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IN VITRO SAFETY EVALUATION OF *Bacillus subtilis* SPECIES COMPLEX ISOLATED FROM VIETNAM AND THEIR ADDITIONAL BENEFICIAL PROPERTIES

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SUMMARY

Bacillus genus are Gram-positive, rod shaped, spore-forming, aerobic or facultative anaerobic bacteria, which have the ability to produce a wide variety of enzymes, antimicrobial compounds, vitamins, and carotenoids. Nowadays, *Bacillus* species have been increasingly proposed for use as probiotics or feed additives as *B. amyloliquefaciens*, *B. licheniformis, B. subtilis,* and *B. pumilus* because of their advantages such as heat-stability of the spores, storage capacity at ambient temperature, and beneficial properties for health. Since microbes are used for customers and livestock, it is critical to substantiate not only the health benefits and efficacy of unique strains but also their safety for the hosts. This study aims to evaluate the *in vitro* safety and some beneficial properties of *Bacillus* strains to select potential strains for use in humans or animals. A total of 76 *Bacillus* strains belonged to the *B. amyloliquefaciens* and *B. subtilis* groups were preliminary evaluated for their safety via hemolytic activity. Six non-hemolytic strains were identified and their antibiotic susceptibility, cytotoxicity on the growth of HT29 and Vero cells, extracellular enzyme production, antimicrobial activity, and identification based on 16S rRNA and *rpoB* gene sequences were studied. Two strains, *B. subtilis* VTCC 10963 and *B. subtilis* VTCC 11039, should be considered safe. In addition, these two strains exhibited good extracellular enzyme production (amylase, cellulase, and protease) and strong antibacterial activity against *Listeria monocytogenes*. Our results support the use of *B. subtilis* VTCC 10963 and *B. subtilis* VTCC 11039 for the development of probiotic products and feed additives in the future.

Keywords: *Bacillus subtilis*, beneficial properties, probiotic, *rpoB* gene, safety, toxicity

INTRODUCTION

Bacillus species are ubiquitous, rod-shaped, Gram-positive, catalase-producing bacteria, found in soil, plant materials, fermented food products, and intestinal tracts of humans and animals (Nicholson 2002). Among them, members of the *B. subtilis* species complex are commonly recognized producers of secondary metabolites and have great potential applications in the pharmaceutical and food processing industries such as the production of vitamins (e.g., riboflavin, cobalamin, inositol) (Tanaka *et al*. 2014) and food-grade amylase, glucoamylase, protease, pectinase and cellulase in varying foodstuffs (Su *et al*. 2020). Recently, they were widely exploited for the production of their metabolites such as amino acids, antibiotics, bacteriocins, surfactants, and bioactive peptides (Choi *et al*. 2021). In addition, some species such

as *B. amyloliquefaciens*, *B. licheniformis, B. subtilis,* and *B. pumilus* have been increasingly proposed for use as probiotics or feed additives (Hong *et al*. 2005, Choi *et al*. 2021). The *B. subtilis* species complex includes approximately 20 closely related species. It is now divided into four monophyletic groups including *B. subtilis, B. amyloliquefaciens, B. licheniformis,* and *B. pumilus* groups (Fan *et al*. 2017).

Bacillus subtilis species complex can be considered as a group that agreed with "Qualified Presumption of Safety" (QPS) (Koutsoumanis *et al*. 2020). For centuries, the representative species *B. subtilis* has been safely used in fermented foods such as *natto*, *miso*, *doenjang*, *thua nao*, or *kimchi* in Asia countries (Spears *et al*. 2021). However, some studies reported that several strains in the *B. subtilis* species can cause symptoms that mimic listeriosis, food poisoning (Workowski and Flaherty 1992, Salkinoja-Salonen *et al*. 1999) or produce the hemolytic enterotoxin Hbl, making such strains unsuitable for use in foods and supplements (Rowan *et al*. 2001, Spears *et al*. 2021). Therefore, safety concerns of *Bacillus* products for consumers and livestock are very important and the use of these bacteria for humans or animals requires careful studies on their safety on a strain-by-strain basis before being placed on the market. *In vitro* safety evaluation of *Bacillus* strains includes hemolytic activity, antibiotics susceptibility, and cell cytotoxicity (Hong *et al*. 2008, Kotowicz *et al*. 2019, Spears *et al*. 2021).

In Vietnam, the safety assessment of *Bacillus* strains used as probiotics or feed additives has not been paid enough attention yet. Currently, very limited information on the safety of indigenous microbes is available. In this work, we evaluated the *in vitro* safety of 76 *Bacillus* strains (*B. subtilis* and *B. amyloliquefaciens* groups within the *B. subtilis* species complex) via hemolytic activity. Non-hemolytic strains were selected for further investigation on their *in vitro* safety of antibiotic susceptibility and cell cytotoxicity. Extracellular enzyme production, antibacterial activity, and accurate identification of those strains based on *rpoB* gene sequencing were also studied. The obtained results will provide information for the selection and development of *Bacillus*-based products in the food and feed industries in the future.

MATERIALS AND METHODS

Bacterial strains and cell lines

Seventy-six strains of *B. amyloliquefaciens* (29 strains) and *B. subtilis* (47 strains) isolated from various sources were obtained from the Vietnam Type Culture Collection (VTCC), Institute of Microbiology and Technology, VNU (Table 1). The identification of strains was previously performed based on their cultural, morphological, and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology, followed by 16S ribosomal RNA analysis. The strains were stored at -70°C in a Nutrient Broth medium containing 20% (w/v) glycerol.

A human colon adenocarcinoma cell line (HT29) and monkey kidney cell (Vero) were obtained from Hanoi University of Science, and National Institute for Control of Vaccine and Biologicals.

Hemolytic activity

The hemolytic activity of *Bacillus* strains was determined by using Columbia agar containing 5% (w/v) sheep blood (MELAB, Vietnam). After 24 h of incubation at 37°C, the hemolytic activity of strains was evaluated and classified based on the lysis of red blood cells in the medium around the colonies. Alpha hemolysis (α), beta hemolysis (β), and gamma-hemolysis (γ) appeared as green-hued, clear, and no clear zones around the colonies, respectively. *Staphylococcus aureus* ATCC 25922 strain was used as the positive control (Mangia *et al*. 2019). Strains with γ-hemolysis are considered safe.

Antibiotic sensitivity test

The antibiotic susceptibility of the selected *Bacillus* strains was evaluated by the Kirby-Bauer disk diffusion method according to the performance standards for antimicrobial

susceptibility testing of the Clinical and Laboratory Standards Institute (CLSI). Twenty-one antibiotics (Oxoid, United Kingdom) were tested, including ampicillin (10 µg), streptomycin (10 µg), erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), rifampin (30 µg), ciprofloxacin (5 µg), clindamycin (2 µg), vancomycin (30 µg), gentamycin (10 µg), neomycin (30 µg), trimethoprim (1.25 µg)/sulfamethoxazole (23.75 µg), enrofloxacin (5 µg), and linezolid (30 µg). Bacterial strains were cultured on Tryptic Soy Agar (TSA, BD) plates for 20 h at 37ºC. The colonies of each strain were then suspended in NaCl 0.9% solution at approximately 1×10^8 CFU/ml. This suspension was seeded on Mueller-Hinton (MH, Difco) agar plates using a swab. Antibiotic discs were placed on seeded plates and the zones of growth inhibition were measured after 18 h of incubation at 37ºC. *Escherichia coli* ATCC 25922 was used as quality control organisms according to CLSI protocols (Hong *et al*. 2008, Spears *et al*. 2021).

Cytotoxicity assay

The *in vitro* cytotoxicity activity of the selected *Bacillus* strains was carried out using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay measured the development of blue formazan product as a product of the MTT reduction by mitochondrial dehydrogenase in HT29 and Vero cells, which is the indicator of cell viability and normal functioning of mitochondria. Cell lines (intestinal HT29 and monkey kidney Vero cell) were seeded at 2×10^4 cells per well. Bacteria were grown overnight in brain heart infusion broth (BHIB) at 37ºC. The cell-free supernatant (CFS) was obtained by filtration using sterilized filters (0.2 µm). The medium from the cell culture plate was discarded and 100 µl of the two-fold dilution of each CFS was added to cultured cells (in triplicate). And the plates were incubated at 37ºC in 5% CO_2 for 24 h. After the incubation, the suspension from each well was removed. Fifty microliters of fresh medium and 50 µl MTT (2 mg/ml, Biobasic) was added to each well. After 2 h of incubation at 37ºC in 5% CO_2, the MTT solution was discarded and followed by adding 100 µl of dimethylsulfoxide (DMSO) to dissolve the blue crystals for 15 min. Optical densities (OD) of the suspensions at 490 nm were then measured using a microplate reader (800TS BioTek) and the cytotoxicity of the *Bacillus* strains (expressed as % survival of HT29 and Vero cells) were calculated using these OD values. *Aeromonas veronii* VTCC 70168 was used as a positive control (Hong *et al*. 2008, Yasmin *et al*. 2020).

Screening extracellular enzyme activity of the selected bacterial strains

Extracellular amylase, protease, and cellulase activities of the selected *Bacillus* strains were qualitatively estimated in starch-agar, casein-agar, and carboxymethylcellulose (CMC)-agar media plates, respectively (Amoozegar et al. 2003, Kasana et al. 2008, Niranjana and Bavithra 2020). For screening, the colony of each *Bacillus* strain was spotted on the center of each selective medium agar plate. After incubation, all plates were evaluated and the diameters of the zones of clearance were measured removing the diameter of the bacterial colony. The relative enzyme activity (REA) was determined as follows (Latorre *et al*. 2016): REA = diameter of the zone of clearance divided by the diameter of the bacterial colony in millimeters. Based on the REA test, organisms were categorized into excellent (REA >5.0), good (REA > 2.0–5.0), or weak (REA < 2.0).

Antibacterial activity

The antagonistic activity of the selected *Bacillus* strains against different pathogens was preliminarily tested by the agar plug and well diffusion methods. The tested pathogens were *Aeromonas dhakensis* VTCC 70106, *Enterococcus faecalis* VTCC 70177, *E. coli* VTCC 12272, *Listeria monocytogenes* VTCC 70147, *Salmonella enterica* VTCC 70080, *S. aureus* VTCC 12275, and *Vibrio vulnificus* VTCC 70092.

Briefly, for the agar plug method, pathogenic strains were seeded on MH agar plates using a

swab. Agar plugs with a diameter of 6 mm containing the selected *Bacillus* strains grown in LB agar (Luria-Bertani, BD), NA (Nutrient Agar, BD), and TSA media for 24 h were placed on top of the MH plates containing the pathogenic strain. After 18 h of aerobic incubation at 37°C, the sizes of inhibition zones were recorded.

For the well diffusion method, the potential *Bacillus* strains were inoculated in 60 ml of LB broth, NB (Nutrient Broth), and TSB (Tryptic Soy Broth) media in 250 ml flasks on a shaker (Gyromax™ 747, Amerex Instruments, Inc, USA) at 160 rpm, 37°C for 24 h. After the incubation, the cell-free supernatant was separated by centrifugation at 10,000 rpm for 8 min and followed by filtration using sterilized filters (0.2 µm). Six-millimeter-diameter wells were cut into MH agar plates containing the pathogenic strain and filled with 60 µl of cell-free supernatant from the *Bacillus* strains. The plates were incubated aerobically at 37°C for 18 h. The zone of inhibition was measured and expressed as a millimeter in diameter (Hong *et al*. 2008).

PCR amplification of 16S rRNA and *ropB* genes and phylogenetic analysis

The 16S ribosomal RNA (rRNA) gene was amplified using universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1525R (5'-AAAGGAGGTGATCCA GCC-3'). The DNA products were amplified by PCR under the following conditions: initial denaturation 95°C for 3 min, followed by 35 cycles each consisting of 95°C of 30 s, 55°C for 30 s and 72°C for 2 min, following a final extension step at 72°C for 5 min.

The primer pair used for amplication of *ropB* gene’s conserved regions was used as rpoB-2292f (5’-GACGTGGGATGGCTACAACT-3’) and rpoB-3354r (5’-ATTGTCGCCTTTAACGATGG-3’) with the following PCR program: 95°C for 3 min, 35 cycles at 95°C for 30 s, 53°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 5 min (Rooney *et al*. 2009). To verify the identities and demonstrate the evolutionary relationships between the selected *Bacillus* strains, the 16S rRNA and *rpoB* gene sequences of type strain sequences retrieved from EzBioCloud database (https://www.ezbiocloud.net) and NCBI (https://www.ncbi.nlm.nih.gov/) were subjected to phylogenetic analysis using MEGA X software (Kumar *et al*. 2018).

RESULTS AND DISCUSSION

Hemolytic activity

Expression of hemolytic activity is considered a virulent factor and comprises one of the basic safety tests for prescreening a strain before further investigation as a potential probiotic or feed additive. According to European Food Safety Authority (EFSA), the evaluation of hemolytic activity is strongly recommended to ensure that the bacterial strain is free of the toxigenic potential even if it has GRAS (Generally recognized As Safe) or QPS status (Yasmin *et al*. 2020). Strains showing hemolytic activity may be considered unsafe for human or animal health care applications until the effect of this virulence factor is either eliminated, modified, or confirmed as causing no harm to the eukaryotic host.

In this study, a total of 76 *Bacillus* strains were tested. Among them, 6 strains (7.9%) had no transparent or greenish zones surrounding their colonies on the blood agar plates while 23.68% and 68.42% of strains displayed α-hemolysis, β-hemolysis activities, respectively (Table 1). Thus, non-hemolytic strains (VTCC 10957, VTCC 10963, VTCC 10965, VTCC 10987, VTCC 11039, and VTCC 12316) were selected for further studies.

Antibiotic sensitivity testing

The antibiotic susceptibility of the six selected *Bacillus* strains was evaluated based on the level of resistance to three different groups of antibiotics categorized by their mechanisms. The cell wall inhibitor group includes ampicillin and vancomycin. The protein synthesis inhibitors consist of gentamicin, streptomycin,

erythromycin, clindamycin, chloramphenicol, tetracycline, neomycin, and linezolid. Inhibitors of nucleic acid synthesis are ciprofloxacin, rifampicin, sulfamethoxazole, and enrofloxacin.

Table 1. Hemolytic activity of *Bacillus* strains in this study.

Group	VTCC number	Source of isolation	Hemolytic activity	Group	VTCC number	Source of isolation	Hemolytic activity
B. amyloliquefaciens	**VTCC 10987**	**Seaweed, Hai Phong**	γ	*B. subtilis*	VTCC 10275	Soil, Ha Noi	β
	VTCC 10739	Soil, Ha Noi	α		VTCC 10330	Soil, Ha Noi	α
	VTCC 11027	Soil, Ha Noi	α		VTCC 10349	Soil, Ha Noi	α
	VTCC 11028	Soil, Ha Noi	α		VTCC 10379	Soil, Ha Noi	β
	VTCC 11030	Soil, Ha Noi	β		VTCC 10380	Soil, Ha Noi	β
	VTCC 11031	Soil, Ha Noi	β		VTCC 10485	Soil, Ha Noi	β
	VTCC 11037	Soil, Ha Noi	β		VTCC 10505	Soil, Ha Noi	α
	VTCC 11038	Soil, Ha Noi	β		VTCC 10731	Soil, Ha Noi	α
	VTCC 11041	Soil, Ha Noi	α		VTCC 10803	Soil, Ha Noi	α
	VTCC 11089	Soil, Sa Pa-Lao Cai	β		VTCC 10818	Roof plant, Ha Noi	β
	VTCC 11191	Soil, Con Dao-Ba Ria Vung Tau	β		VTCC 10822	Soil, Ha Noi	β
	VTCC 11192	Soil, Con Dao-Ba Ria Vung Tau	β		VTCC 10881	Soil, Ha Noi	β
	VTCC 11254	Soil, Con Dao-Ba Ria Vung Tau	β		VTCC 10882	Coconut water, Ha Noi	β
	VTCC 11319	Soil, Cuc Phuong National park-Ninh Binh	β		VTCC 10948	Seaweed, Hai Phong	β
	VTCC 11323	Soil, Cuc Phuong National park- Ninh Binh	β		**VTCC 10957**	**Seaweed, Hai Phong**	γ
	VTCC 12304	Soil, Ha Long-Quang Ninh	β		VTCC 10960	Seaweed, Hai Phong	α
	VTCC 12314	Soil, Ha Long-Quang Ninh	α		**VTCC 10963**	**Seaweed, Hai Phong**	γ
	VTCC 910106	Soil, Luong Son, Hoa Binh	β		**VTCC 10965**	**Seaweed, Hai Phong**	γ
	VTCC 910125	Soil, Cu Chi-Ho Chi Minh	β		VTCC 11015	Soil, Ha Noi	β
	VTCC 910126	Soil, Cu Chi-Ho Chi Minh	β		VTCC 11016	Soil, Ha Noi	β
	VTCC 910128	Soil, Cu Chi-Ho Chi Minh	β		VTCC 11034	Soil, Ha Noi	α

	VTCC 910130	Soil, Da Lat- Lam Dong	β		VTCC 11035	Soil, Ha Noi	β
	VTCC 910131	Soil, Da Lat- Lam Dong	β		**VTCC 11039**	**Soil, Ha Noi**	γ
	VTCC 910132	Soil, Cho Gao-Tien Giang	β		VTCC 11040	Soil, Ha Noi	α
	VTCC 910133	Soil, Cho Gao-Tien Giang	β		VTCC 11042	Soil, Ha Noi	β
	VTCC 910134	Soil, Cho Gao-Tien Giang	β		VTCC 11123	Shan Tuyet tea, Ha Giang	β
	VTCC 910051	Soil, Nam Dinh	β		VTCC 11295	Soil, Bach Ma National park, Hue	α
	VTCC 910167	Soil, Cuc Phuong National park-Ninh Binh	α		VTCC 11301	Soil, Bach Ma National park, Hue	β
	VTCC 910168	Soil, Trung Khanh-Cao Bang	α		VTCC 11308	Soil, Bach Ma National park, Hue	β
	VTCC 11017	Soil, Ha Noi	β		VTCC 11309	Soil, Bach Ma National park, Hue	β
	VTCC 11018	Soil, Ha Noi	β		VTCC 11310	Soil, Bach Ma National park, Hue	β
	VTCC 11019	Soil, Ha Noi	α		VTCC 11322	Soil, Cuc Phuong National park, Ninh Binh	β
	VTCC 11023	Soil, Ha Noi	β		VTCC 12315	Soil, Cam Xuyen, Ha Tinh	β
B. subtilis	VTCC 11024	Soil, Ha Noi	α		**VTCC 12316**	**Soil, Quang Binh**	γ
	VTCC 11026	Soil, Ha Noi	β		VTCC 910120	Soil, Cuc Phuong National park, Ninh Binh	β
	VTCC 11029	Soil, Ha Noi	β		VTCC 910123	Soil, Tuyen Quang Dairy Factory	β
	VTCC 11032	Soil, Ha Noi	β		VTCC 910127	Soil, Cu Chi-Ho Chi Minh	β
	VTCC 11033	Soil, Ha Noi	β		VTCC 910129	Soil, Cu Chi-Ho Chi Minh	β

The tested strains were found to be sensitive to most of the antibiotics used (Table 2). Strains VTCC 10957, VTCC 10965, VTCC 10987 were sensitive to 7 antibiotics (vancomycin, gentamicin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol) listed by EFSA except for tetracycline (EFSA 2012) while strains VTCC 10963 and VTCC 11039 were susceptible to all antibiotics except for streptomycin. Strain VTCC 12316 showed resistance to streptomycin, tetracycline, and intermediate resistance to gentamicin. Concerning to the other antibiotics tested, VTCC 10957 was resistant to rifampicin and ciprofloxacin. Strain VTCC 12316 exhibited resistance to various antibiotics including ampicillin, neomycin, and enrofloxacin, and intermediate resistance to ciprofloxacin (Table 2).

The antibiotic susceptibility of each strain should be measured for safety purposes. Antibiotic resistance gene transmission can occur due to transposons, plasmids, and bacterial gene mutations, leading to new antibiotic-resistant strains (Teuber *et al*. 1999). However, this issue is still controversial. Moreover,

resistance to a given antibiotic can be inherent to a bacterial species or genus. For example, a study carried out by Adimpong et al. showed that out of 85 *Bacillus* species used for Sudanese bread production (*B. subtilis* subsp. *subtilis* (n = 29), *B. licheniformis* (n = 38), and *B. sonorensis* (n = 18)), all were resistant to streptomycin (Adimpong *et al*. 2012).

Moderate antibiotic resistance can be regarded as a positive characteristic in specific cases such as when a probiotic – antibiotic combined treatment has to be used. More specifically, if the applied probiotics are fully susceptible to antibiotics, their survival potential will be interfered and their potency lost (Choi *et al*. 2021).

Table 2. Antibiotic sensitivity profiles of six Bacillus strains.

Antibiotic disc*	Inhibition zone (mm)					
	VTCC 10957	VTCC 10963	VTCC 10965	VTCC 10987	VTCC 11039	VTCC 12316
Ampicillin (10)	20 (S)	26 (S)	21 (S)	23 (S)	27 (S)	14 (R)
Streptomycin (10)	18 (S)	8 (R)	18 (S)	18 (S)	9 (R)	10 (R)
Erythromycin (15)	23 (S)	30 (S)	23 (S)	24 (S)	25 (S)	23 (S)
Tetracycline (30)	15 (R)	21 (S)	17 (R)	15 (R)	21 (S)	17 (R)
Chloramphenicol (30)	24 (S)	30 (S)	25 (S)	26 (S)	25 (S)	24 (S)
Rifampicin (30)	14 (R)	24 (S)	20 (S)	19 (S)	20 (S)	19 (S)
Ciprofloxacin (5)	7 (R)	33 (S)	31 (S)	22 (S)	29 (S)	19 (I)
Clindamycin (2)	20 (S)	17 (S)	20 (S)	22 (S)	19 (S)	21 (S)
Vancomycin (30)	20 (S)	21 (S)	21 (S)	17 (S)	18 (S)	20 (S)
Gentamicin (10)	23 (S)	24 (S)	20 (S)	23 (S)	21 (S)	15 (I)
Neomycin (30)	21 (S)	22 (S)	20 (S)	20 (S)	19 (S)	10 (R)
Sulfamethoxazole (23.75) + Trimethoprim (1.25)	27 (S)	34 (S)	31 (S)	34 (S)	28 (S)	24 (S)
Enrofloxacin (5)	28 (S)	33 (S)	29 (S)	25 (S)	28 (S)	7 (R)
Linezolid (30)	27 (S)	35 (S)	30 (S)	29 (S)	33 (S)	28 (S)

*Antibiotic-impregnated discs (6 mm) with the amount, in µg shown in brackets, S-susceptible; R-resistant, I-Intermediate sensitivity.

Cytotoxicity assay

For *Bacillus* species, a cytotoxicity test is suggested by EFSA as an important indicator of potential toxicity in members of the *Bacillus* genus outside of the *B. cereus* group instead of hemolysis testing (EFSA 2014). MTT assay was used to observe the effects of different strains' CFS on the viability of HT29 and Vero cells.

This assay is considered a highly sensitive assay for determining cellular respiration, cell viability, and cytotoxicity because only living cells can produce formazan products from this reaction (Yasmin *et al*. 2020). Using a virulent strain of *Aeromonas veronii* VTCC 70168, which showed highly cytotoxic, we demonstrated that the CFS of six *Bacillus* strains exhibited no toxicity to Vero cells and did not significantly damage HT29 cells (Figure 1). These results are in good agreement with a previous study (Kotowicz *et al*. 2019). Our results showed a difference between HT29 and Vero cells. The

HT29 cells were more sensitive to CFS of *Bacillus* strains than Vero cells.

Since HT29 cells are of intestinal origin, it would be more suitable for cytotoxicity testing.

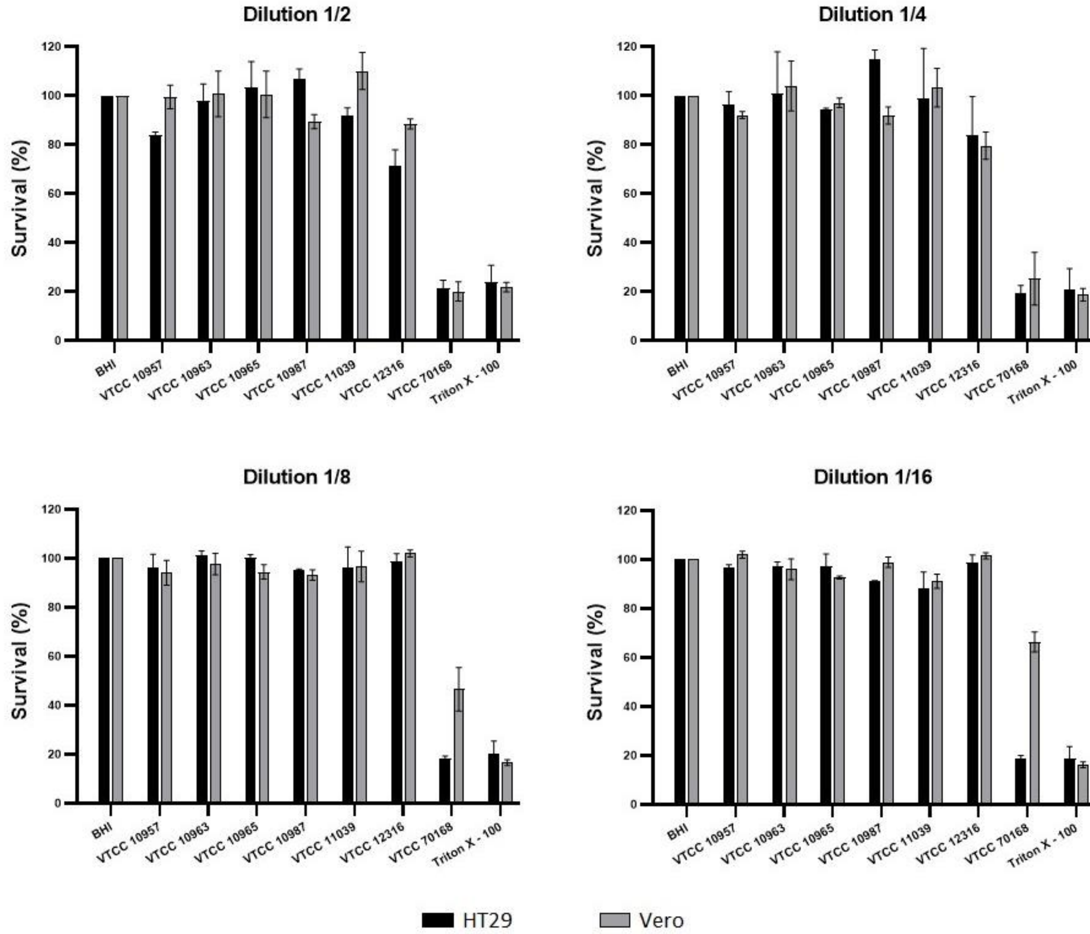

Figure 1. Survival of HT29 and Vero cells after 24 h of incubation with the cell-free supernatants of tested strains (%).

Table 3. Relative enzyme activity values produced by selected *Bacillus* strains.

	Relative enzyme activity (REA)		
VTCC number	**Amylase**	**Cellulase**	**Protease**
10957	1.50	2.85	2.75
10963	1.57	5.33	2.60
10965	2.33	2.00	3.00
10987	1.55	4.00	0.00
11039	1.75	3.60	1.54
12316	1.31	5.00	1.56

Screening extracellular enzyme activity of the selected bacterial strains

There were considerable differences in the REA values of six tested *Bacillus* strains. Two out of six strains (VTCC 10963 and VTCC 12316) showed higher REA value for cellulase production in comparison to other bacterial

strains. Strains VTCC 10963 obtained the highest REA value of 5.33 which is considered an excellent cellulase producer. In the case of amylase activity, strain VTCC 10965 showed a REA value of 2.33 which is considered good (REA > 2.0–5.0), surpassing that of other strains. Protease production was classified as good for the strains VTCC 10965 (REA = 3.00), VTCC 10957 (REA = 2.75), and VTCC 10963 (REA = 2.60) in comparison to the other selected *Bacillus* spp. strains (Table 3). A complete description of the enzyme activity profile of all the selected strains and the appearance of each selective medium are shown in Table 3 and Figure 2, respectively.

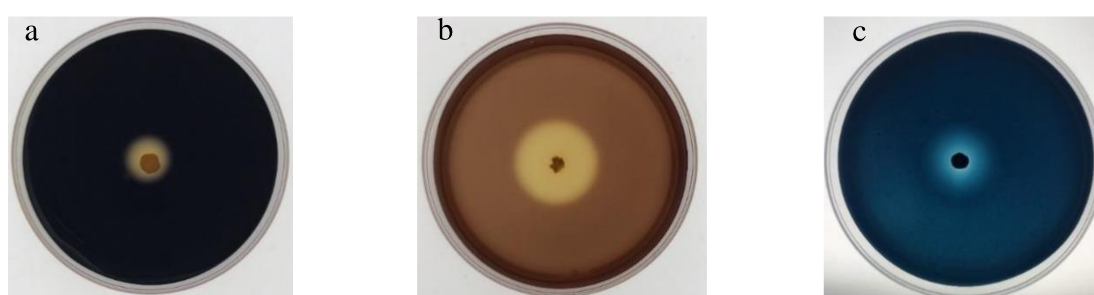

Figure 2. Representative strain VTCC 10963 of enzyme activity using a different selective media for each enzyme under evaluation. An area of clearance around a bacterial colony can be observed, representing enzyme production of amylase (a), cellulase (b), and protease (c).

Table 4. Antimicrobial activity of selected *Bacillus* strains using agar plug and well diffusion methods.

VTCC number	Media	AD	EC	EF	LM	SE	SA	VV
	LB agar/ LB broth	-/-	-/-	-/-	+++/-	-/-	-/-	-/-
10957	NA/NB	++/-	-/-	-/-	+++/+++	-/-	-/-	-/-
	TSA/TSB	-/-	-/-	-/-	++/-	-/-	-/-	-/-
	LB agar/ LB broth	-/-	-/-	-/-	+++/+++	-/-	++/-	-/-
10963	NA/NB	-/-	-/-	-/-	+++/-	-/-	+/-	-/-
	TSA/TSB	-/-	-/-	-/-	+++/-	-/-	++/-	-/-
	LB agar/ LB broth	+++/+++	++/-	-/-	+++/-	++/-	+/-	+/-
10965	NA/NB	+++/+++	+++/-	-/-	+++/-	++/-	-/-	-/-
	TSA/TSB	+++/-	+/-	-/-	++/-	+/-	+/-	-/-
	LB agar/ LB broth	+++/-	+/-	+++/-	+++/-	-/-	-/-	-/-
10987	NA/NB	-/-	-/-	+/-	+++/-	-/-	+/-	-/-
	TSA/TSB	+++/-	-/-	++/-	+++/-	-/-	++/-	-/-
11039	LB agar/ LB broth	-/-	-/-	-/-	+/-	-/-	-/-	-/-
	NA/NB	-/-	-/-	-/-	+/-	-/-	-/-	-/-
	TSA/TSB	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	LB agar/ LB broth	-/-	-/-	-/-	-/-	-/-	-/-	-/-
12316	NA/NB	-/-	-/-	-/-	-/-	-/-	-/-	-/-
	TSA/TSB	-/-	-/-	-/-	-/-	-/-	+/-	-/-

Notes: LB (Luria-Bertani), NA (Nutrient Agar), NB (Nutrient Broth), TSA (Tryptic Soy Agar), TSB (Tryptic Soy Broth); AH-*Aeromonas dhakensis*, EC-*Escherichia coli;* EF-*Enterococcus faecalis;* LM-*Listeria monocytogenes;* SE-*Salmonella enterica;* SA-*Staphylococcus aureus,* VV-*Vibrio vulnificus*; - no inhibition; + growth reduction; ++ zone of inhibition below 10 mm; +++ zone of inhibition above 10 mm inhibition.

Antibacterial activity

In this study, the agar plug method was used for the preliminary detection of antibacterial activity. This method allowed the utilization of a very small amount of medium for the culturing of bacteria and production of bioactive compounds and the detection of antimicrobial activity against several microbial pathogens thereby reducing the costs of experiments and saving resources. The results were presented in Table 4. Four selected *Bacillus* strains VTCC 10957, VTCC 10963, VTCC 10965, and VTCC 10987 exhibited strong antibacterial activity against *L. monocytogenes* in three culture media. Besides, strain VTCC 10987 also showed good inhibitory activity against other pathogens such as *A. dhakensis*, *E. coli,* and *S. enterica*. In the well diffusion method, the CFS of strain VTCC 10957 and VTCC 10963 also displayed a good inhibitory effect against *L. monocytogenes* (Table 4).

Table 5. Identification of six selected *Bacillus* strains by DNA sequencing

Strains	VTCC number (% identity of 16S rRNA gene)					
	10957	**10963**	**10965**	**10987**	**11039**	**12316**
B. amyloliquefaciens DSM 7^T	98.36	98.28	98.37	98.45	98.22	98.44
B. atrophaeus NRRL-NRS 213^T	99.23	99.23	99.23	99.32	99.14	99.32
B. inaquosorum NRRL B-23052^T	99.40	99.91	99.40	99.49	99.83	99.49
B. mojavensis NRRL B-14698^T	99.23	99.74	99.23	99.32	99.66	99.32
B. siamensis KCTC 13613^T	99.93	99.64	99.93	100	99.58	100
B. spizizenii NRRL B-23049^T	99.32	99.83	99.32	99.40	99.74	99.40
B. subtillis NRRL B-4219^T	99.49	100	99.49	99.57	99.91	99.57
B. tequilensis NCTC 13306^T	99.44	99.86	99.44	99.51	99.79	99.51
B. velezensis NRRL B-41580	99.83	99.66	99.83	99.91	99.57	99.91
Strains	**VTCC number (% identity of *rpoB* gene)**					
	10957	**10963**	**10965**	**10987**	**11039**	**12316**
B. amyloliquefaciens DSM 7^T	98.01	90.11	97.96	98.15	90.23	98.30
B. atrophaeus NRRL-NRS 213^T	89.52	91.26	89.46	89.50	91.29	89.17
B. inaquosorum NRRL B-23052^T	89.63	97.16	89.56	89.50	97.20	89.27
B. mojavensis NRRL B-14698^T	89.73	94.63	89.67	89.60	94.61	89.38
B. siamensis KCTC 13613^T	98.33	89.11	98.32	98.29	89.35	98.61
B. spizizenii NRRL B-23049^T	89.21	96.74	89.14	89.09	96.78	88.85
B. subtillis NRRL B-4219^T	89.83	99.47	89.77	98.71	99.48	89.48
B. tequilensis NCTC 13306^T	90.24	96.63	90.10	89.98	96.58	89.98
B. velezensis NRRL B-41580	98.76	89.39	98.75	98.96	89.42	98.96

Phylogenetic analysis of the selected strains based on 16S rDNA and *rpoB* gene sequences

In previous studies, the 16S rRNA gene (~1500 bp) of six strains was amplified and sequenced using the universal primers 27F-1495R for the identification of scientific name. The amplified fragment of the 16S rDNA sequence was blasted using the Basic Local Alignment Search Tool (BLAST) for sequence homology. As shown in Table 5, it is explicit that the 16S rRNA sequence could not discriminate the closely related species in the *B. subtilis* species complex. Almost all selected strains

revealed more than 99% of identity, which is well above the recommended threshold of >98.65% for species delineation (Kim *et al*. 2014). The phylogenetic tree based on the 16S rDNA sequences of the *Bacillus* strains and type or reference strains retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/blast) was constructed (Figure 3).

Figure 3 showed that six strains were located between two clades of a phylogenetic tree. Strains VTCC 10963 and VTCC 11039 were grouped into *B. subtillis*, *B. spizizenii,* and *B. tequilensis*, while strains VTCC 10957, VTCC 10965, VTCC 10987, and VTCC 12316 were identified as *B. siamensis*. Bootstrap values of several clusters were lower than 60% which did not support the robustness of the 16S rRNA phylogenetic tree for accurate *Bacillus* spp. identification. To elucidate more precisely the phylogenetic and taxonomic relationship of the selected *Bacillus*, we sequenced a partial *rpoB* gene sequence. This gene is another molecular marker that has been used as an alternative to rRNA genes sequence analysis. The power and potential of a *rpoB* gene sequence in taxonomic, phylogenetic, and evolutionary studies have previously been reported (Sharma, Patil 2011).

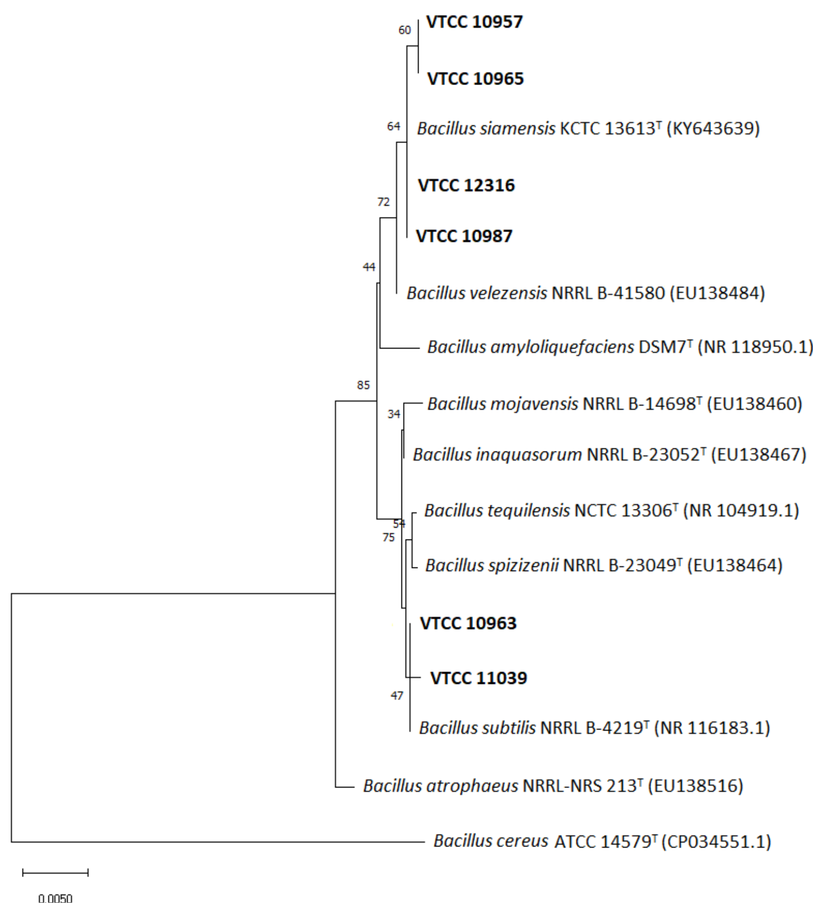

Figure 3. Neighbor-joining phylogenetic tree based on the 16S rRNA gene sequences showing relationships between selected *Bacillus* strains and representative type strains. The numbers at the nodes indicate Bootrap value as percentages of 1,000 replicates. *B. cereus* was used as the outgroup taxon to root the tree.

The *rpoB* gene sequence homology analysis (Table 5) and the phylogenetic tree (Figure 4) revealed quite a similar pattern of *Bacillus* identity compared to 16S rRNA sequence analysis, albeit with a few modifications. Strains VTCC 10963 and VTCC 11039 were clearly placed into *B. subtilis* species. However, the discrimination of strains VTCC 10957, VTCC 10965, VTCC10987, and VTCC 12316 was ambiguous through the *rpoB* sequence analysis. Although *rpoB* gene sequencing is useful to discern highly similar rRNA genes in bacteria, in some cases, it may not be suitable for identification purposes. This obtained result is in good agreement with observations from another author (Mohkam *et al*. 2016). For this reason, it is important to investigate a combination of different conserved gene sequences such as *gyrA*, *recA,* etc*.,* before deciding on the identification and resolving the ambiguousness.

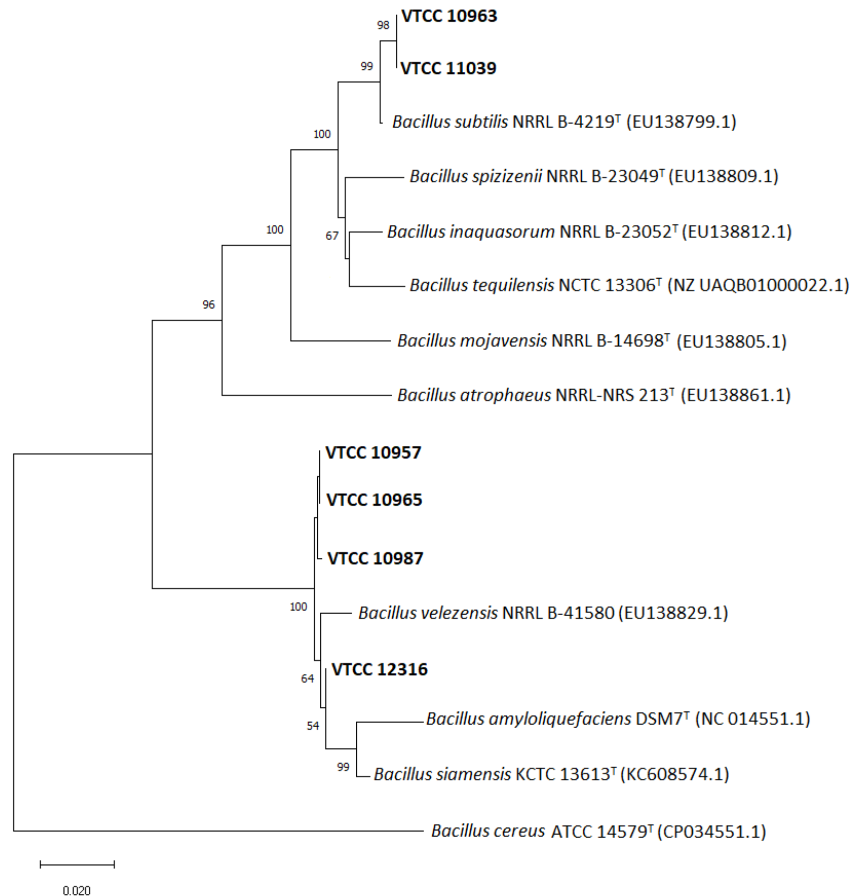

Figure 4. Neighbor-joining phylogenetic tree based on the *rpoB* gene sequences showing relationships between selected *Bacillus* strains and representative type strains. The numbers at the nodes indicate Bootrap value as percentages of 1,000 replicates. A value higher than 50% is expressed. *B. cereus* was used as the outgroup taxon to root the tree.

CONCLUSION

In this study, we evaluated the safety of 76 *Bacillus* strains via their hemolytic activity. Six non-hemolytic strains were identified and further studies on their safety (antibiotic susceptibility, cytotoxicity), beneficial properties (extracellular enzyme production and antibacterial activity

against pathogens) as well as accurate identification based on their 16S rRNA and *rpoB* genes. Among all the strains, *B. subtilis* VTCC 10963 and *B. subtilis* VTCC 11039 had the potential for practical applications due to their strong extracellular enzyme activities and antibacterial activity against pathogens. In addition, *B. subtilis* VTCC 10963 and *B. subtilis* VTCC 11039 also displayed susceptibility to most of the tested antibiotics and showed no cell cytotoxicity. The two strains are highly recommended for use in the development of probiotic products and feed additives.

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