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Investigation of *B. subtilis* viability at different pH ranges for use in microbial cleaner formulation

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Abstract: Until today, several disinfecting methods have been proposed and studied for cleaning and disinfection, containing heavy metals and chlorine, to reduce the biological load on environmental surfaces. Most of the proposed techniques are based on the use of chemical compounds. These methods have proven to be effective in reducing the majority of pathogens, but have been ineffective in preventing the persistence of pollutant microorganisms. Nowadays, increasing demand for natural and green products has led to recognition of cleaning products containing microbial based cultures. Cleaning products containing live microorganisms as active substances are becoming increasingly common in homes and industrial cleaning applications. These products are called "probiotic or microbial" cleaners. Microbial based cleaning products are environmentally cleaning products that contain bacteria or spores that are suitable as active ingredients. The purpose of using these cleaning systems is to prevent the development of pathogens by tolerating the presence of harmless probiotic microorganisms on surfaces. The aim of this study is to investigate the viability of *B. subtilis* species at different pH values for use in detergent formulation and the effective removal of pathogenic bacteria and fungi on the surface in the long term. As a result, it was observed that the probiotic *B.subtilis*, which can be used in the microbial based cleaner formulation, showed higher growth and vitality at the neutral pH and exhibited high antibiotic resistance, auto-aggregation, antagonistic and antifungal ability. Based on these findings, *B. subtilis* can be used to prevent the development of pathogenic species.

Keywords: *B. subtilis*; Probiotics; Microbial-based cleaning; Probiotic cleaning system

1 Introduction

Increasing demand for natural and green products has led to recognition of cleaning products containing microbial based cultures. Towards the early 2000s, new generation of microorganism-based products began to emerge for cleaning surfaces (Teasdale and Kademi 2018). Cleaning products containing live microorganisms as active substances have become increasingly common in houses and industrial cleaning applications (Spöka et al. 2018). Today, new generation cleaning products containing active microorganisms or spores as active ingredients attract the attention of professional users, consumer organizations and regulators. These products are also called as "biological" cleaners, "probiotic" cleaners or "microbial" cleaners (Teasdale and Kademi 2018; Spöka et al. 2018). In some studies, microbial cleaning products have proven to be effective in preventing contamination of surfaces with various pathogens. In addition, microbial cleaning products have been shown to reduce the presence of pathogens by 80-90% compared to the microbial load detected on surfaces treated with conventional cleaner / disinfectants (Brooke and Selby 2014; Vandini et al. 2014; Caselli et al. 2016; Caselli 2017; D'Accolti 2018; Caselli et al. 2019). The purpose of these microbial cleaning systems is to prevent the development of pathogenic species by tolerating the presence of microorganisms that do not harm for humans, not by a general disinfection that minimizes the presence of microorganisms (Vandini et al. 2014). In other words, instead of removing microorganisms from the environment, it may bring a new understanding of cleaning systems aiming to establish a controlled and less harmful microbiota with microbial cleaning products (Vandini et al. 2014; Caselli 2017). This approach considers that replacing all pathogens with beneficial microbes may be more effective at reducing infections (Caselli 2017). It is generally accepted that beneficial microbes are important for our health and their use can be effective in the prevention and treatment of infectious diseases. Among the potentially beneficial microorganisms for this purpose, "probiotics" are particularly preferred because they are defined as useful microorganisms for health (Vandini et al. 2014; Caselli 2017). Probiotics are beneficial microorganisms that benefit host health when taken in sufficient quantities (Gómez et al. 2016). Because of the therapeutic properties of probiotics, they have been used as food for many years. Probiotics are investigated as their survival in stomach conditions, colonization in the gut, antimicrobial effects against foodborne pathogens and other

functional properties (Lee et al. 2013). New generation cleaning products contain beneficial microorganisms that can compete with pathogenic microorganisms using nutrients on contaminated surfaces. These beneficial microorganisms are preferred because they remain on the surface where they are applied (often spores; bacteria that form spores in many formulations, *Bacillus* spp.) and prevent colonization by pathogenic microorganisms (Spöka et al. 2018). *Bacillus* is a genus of Gram-positive nonpathogenic bacteria, found in many places in nature (such as in soil, water, vegetables, human intestine) and have safe use in humans (Caselli 2017). *Bacillus clausii*, *Bacillus subtilis*, *Bacillus pumilus,* and *Bacillus coagulans* are specific species of the *Bacillus* genus that used as probiotics (Hong et al. 2008). Microbial cleaning products are more susceptible to microbial contamination and degradation due to their mild physical and chemical properties than conventional chemical cleaners with extreme pH and hard components. Therefore, all steps to be performed after fermentation should be carried out carefully to minimize the presence of external microorganisms contaminated detergent (Teasdale and Kademi 2018; Spöka et al. 2018). If storage conditions are not optimal, this cause different levels of stress for microbial components in the products and can cause a decrease in their viability despite the high resistance of *Bacillus* spores (Teasdale and Kademi 2018). In addition to storage and transport conditions, pH, the quality of spores and the biocompatibility of formulation components as well as the nature and concentration of preservative used are factors that can contribute to reducing the stability of *Bacillus* (Teasdale and Kademi 2018; Spöka et al. 2018). pH plays an important role in the stability and maintenance of the vitality of *Bacillus* spores. The optimal stability of *Bacillus* spores was found at pH 6.8 in a liquid medium and pH below 4.8 had a significant effect in reducing their viability over time, in the Teasdale and Kademi's study. The quality of spores also showed an important effect on maintaining their vitality in the liquid environment (Teasdale and Kademi 2018). The aim of this study is to investigate the viability of *B. subtilis* species at different pH values for use in detergent formulation and to investigate the effective removal of pathogenic bacteria and fungi on the surface in the long term.

2 Materials and Method

2.1 Pathogenic test cultures

The bacterial test cultures of *K. pneumoniae* ATCC 43816 and *P. aeruginosa* ATCC 27853 and fungi of the *Aspergillus brasiliensis* ATCC 16404 species were obtained from the culture collection of Algal Biotechnology and Bioprocess Laboratory of Yıldız Technical University. Stock cultures of bacteria were stored at -20 °C in Nutrient agar (NA) medium, supplemented with 25 % (v/v) glycerol. Pathogenic bacteria was incubated for 20-24 h at 37 °C in Nutrient agar (NA) medium (Vandini et al. 2014). Potato dextrose agar (PDA) was chosen as fungal medium for the growth of fungi and it was incubated at 28 °C for 6 days (Vehapi et al. 2018).

2.2 Cryopreservation of probiotic bacteria

Probiotic *Bacillus subtilis* ATCC 6633 was obtained from the culture collection of Algae Biotechnology and Bioprocess Laboratory of Yıldız Technical University. Probiotic strain *B. subtilis* was grown in 10 mL Luria Bertani (LB) medium at 37 °C for 24 h, and then incubated at 37 °C for 24 hours on Tryptic Soy Agar (TSA) plate. The cultures from the agar plate with the loop were transferred to eppendorf tubes that contain 500 µL of double LB and 500 µL of glycerol and stored as stock at -80 °C for future experiments.

2.3 Production of probiotic bacteria

B. subtilis ATCC 6633 stored at -80 °C, was activated by adding to the Luria Bertani (LB) liquid medium. Afterwards, planting was done on solid media containing Tryptic Soy Agar (TSA). Petri dishes were incubated overnight at 37 °C (Teasdale and Kademi 2018). Pure culture was obtained from the colonies grown in Petri dishes. For this, bacteria taken from a single colony were incubated in a 10 mL liquid medium (LB) at 35 °C and at different pH ranges such as pH 3, 5, 6, 7 and 8.

2.4 Investigation of growth kinetics

When the bacterium enters the liquid medium, a short lag phase, which passes to adapt to the medium, was observed. This was followed by the logarithmic phase in which the proliferation continues at a constant rate. As the nutrients in the environment were exhausted, the stationary phase began. By the way, some bacterial cells started to die (Winslow and Walker 1939). *B. subtilis* cell growth was monitored by measuring the optical density at 600 nm by taking samples from the culture grown in 10 mL LB medium hourly. PG Intruments T-60 UV Visible Spectrophotometer device was used for optical density analysis (Bhatta et al. 2006).

2.5 Calculation of plate count

Probiotic bacteria numeration was evaluated by pour plate method with serial dilution. Culture samples were serially diluted with phosphate buffered saline (PBS) according to the pour plate method, and 100 µL probiotic bacteria was spread on TSA plate, and incubated at 37 °C for 20-24 h. The number of Colony Forming Units (CFU) was determined by colony count after incubation of TSA plate. *Bacillus* colonies developing after incubation were counted and expressed in CFU mL^{-1} (Vandini et al. 2014; Bauzad et al. 2019). The numbers of bacteria per mL were calculated using the Eq. (1).

$$CFU\ mL^{-1} = (CFU \times DF) / aliquot \quad (1)$$

CFU: number of colonies (Colony Forming Unit)

DF: dilution factor

2.6 Auto-aggregation ability

Cell-cell interactions were evaluated according to the auto-aggregation test. Probiotic bacterial cells grown for 20 h at 35 °C in LB medium were collected by centrifugation. Optical density (OD 600nm) with phosphate buffered saline (PBS; pH 7.2) was adjust to A_0 (A_0=0.5). The number of bacteria (10^6-10^7 CFU mL^{-1}) was standardized in this way. Each bacterial suspension (8 mL) was vortexed and incubated at 30 and 37 °C for 7 h. Absorbance values were measured at 600 nm after 7 h (A_t) (Collado et al. 2008; Woo and Ahn 2013; Gómez et al. 2016). Auto-aggregation ability was calculated with Eq. (2).

Auto - aggregation (%) = $(1 - A_t / A_0) \times 100$ (2)

A_t: absorbance value measured at 7 th hour

A_0: absorbance value measured at initial time

2.7 Antibiotic susceptibility test

The antibiotic susceptibility test was performed by using the Bauer-Kirby method (Bauer et al. 1966). The Kirby-Bauer test, known as the disc diffusion method, is an antibiotic susceptibility test. For this test, a culture medium was properly and aseptically inoculated with the test organism, and then filter paper discs with a certain antibiotic concentration were placed in the medium. While the organism grows on the agar plate, the antibiotic inhibits growth. If the organism is sensitive to a specific antibiotic, no growth around the disc containing the antibiotic is observed. Therefore, an "inhibition zone" is measured to determine the susceptibility of microorganism to an antibiotic. It is then classified as microorganism Resistant (R), Medium (I), or Sensitive (S) (Bauer et al. 1966).

2.8 Antagonistic activity of *B. subtilis*

This test determines the antagonistic ability of the *B. subtilis* against pathogenic bacteria like as *K. pneumoniae* and *P. aeruginosa*. Antagonistic activity of *B. subtilis* against pathogens were studied by the method of antagonism in solid medium. The antagonistic activity was detected as a zone of pathogens' growth inhibition. *B. subtilis* grown in 10 mL LB medium for 30 h at 35 ºC were centrifuged at 12000 rpm for 15 minutes. Indicator pathogenic strains were subcultured on nutrient broth (NB) for 24 h at 37 °C. Nutrient agar (NA) plates were spread with 100 µL of indicator bacteria. *Bacillus* supernatant was dropped 30 µL into the wells (approximately 6 mm) on the surface of the nutrient agar (NA) plate containing pathogenic indicator strains at 10^8 CFU mL^{-1}, and the plates were incubated at 37 ºC for 24 h. Antagonistic activity was identified as the growth of *B. subtilis* around the well and the inhibition of the pathogen (Hong et al. 2008; Moore et al. 2013).

2.9 Antifungal activity of *B. subtilis*

In disc diffusion test, petri plates for pathogenic fungal microorganism "*Aspergillus brasiliensis* ATCC 16404" containing sterile Potato Dextrose Agar (PDA) growth medium (15 mL $plate^{-1}$), were inoculated with 6 mm diameter mycelium disc taken from 7 days old fungal cultures. Then, it was placed in the middle of the petri dishes (Vehapi et al. 2018, Özçimen 2018). *Bacillus* supernatant was dropped as 30 and 50 µL into the paper disc, and paper disc was placed on the cover of the petri dish. All the plates were inverted and incubated at a temperature of 28 °C for 6 days to allow adequate *Bacillus* - pathogen interaction to take place. All the cultured plates were periodically observed for mycelial growth. After incubation, fungal growth was measured and antifungal activity was evaluated by disc diffusion assay (Killani et al. 2011).

2.10 Statistical analysis

Data was presented as means with ± standard deviations. Analysis of variance was carried using the JMP (release 6.0.0, SAS) analysis program. The significance ratings between the averages were determined by Student's t test ($p < 0.05$ was considered significant).

3 Results and Discussion

3.1 Growth response of *B. subtilis* to variety pH

Growth curves of *B. subtilis* grown in media buffered at pH 3, 5, 6, 7 and 8 were shown in Figure 1. *B. subtilis* cells were incubated at 35 ºC for 30 h at various pH values from pH 3 to 8 in buffered LB nutrient medium and growth curves were obtained.

B. subtilis cultures in buffered modified medium of pH 7 and 8 were growth rapidly, whereas cultures of pH 5 and 6 showed a long lag time (Figure 1). A little difference was observed among the specific growth rates of *Bacillus* at pH 5, 6 and 8, indicating that there is a rather wide pH range for comparable growth of this bacteria under the conditions employed. As one would expect, the growth rate of the culture in the pH 3 medium rapidly decreased (Table 1).

In addition, it was observed that when the cells were grown at pH 3, 5 and 6, the adaptation phase lasted longer and some cells died after the adaptation phase. *B. subtilis* shows a lag in growth following an acidification, and the cells only at pH 3 switched to the death phase after the 6 th hour (Figure 1). Thus, growth in a acid induced adaptation to an acidic pH value such pH 5 and 6. In LB nutrient medium, the highest growth was observed at pH 7 and 8, and the lowest growth was at pH 3 and 35 ºC (Figure 1).

Specific cell growth rates and doubling time of *B. subtilis* that grown at different pH values were calculated using the OD600 values in the logarithmic phase of the growth curve of bacteria according to the Eqs. (3) - (4) (Table 1). The Monod equation is the most common rate expression to describe the growth of microorganisms (Wang et al., 2014; Baranyi and Pin, 1999; Ahmed et al., 2017). During the exponential phase we can write:

$dX/dt = \mu X$

$\ln(X/X_0) = \mu t$

$X = X_0 \times e^{\mu \Delta t}$ (3)

$t_{1/2} = \ln 2/\mu$ (4)

Where; t = time, X = CFU mL^{-1} at time t, X_0 = CFU mL^{-1} at time t_0, μ = specific growth rate constant (h^{-1}) and $t_{1/2}$=doubling time (h).

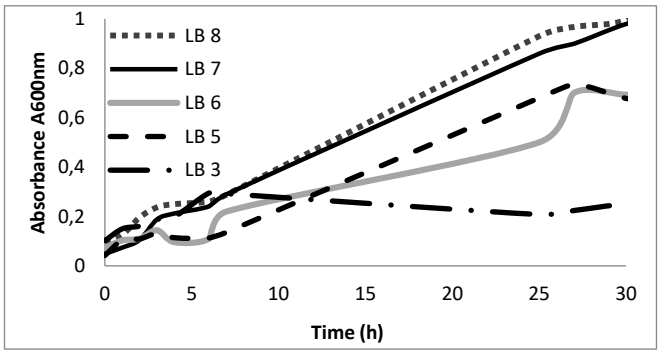

Fig. 1 Growth curves of *B. subtilis* grown in the LB medium buffered at pH 3, 5, 6, 7 and 8

Table 1 Specific growth rates and doubling time of *B. subtilis* at 35 °C in LB medium at different pH values

B. subtilis growth medium	Specific growth rate μ (h^{-1})	Doubling time $t_{1/2}$ (h)
pH 3	0.0125	55.2
pH 5	0.0703	9.81
pH 6	0.0756	9.12
pH 7	0.1112	6.20
pH 8	0.0782	8.82

Table 2 Pour plate count of *B. subtilis* probiotic strain

***Bacillus subtilis* ATCC 6633**							
Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	CFU mL^{-1}	Log CFU mL^{-1}
Count	TNTC	78	20	9	10	2.7×10^{6}	6.44

*TNTC: Too numerous to count

When the cells were grown at pH 3, 5 and 6, the adaptation phase was observed for the first 6 hours. After the adaptation phase, the growth phase was observed at pH 5, 6, 7 and 8, and the death phase was observed at pH 3 (Figure 1). The results showed that all pH values except pH 3 were suitable for cell growth (Figure 1), in addition, the specific cell growth rate at pH 7 was higher than pH 5, 6 and 8 (Table 1).

A similar lag phases in growth of *B. subtilis* was observed when cultures were shifted from pH 6 to pH 8.5 in the study of Wilks et al. 2009. Faille et al. 2002; suggested that when spores were suspended in acidic media, longer lag phases were observed. They compared pH 5 and 7 in their study and they observed better growth at pH 7 compared to pH 5. In addition, they observed the lag phase longer than 100 hours at pH 5 compared to pH 7.

3.2 Pour plate count of *B. subtilis*

The plate count method is commonly used in the pharmacy and food industry to determine the number of microorganism in a certain products. Probiotic based cleaners contain 10^6 CFU mL^{-1} spore probiotic bacteria (Collado et al. 2008; Arcales and Alolod 2018; De Cesare et al. 2019). The counts were listed in the Table 2 and Figure 2.

3.3 Auto-aggregation ability

Probiotic bacteria must accumulate and obtain a sufficient mass to show their beneficial effects (Collado et al. 2008). Its ability to accumulate is directly related to the first binding of bacteria to biotic and abiotic surfaces. The proposed mechanisms for antimicrobial activities of probiotic species include the production of compounds that prevent competition and pathogenic growth with pathogens for adhesion sites and nutrients and competitive exclusion of pathogens (Woo and Ahn 2013). Auto-aggregation ability, which is responsible for the ability to bind between cells, varies with temperature, osmolality and acidic conditions (Woo and Ahn 2013). The auto-aggregation ability of bacterial cells varied according to the incubation temperature. The highest auto-aggregation abilities were observed for *B. subtilis* (69.14%) incubated at 30 ºC in LB medium. However, lower auto-aggregation abilities of *B. subtilis* (65.50%) were observed at 37 ºC of LB medium. As a result, the auto-aggregation ability to collect probiotics is a desirable feature and varies depending on the temperature. Ritter et al. reported auto aggregation as 52.9% in their study with probiotic *Bacillus* isolates (Ritter et al., 2018).

3.4 Antibiotic susceptibility test results

Microbial resistance to antimicrobial agents is due to intrinsic properties of bacteria. Such intrinsic properties could make the bacteria capable of rapid inactivation of antibiotics through, exportation of the antibiotics out of the cell, or alteration of the specific antibiotic target site (Adimpong et al. 2012). The antimicrobial ability was determined against Chloramphenicol C30, Amoxicillin AML10, Ofloxacin OFX5, Fluconazole FCA25 (Table 3). Materials such as a sterile swab, forceps, drug and antimicrobial paper disk were prepared prior to the experiment and fresh 24 hours culture bacteria were used for the assay(Arcales and Alolod 2018). *B. subtilis* has been observed to be intermediate against Chloramphenicol C 30 μg, resistant to Amoxicillin AML 10 μg and Fluconazole FCA 25 μg and sensitive to Ofloxacin OFX 5 μg.

Table 3 Inhibition zone of *B. subtilis* against Chloramphenicol C30, Amoxicillin AML10, Ofloxacin OFX5, Fluconazole FCA25.

	Inhibition zone (mm)			
	C30	AML10	OFX5	FCA25
B.subtilis	15.5±0.70	0.00±0.00	22.5±0.70	0.00±0.00
	I	R	S	R

* Results are given as n = 3 ± standard deviation.

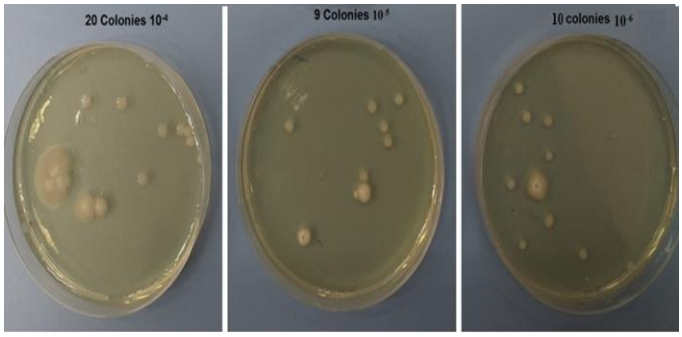

Fig. 2 Plate count of *B. subtilis* dilution with PBS.

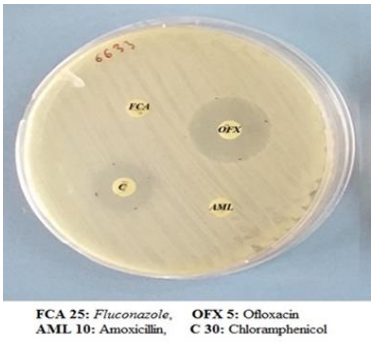

Fig. 3 Antibiotic resistance of *B. subtilis* to antimicrobial agents

Fluconazole FCA25 is used to prevent and treat various fungal and yeast infections. It acts by stopping the growth of certain fungal species (Aher et al. 2009). As seen in our study, Fluconazole FCA25 did not show any inhibitory properties against *Bacillus* species. As a result, it was observed that *B. subtilis* was more resistant to some antibiotics (Figure 3). Doganay and Aydin 1991; observed that the new antimicrobial agent, ofloxacin showed very good activity with MICs of 0.03 mg/L against *Bacillus anthracis* isolates. In our study, it was observed that ofloxacin with 5 µg concentration showed highest antibacterial activity with 22.50 mm inhibition zone against *B. subtilis*. Mazza et al. 1992; suggested that *B. subtilis* in the commercial probiotic product Enterogermina was resistant to chloramphenicol, tetracycline and streptomycin. In our study, it was observed that, *B. subtilis* ATCC 6633 was intermediate susceptible to chloramphenicol 30 µg with 15.50 mm inhibition zone. Weber et al. 1988; observed that the antibiotics such as penicillin, ampicillin, methicillin and cephalothin were highly active against *B. subtilis*.

3.5 Antagonistic activity of *B. subtilis*

Within the soil environment, probiotic bacteria compete for nutrients by the production of antibiotics that serve to inhibit the growth of their competitors (pathogenic bacteria). Indeed, the most of antibacterial compounds are natural products of soil-dwelling microorganisms (Hachmann et al. 2009). It was observed that the supernatant of *B. subtilis*, produced in 10 mL LB medium at 35 ºC for 30 hours at pH 7, inhibits *K. pneumoniae* and *P. aeruginosa* species (Figure 4).

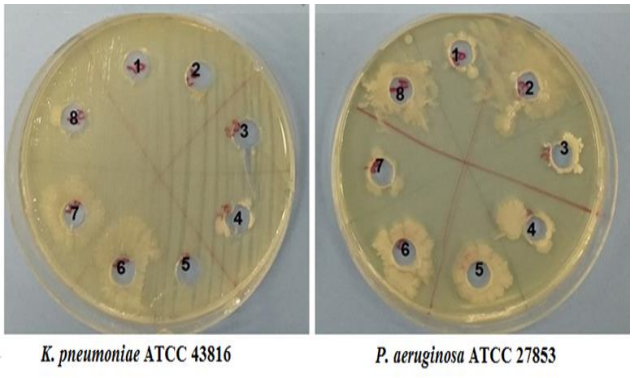

Fig. 4 Antagonistic activity of *B. subtilis* against *K. pneumoniae* and *P. Aeruginosa*

In this study, *B. subtilis* was growth at 35 ºC for 30 h at pH 7, after that, it was centrifuged and its supernatant was used as an antimicrobial agent against pathogenic species. As a result, *B. subtilis* inhibited pathogenic *K. pneumoniae* and *P. aeruginosa* strains. According to Figure 4, it is clear that *B. subtilis* has a higher antigonistic effect against *P. aeruginosa* than *K. pneumoniae*.

3.6 Antifungal activity of *B. subtilis*

Bacillus spp. are used for various biotechnological applications, such as probiotic cleaner, due to their ability to produce antimicrobial compounds inhibitory to pathogenic microorganisms in the surface enviroment (Adimpong et al. 2012).

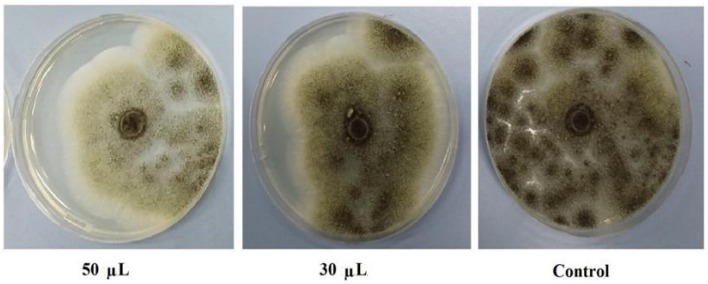

Fig. 5 Antifungal activity of *B. subtilis* at 30 and 50 µL doses against *Aspergillus brasiliensis* at 6 day of incubation

B. subtilis was growth at 35 ºC for 30 hours at pH 7 on LB medium, after that, it was centrifuged and its supernatant was used as an antifungal agent against *Aspergillus brasiliensis* ATCC 16404 mycelial growth. Increasing the doses dropped onto paper discs (30 and 50 µL) of *B.subtilis* supernatant resulted in weaker growth of fungi (Figure 5).

Table 4 Inhibitory effects of *B. subtilis* at different doses and in vitro mycelial growth levels of *A. brasiliensis*
Data are presented as means ± standard deviations (n=3).

	Mycelial growth diameter (mm)		
Incubation period	50 µL	30 µL	Control
3 day	$68.5\pm0.70^{A,a}$	$70.0\pm0.00^{B,a}$	$80.0\pm0.00^{C,a}$
4 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$
5 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$
6 day	$73.0\pm0.00^{A,b}$	$74.5\pm0.00^{B,b}$	$90.0\pm0.00^{C,b}$

a-b: Within each column, different superscript lowercase letters show differences between the *Bacillus* dose within each storage period ($p < 0.05$).

A-C: Within each row, different superscript uppercase letters show differences between the *Bacillus* dose and control ($p < 0.05$).

In-vitro effect of *B. subtilis* on fungal pathogen *Aspergillus brasiliensis* after 3 days showed average diameter of 68.50 - 70.00 mm of the mycelia of the fungal pathogen. In addition, the mycelia of the control fungal pathogen was observed as average 80.00 mm diameter. Results of fumigation application via *B. subtilis* supernatant, which were determined using the variance analysis JMP package program, showed that 50 µL dose of *B. subtilis* had the highest inhibition rates with 73.00 mm, compared with 30 µL dose with 74.5 mm inhibition rate and the the mycelia of the control fungal pathogen was observed average 90.00 mm diameter at 6 day of incubation (Table 4). As a result, *B. subtilis* used in this study successfully inhibited the growth of *Aspergillus brasiliensis*.

5 Conclusion

As a result, it was observed that the probiotic species "*B. subtilis*", which can be used in the detergent formulation, shows higher growth and vitality at the neutral pH value, while it passes into the death phase at acidic pH value such as pH 3. Therefore, it should be noted that surfactants and other additives to be used in the probiotic detergent formulation do not prevent the development of *B. subtilis* and do not lower the pH of the medium. In addition, *B. subtilis* has been observed to exhibit high antibiotic resistance and oto-aggregation ability.

According to antagonistic assay results, *B. subtilis* inhibited growth of indicator strain *K. pneumoniae* and *P. aeruginosa*. In addition, it can be derived from the Table 4 that *B.subtilis* exhibited high inhibitory effects on the growth of *Aspergillus brasiliensis* and the level of antifungal action was proportional to the doses (30µl, 50µl) that pipette onto paper discs. Results of this study corroborated that *B. subtilis* will effectively remove pathogenic bacteria and fungi on the surfaces, and the cleaner must contain the probiotic *Bacillus* species in order to effectively clean the pathogenic microorganisms on the surfaces in the long term.

Therefore, *B. subtilis* is a promising new strategic method in the probiotic/microbial cleaner formulation to remove pathogenic species from the environment and to prevent the development of pathogenic species in the long term.

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Authors' contributions: MV did the experiments of the growth of *B. subtilis* and antimicrobial tests. DÖ commented and reported all experimental results.

Conflict of interest disclosure:

There is no conflict of interest.

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