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ORIGINAL ARTICLE

Interaction between the probiotic *Bacillus subtilis* and *Salmonella* Typhimurium in Caco-2 cell culture

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ABSTRACT

Background and Objectives: *Bacillus* probiotics have been recently considered in biotechnological researches, and food additives. The present study was aimed to investigate the effects of *Bacillus subtilis* probiotics (PY79 and ATCC 6633) and their metabolites on *Salmonella* Typhimurium in Caco-2 cells.

Materials and Methods: Cytotoxicity of *B. subtilis* ATCC 6633 crude supernatant (CS) was evaluated by 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. *S.* Typhimurium invasion assay was performed in the presence of the probiotics. Cell viability, apoptosis, and necrosis were evaluated in presence of *S.* Typhimurium , *B. subtilis* strains, and CS (4%, 8%) using flow cytometry.

Results: Results showed a significant reduction in the invasive ability of *S.* Typhimurium to Caco-2 cells by employing *B. subtilis* probiotics, and CS ($p < 0.05$). The less invasion was indicated in *B. subtilis* PY79 and *Salmonella* co-cultural group. Furthermore, the cell survival rates, and apoptosis/necrosis were respectively increased and decreased in co-culture groups ($p < 0.05$).

Conclusion: Hence, it seems that *B. subtilis* strains could be suggested as beneficial candidates to overcome the invasion and cytotoxicity of *Salmonella* on the intestinal cells. However, additional *in vivo* models are suggested to validate our results.

Keywords: Probiotics; *Salmonella* Typhimurium; Caco-2 cells; Cytotoxicity tests; Immunologic; Flow cytometry

INTRODUCTION

Salmonella is noticed as the main agent of food borne disease. *S.* Typhimurium is known as a facultative intracellular pathogen with adherence and invasion ability to epithelia and macrophages due to its fimbriae and surface proteins. The pathogen damages to the host cells through colonizing, intracellular proliferating, leading to diarrhea (1, 2). As regards, invasion ability plays a key role to initiate the intestinal infection; identification of preventing methods may be helpful in controlling the disease. Probiotics and their metabolites are reported to be useful against enteropathogenic bacteria (3). Among common health promoting probiotics including *Lactobacillus* and *Bifidobacterium* species, *Bacillus* probiotics have been extensively employed as food additives for humans and animals. This is mainly due to surviving in harsh conditions such as high temperature, low pH, and chemicals (4, 5). Some valuable *Bacillus* probiotics features include improving mucosa-associated lymphoid tissue (MALT) in human and animals; applying as insecticide; producing antimicrobial compounds such as amino acids, enzymes, lipopeptides, bacteriocins and bacteriocin-like in-

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hibitory substances (BLIS) (6, 7).

Bacillus subtilis strains 189 and PY79, introduced as generally recognized as safe (GRAS) bacteria (8), are principally isolated from gastrointestinal tract (9). According to previous studies, bacteriocins produced by some strains of *B. subtilis*, have shown inibitory effects against Gram negative, and Gram positive pathogens (7, 10-13).

In vitro intestinal monolayers such as Caco-2 cells shows high similarity to epithelial cells of humans` ileum; the cell line has been widely selected to study interactions of probiotics and human-specific enteropathogens such as *Salmonella* (14-16).

In this study, we used an *in vitro* intestinal model to assess the influences of *B. subtilis* PY79, and *B. subtilis* ATCC 6633, and their antimicrobial substances against *S*. Typhimurium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* PY79, *Bacillus subtilis* subsp. *spizizenii* ATCC 6633, and *S*. Typhimurium (ATCC 14028) were obtained from the Iranian Organizations for Science and Technology (Tehran, Iran). *S*. Typhimurium and the *B. subtilis* strains were respectively cultured in TSB (Merck, Germany) and TSB supplemented with 1% (w/v) yeast extract (Merck, Germany) (TSBYE) for 18 h at 37°C in an aerobic condition. 10^7 CFU/ml of each bacterium was finally re-suspended in cell culture medium for further assays.

Preparation of crude supernatant. TSBYE medium was inoculated with *B. subtilis* ATCC 6633 and incubated at 37°C for 18 h. The supernatant fluid was collected after centrifugation (7000×g, 20 min, 4°C) to supply crude supernatant (CS) and kept as freeze dried (Christ, Germany). Various CS concentrations were dissolved in RPMI medium and adjusted to pH 7 using pH meter (BASIC 20, Cerison, UK). Catalase enzyme (Sigma-Aldrich, USA) was applied to remove probable hydrogen peroxide from the CS which was subsequently sterilized by 0.22 µM filter (17).

Bradford assay was performed to calculate protein concentration of CS using bradford stain (BioIdea, Iran), and bovine serum albumin (18). Optical density (O.D.) of samples was then recorded at 595 nm using microplate reader (BioTek, USA).

Cell culture. Caco-2 ATCC HTB-37 epithelial cell line was purchased from Pasteur Institute of Iran (Tehran, Iran). The cells were routinely grown in RPMI 1640 (BioIdea, Iran), and supplemented with 20% fetal bovine serum (FBS) (Gibco, USA), 1% (v/v) penicillin-streptomycin (10000 IU/ml and 10000 µg/ml; BioIdea, Iran), and amphotericin B (50 mg/10 ml) (Sigma, USA). Cells were incubated at 37°C in 5% CO_2 atmosphere, and detached by 0.02% trypsin-EDTA. The cells were then seeded at approximately 3 × 10^4 cells/well into 96-well tissue culture plate and incubated at 37°C in 5% CO_2 atmosphere. The experiments were performed on the confluent differentiated cells (15 days post-confluence) (19).

CS cytotoxicity on Caco-2 cells. The MTT test was carried out as described by Ozkan et al. (2013). At first, monolayer of each well was washed using PBS and replaced with 100 µl non-supplemented RPMI. Plates were then incubated at 37°C in 5% CO_2 for 1 h. The cells were then exposed to 100 µl CS (4%, 8%) and further incubated for 18 h at 37°C in 5% CO_2. Metabolic activity analysis of the intestinal cells was performed according to 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (BioIdea, Iran). After the incubation period, the culture medium was replaced with 100 µl of RPMI 1640 and 10 µl MTT (12 mM) solution which was subsequently loaded into each well. The plates were incubated at 37°C for 4 h, the medium of each well was then replaced by 50 µl Dimethyl sulfoxide (DMSO) and incubated at 37°C for 10 min. The absorbance of the wells was finally measured at 570 nm by microplate reader (BioTek, USA). The cell toxicity was calculated using the current equation (1- O.D. of the test sample/ O.D. of the negative control) × 100 (20).

***S*. Typhimurium invasion to Caco-2 cells.** The monolayers (in 96 well tissue culture plate) were washed by PBS, and 100 µl (10^7 CFU/ml RPMI) *B. subtilis* ATCC 6633 or PY79 was added to the cells followed by incubation at 37°C for 1 h in a humid atmosphere of 5% CO_2. 100 µl RPMI medium containing 10^7 CFU/ml of *S*. Typhimurium was subsequently loaded into the Caco-2 cell monolayers faced with probiotics. In addition, 100 µl RPMI containing CS (4%) and 100 µl RPMI containing 10^7 CFU/ml *S*. Typhimurium were simultaneously added to the epithelial cells. Negative and positive controls were considered uninfected cells and *S*. Typhimurium

infected cell, respectively. After washing the monolayers with PBS, 100 µl RPMI containing 100 µg/ml gentamicin (Alborz Darou, Iran) was loaded into each well. The plate was incubated for 1 h. The cells were subsequently washed followed by adding 0.1% Triton X-100 for cell lysis. The invaded *S.* Typhimurium bacteria to the cells were counted by surface plate counting method on brilliant green agar which was then incubated at 37°C for 18 h. The experiments were repeated 4 times (19).

Survival rate, early apoptosis, and necrosis of Caco-2 cells treated by *S.* Typhimurium and *B. subtilis* strains. Flow cytometry was applied to examine the survival rate, early apoptosis and necrosis of intestinal cells exposed to various treatments of *S.* Typhimurium and *B. subtilis* strains. 4×10^5 cells initially seeded to T-12.5 flasks and incubated for 48 h at 37°C. After washing the monolayers with PBS, 10^7 CFU/ml *B. subtilis* strains, 1 ml RPMI containing CS (4%) were added to the cells and were incubated in 5% CO_2 at 37°C for 2 h. *S.* Typhimurium suspension (10^7 CFU/ml RPMI) was then added to the flasks containing *B. subtilis* (10^7 CFU/ml) or CS (4%) or monolayers without any previous treatments. The flasks were incubated at the same condition. The cells were trypsinized, centrifuged (200×g, for 8 min), and washed 4 times with PBS. Following the last wash, the treated cells were re-suspended in 1× binding buffer and transferred into 1.5 ml microtubes. Uninfected flasks containing monolayers were considered as negative controls. Negative and unstained controls were prepared conferring to the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, US). For each cell experiment run, data for 100000 events was determined by flow cytometer (BD FACSCalibur, USA) (21, 22).

Statistical analysis. One-way ANOVA test was applied using SPSS (version 16.0) software (SPSS, Inc., Chicago, IL) to analyse the data. Level of statistical significance reported at $p < 0.05$. Flow cytometry data was also analysed by FlowJo (version 7.6) software (Flexera, US).

RESULTS

CS cytotoxicity on Caco-2 cells. CS cytotoxicity (8%, 4%) was evaluated on Caco-2 monolayer. The negative control (3.3%), and 4% CS (6.14%) groups revealed low cytotoxicity. While, the 8% CS concentration showed high cell toxicity (45.09%); it was accordingly not employed in further experiments.

***S.* Typhimurium invasion to Caco-2 cells.** *S.* Typhimurium invasion to Caco-2 cells was significantly reduced in the presence of *Bacillus* probiotics and the CS (4%) ($p < 0.05$). Invasion in co-culture groups including *S.* Typhimurium and *B. subtilis* PY79, *S.* Typhimurium and *B. subtilis* 6633, and also *S.* Typhimurium and CS (4%) were reported as 3.49, 4.02 and 4.02 log CFU/ml, respectively, compared to *S.* Typhimurium alone (5.25 log CFU/ml) (Fig. 1).

Survival, early apoptosis and necrosis of Caco-2 cells treated by *S.* Typhimurium and *B. subtilis* strains. Cells survival, apoptosis, and necrosis were evaluated using flow cytometry in Caco-2 cells treated with *S.* Typhimurium, *B. subtilis* strains, and CS (4%, 8%) (Table 1). According to the results, *B. subtilis* probiotics and CS played effective roles to increase the Caco-2 cells viability. As shown in Table 1, mixtures of CS (4%) or *B. subtilis* PY79 or *B. subtilis* 6633 with *S.* Typhimurium, showed respectively 60.04%, 65.29%, and 58.61% cell viability. Furthermore, the highest record of cell viability belonged to both strains of *B. subtilis* including PY79 (96.5%), 6633 (92.71%), and 4% of CS (95.3%). Results were not statistically significant when they compared to the negative control group (97.79%) ($p > 0.05$). Moreover, the lowest level of cell viability was shown in the *S.* Typhimurium group (48.64%).

DISCUSSION

Currently, interests are extensively focused on probiotics as safe alternative approach in preventing food borne pathogens rather than antibiotic therapy (23). *Bacillus* probiotics, and their metabolites have shown potential effects to combat *Salmonella* infections according *in vitro* and *in vivo* studies (11, 19, 24). In this study, the potency of two *B. subtilis* strains was investigated to control invasive enteropathogen such as *S.* Typhimurium in the intestinal epithelial cells. We initially evaluated the cell toxicity of *S.* Typhimurium, the *Bacillus* probiotics, and their metabolites (CS 4% and CS 8%) on Caco-2 cells using MTT assay. Results showed low cytotoxicity rate

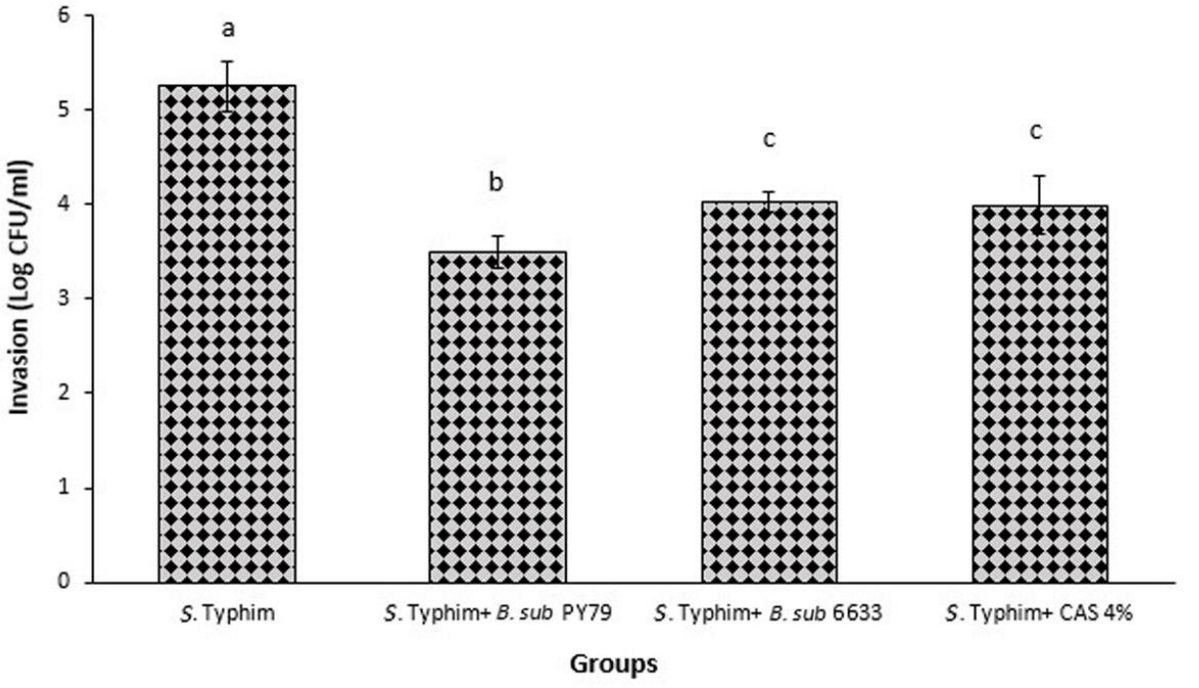

Fig. 1. Invasion inhibition of various treatment groups in Caco-2 cells. Bars represented standard deviation (SD) values of triplicates. Different letters on bars indicate significant statistical differences ($p < 0.05$).

Table 1. Percentages of viable, early, and late apoptosis (necrosis) of Caco-2 cells in different experimental groups using flow cytometry assay.

Experimental groups	Viable cells	Early apoptotic cells (%)	Necrotic cells (%)
Negative control	97.8 ± 3.7 ^{a}	1.63 ± 0.34 ^{aA}	0.57 ± 0.37 ^{aA}
CS* 4%	95.32 ± 4.77 ^{a}	2.83 ± 0.81 ^{aA}	1.85 ± 0.52 ^{aA}
B. subtilis PY79	95.6 ± 5.16 ^{a}	2.20 ± 0.50 ^{aA}	1.3 ± 0.33 ^{aA}
B. subtilis ATCC 6633	92.72 ± 3.77 ^{a}	4.96 ± 0.85 ^{abA}	2.33 ± 0.56 ^{aA}
CS 8%	65.52 ± 5.35 ^{b}	15.30 ± 3.45 ^{cA}	20.47 ± 4.70 ^{dA}
S. Typhimurium	48.64 ± 3.74 ^{c}	34.70 ± 2.47 ^{eA}	16.29 ± 2.86 ^{cdB}
S. Typhimurium + *B. subtilis* ATCC 6633	58.61 ± 5.22 ^{d}	30.72 ± 4.9 ^{deA}	9.67 ± 0.47 ^{bB}
S. Typhimurium + *B. subtilis* PY79	65.28 ± 4.47 ^{b}	28.87 ± 5.37 ^{dA}	6.85 ± 4.36 ^{abB}
S. Typhimurium + CS 4%	60.04 ± 4.97 ^{d}	26.84 ± 5.54 ^{dA}	12.59 ± 0.48 ^{bcB}

*Crude supernatant. Values are mean ± SD of triplicates for flow cytometry assay. Different small and capital letters represent the statistical difference between each column and rows, respectively ($p < 0.05$).

for *B. subtilis* PY79, and the CS 4% groups; while high cytotoxic percentage was observed in *S.* Typhimurium group. Previous reports showed numerous cytotoxicity rates of *Bacillus* species on different cell lines (25). The supernatants of *B. subtilis* PY79 cultured in BHI broth, and *B. subtilis* 6633 cultured in NB were respectively shown 0 and 4.5% cytotoxicity to Caco-2 and HT-29 cells (20, 26) which supported our results. In contrast, the CS with the concentration of 8% showed a high cytotoxicity which may be attributed to increased concentration of toxic protein compounds and remained culture medium in the antimicrobial substances obtained from the probiotic. Our results corroborate with some former reports (20, 26, 27). Moreover, the highest cytotoxicity rate was obtained in *S.* Typhimurium group in our study which was similar to those previously reported (19, 28).

The invasive ability of *Salmonella* to mammalian cells was repeatedly addressed by others. For in-

stance, the bacteria invaded into Caco-2 monolayers were found 5.25 log CFU/ml in the present study. The result is compatible with the reports of other studies (1, 14, 29, 30). Lin et al. (2008) showed that of 8 log CFU/ml the *S. choleraesuis* 2a initially inoculated into Caco-2 cells, 5 log CFU/ml of the bacteria were internalized (31). The results were similar for *S*. Typhimurium (strains C52 and SL1433) when it was inoculated into the intestinal epithelial cells (29).

Identification of preventive methods against invasion of pathogens into the mammalian cells is considered the first step to inhibit the infection (2). Specific and nonspecific protective mechanisms suggested for probiotics against *Salmonella* include competition for adhesion, mucosa colonization, competitive exclusion, immunogenic effects, secreting antimicrobial substances, steric hindrance and other health impacts (7, 23, 32, 33). According to earlier researches, *B. subtilis* spp. have shown effective competitive exclusion role to control the enteropathogens (7, 34, 35). For instance, the intestinal colonization of bacteria was significantly decreased followed by administration of PY79 and RX71 strains of *B. subtilis* to chickens (24, 35). Furthermore, prior studies have been declared that some strains of *B. subtilis* isolated from various sources were capable to produce the antimicrobial substances (11, 13, 36). *B. subtilis* ATCC 6633 has revealed inhibitory ability against several Gram negative and positive pathogens followed by producing lantibiotic peptides (including subtilin, subtilosin A) and some protein-like inhibitory substances (37). As the present study showed, the invasive ability of *S*. Typhimurium into Caco-2 cells was significantly reduced by using *B. subtilis* probiotics and CS ($p < 0.05$), among which the less pathogen invasion was indicated when *B. subtilis* PY79 was co-cultured with the *Salmonella*. Our results were similar to previous published data based on applying probiotics and their metabolites in excluding *Salmonella* species (1, 19, 31, 32).

The early and late apoptosis (necrosis) were significantly decreased in the most co-culture groups ($p < 0.05$). As demonstrated in Table 1, the lowest rate of apoptosis and necrosis were shown in *B. subtilis* PY79, *B. subtilis* 6633, and the CS (4%), whereas, the highest apoptotic rate were found in *S*. Typhimurium group and the combined group of *S*. Typhimurium and *B. subtilis* 6633 ($p < 0.05$). The highest rate of necrosis also belonged to the groups including CS (8%), *S*. Typhimurium , and combination of *S*. Typhimurium and 4% of CS ($p < 0.05$). In addition, apoptotic rate was significantly higher or the same as the rate of necrosis in whole groups ($p < 0.05$).

The ability of *Salmonella* to induce apoptosis in the initial step of infection, overcoming the immune system and consequence surviving are extensively noticed, because the signals correlated with programed cell death can be affected by the pathogen (38). Flow cytometry assay which has been considered as powerful, sensitive and specific technique (39), was eventually applied to further investigate the viability rate, apoptotic and necrotic of cells in this work. Various rates of apoptotic cells have been reported based on types of bacteria and cell cultures (40), for instance, 67% and 13% of apoptotic cells were respectively observed in the macrophages and in RAW264.7 cell line infected with *S*. Typhimurium (41). In the present study, apoptotic and necrotic cells were 34% and 16.29%, respectively. When *S*. Typhimurium was co-cultured with *B. subtilis* probiotics or CS (4%), the necrosis and apoptosis rates were significantly reduced ($p < 0.05$) and as a consequence, the survival rates of Caco-2 cells were increased. Poormontaseri et al. (2017), were also found less apoptosis and necrosis of Caco-2 cells in co-cultured CS of *B. subtilis* ATCC 6633 and *C. perfringens* group in comparison to *C. perfringens* group in cell culture using flow cytometry assay (22). As such, results presented here clarified the inhibitory role of the *Bacillus* probiotics (especially PY79 strain) and their antimicrobial substances over *Salmonella* cytotoxicity. Co-culture of *Lactobacillus casei* with *S*. Typhimurium was reduced the rate of apoptosis in the mice intestinal cells (42).

CONCLUSION

B. subtilis probiotics (PY79 and ATCC 6633 strains) is recommended as beneficial candidates to prevent the invasion of *Salmonella*. It seems that they probably confer their antagonistic activities by steric hindrance mechanism and producing antimicrobial substances which can prohibit the adherence and invasion of the pathogen to its cell epithelial receptor. However, further *in vivo* and *in vitro* assays using animal models and non-cancerous human cell culture should be employed to validate our results.

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