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Full Length Research Paper

Colonization potential and Cytotoxic Effect of Probiotic *Bacillus Species* on Human Colon Adenocarcinoma Caco-2 Cell lines

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Adherence to mucus and intestinal epithelial cells is an essential criterion for selection of probiotic strains for human use. In this study, *Bacillusmegaterium* (TA008 and TA009), *Brevibacillus brevis* (TA010) and *Bacillus subtilis* TA049 isolated from local honey samples previously, were investigated for their adhesive (auto-aggregation and co-aggregation), cell surface hydrophobicity and cytotoxic properties in *invitro* conditions. According to the results, the auto-aggregation, co-aggregation and hydrophobicity percentage varied significantly among the tested isolates. *B.megaterium* TA008 and *B.subtilis* TA049 possessed the highest autoaggregation and hydrophobicity percentages, while *B.megaterium* TA009 and *B.brevis* TA010 demonstrated least auto-aggregative and hydrophobic properties, respectively. The co-aggregation properties of the species differed with the pathogenic strains as *B.subtilis* TA040 co-aggregated strongly with *Salmonella typhi*, whereas *B.megaterium* TA008 showed strong co-aggregation phenotype with *Enterococcus faecalis*. Auto-aggregation property of the isolates in study appeared directly related to adhesion ability and the isolates possessing high auto aggregation percentage were also highly adhesive to human enterocyte-like Caco 2 cell lines. All three isolates in study appeared non cytotoxic to the intestinal epithelial cells and their cytotoxicity percentage was recorded below 15%, compared to the high cytotoxic nature of *B.cereus* ATCC 14579 (85.59%). The results of this study suggest that *Bacillus megaterium* TA008 and *B.subtilis* TA049 possessing significant *invitro* adherence and invitro colonization potentials and non-cytotoxic to Caco-2 cell lines, might be candidate probiotics available for future applications in food, feed or biotechnology industry.

Keywords: Aggregation; *Bacillus megaterium; Bacillus subtilis; Brevibacillus brevis;* Caco-2 cell lines; Cytotoxicity; Hydrophobicity.

INTRODUCTION

In last decade, the spore forming bacteria mainly *Bacillus* species have been highly acknowledged as effective probiotic candidates owing to their superior ability to withstand variable environmental condition, adapt easily to diverse habitats, possess adhesion abilities, produce bacteriocins (antimicrobial peptides) and provide immuno stimulations. One of the essential criterion for selecting a suitable probiotic bacteria is their ability to adhere to the intestinal mucosa (Teitalbaum et al., 2002), as according to postulations strong adherent strains would easily colonize the intestine.Survival and adherence of these bacteria in different parts of the intestinal tract is genus and strain dependent, and while some of them are destroyed during passage in the stomach, others have a high survival until they are shed in the feces (Marteau and Vesa, 1998). Lin et al (2012) and Rinkinen (2003) suggested adhesive ability of probiotic strains to be species specific and not host-specific.

A number of *invitro* and *invivo* models have been used for studying the colonization potentials and competitive exclusion of the pathogens by the probiotic bacteria. Among invitro tests, cell surface hydrophobicity and cell aggregation properties of the target bacteria are most widely studied. It is postulated that bacteria exhibiting good hydrophobicity and cell aggregation percentages might be able to neutralize the effect of pathogens by inhibiting their toxic effects and prevent their colonization with mucosal surface (Tambekar and Bhutada, 2010).The auto-aggregation and hydrophobicity abilities of probiotics can be strongly related. Some probiotic strains can inhibit adherence of pathogenic bacteria to intestinal mucosa either by forming a barrier via auto-aggregation or by direct co-aggregation with the pathogens (Ferreira et al., 2011). It was reported that the proteins, glycoproteins, techoic of lipotechoic acids on the cell wall surface of bacteria play important role in the auto-aggregation and hydrophobicity of the strains (Lahtinen et al., 2009). A correlation between adhesion ability and hydrophobicity has been observed in some probiotic Lactobacillus strains (Kos et al., 2003).

Studying the adherence ability of the probiotic strains to the colonic or intestinal origin cell lines such as human enterocyte-like CaCo2 or HT29 have been regarded as the most suitable model for predicting in vivo adhesions (2, 7, 9, 13). Besides Caco2 and HT29, other cell lines or colon tissues have also been proposed for studying the adherence and colonization potential of some of these probiotic bacteria (Servin and Coconnier, 2003) (42). The epithelial adhesion property differs between strains (2, 7, 9, 13, 42), and this property might be correlated with competitive exclusion properties and immunomodulatory activities in vivo. Until now, competitive exclusion properties of adhering strains have only been shown *in -vitro*. Although, the adherence ability of a bacteria facilitates colonization of the host and exert antagonism against pathogens in the gastrointestinal tract (GIT), but could also be considered as a risk for translocation especially in immune depressed patients (sanders et al., 2010).

In a previous study conducted by the authors, *Bacillus megaterium*TA008.TA009, Brevibacillus *brevis*TA010 and *B.subtilis* TA049 isolated from local honey samples, were shown to possess probiotic characteristics including acid and bile resistance, antagonistic actions against a number of human pathogens and sensitivity to the most commonly used therapeutic antibiotics. Realizing the importance of the adherence ability of the probiotic bacterial strains to the intestinal mucosa, in this study we investigated the colonization potential and cytotoxicity of the selected spore forming bacillus species. The isolates were tested for their auto-aggregation, co-aggregation, and cell surface hydrophobicity percentages. Additionally, the adhesive ability, pathogen displacement and competitive exclusion in the Caco-2 cell lines were studied and results evaluated statistically.

MATERIAL AND METHODS

Strain isolation

The locally isolated strain of *Bacillus megaterium* TA008, *Bacillus megaterium* TA009, *Brevibacillus brevis* TA010 and *B.subtilis* TA049 isolated previously from local honey samples were selected for these studies. The isolates were cultured in BHI overnight at 37ºC in aerobic condition with shaking. Stock cultures were prepared by freezing the strains in 20% glycerol. The cultures were activated by sub-culturing twice to prepare fresh cultures prior to test.

Autoaggregation

Auto-aggregation assay was performed according to the methodology described by Kos et al. (2003). Overnight culture (4ml) containing approximately 10^8 cfu per ml of bacterial cells were homogenized by vortexing (15 seconds), and incubated at room temperature (25ºC) for 5 hours. At every 1 hour intervals, 0.1 ml of upper suspension was carefully removed and transferred to a new tube containing 3.9 ml of phosphate buffered saline (PBS), and absorbance read at 600 nm. The Auto-aggregation percentage was expressed as a function of time until it was constant, using the below mentioned formula:

$A\% = 1-(A_t/A_0) \times 100$

Where A_t represents the absorbance during time intervals of 1, 2, 3, 4 and 5 h, while A_0 is the absorbance at time 0 h.

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Co-aggregation

Co-aggregation assay was optimized by the methodology of Del Re et al. (2000) with slight modification. Bacterial cells were harvested by centrifuging overnight bacterial cultures at 5000 rpm for 15 minutes. The obtained cell pellets were washed with phosphate buffered saline (PBS) twice, and approximately 10^8 cfuml^{-1} of cells were mixed with same volume of selected pathogen (*Enterococcus faecalis* ATCC 51299 *and Salmonella typhi local strain*). The suspension was vortexed (15 seconds) and incubated at room temperature (25ºC) and absorbance measured at hourly intervals during 5 hours, at 600 nm. The Co-aggregation percentage was expressed as a function of time until it was constant, using formula:

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

Where X and Y represents the absorbance at control tube and (X+Y) represent the absorbance of bacteria and pathogens together.

Cell Surface Hydrophobicity

Hydrophobicity assay was evaluated by the method of Van Loosdrecht et al. (1987) with slight modification. Overnight cultures of the mentioned bacteria were centrifuged at rpm 5000 for 15 minutes, and harvested cell pellets washed twice with phosphate buffered saline (PBS) pH 6.5. The harvested cells were suspended in 4 ml PBS, and the absorbance recorded at 620 nm (A_0). Xylene (1ml) was added to the suspension and vortex for 2 min. The aqueous phase was removed after 1 hour of incubation at room temperature and the Hydrophobicity percentage was calculated by the below mentioned formula:

$H\% = [(A_0 - A)/ A_0] \times 100$

Caco-2 Cell Cultures

The Caco-2 cells were obtained from The American Type Culture Collection (No HTB 37). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, UK) with 1% non essential amino acids, and 10% (volume fraction) of fetal bovine serum (Gibco, UK) inactivated (30 min, 56°C), 100 µg/ml streptomycin and 100 U ml^{-1} penicillin (Sigma, UK). Incubation parameters included 37ºC in atmosphere of 5% CO_2 and 95% air.

Adhesion Assay

For assessment of adhesion, monolayers of Caco-2 cells were prepared on glass coverslips which were placed in six-well tissue culture plates. 2 ml of respective cell suspensions at the concentration of 2×10^5 cells cm^{-2} were transferred to each well. The medium was changed on every alternate day. The number of adhesive cells was determined by treating the cells with trypsin for 10 min and later counting the bacterial cells by Hemocytometer.

Twenty-one day old, fully differentiated cells, cultured in 12 well tissue culture plates were used for the cell adhesion experiments. A day prior to the experiment, the monolayer were washed twice with phosphate buffered saline (PBS) in order to remove traces of the medium and then 500λ of DMEM without antibiotics were added to each well. Later 150λ of individual bacterial suspension at the concentration of 1×10^8cfu/ml (Mc Farland Standard) were transferred to each well and incubated at 37°C for 2 hours with 5% CO_2. The supernatant was removed by micropipette and monolayer cells were washed 4 times with 1 ml sterile PBS.

Adhesion scores

Two ml of methanol was added to each well and incubated for 15 min at room temperature. Methanol was completely removed and fixed cells were stained with Gram staining. The plates were air dried and examined under oil immersion microscope. The number of bacteria were counted in 20 random microscopic fields and grouped into 3 categories (Candela et al., 2008; Jacobson et al., 1999). Based on Jacobson's and his colleagues reports, the adhered bacteria were grouped into three classes including; non adhesive (≤40 bacteria), adhesive (41-100 bacteria) and strongly adhesive (>100 bacteria). While, Candela and his coworkers categorized the adhered cells into; non-adhesive when less than 5 cells adhered to Caco-2, 2) adhesive, 5-40 cells adhered to one Caco-2 cell, and 3) highly adhesive, when the level of adhesion exceeds 40 cells per one epithelial cell.

Percent Adhesions

In order to calculate the adhesion percentage, the cells were gently detached by trypsinization (1 ml 0.25 percent trypsin-EDTA solution) from the plate. Harvested cells were centrifuged and trypsin was separated immediately.The adhesion percentage was determined by addition of 1 ml of BHI broth to the pellet and preparing serial dilutions of the suspension in sterile phosphate buffered saline (PBS). The suspension was plated on respective agar plates and incubated at 37 °C for 24-48 hours, and the number of colonies counted (B_1 cfu/ml). Bacterial cells initially added to each well of six-well plates were also counted (B_0 cfu/ml). The adhesion percentage was then calculated by the below mentioned formula:

$\%\ \text{Adhesion} = (B_1/ B_0) * 100$

Inhibition of pathogen adhesion to intestinal mucus

The ability of the three *Bacillus* species in study to inhibit the adhesion of *E.faecalis* ATCC 51299 and *S.typhi* (local isolate) to the intestinal mucus, was studied by the

procedure described by Collado et al. (2005). The adhesion inhibition was calculated as the difference between the adhesion of the pathogen in the absence and presence of *Bacillus* strains.

Pathogen displacement Assay

The ability of the bacillus species to displace the pathogens adhered to the intestinal mucus was studied according to the methods described by Collado et al. (2007). Displacement of pathogens was calculated as the difference between the adhesion after the addition of the respective bacillus species and the corresponding control buffer.

Competitive inhibition Assay

Competitive exclusion of the pathogenic bacteria by the mentioned *Bacillus* species was evaluated by the method described by Xu and his colleagues (2009). *E.faecalis* ATCC 51299 and *S.typhi* (local isolate) freshly grown cultures were co-cultured with the respective bacillus species, in the wells of Caco-2 cell monolayers for 2 hours. After cultivations the suspensions were discarded, washed twice with 0.1% peptone water, and treated with0.05% trypsin-EDTA to detachthe adhered bacteria. The total bacterial count was made by making serial dilutions of the suspensions in peptone water and plating on BHI agar plates.Competitive exclusion was calculated as the percentage of the pathogens bound after the combination with probiotic strains relative to the pathogens bound in the absence of probiotic (control).

Cytotoxicity assay

The survival and or death of Caco-2 cells were determined by the method of Ramarao and Lereclus (2006) with slight modifications. One ml of the mentioned cells at a density of 10^6 were seeded into 24 well tissue plates and incubated overnight at 37ºC in the atmosphere of 5% CO_2 and 95% air. The supernatant fluids of freshly grown culture medium were filter sterilized (0.2um) and added to the wells individually and incubated for 24hrs. Filter-sterilized BHI broth without any inoculations was used as negative control while *B.cereus* ATCC 14579 was used as the pathogenic control. The number of dead and live Caco-2 cells were measured by trypan blue (Sigma, Germany) staining using Neubauer hemocytometer. The cytotoxicity percentage was determined using the below mentioned formula (Ramaro and Lereclus, 2006; Thirabunyanon et al., 2009).

Cytotoxicity% = (No of dead cells/total cell count) * 100.

Statistical Analysis

Each experiment was performed with three replicates. Data were analyzed with Statistical Analysis System Software (SAS) with glm procedure at significant level %0.05.

RESULTS

Auto-aggregation and Co-aggregation

Significant differences in auto and co-aggregation properties ($p< 0.05$) were observed among the studied *Bacillus* species. *B.megaterium* TA008and *B.brevis* TA010 showed the highest auto-aggregation ability followed by*B.subtilis* TA010 and*B.megaterium* TA009, respectively. As illustrated in Figure 1, a significant increase ($p< 0.05$) in auto-aggregation percentage was observed with passage of time from 0 to 5 hrs, in the studied bacterial species.The co-aggregation ability of the bacillus species to pathogens like *E.faecalis* and *S.typhi*s demonstrated in Table 1. *B.megaterium* TA008 demonstrated highest co-aggregation with *S.typhi*, while the other three bacillus species in study showed significant co-aggregation ability with *E.faecalis*. A direct relationship between auto-aggregation and co-aggregation ability were demonstrated by all the bacillus species except for *B.brevis*TA010 which in contrast to its high auto-aggregation ability demonstrated low co-aggregation percentage with the tested pathogens. Overall, auto aggregation percentages were significantly higher than the co-aggregation percentages.

Hydrophobicity

Table 2, shows the obtained hydrophobicity percentage of the tested *Bacillus* species. Based on the results, significant differences in hydrophobicity percentage were observed between the tested isolates ($p< 0.05$). *B.megaterium* TA008 showed the highest hydrophobic activity ($P <0.05$) followed by *B.subtilis* TA049. The hydrophobic percentage of the mentioned Bacillus species was above 80%, while *B.megaterium* TA009 and *Brevibacillus brevis* TA010had hydrophobic percentage below 10%.

Adhesion of strains to Caco-2 cell lines

Table 3 shows the number of adhesive bacteria, percentage of adhered bacteria, and average number of bacteria adhered to Caco-2 cells. All tested isolates possessed the ability to adhere to the Caco-2 intestinal cell lines however with significant variations in their adhesive percentages (Figure 2). *B.megaterium* TA008 and

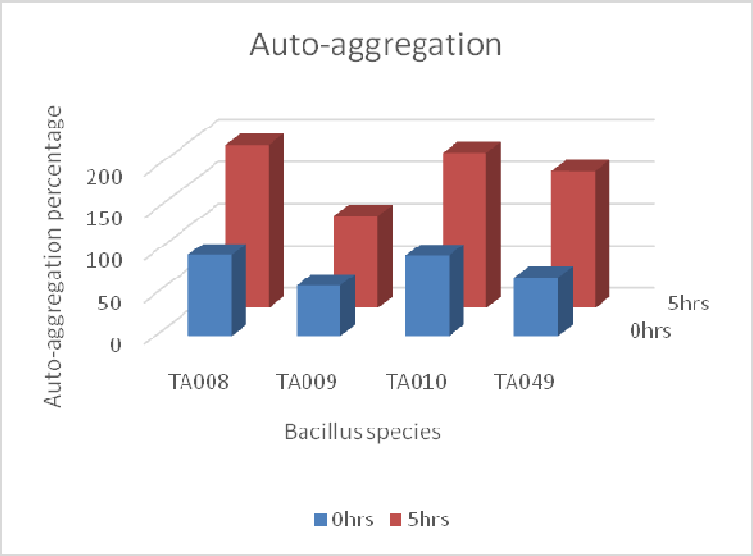

Figure 1: Auto-aggregation percentage of the studied *Bacillus* species at time 0 and after 5 hours

Table 1: Auto-aggregation and Co-aggregation percentage of the studied *Bacillus* species

Bacteria Species		Co-aggregation%	
		E. faecalis	*S. typhi*
B. megaterium TA008		39.38±3.1	62.02±2.7
B. megaterium TA009		26.66±2.6	29.21±4.8
B. brevis TA010		22.93±1.9	12.41±7.8
B. subtilis TA049		54.12±7.4	28.65±4.3
P value		<0.0001	<0.0001
SEM		0.36	0.06
Relationship between time and Co-aggregation percentages			
B. megaterium TA008	h5	61.00±5.1	78.52±7.2
	h0	9.76±6.3	34.87±9.1
B. megaterium TA009	h5	46.00±3.4	14.33±5.1
	h0	7.32±5.8	9.42±4.8
B. brevis TA010	h5	43.28±4.6	13.42±3.1
	h 0	7.58±4.3	3.45±2.7
B. subtilis TA049	h 5	52.43±3.2	45.11±1.2
	h 0	4.58±2.4	3.45±2.0
P value		<0.0001	<0.0001
SEM		0.36	0.06

h: time in hours (co-aggregation results recorded at time 0 and 5 hrs.)
Results are shown as median ±standard deviations (three replicates).

Table 2- Hydrophobicity percentage of the studied *Bacillus* species

Bacteria Species	Hydrophobicity%
B. megaterium TA008	86.38±5.1
B.megaterium TA009	6.30±1.9
Brevibacillus brevis TA010	3.98±2.4
B. subtilis TA049	85.56±7.0
P value	<0.0001
SEM	0.03

Results are shown as median ±standard deviations (three replicates).

Table 3- Adhesive percentage of studied *Bacillus* species to Caco-2 cells

Bacteria Species	Average number of bacteria adhesive to one Caco-2 cell	Adhesion[a1]	*Bacteria adhesive percentage
B. megaterium TA008	19.52 ±4.9	314.25±4.9	35.88±4.9
B. megaterium TA009	11.58±4.9	197.53±4.9	25.22±4.9
B. brevis TA010	6.55±4.9	115.25±4.9	13.24±4.9
B. subtilis TA049	10.91±4.9	311.10±4.9	33.64±4.9
P value	0.0008	<0.0001	<0.0001
SEM	0.59	0.53	0.02

*Expressed as the number of bacteria adhering per 100 epithelial Caco-2 cells; mean standard deviations of three assays, counts were carried out on 20 randomized microscopic fields for each sample per assay.

Adhesion[a1]: average number of adhered bacteria counted in 20 random fields of hemocytometer.

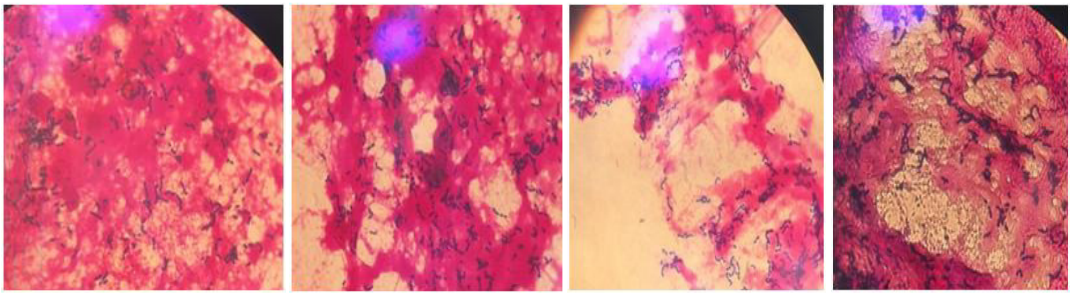

Figure 2: Adhesion of *B. megaterium* TA008, TA009, *B. brevis* TA010 and *B. subtilis* TA049 to Caco-2 cell lines

B.subtilis TA049 showed significantly higher percentage of adhesion to Caco-2 cells ($P < 0.05$) compared to the other two isolates in study. *E.faecalis* and *S.typhi* showed 84.23 and 78.91 % adhesionto the mentioned cell lines.

Inhibition of pathogen adhesion to intestinal mucus

The adhesion inhibition of the isolates in study was calculated as the difference between the adhesion of the pathogen in the absence and presence of respective bacillus species. Significant differences in the ability of the tested bacillus species to inhibit the adhesion of *E.faecalis* and *S.typhi* to the intestinal mucus was found ($P < 0.05$). In contrast to *B.subtilis* TA0449 which demonstrated least inhibition of pathogen adhesion, the other tested isolates showed significantly enhanced inhibition percentages (Table 4).

Table 4: Percentage of adhesion inhibition, displacement of pathogen adherence and competitive exclusion of the pathogens by the selected Bacillus species

Test method	TA008		TA009		TA010		TA049	
	E. f	*S. t*	*E. f*	*S. t*	*E. f*	*S. t*	*E. f*	*S. t*
Inhibition of pathogen adhesion	29.9±7.8	41.4±8.6	29.7±6.7	38.6±9.6	26.8±2.6	44.51±3.6	21.9±7.4	13.41±2.6
Displacement of pathogen adhesions	48.2±4.9	56.8±7.4	16.1±5.4	23.0±6.6	-8.6±2.8*	14.85±3.7	54.3±4.1	15.54±4.9
Competitive exclusion inhibition	17.6±3.4	44.9±5.1	-3.6±2.4*	16.5±6.1	-5.1±4.1*	-12.3±3.2*	32.9±8.6	7.80±6.4

E.f: Enterococcus faecalis ATCC 51299; *S.t: Salmonella typhi* (local isolate)
Results are shown as median ±standard deviations (three replicates).
*No significant difference for the corresponding controls (P > 0.05), considered as 0% pathogen adhesion

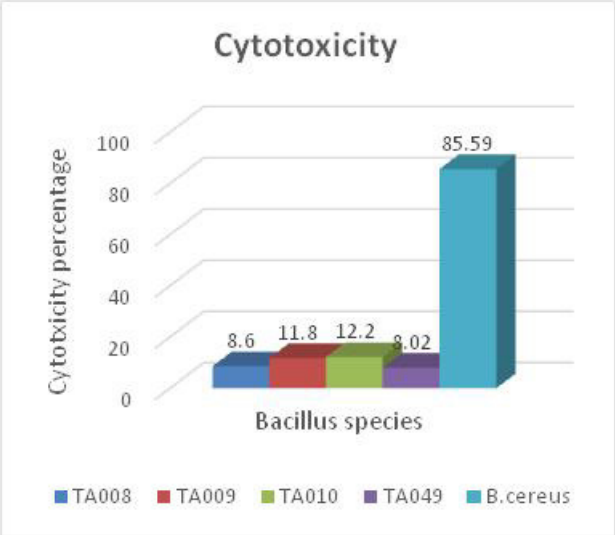

Figure 3: Cytotoxicity percentage of studied *Bacillus* species compared to *B.cereus* ATCC 14579

Pathogen displacement Assay

B.megaterium TA008, *Brevibacillus brevis* TA010 and *B.subtilis* TA 049 were able to displace the pathogen adhered to the intestinal mucus ($P < 0.05$), whereas, *B.brevis* TA010 did not show displacementof *E.faecalis* adhesion but was able to displace the adhered *S.typhi* bacterial cells. According to the results, highest displacement of *S.typhi* was shown by *B.megaterium* TA008 (48.2%), whereas *B.subtilis* TA049 showed highest (54.3%) displacement of *E.faecalis* adhesions to the intestinal mucus.

Competitive Inhibition Assay

The competitive inhibition of adhesion of*E.faecalis* and *S.typhi* to Caco-2 cells by the respective bacillus species is shown in table 4. *B.megaterium* TA008 (17.6%) and *B.subtilis* TA049 (16.9%) were able to competitively inhibit the adhesion of the tested pathogenic bacteria, whereas, *B.megaterium* TA010 and *Brevibacillus brevis* TA010 showed negative inhibition rates.

Cytotoxicity

The cytoxicity percentages of the isolates in study were analyzed by comparing with the cytotoxicity of pathogenic *B.cereus* ATCC 14579. According to our results, the cytotoxicity of the studied *Bacillus* species were less than 15% and were considered non toxic to the Caco-2 cell lines. *B.megaterium* TA008 appeared least toxic (8.6%) to the tested cell lines, followed by *B.subtilis* TA049, *B.megaterium* TA009 and *Brevibacillus brevis* TA010, respectively (Figure 3). The pathogenic strain *B.cereus* ATCC 14579 was graded as highly toxic as it showed significantly increased cytotoxicity (85.59%) to the intestinal epithelial cells, compared to negative control ($P<0.01$).

DISCUSSION

The pharmacokinetics studies on the probiotic bacteria includes investigations on the concentrations of bacteria that adhere, colonize, survive and exert antimicrobial effect on the pathogens during their passage in the gastrointestinal tract (GIT).

Adhesion is a complex process including non-specific and specific ligand-receptor mechanisms, and is considered a significant factor in promoting probiotic bacteria to colonize the gastrointestinal tract and confer healthy benefits to the host. Many factors which might influence the adhesion of probiotic microorganisms include the bacterial concentration, buffer composition, incubation time and growth medium. While in vivo conditions: normal intestinal microbiota, digestion and the food matrix are the main factors affecting the adhesion and colonizing ability of these bacteria (Ouwehand and Salminen, 2003).

Auto-aggregation of potentially probiotic strain seems necessary for adhesion to intestinal epithelial layers, whereas co-aggregation of non-pathogens and pathogens interferes with the ability of the pathogens species to infect the host. Aggregation ability has been suggested to be an important property of many bacterial strains used as probiotics and plays an important role in the formation of biofilm to protect the host from colonization by pathogens (An et al., 2000; Botta et al., 2015). Factors such as the proteins, glycoprotein, teichoic and lipoteichoic acids on the cell wall surface of bacteria were involved in the adhesion, auto-aggregation and hydrophobicity abilities (Ramiah et al., 2008). Li and his co-workers (2015) by removing s-layer protein showed that surface related protein played a partial role in the adhering and Auto-aggregation abilities of the strains. Similarly, co-aggregation of bacterial strains has known to play a significant role in some ecological niches, especially in the human gut. It was reported that co-aggregation abilities may allow probiotic bacteria strains inhibit the growth of pathogenic strains in the gastrointestinal and urogenital tracts in very close proximity (Botes et al., 2008). As reported by Kaewnopparat and his co-researchers (2013), probiotic bacteria like acid lactic bacteria could control microenvironment around the pathogens and increase the concentration of the excreted antimicrobial substances in the process of co-aggregation.The positive correlations among hydrophobicity, auto-aggregation and intestinal epithelial adhesion, as well as between co-aggregation and pathogenic antagonism, allows using Auto-aggregation and Co-aggregation assays as reliable in vitro systems for preliminary screening of potential probiotic strains (Del Re et al., 2000; Dunne et al., 2001; Xu et al., 2009).In this study the adhesive properties of previously isolated bacillus species shown to possess *invitro* probiotic characteristics (data not submitted) was evaluated by determining their auto and co-aggregation abilities and hydrophobicity percentage.

We were also able to prove the adhesive properties of the tested *Bacillus* species and their auto-aggregation and co-aggregation percentages appeared highly significant. Similar to our results, a number of *Bacillus spp*. has been shown to possess adhesion abilities (Barbosa et al., 2005; Cherif et al., 2001).

The key role of hydrophobic interaction in bacterial adhesion is well established since important aspects of microbial behavior are controlled by physicochemical properties of the cell wall (Van Loosdrecht et al., 1987). The high values of hydrophobicity could be a sign of a greater capability of bacteria to adhere the epithelial cells and thus enhancing their useful property in competitive exclusion of pathogens (Rosenberg et al., 1980). Cell surface hydrophobicity (CSH) of the bacteria is one of the most important factors which govern the mechanism of bacterial adhesion to in animate and biological surface. In a study *Bacillus spp*. tested for hydrophobicity was able to adhere to glass surface in abiotic culture (Merghni et al., 2014).Compared to the reports of Thirabunyanon and Thongwittaya, (2012) who showed 31- 62% hydrophobicity of *Bacillus* isolates in xylene, we observed highhydrophobic percentage of 91.30 and 85.56 % in *B.megaterium*TA008 and *B.subtilis* TA049, respectively.A significant difference in the hydrophobic percentage observed among the tested bacillus isolates in our study, indicates variations in their capacity to adhere to the substances which might be explained by the fact that surface charge and hydrophobicity of bacteria are influenced by the environmental condition (Vesterund et al., 2005).

Usually cancers cells are recommended for investigating the adherence and safety properties of probiotic bacteria. Among these, Caco2 cell lines are the most commonly used cells, as they have morphological and functional properties similar to mature enterocyte (Deepika, 2012). Not many reports are present regarding the adherence potential of spore forming *Bacillus spp*. to Caco-2 epithelial cells. *Bacillus spp*. are thought to be less adhesive than *Lactobacillus spp* and in a report, extremely low adhesion rates (0.01 bacteria cell per each Caco-2 cell) of Bacillus strains (BS3 and BL31) to Caco-2 cells was stated (Sorokulova et al.,2008). In contrast to the mentioned reports the adherence ability demonstrated by the *Bacillus spp*. in this study appeared highly comparable to those in some of the *Lactobacillus* strains. However, significant variations ranging from 2.8 to 4.9 log CFU/well were recorded in the tested *Bacillus* isolates. These variations in the adhesion were seen not only at genus and species level but also differences were observed at strain level. Similar observations were made by Xu and his colleagues (2009), who reported that variations in the adhesion ability of the probiotic species might indicate that adhesion is a strain dependent property.

Although adhesion property alone might not approve the safety of a bacteria, but could be considered indicative and a step closer towards approving the safety of the strain. The ability to inhibit pathogen adhesion appears to depend on the specific probiotics and pathogens and on the mucosal site (Collado et al., 2007; Dimitrov et al., 2014). Interestingly, the pathogens (*E.faecalis* and *S.typhi*) used in this study showed high adherence to the intestinal mucus, indicating their pathogenicity to the human epithelial cells. The capacity to bind the intestinal mucus might assist the pathogens in the invasion into the human intestinal mucosa and lead to detrimental health consequences (Xu et al., 2009). In this context, to find appropriate probiotic strain with the ability to prevent the adhesion of these pathogenic bacteria is important. Treatment of intestinal mucus with the bacillus species, alone or in combination, significantly reduced ($P < 0.05$) the adhesion of the tested pathogens. The ability to inhibit the adhesions of the pathogenic bacteria appears to depend on the specific probiotic strain and the tested pathogen. Chouraqui et al. (2004) reported that the inhibition of the pathogen adhesion is not directly related to the adhesion ability of the strains. Similarly, the displacement of pathogen adherence was also found to be dependent on the probiotic strain and pathogen while, no direct correlation was found between adhesion of probiotics and displacement of pathogen.

The cytotoxicity on intestinal epithelial cells by action of a bacterial culture medium is one method of evaluating whether a bacterium strain is harmful or non-harmful. In this regard, lysis of the epithelial cells isconsidered an essential sign of toxicity of a bacterial strain (Thirabunyanon and Thongwittaya, 2012). As far as we know, very meager evidence regarding the toxicity of *B.megaterium* exists. However, investigations on other *Bacillus spp*. like *B.subtilis* have shown them to be non toxic based on the experiments which shows they do not carry enterotoxin operon (Sorokulova *et al.,* 2008). Ostensvik *et al.,* (2004), screened twenty-one isolated belonging to the *Bacillus cereus* and *B.subtilis* groups for cytotoxicity. They reported 9 strains of *B.cereus* and 5 strains belonging to the *B.subtilis* groups cytotoxic. In another research, Thirabunyanon and colleagues (2009) showed *B. subtilis* to possess low cytotoxicity rate with cytotoxic percentage of 12.3%.The presence of cytotoxic *Bacillus spp*. in surface water represents a possible source for food contamination.*B.megaterium* TA008 showed no toxic effect on the epithelial cells during*in-vitro* experiments. The results of this study and our previous results indicate the possible role of *B.megaterium* TA008 as a potential probiotic and that it might be exploited for use in biotechnology industry in future.However, prior to that *in-vivo* study in appropriate animal models are also essential, which is in progress in our laboratory.

CONCLUSIONS

B.megaterium TA008 and *B.subtilis* TA049 were able to inhibit, displace and compete significantly with the pathogens and thus might be suitable candidates for use in specific conditions involving the tested pathogens. However, in order to develop the most appropriate probiotic strains, the specificity of the process, the strains and the pathogens involved with the target groups should be taken into account. These studies allow us to better understand the mechanism of actions and the functions of probiotic strains in the intestinal conditions which reciprocally allows selection and development of more effective probiotic cultures with target specific functions.

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