Original Article

Preliminary *in vitro* assessment of probiotic properties of *Bacillus subtilis* GM1, a spore forming bacteria isolated from goat milk

Daneshazari, R.[1]; Rabbani Khorasgani, M.[2*] and Hosseini-Abari, A.[2]

[1]Ph.D. Student in Microbiology, Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran; [2]Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, Iran

[*]***Correspondence:*** *M. Rabbani Khorasgani, Department of Cell and Molecular Biology & Microbiology, Faculty of Biological Science & Technology, University of Isfahan, Isfahan, Iran. E-mail: m.rabbani@biol.ui.ac.ir*

10.22099/IJVR.2023.43891.6441

(Received 26 May 2022; revised version 8 Jan 2023; accepted 9 Jan 2023)

This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

Abstract

Background: Species of the *Bacillus* genus have a long history of use in biotechnology. Some *Bacillus* strains have recently been identified for food applications and industrial as safe bacteria, which mostly have been recognized as probiotic strains. **Aims:** The primary purpose of the current study was to evaluate the probiotic characteristics of *Bacillus subtilis* strains isolated and identified from the goat milk samples. **Methods:** After sampling from 40 goat milk and cultivation, suspected colonies were subjected to biochemical and molecular identification. Then, the confirmed isolate was assessed for *in vitro* probiotic tests, including hemolysis and lecithinase properties, bile salt, acid, and artificial gastric juice resistance, antioxidant activity, antibiotics susceptibility, enterotoxin genes detection, and attachment capacity to the HT-29 cells. **Results:** Among 11 suspected isolates evaluated, only one isolate was identified as *B. subtilis*. *In vitro* tests for this strain showed similar results to other probiotic strains. The *B. subtilis* strain was susceptible to various antibiotics. The enterotoxin genes were not detected based on PCR assay. Concerning its probiotic characteristics assessment, especially tolerance to bile salts and acidic conditions, the *Bacillus* strain could have the potential to consider as a probiotic. **Conclusion:** Goat milk can be recommended as a source of *Bacillus* isolates. Also, the isolated strain showed high adaptability to the gastrointestinal environment, relatively equal percentages of adhesion properties, and some safety aspects, having the potential to be considered as an appropriate probiotic.

Key words: *Bacillus subtilis*, Goat milk, Probiotic properties

Introduction

Today, foods, in addition to satisfying hunger, can also improve the mental and physical functions of consumers, well-being (Ismail *et al*., 2018), so over the last two decades, scientific interest in probiotics has received to increasing level (Seo *et al*., 2011; Sridevi *et al*., 2015). Also, consumer demands in food production, especially dairy-containing probiotics, have changed considerably (Ismail *et al*., 2018) owing to increasing knowledge about their benefits on immunity and gastrointestinal health (Abid *et al*., 2019).

Live microorganisms that, if administered in adequate amounts, could provide health benefits to the host are defined as probiotics (Olmos and Paniagua-Michel, 2014; Ranadheera *et al*., 2019). These microorganisms could affect the host beneficially by improving the balance and restoration of the gut microbiota, immunomodulation, managing lactose intolerance, reducing blood pressure and cholesterol, lowering allergic, preventing urogenital symptoms, and so on (Seo *et al*., 2011; Sridevi *et al*., 2015; Pique *et al*., 2019). Common probiotic bacteria are *Lactobacillus* and *Bifidobacteria* strains (Igbal *et al*., 2014) since they are indigenous microflora of the animals and many studies represent their beneficial properties (Lee *et al*., 2012), however manufacturing formulations of these probiotics have some challenges. Both of them are slow growers and sensitive to temperature, having short shelf-life in products. Moreover, these bacteria are strict anaerobes or microaerophiles; hence their handling and production require complicated procedures (Suva *et al*., 2016). Thus, researchers and food industries are constantly looking for new strains with better properties, such as more acid and bile tolerance, and longer shelf-life (Abid *et al*., 2019).

A group of highly diverse microorganisms are *Bacillus* species (Suva *et al*., 2016) that contain industrial grade strains, some of which are recognized as safe and known as probiotics (Hanafy *et al*., 2016). *Bacillus* spp. is closely related to *Lactobacillus* spp., and

both are categorized in the same class (Elshaghabee *et al*., 2017). *Bacillus* strains have the stability against harsh conditions due to spore formation (Lee *et al*., 2019), which can then germinate and exert probiotic activities in the gastrointestinal tract (Kim *et al*., 2014). *Bacillus* spp. form resistant spores under environmental stress with the ability to resist physical and chemical conditions such as high temperature or pressure, air-drying activity, and UV light (Elisashvili *et al*., 2019). In addition to spore formation, some properties such as fast growth rate, diversity of nutrients consumption, growth in anaerobic and aerobic conditions, and high level of enzyme production give unique characteristics to *B. subtilis* (Sorokulova, 2013; Suva *et al*., 2016). Also, *B. subtilis* genome is sequenced, generating great amount of essential knowledge and developments of molecular techniques (Olmos and Paniagua-Michel, 2014). This bacterium has been used in fermented foods for several centuries without harmful effects, generally recognized as safe (GRAS) (Kim *et al*., 2014). Taking into account the beneficial properties of *B. subtilis*, these strains are potential probiotic candidate, and recent decade studies in which the strains were isolated from various sources have shown several proprietary strains, marketed as supplements for human consumption in Asia, Europe, and the US (Elshahabee *et al*., 2017). Therefore, isolations of more new strains of *B. subtilis* as good probiotics have received growing attention.

Milk is a highly nutritious food with different commercial origins, such as cow, sheep, camel, and goat, produced worldwide for human consumption (Hernandez-Saldana *et al*., 2016). The valuable nutritious contents of milk, as well as high-water activity and natural pH, provide a suitable environment for supporting the growth of different microorganisms. The easily digested goat's milk could be an alternative dairy product to cow's milk, today. It has gained much attention and interest (Quigley *et al*., 2013; Zhang *et al*., 2017a). It possesses other beneficial characteristics such as low allergenicity, smaller fat globules, a higher concentration of fatty acids, higher levels of iron bioavailability, and buffering capacity that potentially make it useful in medicine and human nutrition (Quigley *et al*., 2013; Hernandez-Saldana *et al*., 2016; Ranadheera *et al*., 2019). Moreover, goat's milk has been interested as a suitable source for isolating microorganisms with potentially desired characteristics, so current research aimed to isolate and identify *B. subtilis* strains from raw goat's milk and then screening them for probiotic traits.

Materials and Methods

Bacterial isolation from goat's milk

Under aseptic conditions, 40 milk samples were firstly collected from goats of Varamin in Tehran province of Iran. Desired dilutions were prepared in sterile saline buffer, the samples were pretreated for 15 min at 80°C for isolation of the spore-forming bacteria. Then, 100 µL of each dilution was streaked on nutrient agar (Merck, Germany). Different colonies of bacteria based on their morphologies were selected, and sub-cultured onto nutrient agar to pure the colonies (Ragul *et al*., 2017; Talebi *et al*., 2018).

Identification of *Bacillus* isolates

Preliminary screening of *Bacillus* species was determined based on morphological and biochemical analysis. The polymerase chain reaction was done to bacterial identification by 27F (5´-AGA GTT TGA TCC TGG CTC AC-3´) and 1492R (5´-CGG TTA CCT TGT TAC GAC TT-3´) as forward and reverse primers, respectively. The obtained sequence was submitted to the National Center for Biotechnology Information (NCBI) (Davati *et al*., 2015; Zulkhairi Amin *et al*., 2020).

Screening of probiotic properties of the isolate

Hemolysis assay

For determination of hemolysis pattern, *Staphylococcus aureus* ATCC 25923 as a control strain and GM1 isolate were streaked on blood agar (Merck, Germany) plates with 5% (W/V) defibrinated blood, and incubated at 37°C for 24 h (Manhar *et al*., 2016).

Lecithinase activity

A loop-full of an overnight culture of selected bacteria and *Bacillus cereus* ATCC14579 as positive control were streaked on egg yolk agar (Biomark, India) and incubated at 37°C. The plates were checked for lecithinase activity after 24-48 h (Lakshmi *et al*., 2017).

Bile salts tolerance assay

Bile salts (Sigma-Aldrich, USA) tolerance of GM1 isolate was evaluated by inoculating 100 µL of bacterial suspension in nutrient broth (Biolife, Italy) comprising 0.3% salts and incubated for 8 h at 37°C. Before and after the incubation period, the absorbance at the wavelength of 600 nm was recorded. The absorbance of a medium without bile salts was measured as a control sample, and the rate of inhibitory was calculated as follows:

$$C_{inh} = \frac{(T8 - T0)control - (T8 - T0)treatment}{(T8 - T0)control}$$

T_0: The OD600 at time zero
T_8: The OD600 after 8 h incubation

For a suitable probiotic candidate, C_{inh} (inhibitory) of less than 0.4 was considered significant (Ebnetorab *et al*., 2020).

Resistance to artificial gastric juice

Gastric juice resistance of the selected isolate was determined using nutrient broth with 0.3% w/v pepsin (Sigma-Aldrich, USA) and 0.5% w/v sodium chloride (Merck, Germany) adjusted to pH 2.5 with HCl (Merck, Germany) 5N and to pH 7 as control. The cell pellet of the fresh culture of *B. subtilis* was washed with phosphate buffered saline (PBS), inoculated in artificial gastric juice and incubated at 37°C for 4 h. The viability percentage of cells was determined by culturing on

nutrient agar and calculated as the log CFU/ml before (0 h) and after (4 h) incubation.

$$\text{Viability (\%)} = (\log Nt/\log N0) \times 100$$

N_0: The initial viable cells
N_t: The final viable cells (Lavanya and Dayakar, 2017)

Acid tolerance

The tolerance of *B. subtilis* strain to acid was assessed in several pH solutions. The strain was grown in nutrient broth, and the cell pellet of an overnight culture was harvested by centrifugation, washed, and added to PBS with pH values of 2 and 4. The plate count method was used for the determination of viable cells after 4 h incubation at 37°C, and the viability rate was calculated as follows:

$$\text{Survival (\%)} = (\log Nt/\log N0) \times 100$$

Where,
N_0: The counts of viable bacteria at time zero
N_t: The counts of viable bacteria after 4 h treatment in acidic conditions (Thirabunyanon and Thongwittaya, 2012)

Auto-aggregation

Auto-aggregation characteristic of bacteria was measured based on the method of Shivangi *et al.* (2020) with some modifications. Cell pellet of overnight culture was harvested, washed, and resuspended in PBS until the optical density of suspension reached 0.3±0.05 at 600 nm. The cell suspension vortexed for 10 s and incubated for 4 h and 24 h at 37°C. After incubation time, optical density at 600 nm was recorded to calculate auto-aggregation as follows:

$$\text{Auto-aggregation (\%)} = \left(1 - \frac{At}{A0}\right) \times 100$$

Where,
A_0: The absorbance at time zero
A_t: The absorbance at the indicated time (4 h or 24 h)

Hydrophobicity assay

The selected strain was grown in nutrient broth (18 h, 37°C). Then, harvested pellets were washed with PBS, resuspended in 2 ml of the same buffer, and their optical density was measured at 600 nm (A0). About 2 ml of bacterial suspension was added to 2 ml of chloroform (Merck, Germany), ethyl acetate (Merck, Germany), and toluene (Merck, Germany), then mixed for 5 min. After the separation of a mixture into two phases, the optical density of the aqueous phase was measured at 600 nm (A1). Hydrophobicity (%) was estimated by the following equation (Kuebutornye *et al.*, 2020):

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{A1}{A0}\right) \times 100$$

Adherence assay

The attachment ability of the selected isolate to the epithelial cells was performed with human colon adenocarcinoma cells obtained from the Iranian Biological Resource Center (HT-29 IBRC C10097). First, 1×10^5 cells/ml were seeded in a 24-well plate and grown at 37°C (humidified atmosphere, 5% CO_2) till to 80-90% confluency. Before adding the bacterial suspension, the cells of each well were washed with PBS (Bioidea, Iran) to remove antibiotics. Then, *B. subtilis* GM1 was inoculated to cells in each well and incubated for 3 h (37°C, 5% CO_2). Finally, the non-adherent bacteria were removed by washing with buffer, and a 0.25% trypsin-EDTA (Bioidea, Iran) solution was used to detach the adherent cells. The colony count method was used for the enumeration of the attached bacteria.

$$\text{HT-29 cell attachment (\%)} = (\log Nt/\log N0) \times 100$$

Where,
N_0: Initial colony counts
N_t: Final colony counts after 3 h (Kim *et al.*, 2014)

Antibiotic resistance

Susceptibility of the GM1 strain to antibiotics was characterized according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI) for the disc diffusion method. The antibiotic discs (Padtan Teb Co., Iran) used in this assay were tetracycline (30 µg), chloramphenicol (30 µg), vancomycin (30 µg), gentamycin (10 µg), erythromycin (15 µg), streptomycin (10 µg), clindamycin (2 µg), and penicillin (10 µg). Cells from bacterial culture were seeded on Mueller-Hinton agar, discs were placed, and the diameter of the inhibition zone was determined after incubation at 37°C for 24 h (Talebi *et al.*, 2018).

Detection of toxic genes

First, a simple boiling method was used for DNA extraction. The fragments of the enterotoxin genes non-hemolytic gene (*nhe*), and hemolysin BL (*hbl*) were amplified from the isolated samples, and *B. cereus* ATCC14579 was used as a positive control. The sequences of the used primers are provided in Table 1 (Kim *et al.*, 2011).

DPPH scavenging activity assay

Antioxidant activity of bacterial isolate was carried out by mixing 100 µL of the sample (cell-free supernatant) with an equal volume of DPPH solution (Merck, Germany) (0.2 mM) in a 96-well plate and left in darkness for 30 min at 30°C. The absorbance of samples was determined at 517 nm using Synergy HTX multimode reader. Control was a sample containing deionized water with DPPH, and the scavenging ability was measured as the following equation:

$$\text{DPPH scavenging ability (\%)} = \left(\frac{Ac - As}{Ac}\right) \times 100$$

A_s: Absorbance of the sample
A_c: Absorbance of the control (Xing *et al.*, 2015; Talebi *et al.*, 2018)

Table 1: Primers and PCR conditions used in this study

Primer	Target size (bp)	Sequences (5´-3´)	Reaction conditions	References
hblA F hblA R	1154	AAGCAATGGAATACAATGGG AGAATCTAAATCATGCCACTGC	94°C, 120 s → (94°C, 1 min → 56°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)
hblC F hblC R	740	GATACCAATGTGGCAACTGC TTGAGACTGCTCGCTAGTTG	94°C, 120 s → (94°C, 1 min → 58°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)
hblD F hblD R	829	ACCGGTAACACTATTCATGC GAGTCCATATGCTTAGATGC	94°C, 120 s → (94°C, 1 min → 58°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)
nheA F nheA R	499	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	94°C, 120 s → (94°C, 1 min → 56°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)
nheB F nheB R	769	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	94°C, 120 s → (94°C, 1 min → 54°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)
nheC F nheC R	581	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA	94°C, 120 s → (94°C, 1 min → 58°C, 1 min → 72°C, 2 min) 35 cycles → 72°C, 300 s	Kim *et al*. (2011)

Statistical analysis

IBM SPSS Statistics Software (ver. 26.0, SPSS Inc., USA) was used to analyze results that were presented as mean±SD. Analysis of variance followed by the Post Hoc method (Duncan) was used to find the differences between means (significant difference, P<0.05). All experiments were performed in triplicates.

Results

Bacterial isolation and identification from goat milk

Among 11 pure colonies of bacteria, only 1 isolate, due to the results of Gram-staining besides the biochemical tests, was generally indicated as *B. subtilis* strain. This isolate was further analyzed by sequencing of *16S rRNA* gene. So, this strain was finally identified with biochemical and *16S rRNA* partial sequencing tests as *B. subtilis* GM1. It was deposited under accession number MK818228.1 in the GenBank database (https://www.ncbi.nlm.nih.gov/nuccore/MK818228.1) and then was characterized for its probiotic capacity. As a control, *B. subtilis* G2 recovered from the commercial probiotic product was used to compare the results. Table 2 shows characteristics of *B. subtilis* GM1 and *B. subtilis* G2.

Hemolysis and lecithinase activity

B. subtilis GM1 showed α-hemolytic activity. For lecithinase production assay, there was no precipitation around the colony of bacterial strain even after 48 h of incubation, indicating that *B. subtilis* GM1 is lecithinase negative, while *B. cereus* ATCC14579 was lecithinase positive (Table 3).

Resistance of bacteria to acid and bile

To evaluate the tolerance of isolate to acid and bile salts, *B. subtilis* GM1 was subjected to two ranges of acidic pH (2 and 4) and bile salts (0.3% concentration) for 4 h and 8 h, respectively. The results showed that more than 80% of the bacterial cells survived at two pH values for 4 h, and the *Bacillus* strain was also resistant to bile salts after 8 h of exposure (Table 3).

Gastric juice tolerance

Exposure of the selected isolate to artificial gastric juice (pH 2.5) revealed a viability count of more than 70%. The result showed the ability of the strain to pass through the stomach conditions (Table 3).

Hydrophobicity and aggregation assays

To get further insight into the probiotic characteristics of the isolate, its surface properties were evaluated. The results of using chloroform, toluene, and ethyl acetate for evaluation of the hydrophobic potential of *B. subtilis* strain are shown in Table 4. Also, auto-aggregation which is strongly correlated with adherence to the gastrointestinal system, showed that the selected isolate had about 42% cell adhesion ability at the first 4 h; However, cell adhesion increased to 90.25% after 24 h of incubation.

Table 2: Biochemical characterization of *Bacillus* isolated from goat milk and commercial product

Tests	GM1	G2	Tests	GM1	G2
Gram-staining	+	+	Glucose	+	+
Spore formation	+	+	Maltose	+	+
Starch hydrolysis	+	+	Rhamnose	+	+
Simon's citrate	+	+	Nitrate reduction	+	+
Methyl red	-	-	VP	+	+
Catalase	+	+	Growth at 50°C	+	+
Urease	-	-	SIM	-/-/+	-/-/+

Plus sign (+) indicates the positive, and minus sign (-) indicates the negative results of reaction/test

Table 3: Hemolysis, lecithinase activities, and the results of the viability of *Bacillus* species isolated in different conditions

Bacteria	Growth at (%)			Biochemical activities		
	pH 2	pH 4	Gastric juice tolerance	Bile 0.3% (rate of inhibition)	Lecithinase activity	Hemolytic activity
B. subtilis strain GM1	80.12±0.24[a]	89.82±0.84[a]	75.29±1.25[a]	0.33±0.02[a]	-	α
B. subtilis strain G2	73.88±0.80[b]	72.15±0.61[b]	90.71±0.85[b]	0.36±0.02[a]	-	α

Data are presented as mean±SD, and n=3. Mean within the same column followed by different superscript letters differ significantly (P<0.05). Minus sign indicates the negative results. Alpha symbol (α) indicates partial hemolysis

Table 4: Surface characteristics of the *Bacillus* isolates

Strain	Autoaggregation (%)		Hydrophobicity (%)			HT-29 attachment
	4 h	24 h	Chloroform	Toluene	Ethyl acetate	
B. subtilis GM1	41.97±2.26[a]	90.25±1.04[a]	67.21±1.10[a]	62.69±2.08[a]	50.39±1.08[a]	49.00±0.70[a]
B. subtilis G2	48.83±0.84[b]	72.46±0.98[b]	61.32±1.76[b]	60.89±4.95[a]	62.19±1.02[b]	50.42±0.70[a]

Cell surface characteristics are presented as mean±SD, and n=3. Mean within the same column followed by different superscript letters differ significantly (P<0.05)

Table 5: Antibiotic-resistant of the *B. subtilis* isolates

Strain	Chloramphenicol	Tetracycline	Erythromycin	Streptomycin	Vancomycin	Gentamycin	Clindamycin	Penicillin
B. subtilis GM1	32.00±3.00[S]	25.00±3.00[S]	27.33±2.52[S]	20.33±3.05[S]	23.00±2.00[S]	24.00±1.73[S]	20.33±1.53[S]	32.00±2.65[S]
B. subtilis G2	31.00±1.00[S]	30.67±0.58[S]	27.33±1.15[S]	17.33±1.15[S]	22.33±0.58[S]	24.67±0.58[S]	18.67±0.58[S]	29.00±1.73[S]

Data are presented as mean±SD for the zone of inhibition diameter (mm), three replications. S: Sensitive, and R: Resistance (No resistant isolate was detected)

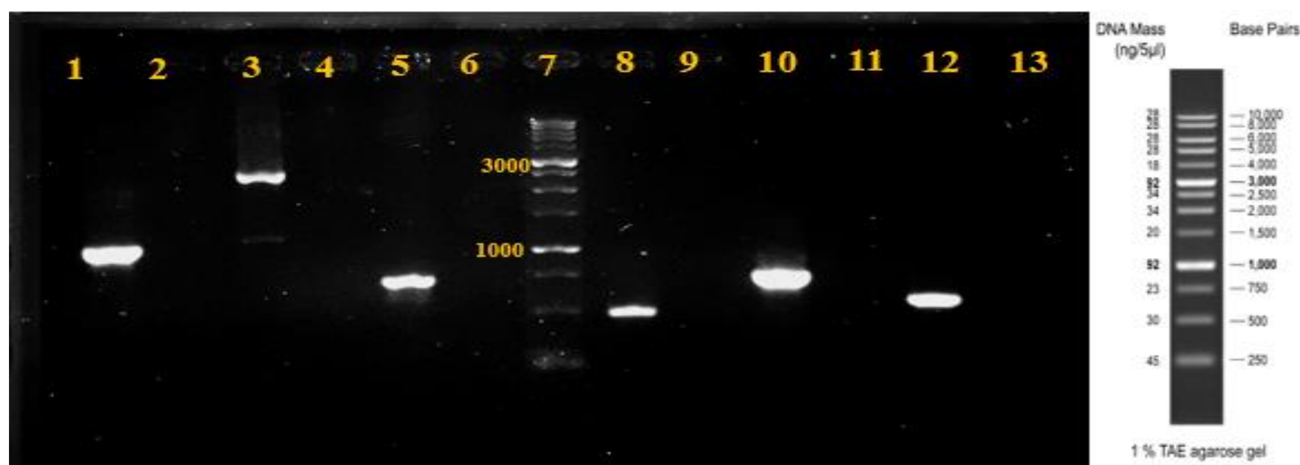

Fig. 1: PCR products of enterotoxin genes from the isolated *Bacillus subtilis* strain and *Bacillus cereus*. Lane 7: 1 kb DNA ladder; Lanes 1, 3, and 5: *hblA*, *hblB*, and *hblC*; Lanes 2, 4, and 6: Negative results of *hbl* genes from isolated strain; Lanes 8, 10, and 12: *nheA*, *nheB*, and *nheC*; and Lanes 9, 11, and 13: Negative results of *nhe* genes from isolated strain

Adhesion to HT-29 cell line

The adhesion of *B. subtilis* GM1to HT-29 cells was studied. The results demonstrated that *B. subtilis* G2 (commercial probiotic strain) and *B. subtilis* GM1 presented relatively equal percentages of adhesion properties (Table 4).

Safety assessment

Results of the antibiotic resistant test showed the susceptibility of *B. subtilis* GM1 to all antibiotics using different mechanisms, including cell wall inhibitors and protein synthesis inhibitors (Table 5). Also, 44.80 ± 1.68 and 41.42 ± 2.11 antioxidant activity was recorded as a result of radical scavenging DPPH assay for cell-free supernatant of *B. subtilis* GM1 and G2, respectively. In addition, all six enterotoxin genes were detected in *B. cereus* ATCC14579 as a reference strain with the PCR method but were not observed in the isolated strain (Fig. 1).

Discussion

Recently, consumers' interest in products containing probiotics has risen owing to their beneficial effects (Yadav *et al*., 2015; Abid *et al*., 2019). So, the natural products and food industries are constantly looking out for strains with probiotic potential (Abid *et al*., 2019). Among the microorganisms, the species of genus *Bacillus*, for their varied range of physiological properties and capability to the production of different types of metabolites, antibiotics, enzymes, and so on, are utilized in most the pharmaceutical, agricultural, and industrial processes (Celandroni *et al*., 2019); Hence isolation of *Bacillus* strains and selection of them according to their ability, are carried out in several laboratories all over the world (Milian *et al*., 2014).

Goat milk, as a valuable natural product, is known to have a buffering capacity and better digestibility than cow and human milk (Atanasova *et al*., 2014). It contains complex and rich autochthonous microbiota that, the important genera of microbiota in goat milk belonged to lactic acid bacteria (Quigley *et al*., 2013; Hernandez-Saldana *et al*., 2016; Makete *et al*., 2016; Pisano *et al*., 2019), Although, there are few studies about the microbial community of milk in different lactation phases (McInnis *et al*., 2015; Niyazbekova *et al*., 2020). Also, it should be noted that geographic locations, genetic specificity, feedings, milking equipment, milk transportation, and storage are essential factors that could

affect the microbial composition of milk (Niyazbekova *et al*., 2020). The predominant bacterial flora reported from the farms of the Languedoc Roussillon and Midi-Pyrenees regions in France belonged to *Staphylococcus*, *Serratia*, and *Arthrobacter* genera (Tormo *et al*., 2011). Also, the cell-free supernatant of all eight strains of *Bacillus amyloliquefaciens* isolated from Aloqt (goat milk-based product) possesses inhibition properties against *Escherichia coli* and *S. aureus* (Hanafy *et al*., 2016). Obtained results by Abid *et al*. (2019) support that *Bacillus tequilensis* GM, isolated from Tunisian spontaneously fermented goat milk, could be a probiotic candidate strain. To the best of our knowledge, there is no research on the isolation of *B. subtilis* with probiotic characteristics from goat milk. Thus, the main objective of the current study was to assay the probiotic properties of the newly isolated *Bacillus* strain via several *in vitro* tests.

Probiotics, for exerting their effects, have to remain alive in the host during a pass from the stressful conditions of the upper intestine that contains bile and the acidic pH of the stomach (Makete *et al*., 2016). Because of the toxic effect of bile salts on living cells, tolerance to these salts is a primary characteristic of probiotic isolates (Ragul *et al*., 2017). In this study, the isolated strain showed tolerance to two sets of acidic pH values 2 and 4 after 4 h. Also, it revealed viability through the artificial gastric juice that showed strain tolerance to stomach conditions. In addition, the results showed the resistance of isolate to bile salts. Our findings are similar to the reports of Wang *et al*. (2010), Lee *et al*. (2017), and Zulkheiri-amin *et al*. (2020), who noted the ability of *Bacillus* species to remain viable in the digestive tract.

The potency of probiotics to the attachment to the target sites for expressing optimal functionality is another important property. The adherent probiotic strains can inhibit the colonization of pathogens and establish competition in the gastrointestinal tract (Mahmoudi *et al*., 2019). Several *in vitro* tests, such as the hydrophobicity of the cell surface, auto-aggregation properties, and Caco-2 or HT-29 adhesion assays, have been considered to evaluate the colonization ability of a strain to interact with the host (Sagheddu *et al*., 2019). To find the adhesion ability of bacteria, the cell surface hydrophobicity assay could offer a competitive advantage to probiotic strains during the adhesion, which helps them colonize in the gastrointestinal tract (Shivangi *et al*., 2020). Here, the adhesion capacity was observed 67.21 ± 1.10% in chloroform, 62.69 ± 2.08% in toluene, and 50.39 ± 1.08% in ethyl acetate for *B. subtilis* GM1. The adhesion capacity of the isolate was approximately in line with Fc6 and much higher than Fc3 and Fs1 when compared to the report of Kavitha *et al*. (2018). Therefore, the isolated strain showed good affinity to different solvents, implying that it possesses hydrophobic cell surface characteristics. In contrast, in a study by Mohkam *et al*. (2016), most of the isolates showed good affinity to xylene, still, they were weak electron donors (basic characteristic) and weak electron acceptors (acidic characteristic), which indicated they had low hydrophilic surface. Auto-aggregation, the bacterial aggregation between microorganisms of the same strain, is important in the human gut (Lim *et al*., 2021). *B. subtilis* GM1 showed auto-aggregation ability of 90.25 ± 1.04 after 24 h. The auto-aggregation percentages of *B. subtilis* MKHJ 1-1, *B. subtilis* p223, *B. clausi* ATCC 700160 were reported to be 91.32 ± 0.74%, 86.03 ± 2.46%, and 93.42 ± 0.86% after 24 h incubation, respectively. So, our finding is nearly in line with the auto-aggregation ability of previously reported *Bacillus* strains (Jeon *et al*., 2017; Lim *et al*., 2021).

Moreover, *in vitro* studies using cell lines such as HT-29 and Caco-2 have helped evaluate the efficacy of gastrointestinal conditions and probiotic bacteria on the adhesive ability of strains (Monteagudo-Mera *et al*., 2019). The adhesion ability of the commercial and selected strain was assessed using the HT-29 cell lines. Based on the adhesion score percentage, *B. subtilis* GM1 isolate was a good adhesive strain, and there was no significant difference with *B. subtilis* G2 as the control strain. Still, the HT-29 cell adhesion rate of *B. subtilis* GM1 strain was higher than that of *Bacillus* isolates reported on Caco-2 cell line by Talebi *et al*. (2018). Based on the results emerging from their study, 31.8 ± 0.5, 27.4 ± 0.3, and 23.9 ± 0.7% of 437F, 1630F, and 1020G strains can adhesion to the Caco-2 cell line, respectively. The type of cell line used (Caco-2 or HT-29), and the mechanisms by which the bacteria interact with the superficial components of intestinal cells could affect the adhesion capacities of strains (Fonseca *et al*., 2021).

Moreover, medical and food industries are common purposes for probiotics usage, so evaluating bacterial safety for selecting one strain as a probiotic is necessary (Deng *et al*., 2021). *In vitro* tests were usually used for the safety assessment of probiotics such as hemolytic activity, the synthesis of certain enzymes, production of enterotoxin genes and biogenic amines, as well as transferability of antibiotic resistance genes (AlGburi *et al*., 2016; Sagheddu *et al*., 2019). Lecithinase production and hemolytic activity are associated with the virulence of bacterial strains (Lakshmi *et al*., 2017). The current report confirmed that the selected strain did not have lecithinase activity. Also, the isolate showed α-hemolytic activity that γ- and α-hemolysis is considered safe (Zulkhairi Amin *et al*., 2020). The current results correlate with reports from Sorokulova *et al*. (2008), Kuebutoraye *et al*. (2020), and Park *et al*. (2020). In comparison, Duc *et al*. (2004) showed lecithinase and hemolysis activity in three products based on *B. cereus* probiotic (Bactisubtil, Subtyl, and BioSubtyl ^{DL}). Even though the isolates with hemolytic or lecithinase activity may be considered unsafe for personal healthcare and food applications but it is essential to know that these products have not been documented as probiotics until elimination or modification of these factors have done and confirmed that there are no harmful effects for eukaryotic cells (AlGburi *et al*., 2016).

Identification of antibiotic susceptibility characteris-

tics is another factor that should be considered for the safety of probiotics. The transferability of resistance gene(s) from probiotic bacteria to other ones, including pathogenic or commensal bacteria, is also essential (Zhang *et al*., 2017b). However, resistance to the antibiotics was reported for some probiotics (AlGburi *et al*., 2016; Imperial and Ibana, 2016; Li *et al*., 2020), but in our study, strain GM1 showed sensitivity to all antibiotics, as listed in Table 5, which results were similar to the research of Thirabunyanon *et al*. (2012), Thankappan *et al*. (2015), and Lefevre *et al*. (2017) which they reported some *Bacillus* strains as a probiotic candidate that be sensitive to antibiotics.

The occurrence of enterotoxin genes was done by the PCR method, and results showed that *B. subtilis* isolate could not produce selected toxins. Our findings are the same as those obtained by Ouoba *et al*. (2008), Lee *et al*. (2016), and Mohkam *et al*. (2019). So, the absence of enterotoxin genes, lecithinase, and hemolytic activities suggests that the GM1 strain has no risk factors for human health.

Membrane phospholipids of intestinal epithelial cells could be affected by high levels of free radicals that antioxidant potential of probiotics or inducer characteristics of them for signaling intrinsic antioxidant defense could be affecting the oxidative status of the gut (Zolotukhin *et al*., 2017). The *Bacillus* strain represented the antioxidant activity, and the result agrees with that found for *Bacillus atrophaeus* and *Bacillus safensis* by Talebi *et al*. (2018).

In conclusion, although previous studies have carried out the characterization of raw goat's milk that the most prevalent genera in raw goat milk have belonged to lactic acid bacteria, to the best of our knowledge, no study has investigated the probiotic potential of *B. subtilis* isolated from goat milk. So, the present study has revealed that *B. subtilis* GM1, isolated from goat milk, displayed potential probiotic characteristics. Other *in vitro* and *in vivo* properties of this strain such as enzymatic activity, co-aggregation, antimicrobial activity, biofilm formation, cholesterol reduction, and animal models must be evaluated in future for the final decision about its application as probiotic strain.

Acknowledgement

The authors are thankful to Dr. Esmaeli for his assistance in goat milk collection.

Conflict of interest

The authors declare that they have no competing interests.

References

Abid, Y; Azabou, S; Casillo, A; Gharsallah, H; Jemil, N; Lanzetta, R; Attia, H and Corsaro, MM (2019). Isolation and structural characterization of levan produced by probiotic *Bacillus tequilensis*-GM from Tunisian fermented goat milk. Int. J. Biol. Macromol., 133: 786-794.

AlGburi, A; Volski, A; Cugini, C; Walsh, EM; Chistyakov, VA; Mazanko, MS; Bren, AB; Dicks, LMT and Chikindas, ML (2016). Safety properties and probiotic potential of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquifaciens* B-1895. Adv. Microbiol., 6: 432-452.

Atanasova, J; Moncheva, P and Ivanova, I (2014). Proteolytic and antimicrobial activity of lactic acid bacteria grown in goat milk. Biotechnol. Biotechnol. Equip., 28: 1073-1078.

Celandroni, F; Vacchione, A; Cara, A; Mazzantini, D; Lupetti, A and Ghelardi, E (2019). Identification of *Bacillus* species: Implication on the quality of probiotic formulations. PLoS One. 14: e0217021.

Davati, N; TabatabaeeYazdi, F; Zibaee, S; Shahidi, F and Edalatian, MR (2015). Study of Lactic acid bacteria community from raw milk of Iranian one humped camel and evaluation of their probiotic properties. Jundishapur. J. Microbiol., 8: e16750.

Deng, F; Chen, Y; Sun, T; Wu, Y; Su, Y; Liu, Ch; Zhou, J; Deng, Y and Wen, J (2021). Antimicrobial resistance, virulence characteristics and genotypes of *Bacillus* spp. from probiotic products of diverse origins. Food. Res. Int., 139: 109949. https://doi: 10.1016/j.foodres.2020.109949.

Duc, LH; Hong, HA; Barbosa, TM; Henriques, AO and Cutting, SM (2004). Characterization of *Bacillus* probiotics available for human use. Appl. Environ. Microbiol., 70: 2161-2171.

Ebnetorab, SMA; Ahari, H and Kakoolaki, S (2020). Isolation, biochemical and molecular detection of *Bacillus subtilis* and *Bacillus licheniformis* from the digestive system of rainbow trout (*Oncorhynchus mykiss*) and its inhibitory effect on Aeromonas hydrophila. Iran. J. Fish. Sci., 19: 2824-2845.

Elisashvili, V; Kachlishvili, E and Chikindas, ML (2019). Recent advances in the physiology of spore formation for *Bacillus* probiotic production. Probiotics. Antimicro. Prot., 11: 731-747.

Elshaghabee, FMF; Rokana, N; Gulhane, RD; Sharma, C and Panwar, H (2017). *Bacillus* as potential probiotics: status, concerns, and future perspectives. Front. Microbiol., 8: 1490. https://doi.org/10.3389/fmicb.2017.01490.

Fonseca, HC; Melo, DS; Ramos, CL; Dias, DR and Schwan, RF (2021). Probiotic properties of Lactobacilli and their ability to inhibit the adhesion of enteropathogenic bacteria to Caco-2 and HT-29 cells. Probiotics. Antimicro. Prot., 13: 102-112.

Hanafy, AM; Al-Mutairi, AA; Al-Reedy, RM and Al-Garni, SM (2016). Phylogenetic affiliations of *Bacillus* amyloliquefaciens isolates produced by a bacteriocin-like substance in goat milk. J. Taibah. Univ. Sci., 10: 631-641.

Hernandez-Saldana, OF; Valencia-Posadas, M; Delafuente-Salcido, NM; Bideshi, DK and Barboza-Corona, JE (2016). Bacteriocinogenic bacteria isolated from raw goat milk and goat cheese produced in the center of Mexico. Indian J. Microbiol., 56: 301-308.

Igbal, MZ; Qadir, MI; Hussain, T; Janbaz, KH; Habib Khan, Y and Ahmad, B (2014). Probiotics and their beneficial effects against various disease. Pak. J. Pharm., 27: 405-415.

Imperial, ICVJ and Ibana, JA (2016). Addressing the antibiotic resistance problem with probiotics: Reducing the risk of its double-edged sword effect. Front. Microbiol., 7: 1983.

Ismail, MM; Hamad, MF and Elraghy, EM (2018). Using goat's milk, barley flour, honey, and probiotic to manufacture of functional dairy product. Probiotics.

Antimicro. Prot., 10: 677-691.

Jeon, HL; Lee, NK; Yang, SJ; Kim, WS and Paik, HD (2017). Probiotic characterization of *Bacillus subtilis* P223 isolated from kimchi. Food. Sci. Biotechnol., 26: 1641-1648.

Kavitha, M; Raja, M and Perumal, P (2018). Evaluation of probiotic potential of *Bacillus* spp. isolated from the digestive tract of freshwater fish Labeo calbasu (Hamilton, 1822). Aquac. Rep., 11: 59-69.

Kim, BJ; Hong, JH; Jeong, YS and Jung, HK (2014). Evaluation of two *Bacillus subtilis* strains isolated from Korean fermented food as probiotics against loperamide-induced constipation in mice. J. Korean Soc. Appl. Biol. Chem., 57: 797-806.

Kim, JB; Kim, JM; Cho, SH; Oh, HS; Choi, NJ and Oh, DH (2011). Toxin genes profiles and toxin production ability of *Bacillus cereus* isolated from clinical and food samples. J. Food. Sci., 76: 25-29.

Kuebutornye, FKA; Lu, Y; Abarike, ED; Wang, Zh; Li, Y and Sakyi, ME (2020). *In vitro* assessment of the probiotic characteristics of three *Bacillus* species from the gut of Nile tilapia, *Oreochromis niloticus*. Probiotics. Antimicrob. Prot., 12: 412-424.

Lakshmi, SG; Jayanthi, N; Saravanan, M and Sudha, RM (2017). Safety assessment of *Bacillus clausii* UBBC07, a spore forming probiotic. Toxicol. Rep., 4: 62-71.

Lavanya, K and Dayakar, Y (2017). Isolation and characterization of probiotic bacteria from the soil samples of the coastal areas of (Gudur division, Nellore Dt.) for utilization in shrimp farming. Int. J. Fish. Aquat. Stud., 5: 371-376.

Lee, SG and Chang, HC (2016). Assessment of *Bacillus subtilis* SN7 as a starter culture for Cheonggukjang, a Korean traditional fermented soybean food, and its capability to control *Bacillus cereus* in Cheonggukjang. Food Control. 73: 946-953.

Lee, NK; Kim, WS and Paik, HD (2019). *Bacillus* strains as human probiotics: characterization, safety, microbiome and probiotic carrier. Food. Sci. Biotechnol., 28: 1297-1305.

Lee, S; Lee, J; Jin, YI; Jeong, JC; Chang, YH; Lee, Y; Jeong, Y and Kim, M (2017). Probiotics characteristics of *Bacillus* strains isolated from Korean traditional soy sauce. Food. Sci. Technol., 79: 518-524.

Lee, J; Park, I; Choi, Y and Cho, J (2012). *Bacillus* strains as feed additives: *in vitro* evaluation of its potential probiotic properties. Rev. Colomb. Cienc. Pecu., 25: 577-585.

Lefevre, M; Racedo, SM; Denayrolles, M; Ripert, G; Desfougeres, T; Lobach, AR; Simon, R; Pelerin, F; Justen, P and Urdaci, MC (2017). Safety assessment of *Bacillus subtilis* CU1 for use as a probiotic in humans. Regul. Toxicol. Pharmacol., 83: 54-65.

Li, T; Teng, D; Mao, R; Hao, Y; Wang, X and Wang, J (2020). A critical review of antibiotic resistance in probiotic bacteria. Food. Res. Int., 136: 109571. https://doi: 10.1016/j.foodres.2020.109571. PMID: 32846610.

Lim, H; Oh, S; Yu, S and Kim, M (2021). Isolation and characterization of probiotic *Bacillus subtilis* MKHJ 1-1 possessing L-asparaginase activity. Appl. Sci., 11: 4466. https://doi.org/10.3390/app11104466.

Mahmoudi, I; Ben Moussa, O; Khaldi, TE; Kebouchi, M; Soligot-Hognon, C; Leroux, Y and Hassouna, M (2019). Adhesion properties of probiotic *Lactobacillus* strains isolated from Tunisian sheep and goat milk. J. Agr. Sci. Tech., 21: 587-600.

Makete, G; Aiyegoro, OA and Thantsha, MS (2016). Isolation, identification and screening of potential probiotic bacteria in milk from South African Saanen goats. Probiotics. Antimicro. Prot., 9: 246-254.

Manhar, AK; Bashir, Y; Saikia, D; Nath, D; Gupta, K; Konwar, BK; Kumar, R; Namas, ND and Mandal, M (2016). Cellulolytic potential of probiotic *Bacillus subtilis* AMS6 isolated from traditional fermented soybean (churpi): An *in vitro* study with regards to application as an animal feed additive. Microbiol. Res., 186-187: 62-70.

McInnis, EA; Kalanetra, KM; Mills, DA and Maga, EA (2015). Analysis of raw goat milk microbiota: Impact of stage of lactation and lysozyme on microbial diversity. Food. Microbiol., 46: 121-131.

Milian, G; Rondon, AJ; Perez, M; Samaniego, LM; Riano, J; Bocourt, R; Ranilla, MJ; Carro, MD; Rodriguezn, M and Laurencio, M (2014). Isolation and identification of strains of *Bacillus* spp. in different ecosystems, with probiotic purposes, and their use in animals. Cuba. J. Agric. Sci., 48: 347-351.

Mohkam, M; Nezafat, N; Berenjian, A; Zamani, M; Dabbagh, F; Bigharaz, R and Ghasemi, Y (2019). Multifaceted toxin profile of *Bacillus* probiotic in newly isolated *Bacillus* spp. from soil rhizosphere. Biologia. 75: 309-315.

Mohkam, M; Rasoul-Amini, S; Shokri, D; Berenjian, A; Rahimi, F; Sadraeian, M; Khalvati, B; Gholami, A and Ghasemi, Y (2016). Characterization and *in vitro* probiotic assessment of potential indigenous *Bacillus* strains isolated from soil rhizosphere. Minerva Biotechnol., 28: 19-28.

Monteagudo-Mera, A; Rastall, RA; Gibson, GR; Charalampopoulos, D and Chatzifragkou, A (2019). Adhesion mechanisms mediated by probiotics and prebiotics and their potential impact on human health. Appl. Microbiol. Biotechnol., 103: 6463-6472.

Niyazbekova, ZH; Yao, XT; Liu, MJ; Bold, N; Tong, JZ; Chang, JJ; Wen, Y; Li, L; Wang, Y; Chen, DK and Ma, WT (2020). Compositional and functional comparisons of the microbiota in the colostrum and mature milk of dairy goats. Animals. 10: 1955. https://doi.org/10.3390/ani10111955.

Olmos, J and Paniagua-Michel, J (2014). *Bacillus subtilis* a potential probiotic bacterium to formulate functional feeds for aquaculture. J. Microb. Biochem. Technol., 6: 361-365.

Ouoba, LII; Thorsen, L and Varnam, AH (2008). Enterotoxins and emetic toxins production by *Bacillus cereus* and other species of *Bacillus* isolated from Soumbala and Bikalga, African alkaline fermented food condiments. Int. J. Food. Microbiol., 124: 224-230.

Park, H; Lee, M; Jeong, D; Park, S; Ji, Y; Todorov, SD and Holzapfel, WH (2020). Safety evaluation and *in vivo* strain specific functionality of *Bacillus* strains isolated from Korean traditional fermented foods. Probiotics. Antimicro. Prot., 13: 60-71.

Pique, N; Berlanga, M and Minana-Galbis, D (2019). Health benefits of heat-killed (Tyndallized) probiotics: an overview. Int. J. Mol. Sci., 20: 2534. doi: 10.3390/ijms20102534.

Pisano, MB; Deplano, M; Fadda, ME and Cosentino, S (2019). Microbiota of Sardinian goat's milk and preliminary characterization of prevalent LAB species for starter or adjunct cultures development. Biomed. Res. Int., 2019: e6131404.

Quigley, L; Osullivan, O; Stanton, C; Beresford, TP; Roass, RP; Fitzgerald, GF and Cotter, PD (2013). The complex microbiota of raw milk. FEMS. Microbiol. Rev., 37: 664-698.

Ragul, K; Syiem, I; Sundar, K and Shetty, PH (2017). Characterization of probiotic potential of *Bacillus* species isolated from a traditional brine pickle. J. Food. Sci.

Technol., 54: 4473-4483.

Ranadheera, CS; Evans, CA; Baines, SK; Balthazar, CF; Cruz, AG; Esmerino, EA; Freitas, MQ; Pimentel, TC; Wittwer, AE; Naumovski, N; Graca, JS; Sant'Ana, AS; Ajlouni, S and Vasiljevic, T (2019). probiotics in goat milk products: delivery capacity and ability to improve sensory attributes. Compr. Rev. Food. Sci. Food. Saf., 18: 867-882.

Sagheddu, V; Guidesi, E; Galletti, S and Elli, M (2019). Selection and characterization criteria of probiotics intended. Food. Sci. Nutr. Studies. 3: 73-83.

Seo, WT; Nam, SH; Lee, ChK and Cho, KM (2011). Identification of potential *Bacillus subtilis* probiotics from Korean soybean paste and their antimicrobial and immune activities. J. Food. Sci. Nutr., 16: 37-44.

Shivangi, Sh; Devi, PB; Ragul, K and Shetty, PH (2020). Probiotic potential of *Bacillus* strains isolated from an acidic fermented food Idli. Probiotics. Antimicro. Prot., 12: 1502-1513.

Sorokulova, I (2013). Modern status and perspectives of *Bacillus* bacteria as probiotics. J. Prob. Health. 1: e106.

Sorokulova, IB; Pinchuk, IV; Denayrolles, M; Osipova, IG; Huang, JM; Cutting, SM and Urdaci, MC (2008). The safety of two *Bacillus* probiotic strains for human use. Dig. Dsi. Sci., 53: 954-963.

Sridevi, V; Sirisha, R and Swapna, SN (2015). Screening of probiotic goat milk and cow milk isolated for acid resistance, antagonistic activity, and tolerance to antimicrobial activity of spices: molecular identification of potential probiotic goat milk isolate, G8. Int. J. Curr. Microbiol. App. Sci., 4: 406-421.

Suva, MA; Sureja, VP and Kheni, DB (2016). Novel insight on probiotic *Bacillus* subtilis: mechanism of action and clinical applications. J. Curr. Res. Sci. Med., 2: 56-72.

Talebi, S; Makhdoumi, A; Bahreini, M; Matin, MM and Moradi, HS (2018). Three novel *Bacillus* strains from a traditional lacto-fermented pickle as potential probiotics. J. Appl. Microbiol., 125: 888-896.

Thankappan, B; Ramesh, D; Ramkumar, S; Natarajaseenivasan, K and Anbarasu, K (2015). Characterization of *Bacillus* spp. from the gastrointestinal tract of Labeo rohita- towards to identify novel probiotics against fish pathogens. Appl. Biochem. Biotechnol., 175: 340-353.

Thirabunyanon, M and Thongwittaya, N (2012). Protection activity of a novel probiotic strain of *Bacillus subtilis* against *Salmonella* Enteritidis infection. Res. Vet. Sci., 93: 74-81.

Tormo, H; Delacroix-Buchet, A; Lopez, C; Haimoud Lekhal, DA and Roques, C (2011). Farm management practices and diversity of the dominant bacterial species in raw goat's milk. Int. J. Dairy Sci., 6: 29-43.

Wang, Y; Zhang, H; Zhang, L; Liu, W; Zhang, Y; Zhang, X and Sun, T (2010). *In vitro* assessment of probiotic properties of *Bacillus* isolated from naturally fermented congee from Inner Mongolia of China. World J. Microbiol. Biotechnol., 26: 1369-1377.

Xing, J; Wang, G; Zhang, Q; Liu, X; Gu, Z; Zhang, H; Chen, YQ and Chen, W (2015). Determination antioxidant activities of Lactobacilli cell-free supernatants by cellular antioxidant assay: a comparison with traditional methods. PLoS One. 10: e0119058.

Yadav, AK; Kumar, R; Priyadarshini, L and Singh, J (2015). Composition and medicinal properties of camel milk: A review. Asian. J. Dairy & Food. Res., 34: 83-91.

Zhang, F; Wang, ZH; Lei, F; Wang, B; Jiang, S; Peng, Q; Zhang, J and Shao, Y (2017a). Bacterial diversity in goat milk from the Guanzhong area of China. J. Dairy Sci., 100: 7812-7824.

Zhang, M; Zhang, R; Tian, X; Zhou, X; Pan, X and Wong, A (2017b). Assessing the risk of probiotic dietary supplements in the context of antibiotic resistance. Front. Microbiol., 8: 908. https://doi: 10.3389/fmicb.2017.00908.

Zolotukhin, PV; Prazdnova, EV and Chistyakov, VA (2017). Methods to assess the antioxidative properties of probiotics. Probiotics. Antimicro. Prot., 10: 589-599.

Zulkhairi Amin, FA; Sabri, S; Ismail, M; Chan, KW; Ismail, N; Mohd Esa, N; Mohd Lila, MA and Zawawi, N (2020). Probiotic properties of *Bacillus* strains isolated from stingless bee (Heterotrigona itama) honey collected across Malaysia. Int. J. Environ. Res. Public Health. 17: 278. https://doi: 10.3390/ijerph17010278.