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ORIGINAL ARTICLE

Evaluation of the probiotic characteristics of *Bacillus* species isolated from different food sources

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Abstract Probiotics have established their efficacy as dietary adjuncts providing benefits to consumers. However, selection of probiotics before incorporation into diet requires close scrutiny in the form of in vitro as well as in vivo tests. Three bacteriocinogenic *Bacillus* sp., namely, *B. licheniformis* Me1, *B. flexus* Hk1, and *B. subtilis* Bn1 previously isolated from milk, cheese and fermented beans, respectively, were characterized for typical in vitro probiotic criteria. When compared to probiotic *Bacillus coagulans*, all three cultures were found to possess better acid and bile tolerance. Cultures Me1 and Bn1, except Hk1, showed bile salt hydrolase activity. A marked difference in adhesion to hydrocarbons and auto-aggregation properties from 10–80 and 60–99%, respectively, were observed for the tested cultures. Highest antioxidant activity was measured for culture Hk1 (66.6%), whereas least activity of 53% was observed for culture Bn1. Cultures Me1 and Bn1 were sensitive to all the antibiotics tested, whereas Hk1 and *B. coagulans* showed resistance to the penicillin group of β-lactum antibiotics. All the tested cultures showed a broad spectrum of activity against food-borne pathogens. In co-cultivation studies, *B. licheniformis* Me1 completely inhibited the growth of the indicator pathogen *Listeria monocytogenes* ScottA. Overall, the test cultures exhibiting potential probiotic characteristics, particularly *B. licheniformis* Me1, can serve as probiotics of commercial interest.

Keywords Probiotics · *Bacillus* · Functional food · Acid and bile tolerance

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Introduction

Probiotics are live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance (FAO/WHO 2006; Fuller 1989). The documented potential benefits include modulation of the immune system, lowering of serum cholesterol level, prevention of intestinal disorders, such as diarrhea or lactose intolerance, and of antibody-associated diarrhea (Guo et al. 2010). Probiotics have recently become available as novel foods or dietary supplements for human nutrition and as feed supplements for animal and aquaculture use (Rolfe 2000; Rowland 1999). Probiotics can serve as an alternative to antibiotics in farming and aquaculture, and as prophylactics in humans. Moreover, probiotic therapy is very attractive, because it is an effective and non-invasive approach, which attempts to restore the natural flora. Microorganisms studied and commercialized as probiotics are mainly Gram-positive bacteria belonging to the genera of *Lactobacillus, Bifidobacterium* (De Vecchi and Drago 2006; Lee et al. 1999; Vijayendra and Gupta 2011), and *Bacillus* (Hong et al. 2008; Sanders et al. 2003).

For decades, *Bacillus* and their metabolites have found several biotechnological applications, including enzymes, amino acids, antibiotic production, preparation of fermented foods and as pest control agents, etc. Recently, selected strains of *Bacillus* are increasingly being introduced into various food products, because they also possess common probiotic features, such as gut viability (Tam et al. 2006), resistance to bile and acid (Hong et al. 2005), and the ability to synthesize different compounds useful to humans. In addition, spore-forming *Bacillus* possess several advantages over other non-spore-formers, such as *Lactobacillus*, namely,

(1) *Bacillus* can survive in foods requiring harsh processing conditions, such as high temperature and pressure, (2) they survive better under gastrointestinal tract (GIT) conditions, (3) they possess a long shelf-life and remain viable throughout their shelf-life both at room temperature and refrigerated conditions (Cutting 2011), and (4) due to their better survivability, the effective dose required for *Bacillus* as probiotic supplements is less (Durkee 2010). Out of more than 100 *Bacillus* spp. known, only a few are being used as probiotics for human consumption, which include *B. subtilis, B. licheniformis, B. clausii, B. coagulans, B. cereus, B. pumilus*, and *B. laterosporus* (Sorokulova et al. 2008; Urdaci and Pinchuk 2004). Products containing *Bacillus* endospores are used commercially as probiotics containing a single dose of 10^9 spores/g or 10^9 spores/ml (Mazza 1994). Furthermore, the composition of currently used probiotics varies from those containing a mixture of many strains to those containing just a single strain. The cultures *B. subtilis* 3 and *B. licheniformis* 31 are components of probiotic Biosporine® and it is commercially available in Russia and Ukraine (Gracheva et al. 1996; Sorokulova 1996).

Different probiotic effects addressed for *Bacillus* include production of antibiotics, providing protection against wide range of pathogenic bacteria (Urdaci and Pinchuk 2004), production of enzymes (Inatsu et al. 2006), production of essential amino acids and vitamins (Salvetti et al. 2003), decrease in the levels of serum blood cholesterol (Kim et al. 2002), reduction of blood clotting by fibrinolysis (Sumi et al. 1995), antimutagenic effects (Caldini et al. 2002), and stimulation of the immune system (Hosoi and Kiuchi 2004). In human, probiotic *Bacillus* are being used as health supplements for prophylaxis of gastrointestinal disorders (Pinchuk et al. 2001) and as therapeutic agents for the treatment of urinary tract infections (Meroni et al. 1983). In animals, probiotic *Bacillus* is used as growth promoting and prophylactic agents (e.g., Toyocerin) after the European Union (EU) banned the antibiotic growth supplements (Cartman et al. 2007). *Bacillus* probiotics are also being used in aquaculture, particularly in shellfish to enhance growth and resistance to diseases (Verschuere et al. 2000). These findings show the possibility that the *Bacillus* strains with a wide spectrum of biological activity can be selected as probiotic. Moreover, such *Bacillus* can also act as preservatives in food products (Beaumont 2002). Thus, before application as food supplements, bacteriocinogenic *Bacillus* should be tested for their efficacy using in vitro and in vivo methods.

The World Health Organization has developed guidelines for the evaluation of probiotics in food, which indicate the parameters for preclinical testing of probiotics, clinical trials, and labeling (FAO/WHO 2006). The requirements which have been identified for classifying strains to be effective probiotic microorganisms include survival through the gastrointestinal tract (Maruo et al. 2006), resistance to gastric acid and physiological concentrations of bile, production of antimicrobial substances, adherence to intestinal epithelial cells (Schillinger et al. 2005), sensitiveness to antibiotics (FAO/WHO 2006), and co-aggregation to form a barrier which prevents colonization by pathogens. Although in vitro studies partially mimic in situ conditions in the gut ecosystem (Dunne et al. 2001), these studies can be useful tools for the screening of numerous samples and the selection of microbes for further in vivo safety and clinical trials and eventually for use in humans.

From the above understanding of probiotic applications of *Bacillus*, in our present study, we aimed to evaluate the probiotic characteristics of bacteriocinogenic *Bacillus* spp. obtained from different food sources. Various criteria for selection of a probiotic strain were investigated and compared.

Materials and methods

Bacterial strains and culture conditions

Three native *Bacillus* spp. previously isolated from different food sources and identified as *B. flexus* Hk1, *B. licheniformis* Me1, and *B. subtilis* Bn1 were used (Nithya and Halami, communicated). These bacterial cultures were identified by biochemical, 16S rRNA gene sequencing and as well as by phylogenetic analysis. *B. coagulans* which is commercially available as a probiotic was used as a positive control in the study. The pathogens used in this study included *Micrococcus luteus* ATCC9341, *Listeria monocytogenes* ScottA, *Staphylococcus aureus* FR1722, *Bacillus cereus* F4433, *Salmonella typhi* FB231, and *Shigella boydii* (a clinical isolate from Mysore Medical College). All the *Bacillus* cultures and the pathogenic strains used in this study were maintained in 20% glycerol (v/v) and storage at −20°C. *Bacillus* strains were grown in Luria Bertani (LB) broth and the pathogens in Brain Heart Infusion (BHI) broth at 37°C prior to their use in the experiments.

Acid tolerance test

Tolerance to low pH was tested for the bacterial cultures as described by Conway et al. (1987). For this purpose, active cultures (incubated for 16±2 h) were used. Cells were harvested by centrifugation for 15 min at 8,000 rpm and 4°C. Pellets were washed once in phosphate-saline buffer (PBS at pH 7.2), resuspended in PBS (pH 3) and incubated at 37°C. Surviving microorganisms were enumerated at 0, 1, 2, and 3 h by plating in LB agar and the count was expressed in

colony-forming units (CFU) per milliliter. The survival rate was calculated using the formula (Fuller 1989);

$$\text{Survival (\%)} = \frac{\text{Log number of cells survived (CFU/ml)} \times 100}{\text{Log number of initial cells inoculated (CFU/ml)}}$$

Growth at different bile concentrations

LB broth prepared with different concentrations of oxbile (Fluka, USA) (0.1, 0.2, 0.3, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, and 8.0% w/v) was inoculated with overnight grown (incubated for 16 h at 37°C) test cultures. After 24 h of aerobic incubation at 37°C, the growth of the test cultures was monitored by measuring the optical density (OD) at 650 nm to determine the growth of cultures at different bile concentration. LB broth without oxbile served as the control.

Bile tolerance test

Growth in the presence of 0.3% (w/v) oxbile was analyzed as described by Gilliland et al. (1984). Overnight grown (16±2 h at 37°C) test cultures were centrifuged at 8,000 rpm for 15 min at 4°C and the pellet collected was resuspended in same volume of saline (0.85% NaCl). Fresh LB broth (5 ml), without oxbile (for control), and LB broth (5 ml) containing 0.3% (w/v) oxbile was inoculated with 250 μl (5%) of cell suspension. The growth was monitored hourly by measuring the OD at 650 nm using spectrophotometer (Shimadzu, Japan). Time lag to reach log phase at various bile concentrations was determined to ascertain bile tolerance or sensitivity of the cultures (Chateau et al. 1994). The cultures were categorized into four groups according to the observed delay of growth (d) in the presence of oxbile: resistant strains ($d \leq 15$ min), tolerant strains ($15 < d \leq 40$ min), weakly tolerant strains ($40 < d < 60$ min), and sensitive strains ($d \geq 60$ min).

Bile salt hydrolase (BSH) assay

BSH activity of the culture was evaluated using the procedure described by Pereira et al. (2003). The 16±2 h grown cultures were streaked on LB agar plates supplemented with 0.5% (w/v) taurodeoxycholic acid sodium salt (TDCA) and incubated at 37°C for 72 h. LB agar plates without supplementation of TDCA were used as control. After incubation, the cultures showing white precipitate around the colonies were scored as positive.

Phytase activity

Phytate degradation plate assay was performed as described by Raghavendra and Halami (2009), using a modified LB medium containing 6.25 g/l of sodium phytate. The harvested cell suspension (3 μl of 10^7-10^8 CFU/ml) was spotted onto the surface of modified LB agar and incubated for 16±2 h at 37°C. After incubation, the colonies were washed from the agar surface using double-distilled water and flooded with 2% (w/v) aqueous cobalt chloride solution (Bae et al. 1999). After 5 min of incubation at 27±2°C, the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium meta vanadate solution. After 5 min of incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plates were examined for zones of phytate hydrolysis.

Exopolysaccharide production

Exopolysaccharide (EPS) production was examined as reported previously by Mora et al. (2002). Briefly, 16±2 h grown *Bacillus* cultures were streaked on the surface of plates containing ruthenium red milk (10% w/v, skim milk powder, 1% w/v, sucrose, 0.08 g/l ruthedium red, and 1.5% w/v agar). After incubation at 37°C for 24 h, ropy white colonies were scored as EPS-producing strains.

Antibiotic susceptibility test

Antibiogram for the strains were characterized by using the disc diffusion method according to the recommendations of European Committee on Antimicrobial Susceptibility Testing (EUCAST 2011). The antibiotics tested are listed in Table 1. Cells from the 16±2 h-old cultures were suspended to the density of a McFarland 0.5 turbidity standard. This suspension was diluted 1:100 and was seeded on Muller Hinton agar (Hi-media, India) plates using sterile cotton wool swabs. Antibiotic-impregnated discs were placed on seeded plates within 15 min of swabbing and the zone of growth inhibition was measured after 24 h of incubation at 37°C. The results were recorded as sensitive (S) and resistance (R) based on the diameter of zone of inhibition.

Bacterial adhesion to hydrocarbons (BATH) and auto-aggregation tests

BATH and auto-aggregation ability of the cultures were assessed according to the procedure described by Canzi et al. (2005). BATH test was conducted using hydrocarbon xylene. A 16±2 h grown culture (1 ml) was taken and centrifuged at 8,000 rpm for 15 min at 4°C. The collected

Table 1 Antibiotic susceptibility of the *Bacillus* cultures

Antibiotic	*B. flexus* Hk1	*B. licheniformis* Me1	*B. subtilis* Bn1	*B. coagulans*
Chloramphenicol (25 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Erythromycin (5 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Fusidic acid (10 mcg)	S^{++}	S^{+}	S^{++}	S^{++}
Methicillin (10 mcg)	R	S^{+}	S^{++}	R
Novabiocin (5 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Penicillin (1 unit)	R	S^{+}	S^{++}	R
Streptomycin (10 mcg)	S^{+++}	S^{++}	S^{+++}	S^{+++}
Ampicillin (10 mcg)	R	S^{++}	R	S^{+}
Tetracycline (30 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Gentamycin (10 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Kanamycin (30 mcg)	S^{+++}	S^{++}	S^{+++}	S^{+++}
Co-trimoxazole (25 mcg)	R	S^{+++}	S^{+++}	R
Amikacin (30 mcg)	S^{++}	S^{++}	S^{+++}	S^{+++}
Steptomycin (25 mcg)	S^{+++}	S^{+++}	S^{++}	S^{+++}
Cefixime (10 mcg)	S^{+}	S^{+++}	S^{+++}	S^{+}
Cefalaxine (10 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Cephadoxine (10 mcg)	S^{+}	S^{+++}	S^{++}	S^{++}
Norfloxacin (10 mcg)	S^{++}	S^{+++}	S^{+++}	S^{++}
Levofloxacin (10 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}
Ciprofloxacin (10 mcg)	S^{+++}	S^{+++}	S^{+++}	S^{+++}

Resistance (*R*): 0–5 mm; Sensitive (S+) 6–15 mm; Sensitive (S++): 16–25 mm; Sensitive (S+++): 26–35 mm

pellet was washed with phosphate-buffered saline (PBS: 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) and resuspended in the same buffer to an absorbance (A_{600}) of 0.5. To this, an equal volume of xylene was added and the two phase system was thoroughly mixed by vortexing for 3 min. Aqueous phase was removed after 1 h of incubation at 27±2°C and its absorbance was measured at A_{600}. Adhesion percentage was calculated using the formula,

$$\text{Adhesion } \% = [(A_0 - A)/A_0] \times 100$$

where A_0 and A are absorbance (A_{600}) before and after extraction with organic solvents, respectively.

For the auto-aggregation test, 10 ml of test culture at stationary phase was taken and maintained at 15°C for 3 h. After incubation, an aliquot of 1 ml from the upper suspension was transferred to another test tube and the OD was measured at $A_{600.}$ Auto-aggregation was calculated as

$$\text{Auto aggregation } \% = 1 - [\text{OD of upper suspension}/\text{OD of total culture}] \times 100$$

2, 2-diphenyl-1 picrylhydrazyl (DPPH) free radical scavenging activity

DPPH free radical scavenging activity of the cultures was evaluated by the procedure described by Ganeshan et al. (2008). The 16±2 h grown *Bacillus* cultures were centrifuged at 10,000 rpm for 15 min at 4°C and the culture filtrate (CF) was collected. The test sample was prepared by adding 2 ml of DPPH solution (6 mg/100 ml of methanol) to the tube containing 500 µl of CF, which was previously mixed with 1.5 ml of sterile double-distilled water. Similarly, a blank solution was prepared by adding 2 ml of methanol to the tube containing 500 µl of CF, which was previously mixed with 1.5 ml double-distilled water. Negative control consisted of 2 ml each of DPPH and double-distilled water. All the tubes were vortexed for 3 min and incubated at 27°C for 30 min in the dark. After incubation, absorbance was measured at A_{517}. Scavenging effect of the culture was calculated in percentage using the formula,

$$\text{Scavenging effect } (\%) = \{1 - [A_{517} \text{ sample} - A_{517} \text{ blank}/A_{517} \text{ control}]\} \times 100$$

Inhibitory activity and Co-cultivation of cultures with pathogen

The cultures were checked for their potent antibacterial activity against pathogenic strains by the well diffusion method (Tagg and Mac Given 1971). Fifty microlitres of CF of 16±2 h grown *Bacillus* cultures were added to the wells made on the nutrient agar plates overlaid with the BHI soft agar seeded with indicator pathogens. Plates were observed for the formation of zone of inhibition around each

well. For co-cultivation studies, approximately 10^6 CFU/ml of pathogen, *Listeria monocytogenes* ScottA, was added to the 16±2 h grown test culture. Samples were withdrawn every 2 h, over an incubation period of 6 h, and the survival of the indicator was checked by plating on selective medium (*Listeria* Oxford medium).

Results and discussion

Acid tolerance of cultures

Before the strains of probiotics are able to exert proposed benefits in the intestines, they need to remain alive during both ingestion and in the harsh environments of the gastrointestinal tract, which include the acidic condition of the stomach and bile salts. The survivability of *Bacillus* spp. in the gastric juice depends on their ability to tolerate low pH, which is an important probiotic characteristic. All the tested cultures including control showed ≥80% of survivability at pH 3 (Fig. 1). The culture Me1 was found to be the most acid-tolerant strain exhibiting 89 % of survivability after 3 h of incubation at pH 3, whereas the cultures *B. flexus* Hk1 and *B. subtilis* Bn1 exhibited 86 and 85% of survivability, respectively. The culture *B. coagulans* showed less resistance to acidic pH, showing only 80% of survivability after the incubation period. This indicates that all the cultures were able to withstand acidic conditions. The existence of similar heterogeneity in response to acidic environments has previously been suggested within the *Bacillus* species. Hyronimus et al. (2000) carried out acid tolerance tests of *Lactobacillus sporogenes, Bacillus laevolacticus*, and *Bacillus racemilacticus* and demonstrated that only *Bacillus laevolacticus* showed a significant survival rate at pH 2.5.

Bile tolerance test

Resistance to bile salts is of great importance in survival and growth of bacteria in the intestinal tract and thus it is a prerequisite for probiotics (Havenaar and Huis in't Veld 1992). All the tested cultures exhibited growth at different concentrations of oxbile (0.1–8% w/v) (Fig. 2). The culture Me1 showed growth up to 6% w/v of oxbile and exhibited a decrease with further increase of bile concentration, while the cultures Bn1, Hk1 and *B. coagulans* showed stability in growth only up to 1%. Bile tolerance studies are mostly carried out using 0.3% ox bile solution because of its similarity to human bile juice (Brashears et al. 2003; Chou and Weimer 1999) and also because 0.3% is considered to be a crucial concentration to evaluate a bile-tolerant probiotic (Gilliland et al. 1984). It was observed that, in the presence of ox bile (0.3%), the cultures Me1 and Bn1 were tolerant, while the cultures Hk1 and *B. coagulans* were found to be sensitive and weakly tolerant, respectively. Hence, in the present study, *Bacillus* spp. were found in both the 'tolerant' and 'sensitive' groups. These results are in agreement with those observed with other cultures such as *B. subtilis* and *B. toyoi* (Cosson and Deschamps 1994) and *B. coagulans* strains (Hyronimus et al. 2000).

Comparison of cultures for BSH activity

Among the strains screened for BSH activity, the cultures Me1, Bn1 and *B. coagulans* showed positive, whereas culture *B. flexus* Hk1 exhibited negative results. The formation of white precipitate around the colonies shows that these strains were able to enzymatically deconjugate bile salts to primary bile salts, which is one of the mechanisms involved in the reduction of serum cholesterol level (Begley et al. 2006). Thus, when ingested as probiotics, these strains may exert beneficial health effects by lowering the serum cholesterol level.

Antibiogram of cultures

Among the *Bacillus* spp., the cultures Me1 and Bn1 were sensitive to all the antibiotics tested, while the culture HK1 and the control probiotic *B. coagulans* exhibited low

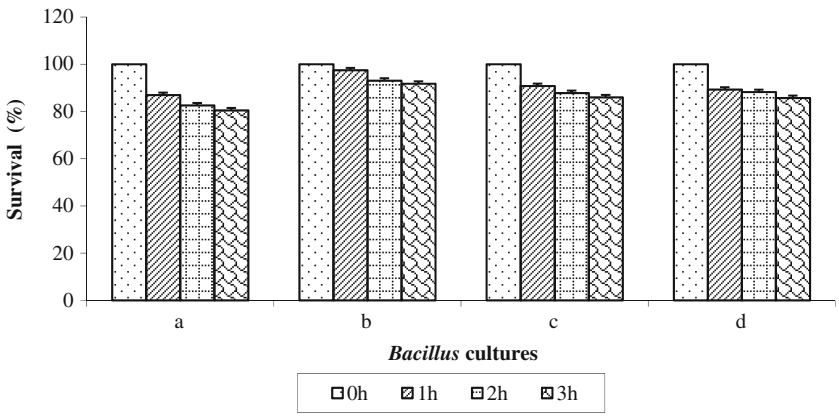

Fig. 1 Acid tolerance of *Bacillus* cultures: *B. coagulans* (**a**), *B. licheniformis* Me1 (**b**), *B. flexus* Hk1 (**c**), *B. subtilis* Bn1 (**d**)

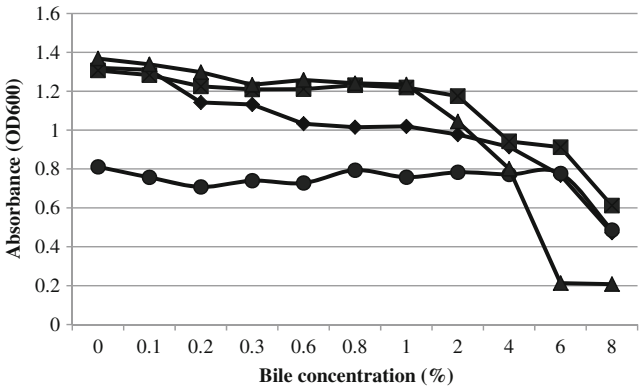

Fig. 2 Growth of *Bacillus* cultures at different concentration of oxbile: *B. coagulans* (♦), *B. licheniformis* Me1 (●), *B. flexus* Hk1 (■), *B. subtilis* Bn1 (▲)

multiple resistances (Table 1). The culture Hk1 was resistant to penicillin, methicillin, and ampicillin, indicating its resistance to the penicillin group of β-lactum antibiotics. *B. coagulans* also showed resistance to penicillin and methicillin and moderate sensitivity to ampicillin (10 mcg). Both of these cultures also exhibited resistance to co-trimoxazole. The resistance of these cultures to β-lactum antibiotics might be primarily due the expression of β-lactamase and hydrolysis of antibiotics, or else due to cellular permeability and mutational events resulting in the modification of PBPs (penicillin binding proteins) of the cultures. Further studies are required to elucidate this perception. However, the intrinsic resistance and susceptibility of these strains to a range of antibiotics is important, since *Bacillus* strains that show resistance to a specific antibiotic can be given at the time of antibiotic treatment. Sorokulova et al. (2008) also reported antibiotic-resistant probiotic *B. subtilis* 3 and *B. licheniformis* 31 strains which are compounds of probiotic Biosporine®. *Bacillus* isolates in the presence of plasmid DNA have frequently been associated with antibiotic resistance determinants and the production of toxins (Bernhard et al. 1978). However, since two of our cultures were found to be sensitive to any of the antibiotics tested, they would not be responsible for transmission of drug resistance genes to other intestinal

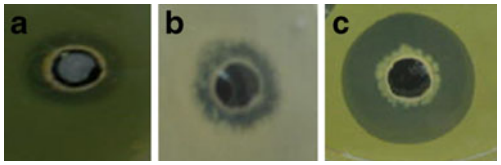

Fig. 4 Inhibitory activity of the culture *B. licheniformis* Me1 against (**a**) *Listeria monocytogenes* ScottA, (**b**) *Shigella boydii* and (**c**) *Micrococcus luteus* ATCC9341

and/or food-borne pathogens, in the food matrix or, more importantly, in the gastrointestinal tract, if introduced as probiotics.

Adhesion to hydrocarbons and auto-aggregation

BATH and auto-aggregation were studied as an index for adhesion property. Bacterial surface characteristics are one of the in vitro properties, which are being studied for knowledge of their probiotic nature. Bacterial surface properties have been associated with attachment to a variety of substrates, which in turn is associated with hydrophobicity (Gilbert et al. 1991). A marked difference in adhesion from 30 to 80% was observed among the tested cultures (Fig. 3). *B. flexus* Hk1 and *B. licheniformis* Me1 showed better hydrophobicity as compared to reference strain *B. coagulans*, suggesting that these native cultures have increased level of adhesion and colonization ability, which can prevent pathogenic access by steric interactions or specific blockage on cell receptors (Otero et al. 2004). This difference in the level of adhesion among the tested strains could be attributed to several factors such as the non-specific reaction by charge and hydrophobicity.

Similar to cell surface hydrophobicity, auto-aggregation activity varied from 60 to 99% (Fig. 3). The culture Hk1 showed the highest auto-aggregation activity (98.2%) and the culture Me1 had the lowest (66.5%). Collado et al. (2007) stated that aggregation is useful for preliminary screening to identify the potent probiotic strains suitable for food, human, or animal use. Thus, the higher aggregation property of the culture *B. flexus* Hk1 represents its

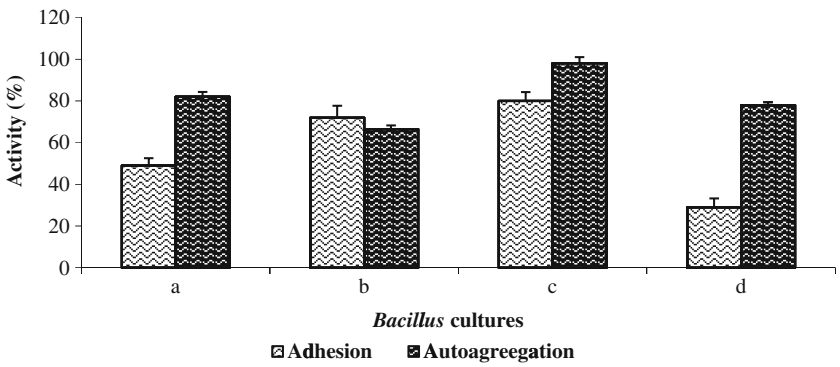

Fig. 3 Adhesion and auto aggregation properties of the *Bacillus* cultures: *B. coagulans* (**a**), *B. licheniformis* Me1 (**b**), *B. flexus* Hk1 (**c**), *B. subtilis* Bn1 (**d**)

characteristic feature for interaction with pathogens, which is of importance from the point of view of both food preservation and the therapeutic impact of food on intestinal microbiota.

DPPH radical scavenging activity

The application of probiotic bacteria with antioxidant activity in foods increases their biological activity and quality, as well as their shelf-life (Nedelcheva et al. 2010). DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle et al. 1996) and is a useful reagent for investigating the free radical scavenging activities of compounds (Duan et al. 2006). All strains tested showed varying degrees of scavenging activity (50–70%) (data not shown). The highest scavenging activity was measured in culture Hk1 (66.6%), while lower activity was observed in culture Bn1 (53%). Scavenging of different types of ROS was thought to be one of the main antioxidant mechanisms exhibited by lactic acid bacteria (Stecchini et al. 2001). Such radical scavenging active fractions and cultures can possibly be used in animal feed formulations as a supplement to relieve oxidative stress.

Antimicrobial activity of the cultures and co-cultivation of the cultures with pathogens

Antagonistic activities of probiotic strains are essential to prevent the infection or invasion of pathogenic bacteria. Members of the genus *Bacillus* are known to produce a wide range of antimicrobial substances, including peptide and lipopeptide antibiotics (Abriouel et al. 2011). The cultures Me1 and Bn1 showed a wide range of inhibitory activity against the pathogenic microorganisms including *Listeria monocytogenes* ScottA, *Staphylococcus aureus* FRI722, *Salmonella boydii*, *Bacillus cereus* F4433, *Salmonella typhi* FB231, and *Micrococcus luteus* ATCC9341 (Fig. 4). The ability of the probiotic to inhibit the ingested pathogenic microorganism in the gastrointestinal tract is an important aspect. The inhibition of pathogens as evidenced by the well diffusion test showed that the inhibitory metabolites produced by these test cultures were extracellular and diffusible, as the test was conducted via diffusion through a layer of agar. Survivability of the pathogen *L. monocytogenes* ScottA by growing the cultures together with pathogenic bacteria was determined by checking the viability in the selective differential medium. The viable count of the indicator organism, *L. monocytogenes* ScottA, decreased with increase in time. When co-cultured with *B. licheniformis* Me1 and *B. coagulans,* complete inhibition of the indicator organism was observed. However, in the case of *B. subtilis* Bn1 and *B. flexus* Hk1, 53 and 56% of the indicator organism survived after 6 h of incubation, respectively. Results indicate that these isolates can also serve as potential cultures for use in biopreservation.

Phytase activity and exopolysaccaride production

Phytase activity of the strains was screened for their ability to degrade myo-inositol hexakisphosphate (IP6) by cobalt chloride qualitative staining method (plate assay). Except for *B. flexus* Hk1, all the cultures were found to produce phytase, observed by the zone of clearance in the sodium phytate-added plate. The cultures *B. licheniformis* Me1 and *B. coagulans* formed white ropy colonies due to the production of EPS. The EPS production is economically important, since it can impart functional effects to foods and confer beneficial health effects.

Conclusion

Results obtained in the present study showed the survivability of the *Bacillus* cultures tested in the conditions of high bile salt concentration and low pH values. This will help strains to reach the small intestine and colon and contribute to the balance of the intestinal microflora. All the tested cultures exhibited good bile salt hydrolase activity, hydrophobicity towards hydrocarbons, and antioxidant properties as well as a wide spectrum of antibacterial activity against food-borne pathogens. In addition, all the cultures were susceptible to the antibiotics tested, which belong to the major classes of antibiotics used in human clinical therapy. Based on the results of our study, we observed that *B. licheniformis* Me1 exhibited remarkable in vitro probiotic properties and thus can be considered a positive trait for supplementation in food products as probiotic. This strain is being further assessed for possible in vivo safety and use as a culture for the development of new food products.

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