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ORIGINAL ARTICLE

Three novel *Bacillus* strains from a traditional lacto-fermented pickle as potential probiotics

S. Talebi[1], A. Makhdoumi[1], M. Bahreini[1], M.M. Matin[1,2] and H.S. Moradi[1]

1 Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
2 Cell and Molecular Biotechnology Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

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Correspondence
Ali Makhdoumi, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran.
E-mail: a.makhdomi@um.ac.ir

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Abstract

Aims: *Bacillus* probiotics recently gained attention due to the production of resistant cells. The *in vitro* probiotic potentials and safety assessment were evaluated for three *Bacillus* strains obtained from traditional pickle.

Methods and Results: Three bacterial strains designated as 437F, 1630F and 1020G were isolated from a traditional pickle and identified as members of the genus *Bacillus*. The novel strains showed high acid and bile tolerance. They exhibited antagonistic activity against various pathogens. Antioxidant activity, auto- and co-aggregation ability as well as their surface hydrophobicity and attachment capacity to the Caco-2 cells were in the range of other well-known probiotic strains. They were susceptible to various antibiotics. The enterotoxin (*HBl and NHe*), cytotoxin (*Cytk1*) and emetic (*Ces*) genes were not detected based on PCR assay. They were not toxic against HT-29 cells.

Conclusion: With respect to their characteristics and safety aspect, these *Bacillus* species may have potential to consider as probiotics for animal and/or human applications.

Significance and Impact of the Study: Nondairy-fermented foods are interesting sources for isolation of novel probiotics. Identification of novel *Bacillus* strains with remarkable probiotic potentials would increase their contribution in food/feed and pharmaceutical industries.

Introduction

Probiotics are known as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.* 2014). The benefits of probiotics have been extended from well-known digestive profits (suppress diarrhoea, alleviate lactose intolerance, antimicrobial and anticolorectal cancer, reduce irritable bowel symptoms) (Tamang *et al.* 2016a) to mental effects (treatment of anxiety and depression) (Bested *et al.* 2013). The host is also not limited to human, and many animals' industries received benefit from probiotics (FAO 2016).

Probiotic micro-organisms must possess certain characteristics according to the FAO/WHO guidelines (FAO/WHO, 2006). Members of various bacterial genera like *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium* and *Streptococcus* harbour these traits and have been used as probiotics (Huys *et al.* 2013). Historically, the strains with the most beneficial effects in either animal or humans belonged to *Lactobacilli* and *Bifidobacteria* (Fijan 2014). However, *Bacillus* probiotics recently gained attention due to the production of resistant cells. The spore-forming strains represent some advantages in comparison to common probiotics such as survival at low pH of the gastric barrier and recognition as the producer of many secondary metabolites like antibiotics and antioxidants which can benefit the host (Duc *et al.* 2004). Besides, fermented food containing the *Bacillus* species can be stored at room temperature without any significant reduction in the bacterial load (Cutting 2011).

Dairy products were considered the most adequate food matrices for probiotics (Silva *et al.* 2018). However,

nonconventional sources such as nondairy-fermented foods, fermented drinks, fruit juices and pickled vegetables were also assessed for their probiotic residents (Sornplang and Piyadeatsoontorn 2016).

Some scientific reports suggest the relation between increased risk of gastric cancer with consumption of pickled vegetables (Ren *et al.* 2012). The generation of potentially carcinogenic *N*-nitroso compounds and mycotoxins from yeasts and moulds in the fermentation process were considered for these undesirable effects. However, others argued this finding with the limited evidence for their carcinogenicity in humans and inadequate evidence in experimental animals (IARC Working Group 1993; Islami *et al.* 2009). Here, we selected Torshi, a traditional lacto-fermented pickle with the high consumption rate in Iran, for isolation of their spore-forming *Bacillus* spp. The *in vitro* probiotic potentials of these strains were evaluated.

Materials and methods

Vegetable pickle description

Torshi (pronounced tursu in some countries) is a common pickled vegetable condiment in Middle Eastern cuisine which is consumed along with food. In general, it is made by families rather than industries and hundreds of recipes according to regional customs are present. The one that we sampled in this study is consumed in the eastern part of Iran and prepared from a mixture of vegetables, including green pepper, green cabbage, red cabbage, cauliflower, carrot, garlic, celery and Persian shallot, pickled in salty vinegar. It is usually developed after few weeks.

Bacterial isolation and identification

Pickle samples were obtained from local stores (Mashhad, Iran) and transferred to the laboratory. Samples were blended and pretreated at 80°C for 15 min in to isolate the spore-forming bacteria. Desired dilutions were prepared in sterile phosphate buffer saline (PBS) and plated in the Nutrient Agar (NA) medium (Merck) by the spreading method. The plates were incubated at 37°C for 1 week and pure isolates were obtained after successive cultivation. Spore formation was detected based on the common Schaeffer–Fulton method (Schaeffer and Fulton 1933). The selected spore-forming isolates were identified after DNA extraction (Thermo Scientific, Lithuania), PCR amplification and sequencing of their *16S rRNA* genes (Macrogen, South Korea) (Reysenbach *et al.* 1994). Phylogenetic analysis was performed using the software package MEGA ver. 6 (Tamura *et al.* 2013).

Probiotic potential characterization

Acid tolerance and bile tolerance

For acid tolerance determination, one per cent (v/v) of overnight grown bacterial culture (Nutrient Broth medium, 37°C, and 150 rpm agitation) (10^9 CFU per ml) was added to the sterile phosphate buffer saline (PBS) (pH: 2, 3, 4 and 7) and mixed. Aliquots were drawn at intervals of 0 and 3 h, serially diluted and plated on NA medium. Plates were incubated at 37°C for 24 h, and viable count was obtained. Survival capacity was calculated as follow (Wang *et al.* 2010):

$$\text{Survival}(\%) = \frac{\log \text{NT}}{\log N0} \times 100 \qquad (1)$$

where NT and N0 are the number of bacteria after and before treatment in acidic condition.

Similarly, 5% (v/v) of overnight grown bacterial culture (10^9 CFU per ml) was inoculated in the Nutrient Broth (NB) (Merck) medium supplemented by 0·3 and 1% (w/v) ox-bile (HiMedia). Media without bile salt was applied as control. Tolerance to bile was evaluated based on the survival capacity and adaptation time. Survival rate was calculated as described above (Eqn (1)), where NT and N0 are the number of bacteria with and without treatment with bile. Adaptation time was defined as the difference between the time that required increasing the absorbance (600 nm) by 0·3 units in NB medium with and without bile salt (Ramos *et al.* 2013).

Auto- and co-aggregation

Auto-aggregation assay was conducted for three *Bacillus* species. Briefly, stationary-phase cells from overnight batch cultures (NB, 37°C, 150 rpm) were centrifuged (12 000 ***g*** for 10 min at room temperature), washed with PBS and resuspended in 4 ml PBS (10^8 CFU per ml). Bacterial suspensions were mixed vigorously and then incubated at 15°C up to 24 h. The upper suspension was carefully removed, and the absorbance at 600 nm was measured. Auto-aggregation percentage was determined using the following equation (Archer and Halami 2015):

$$\text{Auto-aggregation}(\%) = 1 - \frac{A_t}{A_0} \times 100 \qquad (2)$$

where At represents absorbance at different time points (t = 4 h or 24 h) and A0 represents absorbance at the beginning of the assay (0 h).

To determine the co-aggregation ability, bacterial suspension was prepared as stated above. Equal volumes (2 ml) of *Bacillus* strains and pathogenic bacteria (*Escherichia coli and Listeria monocytogenesis)* were mixed by vortexing and incubated at room temperature for 4 h. Control tubes contained only 2 ml suspensions of each strain. The

upper suspension was carefully removed, and the absorbance at 600 nm was measured. The percentage of co-aggregation was determined as follows (Kos *et al.* 2003):

$$\text{Co-aggregation}(\%) = \frac{(Ax+Ay)/2 - A(x+y)}{(Ax+Ay)/2} \times 100 \quad (3)$$

where, Ax and Ay represent the absorbance of each strain (probiotic and pathogen) and A(x + y) represents the absorbance of their mixture.

Hydrophobicity and adherence to Caco-2

The cell surface hydrophobicity was evaluated based on MATH (microbial adhesion to hydrocarbons) measurement. For this, the stationary-phase cells (10^8 CFU per ml) were obtained similarly to aggregation tests, washed with PBS buffer, resuspended in KNO_3 (0·1 mol l^{-1}) and their absorbance were measured at 600 nm (A0). Equal volumes (2 ml) of bacterial suspension and solvent (chloroform, ethyl acetate and hexane) were mixed by vortexing for 1 min and then incubated at room temperature for 1 h to separate two phases. The aqueous phase was removed carefully, and its absorbance was measured at 600 nm (A1). Bacterial hydrophobicity was calculated as (Lee *et al.* 2011):

$$\text{Hydrophobicity}(\%) = \left(1 - \frac{A_1}{A_0}\right) \times 100 \quad (4)$$

Caco-2 cells (human epithelial colorectal adenocarcinoma cell line) were obtained from the Pasteur Institute (Tehran, Iran) and cultured in Roswell Park Memorial Institute (RPMI-1640) medium (Life Technologies, UK) supplemented with 10% fetal bovine serum (FBS, Gibco, Scotland), penicillin (100 U per ml) and streptomycin (100 μg ml^{-1}). Cells were seeded at high density in 96-multiwell tissue culture plates and grown to 80–90% confluence. For adhesion assays, 100 μl of stationary growth phase bacteria (10^8 CFU per ml) was mixed with 100 μl of incomplete DMEM media (without antibiotic), and incubated with Caco-2 cells for 2 h. All experiments were done at 37°C in a humidified atmosphere of 5% CO_2 in the air. After incubation, the nonadherent bacteria were removed by washing the cells with the PBS buffer. Caco-2 cells were lysed with 0·1% (v/v) Triton X-100 for 5 min and the lysate used to count adherent bacteria. Cell adherence capacity was determined based on the viable bacterial count (CFU) at time 0 (N0) and after 2 h (NT) by cultivation on NA (37°C, 24 h) (He *et al.* 2017):

$$\text{Caco2attachment}(\%) = \frac{\log NT}{\log N_0} \times 100 \quad (5)$$

Antimicrobial activity

For studying the antimicrobial activity, selected strains were inoculated into 100 ml of NB broth medium and incubated at 37°C in a rotary shaker at 150 rpm for 2 days. The broth culture was neutralized by NaOH (1 mol per l) and filtrated. Inhibitory activity against test strains including *Staphylococcus aureus* (PTCC1431), *Bacillus cereus* (PTCC1665), *Pseudomonas aeruginosa* (PTCC1074), *Escherichia coli* (PTCC 1330) and *Listeria monocytogenes* (PTCC 1166) was determined by inhibited the growth of pathogenic strains following the well diffusion method (Balouiri *et al.* 2016).

Antioxidant activity

The antioxidant capacity of the bacterial isolates was studied following a method described by Blois (1958). A volume of 100 μl filtrated culture (NB medium, 37°C, 24 h, 150 rpm) was mixed to 1·9 ml of methanol and was added to 2 ml of 0·1 mmol l^{-1} DPPH (1,1-diphenyl-2- picrylhydrazyl) (Sigma, USA) methanolic solution. Tubes were left in darkness at 30°C for 30 min and the absorbance of the resulting solution was measured colorimetrically at 517 nm (A1). Sample containing solvent (methanol) with equal volumes of DPPH was used as control (A0). The scavenging effect was determined as follows:

$$\text{DPPH scavenging effect}(\%) = [(A_0 - A_1)/A_0] \times 100 \quad (6)$$

Safety assessment

Antibiotic susceptibility of *Bacillus* spp. was determined using the disc diffusion method (Balouiri *et al.* 2016). The applied antibiotic agents were (μg/disc): ampicillin (10), clindamycin (2), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), methicillin (5), rifampicin (5), streptomycin (25), tetracycline (30) and vancomycin (30) (EFSA, 2012). The inhibition zones were measured, and the antibiotic susceptibility of the tested strains was evaluated according to the CLSI criteria (CLSI, 2015). Haemolysis was detected by streaking strains on NA medium supplemented with 5% human blood and incubation at 37°C for 24 h. Cytotoxic activity of *Bacillus* strains was evaluated on human colorectal adenocarcinoma cell line (HT-29) by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann 1983). For cytotoxic analysis, $0{\cdot}1 \times 10^5$ HT-29 cells were seeded in 96-well microtitre cell culture plates, in the absence (control) or presence (test) of bacterial filtrated supernatant (prepared as mentioned in the antioxidant assay) in a final volume of 200 μl for 24 h. Cell viability was measured by the MTT assay using the following formula:

$$\text{Viability}(\%) = [(A_T)/A_c] \times 100 \quad (7)$$

where A_C was the absorbance (540 nm) of the control reaction and A_T was the absorbance in the presence of bacterial supernatant.

Treated and nontreated cells were also observed under a light inverted microscope (Olympus, Japan) for monitoring of morphological changes after 24 h. Determination of toxins was conducted by PCR amplification of their relevant genes. Analysed genes were emetic (*ces*), cytotoxic (*cytk1*) and enterotoxin (*hblC*, *hblD*, *hblA* and *nheA*, *nheB*, *nheC*) genes. All protocols including primer sets and PCR conditions were obtained from Wehrle *et al.* (2009). The type strain of *Bacillus cereus* (PTCC1665) was used as control in the amplification experiments.

Statistics

The results of quantitative data are expressed as the mean±SD of three independent experiments. The data analysis was conducted using PRISM software (ver. 6·07 for Windows; GraphPad Software, San Diego, CA) and was subjected to a one-way analysis of variance (ANOVA).

Results

Identification of the isolated bacteria

We cultured a collection of the 18 nonhaemolytic spore former strains from Torshi (pH 4, and salinity 1·5% (v/w)) and a subset of three different isolates designated as 437F, 1630F and 1020G was selected for further analysis. Figure 1 details the phylogenetic identification of strains based on the *16S rRNA* gene sequence. All strains belonged to the *Bacillus* genus. However, as indicated in Fig. 1, none of the previously identified *Bacillus* probiotics (indicated by grey colour in the tree) were placed in the same clade as the novel strains. The closest identified

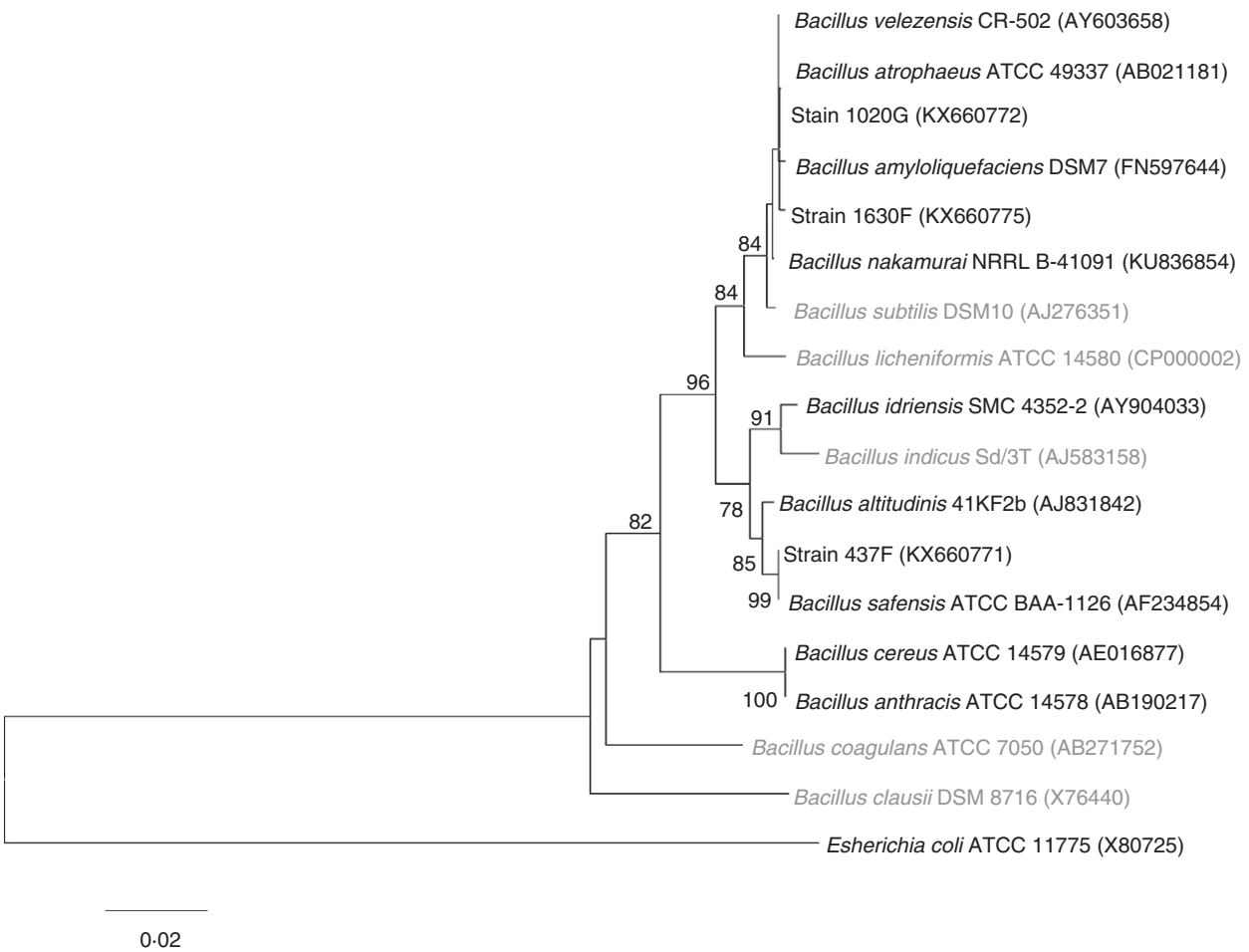

Figure 1 Maximum-likelihood phylogenetic tree based on *16S rRNA* gene sequences showing the relationship of isolated *Bacillus* strains 437F, 1630F and 1020G with other members of the genus *Bacillus*. Accession numbers of the sequences are given in parentheses. Previously reported probiotics were presented in grey. The sequences of the *Escherichia coli* ATCC 11775^T (X80725) were used as an outgroup. Bootstrap values (%) are based on 1000 replicates. Bar, 0·05 substitutions per nucleotide position.

taxa for strains 437F, 1630F and 1020G were *B. safensis* (99·9% similarity), *B. atrophaeus* (99·9%) and *B. amyloliquefaciens* (99·9%) respectively.

Acid and bile tolerance

Acid (pH 2, 3 and 4) and bile (0·3 and 1% (w/v)) tolerance were studied for three obtained *Bacillus* strains (Table 1). All strains showed high acid tolerance and 3-h incubation of bacterial cells in pH 4 and 3 did not affect the bacterial counts in comparison to the neutral condition ($P < 0·05$). More than 80% of these *Bacillus* populations survived the low pH at pH 2. Regarding bile tolerance, all strains showed high bile tolerance and more than 90% of the bacterial cells survived after treatment with 0·3% ox bile. On the basis of adaptation time, all bacterial strains showed an adaptation time of less than 50 min and were classified as bile-tolerant strains.

Cell surface characteristics

The cell surface attributes of the *Bacillus* strains were subjected to study based on auto-aggregation, co-aggregation, hydrophobicity and adherence to Caco-2 cells (Table 2). The auto-aggregation capacity of the strains after 3 h varied from 20·5% (1630F) to 23·2% (437F) and 38·8% (1020G) increasing over time, whereas more than 80% cells of all strains were aggregated after 24 h. The co-aggregation ability with *E. coli* was 45·9%, 52·4% and 56·2% for strains 1630F, 1020G and 437F, respectively, and was relatively higher than with *L. monocytogenes* (28·2, 34·4 and 37·7% respectively). Regarding hydrophobicity, all three strains showed the maximum attachment value for chloroform as it is the most polar protic solvent (4·1 polarity index). The ability of species' attachment to other solvents was strain specific. While for strain 1630F it increased based on the polarity index of the solvents and were 18 and 3% for ethyl acetate (2·8 polarity index) and hexane (0·1 polarity index) respectively; the two other strains represented higher attachment ability to hexane than ethyl acetate (Table 2). The *Bacillus* strains showed strong adhesive properties and *c.* 24, 27 and 32% of primary cells of the strains 1020G, 1630F and 437F were adhered to the Caco-2 cells, respectively (52, 55 and 66 bacterial cells per one Caco-2 cell respectively).

Antimicrobial and antioxidant activity

Antagonistic activity of *Bacillus* strains against five pathogens including *Staphylococcus aureus* (PTCC1431), *Bacillus cereus* (PTCC1665), *Pseudomonas aeruginosa* (PTCC1074), *Escherichia coli* (PTCC 1330) and *L. monocytogenes* (PTCC 1166) is presented in Table 3. All three isolates could inhibit the growth of at least two pathogens. Strain 1020G represented the highest antibacterial activity by the antagonistic effect against four species including *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli*. All *Bacillus* strains could inhibit the growth of *Pseudomonas aeruginosa*. The anti-oxidative nature of bacterial strains was revealed based on DPPH scavenging activity (Table 3). Strain 1020G demonstrated moderate (21%) antioxidant activity, whereas the high antioxidant activity was observed for the supernatants of the strains 437F and 1630F (51 and 42% respectively).

Table 1 Acid and Bile tolerance of the *Bacillus* isolates (log cfu per ml)

	pH tolerance				Bile tolerance			Adaptation time (min)
Strain	Initial counts	pH 2	pH 3	pH 4	Initial counts	0·3%	1·0%	0·3% bile
437F	7·07 ± 0·3	5·62 ± 1·3*	6·87 ± 1·3	6·90 ± 1·3	7·04 ± 0·2	6·54 ± 0·3	6·02 ± 0·4*	56 ± 3
1630F	6·08 ± 0·9	5·74 ± 0·9	5·89 ± 0·8	6·02 ± 1·3	7·47 ± 0·2	7·09 ± 0·1	6·82 ± 0·3*	25 ± 2
1020G	6·27 ± 0·6	5·39 ± 0·9*	5·95 ± 1·3	6·12 ± 1·3	7·48 ± 0·7	7·04 ± 0·3	6·74 ± 0·5*	32 ± 2

Data are presented as mean ± SD. * Statistically significance difference in comparison with the control group $P < 0·05$.

Table 2 Surface characteristics of the *Bacillus* isolates. *: *Listeria monocytogenes*, †: *Escherichia coli*

	Auto-aggregation (%)		Co-aggregation (%)		Hydrophobicity (%)			
Strain	4 h	24 h	L. m*	E. c†	Chloroform	Hexane	Ethyl acetate	Caco-2 attachment (%)
437F	$23·2 \pm 0·1^a$	$80·5 \pm 0·7^a$	$37·7 \pm 0·3^a$	$52·4 \pm 1·3^a$	$57·4 \pm 0·9^a$	$26·2 \pm 0·8^a$	$2·0 \pm 0·1^a$	$31·8 \pm 0·5^a$
1630F	$20·5 \pm 0·4^a$	$94·2 \pm 1·1^b$	$28·2 \pm 0·7^b$	$45·9 \pm 0·2^b$	$98·0 \pm 1·5^b$	$3·0 \pm 0·1^b$	$18·0 \pm 0·6^a$	$27·4 \pm 0·3^b$
1020G	$38·8 \pm 0·8^b$	$89·2 \pm 0·7^b$	$34·4 \pm 0·4^a$	$56·2 \pm 1·9^a$	$83·7 \pm 1·7^b$	$31·0 \pm 1·1^a$	$7·2 \pm 1·5^b$	$23·9 \pm 0·7^c$

Data are presented as mean ± SD. Mean within the same column followed by different superscript letters differ significantly ($P < 0·05$).

Table 3 Antimicrobial and antioxidant activities of the *Bacillus* isolates

Strains	Inhibition zone of pathogens growth (mm)					Antioxidant activity (%)
	Escherichia coli	*Bacillus cereus*	*Staphylococcus aureus*	*Listeria monocytogenes*	*Pseudomonas aeruginosa*	
437F	–	–	18·0 ± 0·2[a]	8·0 ± 0·2	15·1 ± 0·2[a]	51·3 ± 0·8[a]
1630F	–	10·5 ± 0·6[a]	–	–	21·1 ± 0·5[b]	42·6 ± 0·6[a]
1020G	16 ± 0·3	17 ± 0. 8[b]	21·6 ± 0·3[a]	–	15·3 ± 0·1[a]	21·8 ± 0·9[b]

Data are presented as mean ± SD. Mean within the same column followed by different superscript letters differ significantly ($P < 0·05$).

Table 4 Antibiotic susceptibility test of the *Bacillus* isolates. Am: Amikacin, Cl: Clindamycin, Ch: Chloramphenicol, Er: Erythromycin, Ka: Kanamycin, Me: Meticillin, Ri: Rifampin, Te: Tetracyclin, Va: Vancomycin

Strains	Am	Cl	Ch	Er	Ka	Me	Ri	Te	Va
437F	23 ± 1·5[S]	18 ± 2·1[S]	8·0 ± 0·5[R]	31 ± 3·5[S]	30 ± 1·5[S]	37 ± 2·6[S]	21 ± 2·2[S]	50 ± 3·8[S]	18 ± 0·1[S]
1630F	56 ± 3·5[S]	19 ± 1·1[S]	29 ± 1·9[S]	45 ± 3·7[S]	38 ± 2·5[S]	37 ± 3·1[S]	48 ± 3·0[S]	46 ± 3·6[S]	20 ± 1·1[S]
1020G	30 ± 2·3[S]	24 ± 1·4[S]	38 ± 2·2[S]	36 ± 3·2[S]	25 ± 1·2[S]	21 ± 1·5[S]	22 ± 2·8[S]	32 ± 2·1[S]	19 ± 0·7[S]

Data are presented as mean ± SD. S: sensitive, R: resistance.

Safety assessments

The inhibition zones of selected antibiotics against three *Bacillus* species are presented in Table 4. Data were interpreted based on the Clinical and Laboratory Standard Institute. Accordingly, with the exception of chloramphenicol resistance of strain 437F, all *Bacillus* strains obtained from fermented pickle in this study were susceptible to various affected antibiotics. PCR-based method was applied for detection of putative virulence toxic genes. The gel electrophoresis of the PCR product was represented in Fig. 2. On the amplification efforts, we could detect all six enterotoxin genes (*hblA*, *B* and *C*; *nheA*, *B* and *C*) in the reference *B. cereus* strain but were not observed in the isolated strains (Fig. 2, Table 5). Regarding strain 437, the PCR amplification of *hblC* and *nheA* genes resulted in the production of a DNA fragment about 300–550 bp in size (Fig. 2 Lane 6 of the up and middle parts) which was in a different size range expected for toxic genes (Table 5). The sequencing and BLASTX analysis of these fragments revealed that they were not toxic genes and were related to nickel transporter proteins and glycosyltransferase family respectively. We cloud not detect *ces* and *cyk1* genes neither for reference nor for the isolated strains. Results of cytotoxicity of *Bacillus* strains were presented in Fig. 3. Based on the statistical analysis, these strains had no toxic effect on HT-29 cell lines ($P < 0·05$).

Discussion

Although the role of *Bacillus* species in the fermentation process of traditional foods are well determined (Tamang *et al.* 2016b), they received less attention in the functional foods and pharmaceutical industries. Recently, the potentials and concerns about the *Bacillus* probiotic and their health benefits, mechanism of actions, main groups and challenges ahead in their application was reviewed (Elshaghabee *et al.* 2017). The authors concluded spore former species will make a major contribution in global market of probiotics. Here, we sampled a traditional vegetable pickle to explore the probiotic potentials of its *Bacillus* resident. The isolated strains were not phylogeneticaly related to known *Bacillus* probiotics. Two strains (1630F and 1020G) were clustered with *B. atrophaeus* and *B. amyloliquefaciens* and another (strain 437F) was clustered with *B. safensis*. The probiotic potentials of *B. amyloliquefaciens* isolated from mouldy corn (Lee *et al.* 2017) and soil (Wu *et al.* 2017) were recently indicated. Here, we presented the new source for the isolation of *Bacillus* probiotics.

Survival in the gastrointestinal tract (very low pH and presence of bile salt) is necessary for all probiotics to pass from the stomach and duodenum. The novel isolates could tolerate the intestinal pH, and the results are in line with other previous *Bacillus* probiotics (Ragul *et al.* 2017) and relatively higher than some *Lactobacillus* spp. (Wang *et al.* 2010). The bile tolerance ability of three isolates is comparable to other *Bacillus* (Lee *et al.* 2017) and *Lactobacillus* (Liu *et al.* 2018) probiotics, and 0·3% bile salt (physiological concentration of bile) did not affect their survival.

One of the mechanisms by which probiotics benefit their host is the inhibitory effects on pathogenic biofilm formations in terms of competition, exclusion or displacement (Woo and Ahn 2013) and relate to the cell surface characteristics. Hydrophobicity is routinely studied in probiotic characterization and related to the non-specific cell adherence. The results obtained in this study revealed that there was no definite correlation between hydrophobicity and cell attachment capacity, while the

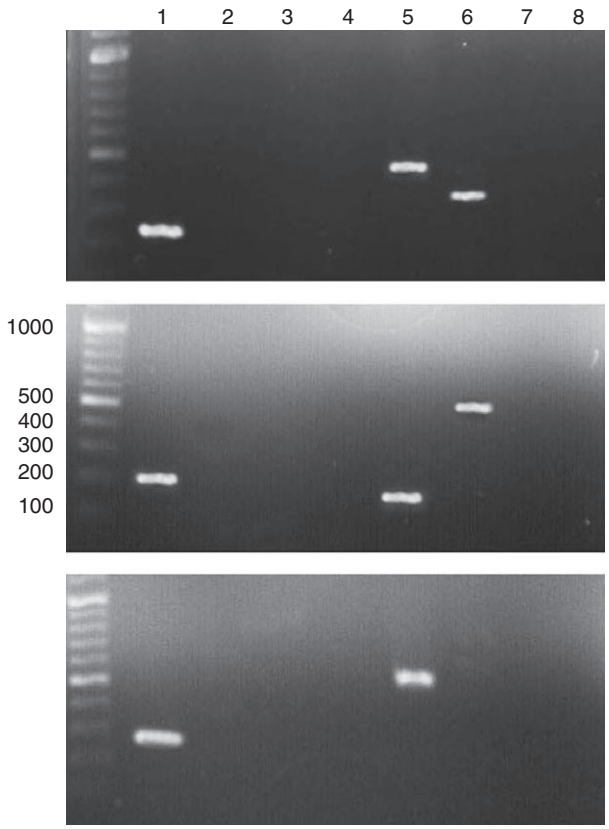

Figure 2 PCR products of toxic genes from the isolated *Bacillus* strains and *Bacillus cereus*. Lane M 1000-bp DNA ladder. Lane 1 and 5: *Bacillus cereus*, Lane 2 and 6: strain 437F, Lane: 3 and 7: strain 1630F. Lane 4 and 8: strain 1020G. Up: Lane 1–4 *hblA*, Lane 5–8: *hblC*. Middle: Lane 1–4: *hblD*, Lane 5–8: *nheA*. Down: Lane 1–4: *nheB*, Lane 5–8: *nheC*.

Table 5 Detection of virulence genes in the *Bacillus* isolates

	PCR product size(bp)				
Target gene	Expected size*	*Bacillus cereus* (PTCC1665)	Strain 437F	Strain 1630F	Strain 1020G
hblA	186	220	ND	ND	ND
hblC	411	400	300	ND	ND
hblD	205	200	ND	ND	ND
nheA	237	200	550	ND	ND
nheB	281	250	ND	ND	ND
nheC	618	600	ND	ND	ND
Cytk1	623	ND	ND	ND	ND
ces	1271	ND	ND	ND	ND

*Data from Wehrle *et al.* 2009. ND: not detected.

most attachment was observed for chloroform, a polar protic solvent. Some previous reports indicated that the charge groups of the cell surface macromolecules were responsible for bacterial attachment to surface (Tarazanova *et al.* 2017) and confirm our data. The isolated *Bacillus* showed high auto- and co-aggregation ability and was in the range that was reported for various *Lactobacillus* probiotics (Tuo *et al.* 2013).

Productions of antibiotic and antioxidant compounds are also desired for probiotics. The obtained *Bacillus* strains could inhibit the growth of both Gram-negative and Gram-positive strains. Antagonistic activity remained after neutralization which concluded that the inhibitory effects were not related to organic acids which were observed in some *Lactobacillus* probiotics (Shokryazdan *et al.* 2014). The most potent strain in the current study, strain 1020G, was phylogeneticaly related to *Bacillus amyloliquifaciens*, where its potential for the antimicrobial production was well determined (Wu *et al.* 2015). Interestingly all three strains could inhibit the growth of *Pseudomonas aeruginosa* and one inhibits the growth of *Candida albicans*, suggesting that these *Bacillus* spp. are good candidates for application as respiratory probiotics (Wang *et al.* 2016). Free radicals which are generated in the body environment adversely alter macromolecules; hence, probiotics with antioxidant activity benefit the host by rampaging and neutralizing free radicals. All *Bacillus* strains represented the antioxidant activity that is similar to amounts reported for potent *Lactobacillus* (Wang *et al.* 2017).

Probiotics should be recognized as safe micro-organisms. *Bacillus anthracis* and *Bacillus cereus* are two well-known pathogenic *Bacillus*. The latter is the cause of gastrointestinal illness like diarrhoea and emetic-type disease. The adverse effects are due to the presence of haemolysin BL (Hbl) and nonhaemolytic enterotoxin (Nhe) as well as Cereulide (ces) emetic toxins. Transfer of these toxic genes to nonpathogenic *Bacillus* species is the most concerns regarding the probiotic application of *Bacillus*. The mentioned virulence genes were not detected in the isolated *Bacillus* and support of their safety as probiotic. Susceptibility to the antibiotics is another important factor for safety evaluation. Antibiotic resistance genes could transfer from probiotics to susceptible resident gut microbiota or release to the environment through faeces. The resistance to the antibiotics were reported for some probiotics (Imperial and Ibana 2016), however, three novel strains were susceptible to all antibiotics tested (except Chloramphenicol for one strains), validating their safety statue.

In conclusion, we isolated three *Bacillus* strains 437F (*B. safensis*), 1630F (*B. atrophaeus*) and 1020G (*B. amyloliquefaciens*) from a traditional lacto-fermented vegetables pickle in Iran. The *in vitro* evaluation of their characteristics showed that the strains had desirable probiotic potentials: they survive at the low pH and high bile concentration, susceptible to antibiotics, have antimicrobial activity, attached to Caco-2 cell, nontoxic for HT-29 cell line and did not contain the virulence genes. The

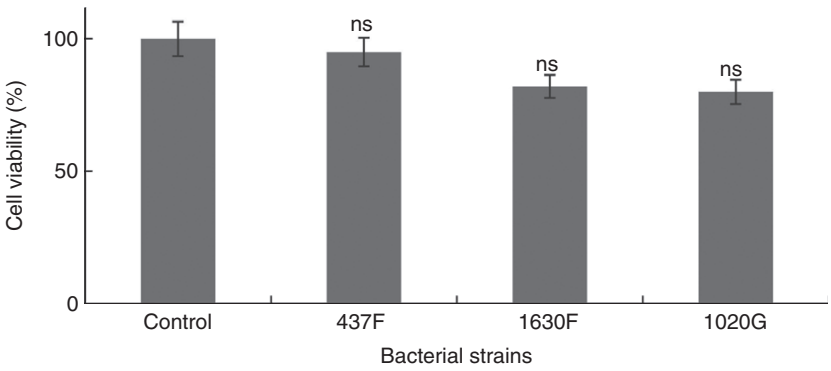

Figure 3 Effect of *Bacillus* strains on the viability of HT-29 cells after 24 h. ns: not significant ($P < 0{\cdot}05$).

isolates were not related to known *Bacillus* probiotics and support that the investigation of a traditional nondairy-fermented product may lead to an extensive probiotics list. The strains can be used as the starter and regarding their tolerance to various environmental conditions they can be added to some dairy-fermented products like cheese. We find torshi is a source of good bacteria; however, the *in vivo* safety and growth benefit of these isolates as well as the presence of health-threatening compounds like carcinogens and/or mycotoxin in this pickled vegetable must be evaluated in the future.

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Conflict of Interest

There are no conflicts of interest.

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