responses (variable slope).

Inhibitory concentration 50 (IC_{50}) determination

The IC_{50} values for levans were determined as previously described [14], with minor modifications. Briefly, in 96-well tissue culture plates, 2.4×10^4 Vero-E6 cells were distributed in each well and incubated overnight at a humidified 37°C incubator under 5% CO_2 condition. The cell monolayers were then washed once with 1x PBS. An aliquot of the SARS-CoV-2 "NRC-03-nhCoV" virus [15] containing 100 $TCID_{50}$ was incubated with serially diluted concentrations of the tested compound and kept at 37 °C for 1 h. The Vero-E6 cell monolayers were treated with virus/compound mixtures and co-incubated at 37 °C in a total volume of 200 µl per well. Untreated cells which were infected with the virus represented "virus control", however cells that were not treated and not infected were designated as "cell control". Following incubation at 37°C in a 5% CO_2 incubator for 72 h, the cells were fixed with 100 µl of 10% paraformaldehyde for 20 min and stained with 0.5% crystal violet in distilled water for 15 min at RT. The crystal violet dye was then dissolved using 100 µl absolute methanol per well and the optical density of the color was measured at 570 nm using Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The inhibitory concentration of 50% (IC_{50}) of the compounds is that required to reduce the virus-induced cytopathic effect (CPE) by 50%, relative to the virus control. The IC_{50} value was calculated using nonlinear regression analysis of GraphPad Prism software (version 5.01) by plotting log concentrations of telaprevir versus normalized responses (variable slope).

Probiotic activities

Hemolytic activity

Isolates were cultivated on a blood agar medium [Oxoid] provided with 5% human blood and was grown at 37 °C for 24 h. The presence (or absence) of hemolysis was investigated visually [16].

Antibiotic sensitivity

The resistance/ sensitivity to some antibiotics of the two strains was tested using the disc diffusion method, as described previously. Four types of different antibiotic discs were used included Flumox, Epigent, Unictam, andDepo-pen. The tested isolates were activated for 24h on nutrient agar (NA). A total of 100 µL of the diluted cultures (after adjusting the optical density for each strain to 0.1 O.D) was diffused in NA. The different antibiotic discs were applied on the surface. The plates were incubated at 4°C for 2h and were then incubated at 37 °C for 24 h. The diameters of the inhibition zones [DIZ] values were measured.

Resistance to low and high pH

The method of Conway *et al.* was used for the pH tolerance study of bacterial isolates [17]. Therefore, the freshly prepared cultures were transferred into the nutrient broth (NB) medium (5%) adjusted to pH 2.0 and pH 3.0 with 2 M HCl and to pH 9 and pH 11 with 1 M NaOH. The flasks were then incubated at 37 °C and culture samples were taken after 1, 3, and 6 h. Medium neutralization was done by serial dilutions in phosphate buffer (0.1 M, pH 7.0) and re-culture on nutrient agar (NA). The plates were then incubated at 37

°C for 24 h and the survival [%] was determined by comparing the viable bacterial count after incubation at pH 2.0, 3.0, 9, and 11 and compared to the control bacterial count incubated at pH 7**.**

Bile salt resistance

Bile tolerance was conducted according to [18,19] where the two strains were grown overnight at 37 °C in NB broth supplemented with 0.3 % (w/v) bile salt (Oxgall, USA). The samples from the broth were then re-incubated at 37°C for 3 hr, 6 hr, and 24 hr, in order to test the growth ability of the bile salt-treated cells. The latter was compared to that of control untreated microorganisms. A spectrophotometer (O.D. at 660 nm) was used to detect bacterial growth. The ratios of bile salt resistance were calculated as follows: Percentage of surviving cells incubated with bile to the cell count of control**.**

Antimicrobial activity

Antimicrobial activity of the two strains was carried out by agar well diffusion method [20] using cell-free culture supernatants (CFCS) of the isolated probiotic strains against pathogenic indicator bacteria: *Staphylococcus aureus, Bacillus cereus, Candida albicans* NRRL Y-477, *Aspergillus niger* NRRL 599, and *Escherichia coli* MC1400. Wells of 5 mm diameter were prepared and loaded with a volume of 100 µl of CFCS of each honey isolate and marked adequately with the isolates' names. The plates were kept for two hours at room temperature, and then incubated for 24 hours at 37 °C. The diameters of inhibition zones (DIZ) were measured. The tests were performed in triplicate and the data were represented with mean ± SD**.**

H_2O_2 tolerance

The tolerance of strains to H_2O_2 was assessed by the method of [21] but with only 30 min incubation time. Overnight grown cultures of the isolates were inoculated (1% v/v) into NA medium and the tow medium containing 0.1% hydrogen peroxide and incubated at 37°C for 30 m.

In vitro, adhesion ability to lung cells

In vitro adhesion ability of the tested microorganisms to A549 Cell Line representing human lung cancer cells was tested. The cell line was supplied by the R&D sector in the Holding Company for Biological Products and Vaccines (VACSERA, Giza, Egypt). The adhesion test was done according to the method of Coconnier *et al.* [22], with some modifications [23].

3. Results

Evaluation of levans types as antivirus

All levan types used in this study were previously prepared as mentioned in material and methods and characterized by thin-layer chromatography as mainly fructose. The ability of the levans to inhibit COVID-19 was evaluated *in vitro* (Fig. 1. A.). The result showed that the most promising levan that caused CoV-19 inhibition was crude levAE (IC_{50}=10.75 mg/ml) and cytotoxicity (CC_{50}> 100 mg/ml). The dialysis process reduced the inhibition activity to a great extent where the IC_{50} of the dialysis form levAE/D was recorded as (7.773e+006 mg/ml). Also, lev13M followed by lev9A showed promising IC_{50} (142.5, 1299 mg/ml) and low cytotoxicity (CC_{50}= 447.9, 5.567e+006 mg/ml) respectively. Finally, levAG and levH had IC_{50} equal to 3155 and 6720 mg/ml respectively.

Comparison between levAE/C, lev9A, and levAE/D FTIR

FTIR for crude and dialyzed samples respectively revealed the major functional groups of levan as seen in Fig. 2, A, B, C. For the crude levan (leAE/C), and lev 9A, a strong broad stretching peaks appeared at 3413.39 and 3290.43 respectively. These bands were attributed to the hydroxyl stretching vibration of the polysaccharide. The same band was shown at 3437.49 cm^{-1} for the dialyzed form (levAE/D) but it was narrow and weak in comparison to that of the levAE/C. The C-H stretching vibration bands for levAE/C, lev9A, and levAE/D were detected at 2929.34, 2923.08, and 2928.38 cm^{-1} respectively. The absorption bands at 1643.05, 1643.02, and 1651.73

cm^{-1} were ascribed to the C=O carbonyl group stretching vibration. The C-H bands were detected at 1422.24, 1419.56, and 1423.21 cm^{-1}. The C-OH characteristic bending bands appeared at 1059, 1039.55, and 1040 cm^{-1}. It was clear from Fig. 2B that most of the bands were greatly reduced after the dialysis process if compared to those of the levAE/C.

Probiotic study

Hemolytic activity

Pseudomonas aeruginosa HI1 showed α blood hemolysis. *Bacillus subtilis 9A* did not show any change in the blood color and consequently no blood hemolysis signs were detected (Fig.3.).

Antibiotic sensitivity

The two isolates showed high sensitivity to different antibiotics that have a broad spectrum of activity. From them, Epigenthad had a similar effect on the two isolates and resulted in the inhibition zones of (7cm) followed by Flumox which caused inhibition zones of (7.1, 5 cm) for *Pseudomonas aeruginosa* and *Bacillus subtilis* respectively. Unictam and Depo-pen showed a good effect on the two strains with some degree of variations (Fig.4.).

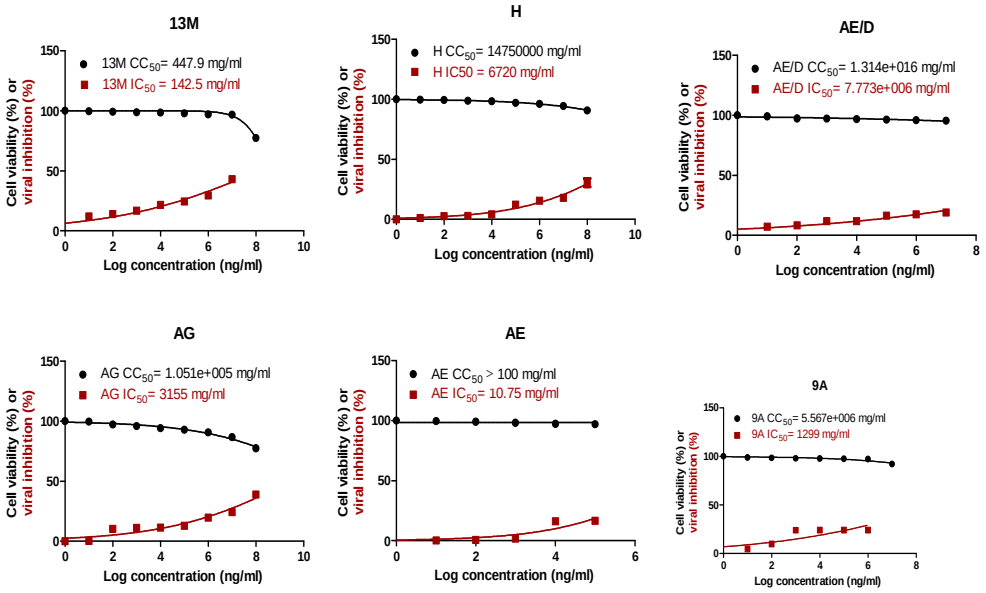
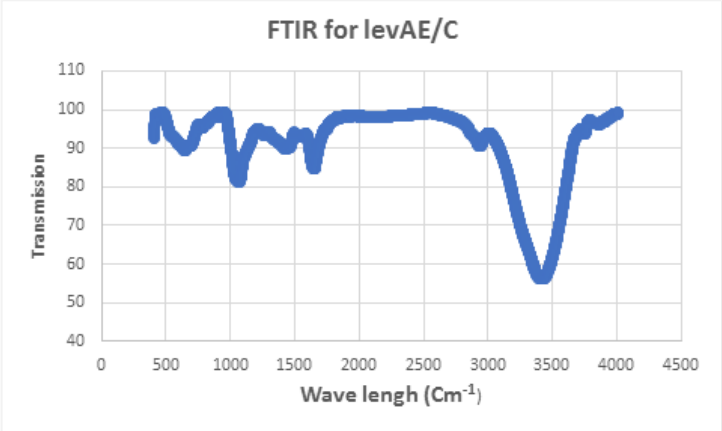
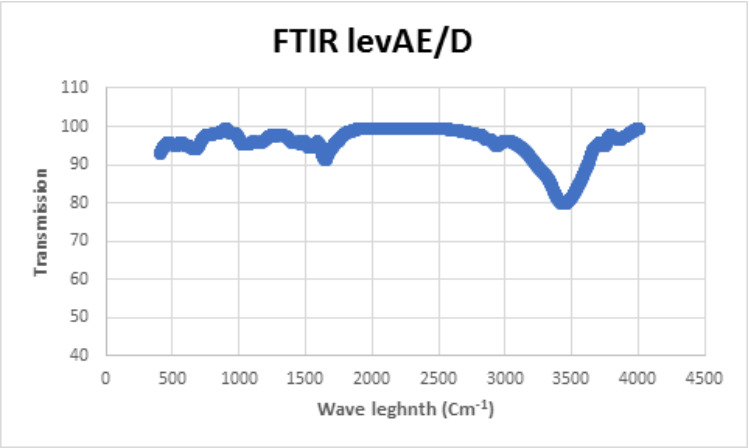


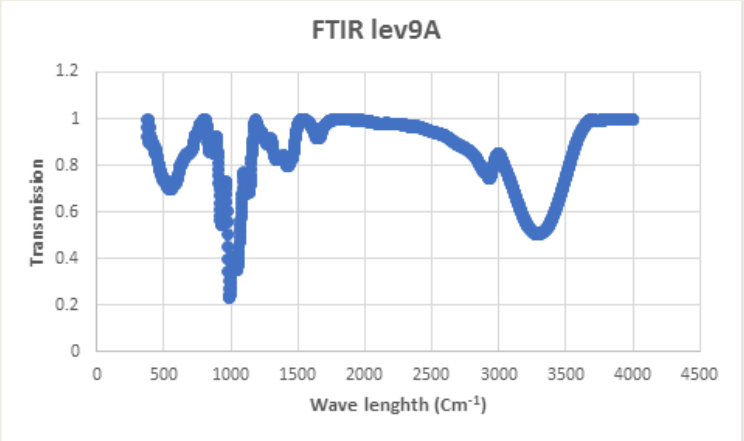
Fig.1. Dose-response, and inhibition curves for levans. Half maximal cytotoxic concentration (CC_{50}) in Vero E6 cells and inhibitory concentration 50% (IC_{50}) against NRC-03-nhCoV were calculated using nonlinear regression analysis of GraphPad Prism.



A.



B.



C.

Fig. 2. A, B, and C. Comparison between the FTIR profiles of levAE/C (A), lev AE/D (B), and lev 9A (C).

AE	**9A**
	
Alpha Hemolytic (Partial hemolysis)	**No hemolysis**

Fig.3. Blood hemolysis test for *Pseudomonas aeruginosa* HI1 and *Bacillus subtilis* 9A.

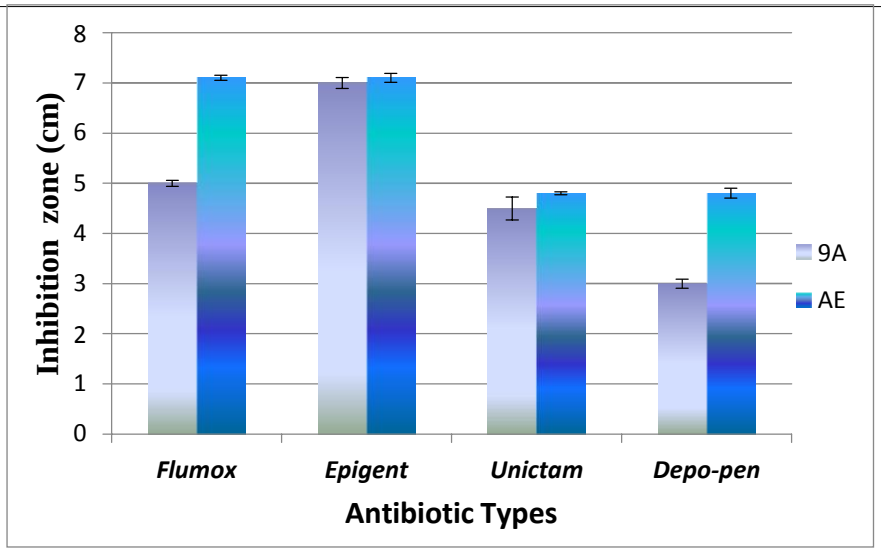


Fig. 4. Antibiotic sensitivity test for the two isolates using different antibiotics

Resistance to low and high pH

Bacillus subtilis 9A maintained its viability for 6 h at pH 9 and pH 11, while it lost from about 42 % and 48 % of its viability at pH 2 and pH 3 after 6 h. *Pseudomonas aeruginosa* could retain its complete survival rate % for 3h at pH 9, 11, and 3. However, after 6h, the surviving % decreased to 69.19%, 64.45%, and 88.62 %respectively (Table 1).

Resistance to bile salt and H_2O_2 tolerance

The bile salt tolerance was evaluated for the two honey isolates. The result (Fig.5A) recorded that *Pseudomonas aeruginosa* lost only 5.41% of its original surviving rate while *Bacillus subtilis* 9A retained its complete surviving rate. The ability of the two isolates to withstand H_2O_2 showed that *Pseudomonas aeruginosa* could keep 77% of its original activity while *Bacillus subtilis* 9A remained at its complete surviving rate (Fig. 5B).

Antimicrobial activity

The antimicrobial activity was achieved using the agar well diffusion method.

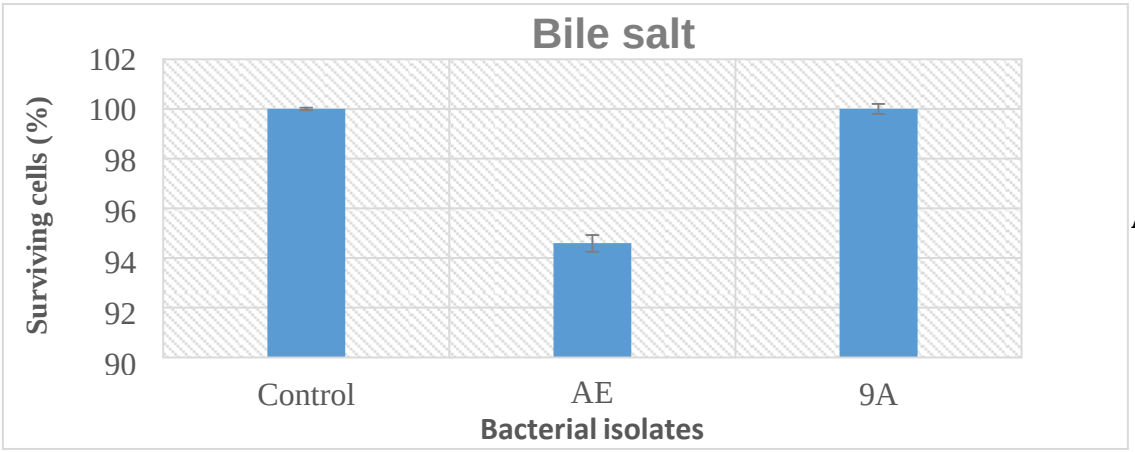
Pseudomonas aeruginosa strain showed antimicrobial activity against all tested microorganisms with different levels. While *Bacillus subtilis* 9A inhibited *Bacillus cereus* (2.60 cm), *C. albicans* (3.00 cm), and *A. niger* strains (3.30 cm) (Table 2).

Adhesion property to A549 Cell Line representing human lung cancer cells

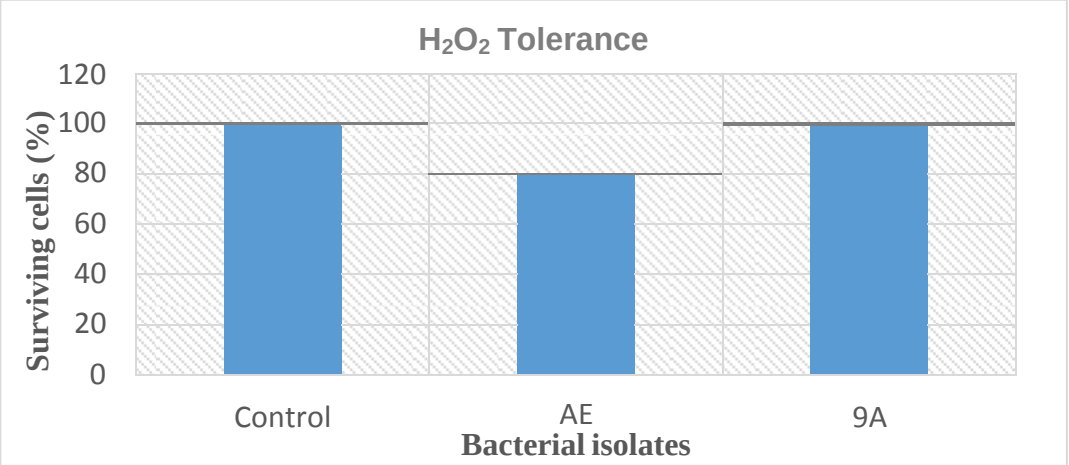
In vitro, the two isolates showed adhesive abilities to A549 Cell Line as seen in Fig.6. The results pointed to the low affinity of the *Pseudomonas aeruginosa* HI1 and *Bacillus subtilis* 9A to colonize the lung cells (0.26% and 2.27% respectively).

The suggested mechanism of levan as antivirus

The diagram (Fig. 7.) illustrates that levan could act as a preventive agent by diffusing through the polycationic surface, attach to the infected lung cells and disrupt the virus through RNA leak out. On the other hand, some levan types work as a protective agent by blocking the virus's active site and inhibiting the virus attachment.



A



B.

Fig. 5. Bile salt and H_2O_2 tolerance

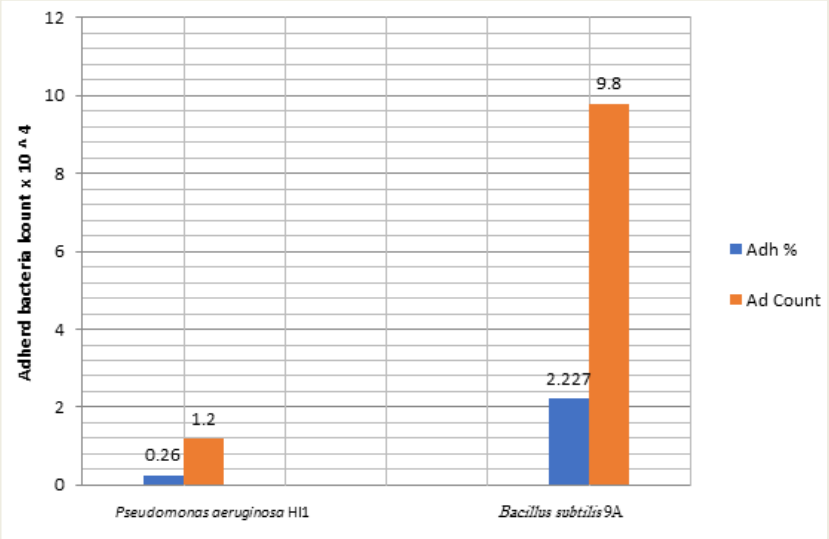


Fig. 6. The adhesion ability (%) and adhesion count for the two isolates to A549 lung cancer Cell Line.

Table 1. Acid and Alkali tolerance (%)

Isolates NO.	Survival (%)											
	pH 11			pH 9			pH 3			pH 2		
	1h	3h	6h	1h	3h	6h	1h	3h	6h	1h	3h	6h
Control	100	100	100	100	100	100	100	100	100	100	100	100
AE	100	100	64.45	100	93.50	69.19	100	100	88.62	97.15	100	100
9A	100	100	100	100	100	100	75.19	53.05	51.14	98.09	96.56	58.01

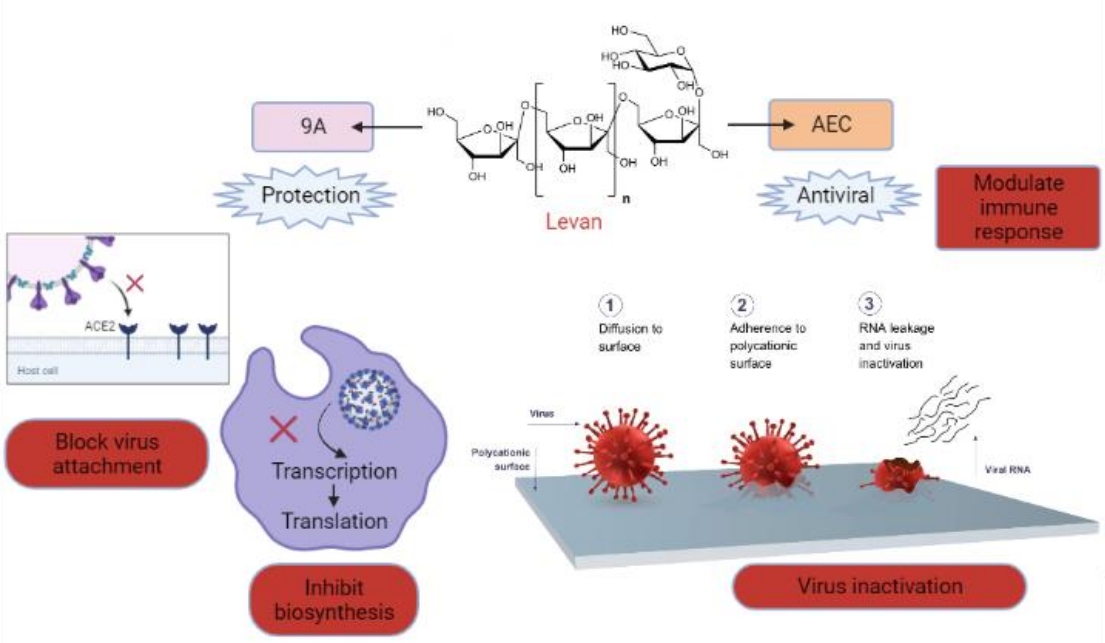


Fig. 7. Suggestion diagram for potential levan mechanism

Table 2. Antimicrobial profile for the two isolates

Isolate No.	Antimicrobial activity (cm)				
	***Bacillus cereus* ATCC 33018**	***Candida. Albicans* NRRL Y-477**	***E. coli* MC1400**	***Staph.aureas* ATCC 29213**	***A.Niger* NRRL 599**
AE	2.80	4.50	2.30	1.70	1.90
9A	2.60	3.00	No zone	No zone	3.30

4. Discussion

Until this moment, no specific drugs for coronavirus treatment were discovered. Researchers do a great effort to formulate vaccines to inhibit COVID-19 growth, but the virus mutates rapidly and develops resistance to these therapies [24]. Levan type fructan was previously identified as a strong antivirus agent against different pathogenic viruses such as pathogenic avian influenza HPAI, H5N1, and New castle disease virus (NDV) [7, 8]. This research study evaluated the effect of different bacterial levans types on CoV-19/Egypt/NRC-03/2020 inhibition. The results detected three levans types, designated as levAE, lev13M, and lev9A, having strong inhibitory effects on the virus. Although the virus half inhibition [IC_{50}] of levAE was higher than that of lev13M by approximately 13-fold and that of levA9 by 120-fold whereas, the cytotoxicity of levA9 was negligible compared to both of levAE and lev13M. The difference in the virus inhibition effect according to the type of levan could be attributed to the difference in fructofuransyl chains as shown by the FTIR. To understand the effect of the dialysis process on the levan virus inhibition activity, FTIR was performed.

The FTIR of levAE/D pointed to the absence and reduction of fructofuranosyl rings after the dialysis process and this was suggested to be the main reason for the loss of levAE virus inhibition activity. The levA9 and levAE showed a broad wide spectrum at 3350-3500 Cm^{-1} and 1000-1100 Cm^{-1} respectively, compared to the same spectrum of the dialyzed form (levAE/D).

The previous researches referred to a firm relationship between the probiotic bacteria from the honey isolates and the levansucrase production [25, 26, 27]. Accordingly, the second part of this study focused on studying of the probiotic activity of *Pseudomonas aeruginosa* HI and comparing it to that of *Bacillus subtilis* 9A, previously studied [data under publication]. The results showed that the two strains had no harmful effect on red blood cells, also they were sensitive to all used antibiotics. These results confirmed the isolates' safety and coincided with those of Ambalam *et al.* [28] who mentioned that the *Lactobacillus rhamnosus* 231 and *Lactobacillus rhamnosus* V92 had no hemolytic activities and showed positive antibiotic sensitivity. The most important probiotic characteristic is the bacterial

ability to withstand the hard stomach environment such as severe acidity. Both tested isolates showed high tolerance to pH values of 2 and 3. Acid tolerance is mentioned as one of the most favorable properties characterizing probiotic strains [29]. Also, the two isolates recorded a satisfactory tolerance to pH 9 and 11. These results exceeded those of Sawatari *et al.* [30] who found that the digestive tract isolates *L. casei* NRIC 1917 and *L. paracasei* subsp. *tolerant* NRIC 1940 showed the highest alkali tolerance at pH 8 and 9. Moreover, both isolates showed approximately complete tolerance to the bile salt. *Lactobacillus casei* was previously found to tolerate bile salt and the acidic medium [31]. In addition, the two isolates could tolerate H_2O_2 with different levels. The antimicrobial activity against the pathogenic strains is an essential criterion of probiotics that allows them to protect the intestine from invading bacteria. *Pseudomonas aeruginosa* HI1 revealed significant antimicrobial activity against all the pathogenic bacteria. In the case of *Bacillus subtilis*, no inhibition was detected against *E. coli* and *Staphylococcus aureas*. Both isolates showed the highest antimicrobial activity against *Candida albicans* which is a common property of most honey isolates [25]. The adhesion of the bacteria to certain cells was found to be controlled by many factors such as polysaccharide secretion. The formation of a polysaccharide such as levan allows managing the bacteria to colonize lung cells. In our result the low adhesion affinity of the two isolates to colonize the A549 Cell Line could be attributed to the unsuitable environment for the isolates to yield the polysaccharide [32]. However, this result suggests the possibility of using *Bacillus subtilis* 9A and its yielded levan to control the lung attack by COVID-19. The suggested Diagram tried to explain the levan virus inhibition mechanism. This suggestion is intended as a brief report derived from our previous experience in investigating the efficacy of levan as an antivirus [7, 8]. Some Levans had the ability to protect the cells from virus attachment by occupying the virus receptors places [7] which has been proven here by the inhibition effect on the virus after incubation of the normal cells with the crude levAE levan for one hour before virus exposure. Due to their amphiphilic nature, levans most probably adhere to the cells via their hydroxyl groups whereas the viruses diffuse into the polycationic region of levan. Once adhered, the levans attack the viruses and disrupt their RNA causing their denaturation [3].

5. Conclusion

This research is a novel trial that evaluated the role of different levan types in hCoV-19/Egypt/NRC-03/2020 inhibition. The results paid much attention to levan as a strong antivirus and protective agent against the COVID-19. Also, the study tried to correlate the probiotic activity of two bacterial honey isolates and their potential levan products, to their antiviral effect.

6. Acknowledgments

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