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ORIGINAL ARTICLE

Screening and selection of novel animal probiotics isolated from bovine chyme

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Abstract Probiotics, gut-colonizing microorganisms capable of conferring a number of health benefits to their hosts, are highly desirable as animal feed supplements. Members of the Gram-positive genus *Bacillus* are often utilized as probiotics, since endospores formed by those bacteria render them highly resistant to environmental extremes and therefore capable of surviving gastrointestinal tract conditions. In this study, 84 distinct bacterial colonies were obtained from bovine chyme and 29 isolates were determined as *Bacillus* species. These isolates were principally screened for their antimicrobial activity against a group of two Gram-positive and four Gram-negative bacteria, including known human and animal pathogens such as *Salmonella enterica, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Staphylococcus aureus.* Seven strains displaying strong antimicrobial activity against the test cohort were further evaluated for other properties desirable from animal probiotics, including high spore-forming capacity and adhesiveness, resistance to pH extremes and ability to form biofilms. The isolates were found to resist simulated gastrointestinal conditions and most of the antibiotics tested. In addition, plasmid presence was checked and cytotoxicity tests were performed to evaluate the potential risks of antibiotic resistance transfer and unintended pathogenic effects on host, respectively. We propose that the bacterial isolates are suitable for use as animal probiotics.

Keywords Antimicrobial · *Bacillus* · Feed supplement · Isolation · Probiotics

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Introduction

The widespread and intensive use of antibiotics for therapeutic purposes has led to a considerable increase in the number of antibiotic-resistant pathogenic strains, resulting in frequent occurrence of serious and hard-to-treat infections in both humans and livestock (Barbosa and Levy 2000; Barbosa et al. 2005; Chaiyawan et al. 2010). As such, there has been an increasing concern about non-therapeutic uses of antibiotics, ultimately culminating in bans of their use as animal feed additives. The European Parliament and the Council of the European Union have encouraged the development of alternative products to replace antibiotics as feed supplements for growth promotion (Santini et al. 2010; The European Parliament and the Council of the European Union 2003). Consequently, there is an ongoing search for replacement products capable of enhancing growth and preventing disease (Chaiyawan et al. 2010; Santini et al. 2010). An effective and safe alternative to antibiotic implementation is the use of probiotics, which protect the animal from pathogens by improving the microbial balance in the gastrointestinal tract to exclude potentially harmful bacteria (Chaiyawan et al. 2010; Modesto et al. 2009; Patterson and Burkholder 2003; Santini et al. 2010).

Probiotics are beneficial live microorganisms primarily including various species of bacteria and fungi (Duc et al. 2004; Lutful Kabir 2009). Probiotics influence the host organisms' health by maintaining the normal intestinal microbiota, preventing the growth of pathogenic microorganisms, promoting digestion and intake of feed, and inducing the immune system (Kim et al. 2009; Lutful Kabir 2009). As such, the use of probiotics on livestock enhances the growth of animals, improves efficiency of feed conversion, and decreases the rate of mortality (Kim et al. 2009; Lutful Kabir 2009). Probiotic microorganisms should ideally be non-pathogenic and non-toxic, improve growth of the host animal, and be stable and

active during processing and storage. In addition, probiotics should be able to survive and continue their metabolic activities in gastrointestinal conditions and produce compounds that inhibit the growth of pathogenic microorganisms (Kim et al. 2009; Patterson and Burkholder 2003).

A number of bacterial species are currently used as probiotics, mostly in genera *Bacillus, Bifidobacterium, Enterococcus, Escherichia, Lactobacillus, Lactococcus*, and *Streptococcus* (Patterson and Burkholder 2003). Some yeast species, such as *Saccharomyces cerevisiae*, are also utilized as probiotics (Lutful Kabir 2009).

The most common probiotics used in humans are species of *Lactobacillus* and *Bifidobacterium*, while *Bacillus, Enterococcus,* and *Saccharomyces* species are frequently used in livestock (Patterson and Burkholder 2003). *Bacillus* species in particular are advantageous for use as probiotics, since members of this genus are spore-formers displaying high resistance to heat, chemicals, and other stress factors (Cartman et al. 2008; Chaiyawan et al. 2010; Nicholson et al. 2000; Setlow 2006., *Bacillus* spores can survive in harsh pH conditions of the gastric fluids (Cutting 2011) and reach the small intestine relatively unharmed, making them suitable for use as feed supplements. In addition, spores can be kept for a long time in desiccated form with little to no loss of viability, allowing for ease of transport (Duc et al. 2004; Mazza 1994). *Bacillus subtilis, B. clausii, B. cereus, B. coagulans*, and *B. licheniformis* are the *Bacillus* species most widely researched and used as animal probiotics (Cutting 2011).

In this study, novel bacterial strains were isolated from bovine chyme samples, and *Bacillus* strains obtained in this manner were characterized and screened for their potential to be used as animal probiotics. Criteria utilized for assessing probiotic capacity include survival in gastrointestinal tract (GIT) conditions, lack of toxicity and invasiveness, ability to adhere to the small intestine, and inhibition of pathogenic bacteria in the GIT by competitive exclusion or synthesis of antimicrobial compounds.

Materials and methods

Bacillus isolation and growth conditions

Microorganisms were isolated from chyme samples of eight male cattle between 2 and 4 years of age. The small intestine was removed as a whole and chyme samples were collected by squeezing the intestinal contents into sterile tubes. Isolation of the microorganisms was performed as described by Lalloo et al. (2007) with minor modifications. An amount of 1 g of chyme fluid from each sample was suspended in 3 ml of 0.9 % NaCl solution and inoculated in 9 ml of nutrient broth (NB). Samples were incubated at 37 °C for 24 h, followed by incubation at 45 °C for 10 min to initiate spore formation. Then, 50 % (v/v) ethanol was added to a volume of 20 ml and the suspension was incubated at 20 °C for 1 h. Centrifugation was performed at 12,000 *g* for 30 s, the supernatant was decanted, and pellets were incubated at 105 °C for 5 min. Dry pellets were resuspended in 20 ml of 0.9 % NaCl solution and serially diluted in ten-fold increments.

An amount of 150 μl of each dilution was spread on MYP (Mannitol–Egg Yolk–Polymyxin) agar plates and incubated at 37 °C for 24 h. *Bacillus* species were differentiated according to their ability to ferment mannitol or degrade lecithin, while polymyxin was utilized to inhibit the growth of Gram-negative bacteria. Single colonies with distinct morphologies were collected and transferred on Luria–Bertani (LB) agar plates. Cultures were incubated at 37 °C and 125 rpm until visible colony formation, and stored at 4 °C to maintain viability. Cultures were maintained at −80 °C in 30 % glycerol for long-term storage.

Antimicrobial activity

Antimicrobial activity assay was adapted from the method described by Saravanakumari and Mani (2010). Overnight incubated cultures of the isolates were horizontally streaked on LB agar plates and incubated at 37 °C for 48 h. After the incubation period, plates were exposed to chloroform vapor for 90 min for the inactivation of active cells. Plates were aerated for 20 min to completely remove residual chloroform by vaporization and plate covers were subsequently changed (Barbosa et al. 2005). Representative bacteria (Table 1) were then streaked vertically on the horizontally streaked colonies and plates were incubated at 37 °C for 24 h. Inhibition on the bacterial growth line (Table 1) was assessed to evaluate the antimicrobial activity of isolates against each representative strain.

Table 1 Microorganisms used in the present study; all strains were obtained from RSHM (Refik Saydam National Type Culture Collection Laboratory, Ankara, Turkey)

Bacterial species	Strains
Bacillus subtilis	ATCC 6633 (RSHM 03013)
Bacillus cereus	RSHM 709
Staphylococcus aureus	ATCC 25923 (RSHM 96090/07035)
Escherichia coli	DH5α, RSHM 888
Pseudomonas aeruginosa	ATCC 29212 (RSHM 03015)
Klebsiella pneumoniae	ATCC 10031 (RSHM 06017)
Salmonella enterica	ATCC 13311 (RSHM 4059, CIP 5858, NCTC 74)

Sporulation efficiency

Sporulation efficiencies of the isolates were measured as described by Barbosa et al. (2005). Isolates were inoculated (0.1 %v/v) in Difco Sporulation Medium (DSM) and incubated at 125 rpm and 37 °C for 24 h. After 24 h, serial dilutions were prepared in ten-fold increments and spread on LB agar plates before and after heat exposure at 80 °C for 20 min. Plates were incubated at 37 °C overnight and colony-forming unit (CFU) numbers were counted; colony growth before and after heat exposure was compared to assess the sporulation capacities of the isolates.

Spore formation

Spores were harvested and purified to test the spore tolerance of each isolate to simulated gastric fluids and bile salts. Cultures were grown in 50 ml DSM medium at 37 °C, 250 rpm for 24 h and centrifuged at 1,500 *g* for 5 min to refresh the medium of spores. Harvested spores were then inoculated in DSM medium and incubated at 37 °C and 250 rpm for 48 h. After the incubation period, spores were centrifuged at 5,000 *g* for 30 min; spore pellets were washed with 20 ml sterile distilled water and resuspended in 5 ml sterile water. Spores of each isolate were stored at 4 °C for further use.

Resistance to simulated gastric fluids

Vegetative cells of the isolates were tested for survival capacity in simulated gastric fluids as described by Barbosa et al. (2005), Hong et al. (2008), and Patel et al. (2009) with minor modifications. Briefly, bacteria grown overnight were inoculated in LB broth (pH=2.0, adjusted with 37 % HCl) and incubated at 37 °C and 125 rpm for 30 min. Samples were then serially diluted, spread on LB agar plates and incubated at 37 °C for 24 h. Colonies were counted and compared with those of control cultures grown in unmodified LB broth.

Spore tolerance to simulated gastric fluids was also tested as described by Barbosa et al. (2005), Duc et al. (2004), Hong et al. (2008), and Patel et al. (2009). Approximately 10^8–10^9 purified spores per ml were resuspended in 0.85 % NaCl solution (pH=2, adjusted with 37 % HCl), incubated and spread on LB agar plates as described for vegetative cells. Spores resuspended in 0.85 % NaCl solution with no pH modification served as controls. Colonies were counted and compared with those of controls to assess the tolerance of spores to simulated gastric fluid conditions.

Bile salt tolerance

Bile salt tolerances of vegetative cells were tested as described by Barbosa et al. (2005). Overnight cultures of isolated bacteria were inoculated in fresh LB supplemented with 0.2 % bile salts (50 % cholic acid sodium salt, 50 % deoxycholic acid sodium salt; Sigma-Aldrich, USA) and incubated at 37 °C and 125 rpm. Bacteria inoculated in fresh LB not containing bile salts were incubated in parallel as controls. Aliquots were taken after 3 h of incubation, serially diluted and plated on LB agar; colonies were counted after incubation at 37 °C for 24 h.

Bile salt tolerances of spores were also assayed as described by Barbosa et al. (2005). Spores were purified as described by Henriques et al. (1995) and approximately 10^8–10^9 purified spores per ml were resuspended in an isotonic buffer [Bott and Wilson salts: 1.24 % K_2HPO_4, 0.76 % H_2PO_4, 0.1 % trisodium citrate, 0.6 % $(NH_4)_2SO_4$, pH=6.7)] containing 0.2 % bile salts. Spores were incubated at 37 °C and 125 rpm, aliquots were taken at 0, 1, and 3 h and colony counts were performed to compare survival efficiencies. Spores resuspended in isotonic buffer not containing bile salts were incubated in parallel as controls.

Biofilm formation

Biofilm formation abilities of isolates were tested as described by Barbosa et al. (2005) with minor modifications. Instead of Sterlini–Mandelstam medium, a biofilm growth medium consisting of LB medium (pH=7) supplemented with 0.15 mol/l ammonium sulfate, 0.1 mol/l potassium phosphate, 0.034 mol/l sodium citrate, 0.001 mol/l $MgSO_4$ and 0.1 % glucose, (Hamon and Lazazzera 2001) was utilized in this study. Isolates were grown in this biofilm growth medium to exponential phase, transferred to 3 ml of the same medium in a culture tube and incubated at 37 °C without agitation to an optical density of ~0.01 at 600 nm. An uninoculated culture tube and a culture tube freshly inoculated with exponential phase cells were utilized as controls. Tubes were rinsed with water and stained with a 1 % crystal violet solution for 15 min. Excess stain was then removed by washing. A ring of staining on the medium suggests that the bacteria are capable of forming biofilm.

Antibiotic susceptibility

Antibiotic resistances of the isolates were tested as described by Chaiyawan et al. (2010) and Santini et al. (2010) with modifications. Ampicillin, kanamycin, chloramphenicol, erythromycin, vancomycin, neomycin, ciprofloxacin, streptomycin, and gentamicin were the antibiotics tested. Two-fold serial dilutions of antibiotics were prepared in concentrations ranging between 1 and 256 μg/ml. Next, 160 μl of fresh LB broth, 20 μl of appropriate dilutions of antibiotics, and 20 μl of overnight grown isolates diluted to 10^6CFU/ml were added in each well of 96-well microtiter plates. Uninoculated LB broth and isolates inoculated in antibiotic-

free LB broth were utilized as negative and positive controls, respectively. Plates were incubated at 37 °C for 24 h and the minimum inhibitory concentrations of each antibiotic were determined by comparing sample turbidity at 620 nm to control wells.

Plasmid DNA extraction and purification

DNA minipreparation was performed to observe plasmid presence in isolates. Isolates were grown overnight at 37 °C and 250 rpm in LB broth, confluent bacterial cultures were centrifuged at 12,000 *g* for 3 min to obtain cell pellets and plasmid DNA was extracted from the pellet of each isolate using QIAprep Spin Miniprep Kit (Qiagen, Germany). Gel electrophoresis was performed to determine whether the isolates carry plasmid DNA.

Adhesion and invasion

Adhesion and invasion assays of *Bacillus* isolates were performed as described by Rowan et al. (2000, 2001) with minor modifications. HT-29 cell monolayers were grown in DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 °C in 5 % CO_2 environment. Cells were seeded in 24-well plates containing approximately 3×10^5 cells per well and incubated overnight at 37 °C in 5 % CO_2. Before adhesion and invasion assays, HT-29 cell monolayers were washed three times with DMEM and inoculated with 1 ml of bacterial cultures previously washed with DMEM containing 10 % FBS. 24-well plates were incubated at 37 °C in 5 % CO_2 for 2 h. After the incubation period, HT-29 cells were washed with DMEM to remove nonadherent bacteria. For the adhesion assay, HT-29 cells with adherent bacteria in 1 ml of DMEM supplemented with 10 % FBS were incubated for another 2 h at 37 °C in 5 % CO_2. For the invasion assay, infected cells were incubated for 2 h in 1 ml of DMEM containing 10 % FBS and 100 μg of gentamicin per ml at 37 °C in 5 % CO_2. Cells in both assay groups were then washed three times with DMEM and lysed with 1 ml of 1 % (v/v) Triton X-100 for 5 min at 37 °C. Samples from lysed cells were serially diluted and spread on BHI agar plates for colony counting. *Bacillus subtilis* RSKK 03013 (ATCC 6633) and *B. cereus* RSKK709 were utilized as controls.

Cell cytotoxicity

Cytotoxic effects of isolates were tested as described by Rowan et al. (2000, 2001) and Hong et al. (2008) with modifications. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) based in vitro toxicology assay kit (Sigma) was used to measure total cellular metabolic activity, the presence of which directly corresponds to the lack of cytotoxic effects. HT-29 cell monolayers were grown in DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin at 37 °C in 5 % CO_2 environment. The cells were then transferred to 96-well plates with approximately 5×10^4 cells in each well and incubated overnight at 37 °C in 5 % CO_2 atmosphere. Filter sterilized (by 0.2-μm pore size membranes) supernatants of overnight grown bacteria (BHI, 37 °C, and 125 rpm) were added to test plates before and after heat treatment (95 °C, 10 min). Four replicates were done for the supernatant of each isolate, and 1 % Triton X-100 (Sigma) and PBS were used as positive and negative controls, respectively. Cells inoculated with bacterial supernatant were incubated overnight at 37 °C in 5 % CO_2. After the incubation period, the medium in each well was removed and 100 μl of DMEM supplemented with PBS containing 0.5 % MTT was added to wells. Plates were incubated at 37 °C for 4 h, after which 100 μl of MTT solubilization solution (Sigma) was added to each well. The absorbance of the wells was measured at 570 nm by using a microplate reader (SpectraMax M5; Spectra Lab.). Background absorbance of well plates was also measured (at 690 nm) and subtracted from 570 nm measurements. Toxicities of the supernatants to HT-29 cells were determined by using the equation (1-optical density of the test sample/optical density of the negative control) × 100, where the resulting percentage represents the ratio of cellular respiration in the sample to respiration in an equal number of healthy cells (Rowan et al. 2001). *Bacillus subtilis* RSKK 03013 (ATCC 6633) and *B. cereus* RSKK709 were utilized as controls.

Results

Bacillus isolation and growth conditions

Chyme samples were collected from eight different male beef cattle for potential *Bacillus* probiotic isolation and 84 distinct isolates were obtained in total. Isolates were grown on MYP agar and 29 were found to be members of the genus *Bacillus*. All 29 isolates were observed to sporulate following heat exposure and inoculation in DSM broth. Light and scanning electron microscopy images were taken to confirm isolate morphology; all isolates were found to be bacilli (data not shown).

Antimicrobial activity

Antimicrobial effects of the selected *Bacillus* isolates were tested against different indicator strains, including four Gram-negative and two Gram-positive bacteria (Table 1). While all isolates displayed some antimicrobial activity, seven (STF4, STF8, STF9, STF10, STF15, STF25, and STF26) showed particularly strong inhibitory activity against the tested bacteria (Table 2). These seven isolates

Table 2 Inhibitory activity of isolates as inferred by diameters of inhibition zones

Isolate	Indicator strains					
	S. aureus	*P. aeruginosa*	*E. coli*	*B. subtilis*	*K. pneumoniae*	*S. enterica*
STF1	−	−	−	+	−	−
STF2	−	−	−	−	−	−
STF3	−	−	−	−	−	−
STF4	++	++	++	−	++	−
STF5	+	±	−	±	±	−
STF6	±	±	−	+	±	±
STF8	++	±	+	+++	−	±
STF9	+	−	−	++	−	−
STF10	+±	±	±	++	−	−
STF11	−	−	−	−	±	−
STF12	±	+	−	±	−	±
STF13	+	−	−	±	+±	±
STF14	+	−	−	−	+	±
STF15	+++	±	±	±	−	−
STF16	±	−	−	±	−	±
STF18	±	−	−	−	−	±
STF19	±	−	−	±	±	±
STF20	−	−	+	±	±	±
STF21	±	+	−	−	−	−
STF22	±	+±	−	−	±	±
STF23	−	+	−	+	+±	−
STF24	−	+	+	−	−	−
STF25	±	±	+	+++	−	−
STF26	+++	±	+++	+±	++±	++
STF27	+	−	−	+	−	−
STF28	−	±	−	±	+	−
STF29	±	±	−	±	−	±

Signs correspond to degrees of inhibitory effect on growth from − (no inhibition) to +++; ± reflects reduction in growth but not complete inhibition

were therefore chosen for further study, identified by 16S rRNA sequencing (Table 3) and imaged by SEM to observe their cell morphologies. STF4, STF8, STF15, and STF26 were all effective in preventing the growth of *S. aureus*, while only STF4 showed inhibitory effect on *P. aeruginosa.* Further, while STF8, STF9, STF10, and STF25 all displayed a clear inhibition zone on the growth line of *B. subtilis,* only STF26 could effectively inhibit the growth of *S. enterica*. STF26 and STF4 also showed stronger antimicrobial activity on *E. coli* and *K. pneumoniae* compared to other isolates.

Sporulation efficiency

Sporulation efficiencies of the isolates STF4, STF8, STF9, STF10, STF15, STF25, and STF26 were tested by inducing spore formation in a sporulation medium and evaluating the number of colonies formed before and after heat exposure at 80 °C. Colony numbers were compared and survival percentages were subsequently calculated from this data. The results are listed as percentages in Table 3. All isolates were spore-formers, although their sporulation efficiencies varied greatly. It is notable that while STF25 and STF26 had relatively low sporulation efficiencies, those isolates proliferated very rapidly in sporulation medium and could therefore have been resistant to the associated stresses. Sporulation rates for those isolates increased substantially after longer incubation times (48 and 60 h), suggesting that STF25 and STF26 were disinclined to sporulate in the medium utilized for a certain time period. While other isolates also yielded higher sporulation rates after longer periods of incubation, those increases were not as drastic as the 1.5- and 1.6-fold increases seen in STF25 and STF26.

Resistance to simulated gastric fluids

Vegetative cells of the isolates STF4, STF8, STF9, STF10, STF15, STF25, and STF26 were tested for survival in simulated gastric fluids. All tested isolates were resistant to simulated gastric fluid conditions, although their survival

Table 3 Identification and characterization of selected isolates

Isolate	16S rRNA sequence analysis (Closest known species)	Sporulation efficiency (%)	Survival in bile salt (Vegetative form)	Survival in simulated gastric fluid (Vegetative form)	Biofilm formation
STF4	*Paenibacillus xylanexedens*	79.9	+	+	−
STF8	*Bacillus subtilis*	127.02	+++	++++	−
STF9	*Bacillus subtilis*	22.75	+++	+++	+
STF10	*Bacillus licheniformis*	173.91	+++	++	+
STF15	*Bacillus pumilus*	15.28	+	+++++	+
STF25	*Bacillus licheniformis*	35.2	+	++++	−
STF26	*Bacillus pumilus*	36.7	++	++++	+

Sporulation efficiency is given as the percentage of survivors

Plus signs under survival columns reflect the degrees of survival in simulated gastric fluid, + denoting the lowest survival and +++++ the highest. Plus signs under the biofilm formation column reflect presence of biofilm formation while minus signs denote absence of biofilm formation

rates were different (Table 3). Tolerance of purified spores of STF4, STF8, STF9, STF10, STF15, STF25, and STF26 to low pH condition of gastric fluids was also tested after 30 min and 1 h of incubation. After 30 min, there was no reduction in the number of spores compared to controls. After 1 h incubation, there were 2-log reductions in the viability of STF15 and STF25 spores and no difference in STF4, STF8, STF9, STF10, and STF26 spores compared to controls.

Bile salt tolerance

Isolates were analyzed for their resistance to intestinal conditions by testing survival of both vegetative cells and spores in bile salts medium. Vegetative cells of all isolates were capable of surviving exposure to bile salts (Table 3). At 0 and 1 h, no reduction was observed in the number of germinating spores of any isolate exposed to bile salts. After 3 h incubation in LB supplemented with bile salts, a 2-log reduction was observed in STF25; 1-log reductions were present in STF4, STF8, STF9, and STF10, a slight reduction was observed in STF26, and no reduction was found in STF15.

Biofilm formation

When grown in LB broth, isolates STF9, STF10, STF15, and STF26 formed highly viscous structures on the bottom of the tubes. After the biofilm assay, these isolates formed a notable ring of crystal violet, suggesting that the isolates are capable of forming biofilms (Table 3).

Antibiotic susceptibility and plasmid presence

Antibiotic selection and determination of resistance or sensitivity of isolates against chosen antibiotics were performed according to the microbiological breakpoints used by SCAN (Scientific Committee on Animal Nutrition) (von Wright 2005). Minimum inhibitory concentration (MIC) values are displayed in Table 4. Of the isolates tested, all except STF4 were resistant to ampicillin, vancomycin and gentamicin; four isolates (STF8, STF9, STF15, and STF25) were resistant to kanamycin while another four (STF9, STF15, STF25, and STF26) were resistant to chloramphenicol. STF4 and STF8 were sensitive to erythromycin, whereas other isolates displayed resistance to this antibiotic. MIC values of STF4 and STF26 against neomycin were lower than the breakpoints mentioned in the SCAN report; therefore, they were considered as sensitive to neomycin. Three cultures (STF15, STF25, and STF26) showed resistance to ciprofloxacin and only STF26 exhibited low MIC values to streptomycin.

Since the isolates were resistant to many of the antibiotics tested, it is possible for one or more isolates to carry plasmids bearing antibiotic resistance genes, which may be then acquired by pathogenic organisms. As this outcome is highly undesirable, plasmid extraction and gel electrophoresis were performed to determine whether the observed resistances were transmissible. No plasmid presence was detected in the isolates STF4, STF8, STF9, STF10, STF15, and STF26; only STF25 displayed a faint band after gel electrophoresis (Fig. 1).

Adhesion and invasion

Abilities of *Bacillus* isolates to adhere to HT-29 cells were tested. We observed that all isolates tested could adhere to HT-29 cells (Table 5). STF9 could not grow in BHI broth; therefore, we could not perform the test for this isolate. STF4 showed the strongest adhesion with a percentage of 2.5 % while STF15 showed the lowest with 0.002 %. Invasion properties of *Bacillus* isolates were also assayed as mentioned above. Assay results demonstrate that none of

Table 4 Minimum inhibitory concentrations (MIC) of select antibiotics against isolates

Isolate	Minimum Inhibitory Concentration (MIC) μg/ml								
	A	K	CH	E	V	N	CIP	S	G
STF4	<1	<1	<1	<1	<1	4	<1	64	<1
STF8	16	64	8	2	4	64	<1	>128	16
STF9	64	64	64	8	4	64	<1	128	8
STF10	128	<1	8	4	8	64	<1	128	16
STF15	16	64	64	16	4	64	8	>128	8
STF25	128	64	64	128	128	128	16	128	16
STF26	32	16	32	32	32	32	4	32	16

A, *K*, *CH*, *E*, *V*, *N*, *CIP*, *S*, and *G* refer to ampicillin, kanamycin, chloramphenicol, erytromycin, vancomycin, neomycin, ciprofloxacin, streptomycin, and gentamycin, respectively

the bacteria were capable of invading HT-29 cells, with the possible exception of STF9, which failed to grow in the assay medium (Table 5). *Bacillus subtilis* ATCC 6633 and *B. cereus* RSHM709 displayed higher adhesion rates compared to our isolates (except STF4), though both reference strains could also invade cells. While *B. subtilis* ATCC 6633 had minor invasion capacity (0.050 %), the *B. cereus* strain was highly invasive (0.338 %).

Cell cytotoxicity

The cell cytotoxicities of the supernatants of 18 h grown isolates in BHI were tested by MTT assay. STF9 could not grow in BHI broth and was therefore not a subject for this test. STF8 and STF15 both had heat-stable toxic materials; though their cytotoxicities were different. In addition, STF4, STF25, and STF26 contained low amounts of heat-stable toxic materials in their cell-free supernatants. It is curious that unmodified STF10 supernatant showed no toxicity to the HT-29 cell line, but the presence of a toxic material was observed after heating (Table 5). *Bacillus subtilis* ATCC 6633 supernatant displayed minor cytotoxicity both prior to and after heating, while *B. cereus* RSHM709 expressed both heat-labile and heat-stable toxins.

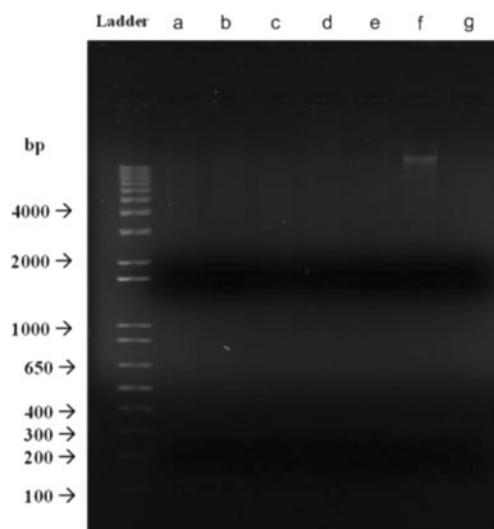

Fig. 1 Agarose gel electrophoresis image of plasmid profile of isolates. **a**, **b**, **c**, **d**, **e**, **f** and **g** represent STF4 (*Paenibacillus xylanexedens*), STF8 (*Bacillus subtilis*), STF9 (*Bacillus subtilis*), STF10 (*Bacillus licheniformis*), STF15 (*Bacillus pumilus*), STF25 (*Bacillus licheniformis*), and STF26 (*Bacillus pumilus*), respectively

Discussion

Bacillus species are commonly used as probiotics, as these spore-forming bacteria can tolerate the harsh environmental conditions associated with the gastrointestinal tract. *Bacillus* species have been incorporated in commercial probiotic products, either alone or alongside consortia of other bacteria (Cutting 2011). Bactisubtil (*B. cereus* strain IP5832), Enterogermina (*B. clausii*), Biosubtyl Nha Trang (*B. pumilus*), and Bispan (*B. polyfermenticus*) are examples of commercially available *Bacillus* probiotics (Duc et al. 2004; Cutting 2011). Further, the production of natto, a fermented soybean product originating in Japan, involves the use of *B. subtilis* to facilitate the fermentation process, indicating that *Bacillus* species have been safely used in foodstuffs for a considerable period of time (Hong et al. 2008).

Bacillus species potentially suitable for use as probiotics were previously reported from the chicken and fish gastrointestinal tracts (Barbosa et al. 2005; Vijayabaskar and Somasundaram 2008). In a similar vein, we isolated *Bacillus* strains from bovine chyme and screened the isolates for probiotic potential, primarily utilizing antimicrobial activity as a measure of efficiency. Isolation of novel strains with high antimicrobial activity against pathogenic bacteria such as *S. aureus, P. aeruginosa, S. enterica*, and *K. pneumoniae* is highly desirable, as prevention of intestinal colonization by pathogenic bacteria is one of the principal ways by which probiotics bolster human and animal health. Other characteristics, such as the ability to survive the passage through the digestive tract and to successfully adhere to the intestinal mucosa, should also be considered in order to evaluate the capacity of a novel isolate as a potential probiotic.

Table 5 Adhesion, invasion rates and cytotoxicity of *Bacillus* isolates against HT-29

Isolate	Bacterial species	Adhesion (%)	Invasion (%)	Cytotoxicity (%)	
				Normal	Heat treated
STF4	*Paenibacillus xylanexedens*	2.500	0	2.9	0
STF8	*Bacillus subtilis*	0.010	0	95.2	68.6
STF9	*Bacillus subtilis*	–	–	–	–
STF10	*Bacillus licheniformis*	0.010	0	0	3.8
STF15	*Bacillus pumilus*	0.002	0	11.6	7.5
STF25	*Bacillus licheniformis*	0.100	0	7.8	0
STF26	*Bacillus pumilus*	0.008	0	8.5	0
ATCC 6633	*Bacillus subtilis*	0.466	0.050	4.5	2.7
RSHM709	*Bacillus cereus*	0.126	0.338	81.8	14.5

All results are given as the percentage of the survivors

– sign indicates that no growth on BHI was observed

Spores of certain prospective *Bacillus* probiotics were found to be sensitive to the gastrointestinal tract (GIT) conditions (Duc et al. 2004). However, the ability of probiotic strains to survive in the GIT of the animal on which they are used as feed supplement is of great importance, as the probiotic bacteria cannot demonstrate their probiotic activities if they cannot colonize the gastrointestinal tract. Therefore, resistance of isolates to GIT conditions was tested with incubation in simulated gastric and intestinal fluids. Our results revealed that spores of the isolates were resistant to gastric fluid conditions after a 30-min incubation period, exhibiting 100 % survival rates. However, survival rates of STF15 and STF25 spores displayed a marked decrease after 1 h incubation in simulated gastric fluids, while no reduction in viability was observed in the other 5 isolates. Similarly, spores of all isolates were found to be tolerant to simulated intestinal fluid conditions, with 100 % survival rates after a 1-h incubation period. However, survival rates decreased in spores of some isolates for longer incubation periods. Vegetative cells, on the other hand, were not as resistant as spores to stress conditions. This result is expected, as spores of the genus *Bacillus* have protective layers around the nucleoid in the spore core, making spores extremely resistant to adverse environmental conditions (Barbosa et al. 2005). Moreover, it has been reported that, once ingested, spores of *Bacillus* isolates could germinate in the GIT and display activity as vegetative cells in the host animal (Chaiyawan et al. 2010; Casula and Cutting 2002).

Our results demonstrate that STF9, STF10, STF15, and STF26 are capable of forming biofilm layers. Biofilm production is desired for prospective probiotics, since it allows the probiotic to remain longer in the GIT (Hong et al. 2008). Biofilm formation provides protection for probiotic bacteria against the harsh conditions of the GIT, supports spore formation, and prevents pathogenic bacteria from holding on to the GIT surface (Barbosa et al. 2005).

Antibiotic susceptibility is considered as one of the most important characteristics for probiotic bacteria, since the resistance genes could be transferred to pathogenic bacteria in the intestinal tract and thus contribute to the creation of multidrug-resistant pathogens (Patel et al. 2009; Schwarz et al. 2001; Hong et al. 2005; Barbosa et al. 2005). This transfer mostly occurs by plasmid-borne resistance genes (Schwarz et al. 2001). In this study, seven isolates chosen after the initial screening were tested for antibiotic resistance against nine widely utilized antibiotics. Of those isolates, only STF4 had lower minimal inhibitory concentrations (MICs) than the breakpoints used by SCAN (von Wright 2005) and could be considered susceptible to the antibiotics tested. Other isolates (STF8, STF9, STF10, STF15, STF25, and STF26) were found to be resistant to at least one antibiotic tested. After the observation that most of the isolates displayed resistance to antibiotics, plasmid isolation and visualization was performed to determine whether this resistance is due to plasmid-borne resistance genes. If such genes are not integrated in genomic DNA and are instead carried on plasmids, they may be transferred to other bacteria in the environment and confer on them resistance to the same antibiotic. This constitutes a potential health hazard if the recipient bacteria are pathogenic, and the absence of plasmid DNA should therefore be confirmed for an antibiotic-resistant strain to be recommended as a prospective probiotic. As such, plasmid isolation was performed for all seven bacteria and the presence of plasmid was tested by performing gel electrophoresis. Our results suggest that all the samples tested, with the possible exception of STF25, carry potential antibiotic resistance genes in their genomic DNA and bear no risk of transferring them to other pathogenic or non-pathogenic bacteria that may be present in the environment (Hong et al. 2008). While it is possible that large, low-copy number plasmids have not been recovered by our isolation protocol, we find this possibility unlikely as cultures were grown to confluence several times in media lacking in antibiotics and, without the environmental stresses selecting for their presence, plasmids encoding antibiotic resistance genes would likely have been lost.

The ability to adhere to and colonize in the GIT is an important requirement for potential probiotics, as the capacity to adhere increases the mean time of bacteria in the GIT and

allows greater periods of probiotic activity after oral introduction of the probiotic strain (Jacobsen et al. 1999; Saarela et al. 2000). Therefore, probiotic strains displaying stronger adhesion to the GIT are considered to better modify the normal intestinal microbiota, prevent the growth of pathogenic bacteria by competitive exclusion, and induce immune system compared to the non-adherent ones (Saarela et al. 2000; Patel et al. 2009). The HT-29 cell line is used as a model for the testing of adhesion of probiotic bacteria, as these cells differentiate into enterocytes (Saarela et al. 2000; Patel et al. 2009). Thus, we used the HT-29 colon carcinoma cell line for the assessment of cell adhesion by the prospective probiotic strains. In vitro adhesion assays suggest that all isolates except STF9 were able to partially adhere on the colon carcinoma cells, while STF4 had significantly higher adherence ability than the others. The adhesion rates observed were generally similar to the results obtained in other studies related to *Bacillus* adhesion and our own results on reference strains of *B. subtilis* and *B. cereus* (Hong et al. 2008; Rowan et al. 2001). Though our isolates had relatively low adhesion rates compared to previous records, STF4 again displayed higher adhesion than many strains in the literature.

Recent research suggests that members of *Bacillus* species might induce important systemic diseases such as septicemia, peritonitis, endocarditis, liver failure, and meningitis (Rowan et al. 2001). Therefore, invasion studies were conducted for our candidate probiotics. The test results suggest that none of the isolates invaded the HT-29 cells, displaying similar results to our *B. subtilis* reference strain. *B. cereus* RSHM709, however, was highly invasive. This result shows that our isolates are non-invasive and incapable of causing invasion-related diseases, though live animal tests should be conducted for further confirmation.

MTT assay was used for detection of toxic materials in bacterial supernatants. This assay was chosen as it has been determined to be more reliable and sensitive than other cell viability assays (European Commission 2000). In contrast to previous methods, which were based on visual evaluation of toxin-induced cell damage, MTT has the advantage of removing the subjective visual assessment from the assay. MTT is a yellow, water-soluble tetrazolium salt, which is cleaved in the mitochondria of metabolically active cells. In the presence of living cells, MTT solution displays a color change as the insoluble purple formazan is formed due to intramitochondrial metabolization of MTT (Rowan et al. 2001; Finlay et al. 1999; Beattie and Williams 1999; European Commission 2000). MTT assay is therefore highly sensitive for cellular respiration and suitable for assessing cell viability and cytotoxicity, since only living cells produce formazan reaction products (Beattie and Williams 1999; Finlay et al. 1999; Rowan et al. 2001). In this study, the cytotoxicity of cell-free supernatants was assessed by using MTT salts. STF8 had high levels of heat-stable cytotoxic materials in its cell-free supernatant, while other isolates did not produce a significant level of toxic materials. STF10 in particular displayed 0 % cytotoxicity. For STF4, STF25, and STF26, heat treatment of the culture supernatants eliminated toxicity to HT-29, suggesting that toxic materials produced by those isolates are protein-based. The relatively low toxicities of our isolates (bar STF8) is expected as our isolates generally belong to the *B. subtilis* group, which are generally considered safe for human and animal use. Our *B. subtilis* reference strain likewise displayed low cytotoxicity, while the *B. cereus* strain was highly toxic prior to heating and possessed a heat-stable toxin that allowed it to retain some toxicity after heat treatment.

In conclusion, *Bacillus* species were isolated from the GIT of bovine and assessed with regards to their potential capacity as probiotics. Among the isolates, STF4, STF10, STF15, and STF26 appeared to have the highest potential as probiotic candidates compared to other isolates, taking into account their antimicrobial effects, resistance to simulated gastrointestinal conditions, antibiotic susceptibility, colonization capacity, and lack of toxicity.

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