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Safety evaluation of *Bacillus subtilis* strains

Introduction

The *Bacillus subtilis* group is generally considered as safe (so-called "GRAS" status), and comprises the closely related taxa *Bacillus subtilis* subsp. *subtilis*, *Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus atrophaeus, Bacillus mojavensis, Bacillus vallismortis, Bacillus subtilis* subsp. *spizizenii* and *Bacillus sonorensis* (Wang et al., 2007). Their phenotypic differentiation is difficult, but DNA-DNA hybridization and comparative sequence analysis of 16S rRNA provide a firm basis for their identification.

The safety of microorganisms that have been used traditionally in probiotics has been confirmed through a long period of cultural tradition and experience. Strains of species such as *B. subtilis, B. licheniformis* and *B. sonorensis* have been used extensively in (or are associated with) food processing throughout human history and ingestion of foods containing such live or dead bacteria and their metabolites has occurred over a long period of time (see: FAO Background Study Paper No. 65: http://www.fao.org/docrep/meeting/028/mg309e.pdf).

So far, the safety of *B. subtilis* group has not been questioned in general, and reports of possible harmful effects of these microbes to the host are extremely rare. Rare cases are in fact related to "opportunistic" situations of immune compromised persons and patients at risk. On one hand, currently some authorities require proof of safety of newly isolated *B. subtilis* group strains before their commercialization and use in food biotechnology. Applications of such strains include their use as starter cultures for bio-preservatives and as probiotics. The assumption of "GRAS" status is commonly based either on a long period of safe use of a strain or as a reflection of the wide acceptance of indigenous food fermentation throughout human history (Holzapfel, 2002). In the USA, the Food and Drug Administration (FDA) and its Division of Biotechnology and "GRAS Notice Review", have the responsibility of safety ("GRAS") regulation a new microbial strain under the conditions of its intended use in food production or processing (Franz, Hummel & Holzapfel, 2005). On the other hand, the novel "decision-tree" approach of the EU was introduced for the pre-market safety assessment of microorganisms intended for application in food biotechnology. By this "Qualified Presumption of Safety" (QPS) approach, the safety of a defined taxon (genus or group of related species) can be made on the basis of four "pillars": (a) established identity, (b) body of knowledge, (c) possible pathogenicity, and (d) end use. Exclusion or qualification of safety concerns should result in granting QPS status for a given taxonomic group (EFSA, 2007).

Inhalation infection test

It is well known that respiratory tract infection by pathogenic microorganism in human is serious and threatening problem these days, however, the study of inhalation infection caused by microorganism is not fully reported. The animal inhalation infection study by intranasal injection of solubilized microorganisms in liquid can confer the basis for evaluating the safety of microorganisms and understanding their interaction with the host respiratory tract. The method is explained by *Harold S. Ginsberg* et al., and the procedure is as below.

Efficacy tests

Bacillus subtilis strains have also shown the ability to antagonize pathogenic bacteria genera such as *Lactobacillus*, *Xanthomonas*, *Pseudomonas* and *Bacillus* and molds such as *Fusarium*, *Botrytis cinerea*, *Phoma tracheiphila*. Several bioactive components such as enzymes that degrade fungal structural polymers (protease and chitinase), lipopeptides (iturin, surfactin and fengycin), antibiotics (fengycin, surfactin, iturin and bacillomycin D) and antifungal volatiles have been identified as the main antagonistic products. This characteristic has allowed them to be used as a biological control agents (Xu et al. 2015).

In this report, we confirm the taxonomical identity of *B. subtilis* strain number 3, 281 and 298 using 16S rDNA sequencing, and inform on the assessment of possible pathogenicity using in-vitro methods such as lecithinase activity, hemolysis tests and resistance to therapeutic antibiotics. *B. subtilis* strains safety was also evaluate in-vivo through a lung infection model in mices. Lastly, the efficacy of *Bacillus* strains to inhibit the growth of *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhi*, *Listeria inocua*, *Alternaria alternata*, *Cladosporium sphaerospermum* and *Penicillium chrysogenum* was evaluated in-vitro.

Materials and methods

16S rDNA sequencing

Pure cultures of B. subtilis strains 3, 281 and 298 were grown on Luria Bertani Miller agar (BD Difco) at 37°C for 24 hours. The plates were sent to Solgent Inc. (Deajun, South Korea) for bi-directional 16S rDNA sequencing using (5′-AGA GTT TGATCMTGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACTT-3′) primers by (Reysenbach et al. 1992). Bi-directional sequencing results were assembled using Codon Code Aligner (Codon Code Corporation, USA) and compared with reference sequences of B. subtilis on the GenBank database (http://www.ncbi.nlm.nih.gov/Blastn/).

Hemolysis Test

B. subtillis strains 3, 281 and 298 were grown at 37°C for 18 hours in LB broth and then streaked onto 5% sheep blood agar (Hanil Komed) and incubated for 24 h at 37 °C. Alpha (α) hemolysis was considered as the partial decomposition of the hemoglobin of the red blood cells, beta (β) hemolysis as the complete breakdown of the hemoglobin of the red blood cells observed as a clear zone in the agar plate and gamma (γ) hemolysis as the lack of hemolysis. *B. cereus* ATCC 27348 was used as a positive control.

Lecithinase Test

B. subtilis strains 3, 281 and 298 and *B. cereus* ATCC 27348 (positive control) were grown at 37°C for 18 hours in LB broth and streaked out onto egg yolk agar. The plates were incubated for 24 h at 37°C and the formation of a

white precipitate around the colonies was considered as positive for lecithinase activity (Sorokulova et al., 2008, Hong et al., 2008).

Antibiotic resistance test

Pure cultures of *B. subtilis* strains 3, 281 and 298 were cultivated at 37°C for 18 h in LB agar. The agar dilution method was used to assess the minimal inhibitory concentration (MIC) of the strains against antibiotics. A single colony of each strain was resuspended in phosphate buffer saline (PBS) 1X and adjusted 0.01 optical density (OD). Ten microliters ($1x10^5$ CFU/mL) of each strain were inoculated on the plates using multipin-inoculator and incubated at 37°C for 24 hours. The strains were considered susceptible when they were inhibited at a concentration for a specific antimicrobial equal or lower than the established cut-off value and resistant bacterial strain when it was not inhibited at a concentration of a specific antimicrobial higher than the established cut-off value according to the parameters stablished by the European Food Safety Authority (EFSA).

Inhalation infection test

During the experiment the mice were divided on six groups; negative control, positive control which was infected with the pathogenic bacteria (*Pseudomonas aeruginosa* PAO1), and four *B. subtilis* strain 3 infected groups. The *B. subtilis* 3 infected dose was $1X10^6$, $1X10^7$, $1X10^8$ CFU/mouse with pre-treatment of cyclophosphamide for inducing immunosuppression. The last *B. subtilis* 3 group was infected with $1X10^8$ CFU/mouse without pre-treatment with cyclophosphamide. *P. aeruginosa* PAO1 was used as positive control to confirm that the infection was done properly. This group was also previously treated with cyclophosphamide to suppress the intrinsic immune system of the mice. The use of cyclophosphamide for mice immunosuppression is justified when, non-virulent strains may also cause death at high dose on healthy mice. However, infection of immunocompromised the mice can provide a clearer difference between virulent and non-virulent strains.

B. subtilis strain 3 were grown for 18 hours in LB broth (BD Difco) at 37°C. *P. aeruginosa* PAO1 was cultured on Muller-Hinton Agar (BD Difco) and subcultured to Muller-Hinton II Broth (BD Difco) an incubated at the same conditions. The bacterial solution was diluted in 1X PBS to $1x10^7$, 10^8 and 10^9 CFU/mouse. The inoculum concentration was determined by enumeration of cultured bacteria on agar plates.

The animal lung infection model was carried out in ICR 4-week-old female mice (Hyo-chang science). The mice were anesthetized during anesthesia 50 uL of bacterial suspension, according to the concentration of each group, were injected through intranasal route. After 24 h post infection, 2 mice of each group were selected randomly and sacrificed with diethyl ether for enumeration of viable colonies in lung. The lung was extracted and homogenized with 10 mL of PBS. The homogenized lung samples were diluted and spreaded on LB agar. to count the number of CFU in lungs. The LB plates were incubated for 18 hours. The rest of mice were monitored during 2 weeks to measure their survival rates.

Efficacy antagonism test

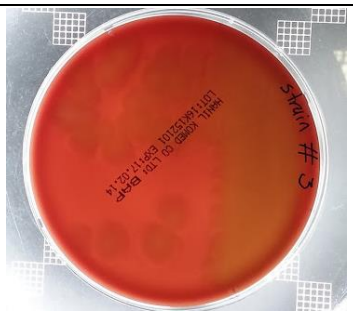
The agar well diffusion assay was done to test the antagonistic activity of *Bacillus subtilis* strains 3, 281 and 298 on the growth of pathogenic bacteria such as *Escherichia coli* ATCC 22922, *Pseudomonas aeruginosa*, *Bacillus cereus* ATCC 27348, *Staphylococcus aureus* ATCC 11335, *Salmonella typhi* ATCC 14028 and *Listeria inocua* ATCC 3586. The antagonistic activity of *Bacillus subtilis* strains was also verified against molds such as *Alternaria alternata* (Fries) Keissler (ATCC® MYA-4642™), *Cladosporium sphaerospermum* Penzig (ATCC® MYA-4645™) and *Penicillium chrysogenum* Thom (ATCC® MYA-4644™). Brain Infusion Heart Agar (BHI) was used for the antagonism against pathogens and Potato Dextrose Agar (PDA) for the essay against molds. Pathogens were cultured on BHI broth and *Bacillus subtilis* strains on LB broth at 37°C for 18 h. Molds were grown on PDA at 25°C with 85% HR for 7 days. Mold spores, pathogens and *Bacillus* strains were harvested and resuspended in PBS. Pathogens and *Bacillus* were adjusted at 0.2 OD and 100 uL of pathogen suspension was spreaded on BHI agar plates. The same amount of spore suspension was inoculated on PDA. Wells were made in all the plates and 20 uL of *Bacillus subtilis* strains suspensions were inoculated in the holes. The BHI plates were incubated at 37°C for 24h and PDA plates at 25°C for 72 h. The inhibition of pathogens and molds was observed as a clear zone around the wells. Hydrogen peroxide at 10% was used as a positive control.

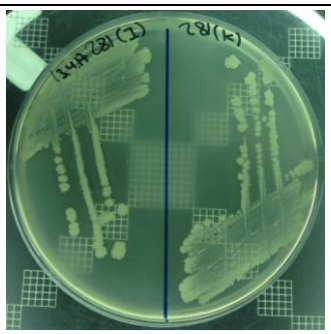
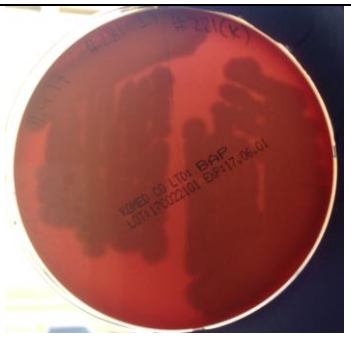
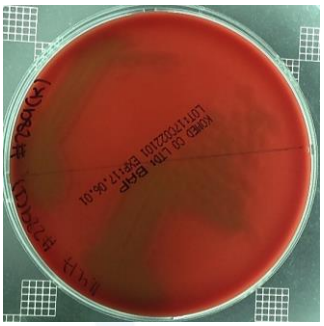
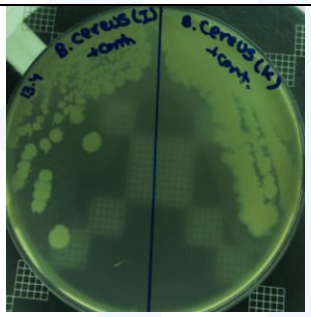
Results

In-vitro safety evaluation of lecithinase and hemolysis activity

B. subtilis strains 3, 281 and 298 did not show lecithinase activity observed as the absence of withe precipitated around the *Bacillus* colonies. Regarding to the hemolytic activity, the agar blood plates inoculated with the *Bacillus subtilis* strains did not shown partial or complete disruption of the red blood cells on the agar compared with the positive control of *B. cereus* ATCC 27348 (Table 1).

Table 1. Lecithinase and hemolysis activity of *B. subtilis* strain 3, 281 and 298 and *B. cereus* ATCC 27348.

Strain / **Test**	**Lecithinase**	**Hemolysis**
***Bacillus subtilis* 3**		
Result	Negative	Gamma

***Bacillus subtilis* 281**		
Result	Negative	Gamma
***Bacillus subtilis* 298**		
Result	Negative	Gamma
***Bacillus cereus* ATCC 27348**		
Result	Positive	Beta

In-vitro evaluation of antibiotic resistance

The agar dilution was used to assess the minimal inhibitory concentration (MIC) of antibiotics. *B. subtilis* strains 3, 281 and 298 were found to be sensitive to erythromycin, gentamicin, tetracycline, streptomycin, vancomycin, chloramphenicol, kanamycin and clindamycin according to the European Food Safety Authority MIC breakpoints for *Bacillus* species. The determined MIC values are clearly below or equal to the EFSA breakpoint values (Table 2).

Table 2. Minimum inhibitory concentrations (MIC) of *B. subtilis* strains number 3, 281 and 298.

Antibiotic resistance test								
Strain	**Minimum inhibitory concentration (mg/L) of antibiotics**							
	Ery	**Gen**	**Tet**	**Str**	**Van**	**Chl**	**Kan**	**Cli**
***B. subtilis* 3**	≤2	≤2	≤4	8	≤2	≤4	≤4	4
***B. subtilis* 281**	≤0.125	≤2	≤0.125	8	0.25	≤4	≤4	2
***B. subtilis* 298**	≤0.125	≤2	≤0.125	8	0.25	≤4	≤4	0.5
EFSA breakpoint	4	4	8	8	4	8	8	4

Ery: Erythromycin; Gen: Gentamicin; Tet: Tetracycline; Str: Streptomycin; Vm: Vancomycin; Chl: Chloramphenicol; Kan: Kanamycin; Cli: Clindamycin.

Inhalation infection test

It was observed that the survival rate of the mice in the inhalation test in the groups infected with *Bacillus subtilis* strains was 100%, which means that all the mice survived to the infection. In contrast, on the group infected with *Pseudomonas aeruginosa* PAO1 any mice survived after 24 hours, 100 % mortality (Figure 1). The lungs of the group of mice infected with *Bacillus subtilis* 3 and the lungs of the mice on negative control group that were sacrificed after 24 h post infection did not grow colonies of bacteria (data are now shown).

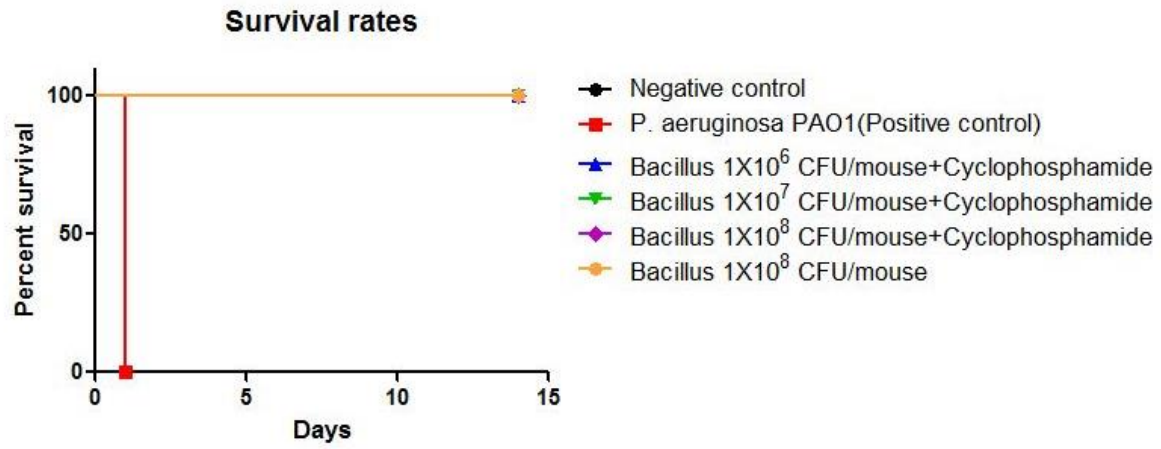


Figure 1. Graphic representation of the survival rates of mice for the inhalation test.

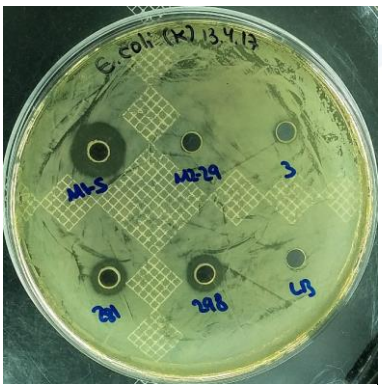
Efficacy tests

The antagonism test results of *B. subtilis strains* against pathogenic bacteria indicate that *B. subtilis* 3 reduce the growth of *E. coli* ATCC 22922, *S. aureus* ATCC 11335 and *L. inocua* ATCC 3586 at various levels. Regarding to *B. subtilis* 281 and 298, they seem to antagonize the growing of *E. coli* ATCC 22922, *P. aeruginosa*, *B. cereus* ATCC 27348 and *S. typhi* ATCC 14028 also at different degrees. In addition, *B. subtilis* 298 has also the ability of slightly reduce the growth of *S. aureus* ATCC 11335. In general, *B. subtilis* 298 could be more effective against pathogenic bacteria than the other two strains (Figure 2).

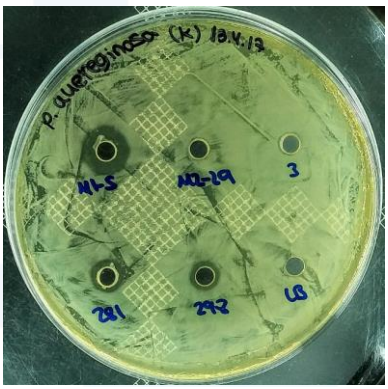
Table 3. Antagonistic activity of *Bacillus subtilis* strains against pathogens.

Pathogens / **Bacillus strains**	**Inhibition zone (cm)**		
	Bacillus subtilis 3	*Bacillus subtilis* 281	*Bacillus subtilis* 298
Escherichia coli ATCC 22922	0.1	0.2	0.2
Pseudomonas aeruginosa	NA	0.1	0.1
Bacillus cereus ATCC 27348	NA	0.1	0.2
Staphylococcus aureus ATCC 11335	0.1	NA	0.1
Salmonella typhi ATCC 14028	NA	0.2	0.2
Listeria inocua ATCC 3586	0.2	NA	NA

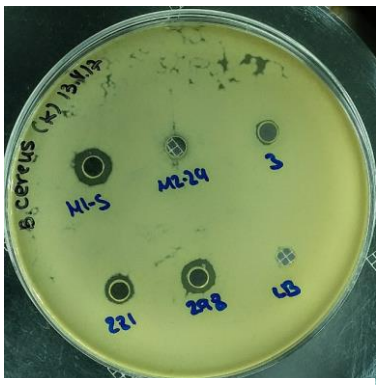
NA: Not antagonism was observed



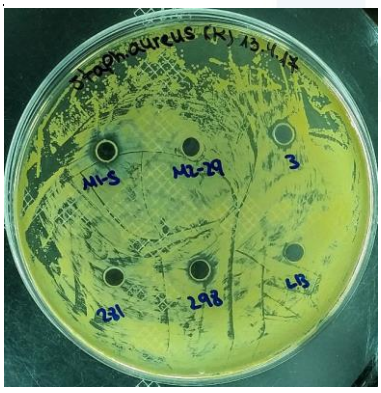
Escherichia coli ATCC 22922



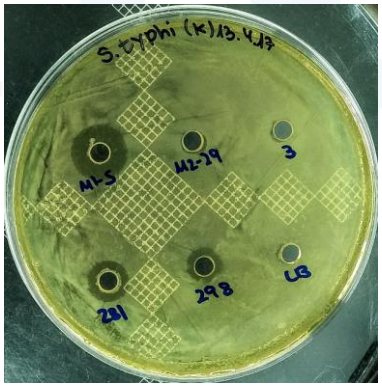
Pseudomonas aeruginosa



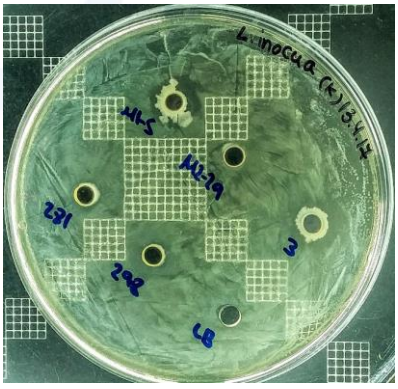
Bacillus cereus ATCC 27348



Staphylococcus aureus ATCC 11335



Salmonella typhi ATCC 14028



Listeria inocua ATCC 3586

Figure 2. Antagonism of pathogenic bacteria by *Bacillus subtilis* strains 3, 281 and 298 evidenced for the formation of a clear zone around the wells.

Concerning to the challenge of Bacillus subtilis strains against molds. The results indicate the strain 3, 281 and 298 has antagonism against *Alternaria alternata* (Fries) Keissler (ATCC® MYA-4642™) and *Cladosporium sphaerospermum* Penzig (ATCC® MYA-4645™) at variable level (Table 4). The inhibition of the mold growth is evidently expressed as a clear zone around the wells inoculated with the Bacillus subtillis strains (Figure 3). *Penicillium chrysogenum* Thom (ATCC® MYA-4644™) was not inhibited at all for any of the strains used on this experiment, however the mycelium color around the well inoculated with strains number 281 and 298 show and slightly decrease in the intensity (data non-shown) from green to white.

Table 4. Antifungal activity of *Bacillus subtilis* strains against molds.

Strain	**Inhibition zone (cm)**		
	Alternaria alternata (Fries) Keissler (ATCC® MYA-4642™)	*Cladosporium sphaerospermum* Penzig (ATCC® MYA-4645™)	*Penicillium chrysogenum* Thom (ATCC® MYA-4644™)
Bacillus subtilis 3	0.2	0.4	NA
Bacillus subtilis 281	0.5	0.5	NA
Bacillus subtilis 298	0.6	0.6	NA
Hydrogen peroxide 10%	0.8	1.2	1

NA: Not antagonism was observed

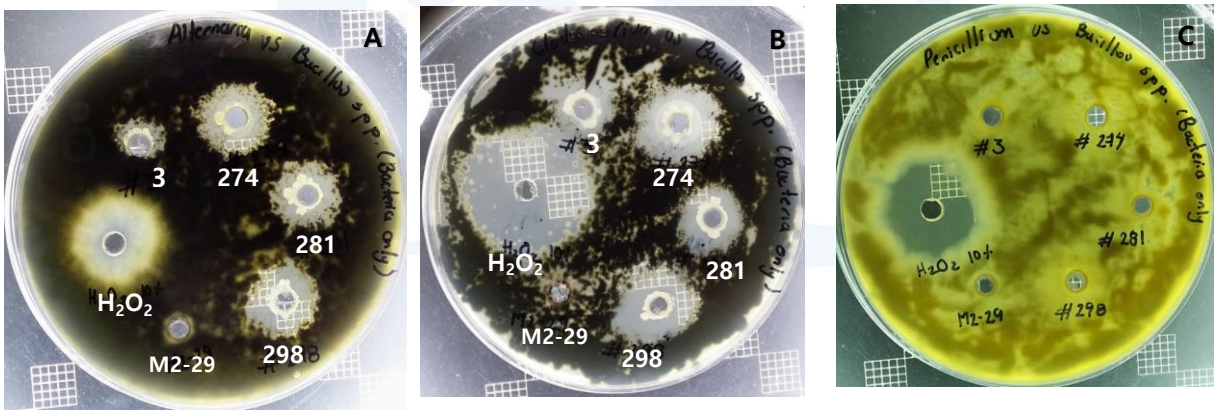


Figure 3. Results for the antagonism test of *Alternaria alternata* (Fries) Keissler ATCC® MYA-4642™ (A), *Cladosporium sphaerospermum* Penzig ATCC® MYA-4645™ (B) and *Penicillium chrysogenum* Thom ATCC® MYA-4644™ (C) against *B. subtilis* strains 3, 281 and 298. 10% hydrogen peroxide was used as a positive control.

Conclusions

The major objective of this study was to identify genotypically *B. subtilis* strain number 3, 281 and 298 and evaluate their safety and efficacy against pathogenic bacteria and molds. The identity of the strains was confirmed as *B. subtilis* according to the National Center for Biotechnology Information (NCBI) database. *B. subtilis* strain

number 3, 281 and 298 were negative for both of lecithinase and hemolytic (γ) activity while the positive control, *B. cereus* ATCC 27348, showed positive reactions in both safety tests. *B. subtilis* strain number 3, 281 and 298 showed susceptibility to the antimicrobial agents tested on this study (erythromycin, gentamicin, tetracycline, streptomycin, vancomycin, chloramphenicol, kanamycin and clindamycin) according to the breakpoints suggested by European Food Safety Association. Regarding to the results of the Inhalation test, *Bacillus subtilis* 3 is no virulent on mice which is supported by the high survival rates on mice and the non-detected colonies on the lungs after sacrifice. The absence of *B. subtilis* 3 on lung suggest that it did not colonize the mice lung and that it could have been removed from the respiratory tract by breathing. In addition, the Bacillus subtilis 3, 281 and 298 shown to have efficacy in the inhibition of a broad spectrum of gram positive, gram negative and molds a t diverse levels with suggest their possible use as control biological agents. Summarizing, *B. subtilis* 3, 281 and 298 can be considered as a safe and functional strains with high potential to be used for industrial applications.

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