

Original Article

Probiotic *Bifidobacterium Lactis* Bacteria Inhibit the Invasion Phenotype of *Shigella Dysenteriae* Induced By Invasion Plasmid Antigen C

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ABSTRACT

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Background and Aims: Shigellosis is an acute gastroenteritis and Invasion plasmid antigen C (IpaC) is the first effector protein for *Shigella* invasion of intestinal cells. Among lactic acid bacteria, *Bifidobacterium lactis* (*B. lactis*) has received increasing attention for protection of a potential host against gastrointestinal infections. The aim of this study was to investigate the inhibitory activity of *B. lactis* against *Shigella dysenteriae* (*S. dysenteriae*) harboring *IpaC* gene strains from clinical specimens.

Materials and Methods: Sixty stool samples of patients with bloody diarrhea were collected from three teaching hospitals in Tehran and subjected to further analysis for the identification of *Shigella* colonies by biochemical tests. DNA was assessed for *IpaC* gene by polymerase chain reaction (PCR). Furthermore, *IpaC* gene expression analysis by real-time PCR was carried out to investigate whether probiotic *B. lactis* can inhibit the invasion phenotype of *S. dysenteriae* induced by *IpaC*.

Results: Analysis of gene expression in *S. dysenteriae* harboring *IpaC* gene strains showed the expression of *IpaC* gene in treated *S. dysenteriae* with *B. lactis* being much lower than that of non-treated group ($p < 0.000$). The results revealed that *B. lactis* at the concentration of 500 $\mu\text{g/ml}$ bears strong inhibitory activity on the growth of *S. dysenteriae* by decreasing *IpaC* expression.

Conclusions: Our results revealed the positive role of *B. lactis* in reducing the expression of the *ipaC* gene and inhibition of epithelial cell invasion by *S. dysenteriae*. Therefore, probiotics can be used as a complementary biotherapeutic agent in severe *Shigella* infection.

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Introduction

Shigellosis, or "bacillary dysentery", is a serious gastrointestinal infection caused by a family of bacteria called *Shigella*. Shigellosis is a major public health problem in many underdeveloped and developing countries occurring mostly in children aged under five [1-3]. For severe shigella infection, antibiotics may shorten the duration of the illness. However, in the treatment of severe infections, failure to respond to antibiotics because of bacterial resistance is an unfortunate reality experienced in some countries such as Iran. [4, 5]. Genetic factors of the *Shigella spp.* invasive phenotype are encoded by large (180- to 210 kilobase) non-conjugative plasmids [6, 7]. Numerous plasmid-encoded antigens have been identified as essential bacterial ligands that facilitate bacterial attachment and invasion of colonic epithelial cells [6]. Invasion plasmid antigens (Ipa proteins), required for invasion of the colonic and rectal epithelial cells, are encoded by the virulence plasmid. Invasion plasmid antigen C (IpaC) and IpaB are multifunctional and essential virulence agents in the infection process [8]. IpaC is involved in the expression of the invasion phenotype in all *Shigella* species [9, 10]. It has been shown that the amount of IpaC protein produced by a type of pathogenic schigella dysentery during the infection process is more than a non-infectious status [11]. IpaC, by actin polymerization and regeneration of the skeletal system, causes *Shigella* entry into epithelial cells [12]. Today, with the increasing awareness of the benefits of probiotic bacteria, lactic acid bacteria, especially Bifidobacterium

strains are considered as the most important probiotics used in food and pharmaceutical products [13-15]. *Bifidobacteria* are gram-positive in various forms and anaerobic that secrete lactic acid, which form a large part of human intestinal microflora and other animals [16]. Various reports have demonstrated the ability of *bifidobacteria* to exert beneficial effects, including protection of a potential host against infectious diseases caused by enteric pathogens and prevention of intestinal disorders [17, 18].

Bifidobacterium lactis (*B. lactis*), a multi-purpose and powerful transient probiotic bacteria, plays an essential role in limiting the formation of exogenous and pathogenic colonies [19]. Previous experiments have shown that probiotic *B. lactis* protects mice against bacterial gastrointestinal infections caused by *Salmonella* and *Escherichia* infection and reduces infection severity [20-22]. The objective of the present study was the isolation of the *IpaC* gene from the *Shigella dysenteriae* samples of patients with bloody diarrhea via PCR and the inhibitory effect of probiotic *B. lactis* bacteria on the expression of this gene in bacteria by real-time PCR (RT-PCR).

Materials and Methods

Sample collection and bacterial isolates

During a period of 6 months, from April to October 2017, a total of 60 stool specimens were collected from patients with diarrhea presenting at three teaching hospitals (Children's Medical Center, Milad and Shahid

Modarres Hospitals) in Tehran, Iran. Patients included for this study had dysentery lasted ≤ 7 days, and blood was evident by stool examination using an occult blood test. All samples were collected in sterile containers and transported to the clinical microbiology laboratory for processing within 2 h of collection. *Shigella* spp. in stool samples were cultured in Cary-Blair medium, MacConkey, xylose-lysin-desoxycholate agar and Salmonella-Shigella agar and incubated at 37°C for 24 h. Non-lactose-fermenting colonies were tested by biochemical routine tests including citrate, Methyl Red, Voges Proskauer, indole production, and lysine decarboxylation for identification of *Shigella dysenteriae* (*S. dysenteriae*). All *S. dysenteriae* isolates were stored at -20 in Luria Bertani broth (Merck, Germany) with 20% glycerol for molecular procedures. This study was approved by the Research Ethics Committee of the Islamic Azad University, North Tehran Branch and participants provided written voluntary informed consent.

DNA extraction and detection of *Shigella* virulence gene *IpaC* by PCR assay

DNA was extracted from *S. dysenteriae* by a commercial genomic DNA extraction kit (CinnaPure DNA Kit, CAT NO: PR881612) according to the manufacturer's recommendations. Total DNA was isolated from 0.5 ml of Luria Bertani broth culture grown overnight for all the bacterial isolates. The concentration of DNA samples was measured as micrograms per milliliter based on A260 on A280 values by the Nanodrop system (Thermo, USA). The isolates were examined for the presence of the virulence gene *IpaC* by PCR assay using

specific primers. The primer sequences used in this assay were:

F: 5'-CCTTCTGATGCCTGATGGAC-3', and R: 5'-TGGAAAACTCAGTGCCTCT-3' (142 bp). PCR amplification was performed in a 25 μ L reaction mixture containing 200 ng of DNA template (2 μ L), 5.5 μ L ready to use Mastermix (Fermentas, Germany), 17.5 μ L of distilled water and 0.1 μ L of each 20 pmols forward and reverse primers. DNA amplification was carried out with a thermal cycler (Eppendorf® Mastercycler Gradient, Germany). Positive and negative controls were amplified in parallel to assess the validity of the procedure. The final amplified products were visualized after electrophoresis on a 1% agarose gel stained with ethidium bromide for 20 to 25 minutes and a voltage of 100. GeneRuler 100 bp Plus DNA ladder (Fermentas, Germany) was used as a size marker. The gels were viewed under UV light and photographed using gel documentation system.

Determination of minimum inhibitory concentration (MIC) of *B. lactis* by broth microdilution method

For determining MIC of probiotic *B. lactis* bacteria required for the inhibition of bacterial growth, broth microdilution method was performed according to standard broth dilution method of Clinical and Laboratory Standards Institute (CLSI). For this purpose, 100 μ L sterilized deionized water containing probiotic bacteria (at different doses ranging from 86 μ g/mL to 1024 μ g/mL in serial two-fold dilutions) were added to 100 μ L of sterile Tryptic Soy Broth (TSB) (Merck, Germany) in each well of the 96-well microtiter plate,

followed by about 24 h of incubation with gentle agitation at 37°C using a shaker incubator. Bacterial growth was tested based on the broth's turbidity, where lack of turbidity was considered as evidence of successful antimicrobial susceptibility. MIC of *B. lactis* was reported as the lowest concentration of the probiotic bacteria that successfully inhibit bacterial growth.

Quantification of *IpaC* gene expression by qRT PCR

After 24 h of incubation with probiotic bacteria by dilution broth method, total RNA was extracted from *S. dysenteriae* isolates with the RNeasy kit including DNase digestion (Qiagen) following the manufacturer's instructions. To remove genomic DNA, the extracted RNA was treated with RNase-Free DNase Set (Qiagen, Germany). Samples for RNA extraction were collected during *in vitro* growth in the logarithmic phase (OD₆₀₀=0.4-0.6). The ratio of absorbance at 260 /280 nm and 260/230 nm was used to assess the purity of RNA. Reverse transcriptase AMV at 25 U/μl (Roche Life Science) was used for cDNA synthesis, which was carried out at 42°C. Real-time PCR analyses were performed using the Corbett Research Rotor-Gene 3000 thermal cycler (Westburg, Leusden, the Netherlands) according to the following protocol: 1 min. at 95°C, followed by 35 cycles of amplification with 40 s annealing at 59°C, 1 min extension at 72°C and denaturation at 95°C for 30 s. For each sample, the reaction mixture was comprised of the following components: 10 μL 2X Prime Q-Master Mix with SYBR Green I

(Genet bio CAT. NO: Q9210), 1 μL of each primer (final concentration 1μM), 1 μL Rox Dye, 5 μL RNase-free water and 2 μL cDNA, in a final reaction volume of 20 μl. Normalized expression levels were calculated using the expression of 16s rRNA gene as the normalization reference. The ΔCT method was used to calculate the relative expression of *IpaC* gene.

Statistical analysis

Statistical package for the social sciences (SPSS) software, v. 16 (SPSSInc, Chicago, IL, USA) was used for the analyses. The results of gene expression were statistically tested using an independent t-test to determine any significant difference. A p-value of <0.05 was considered to be statistically significant.

Results

Characterization of bacteria

The results of standard bacteriological methods and biochemical testing exhibited that all the isolated bacteria were *S. dysenteriae*. The results of DNA amplification by the PCR method based on the primers used in this study showed the presence of a 142 bp fragment for the *IpaC* gene (Fig. 1).

Results for MIC

After treatment of *S. dysenteriae* harboring *IpaC* gene strains with concentrations of 86 to 1024 μg/mL of *B. lactis*, exploration the antimicrobial propensity was measured by broth microdilution method. The results showed that *B. lactis* at the concentration of 500 μg/ml have maximal inhibitory activity on the growth of *S. dysenteriae* by decreasing *IpaC* expression.

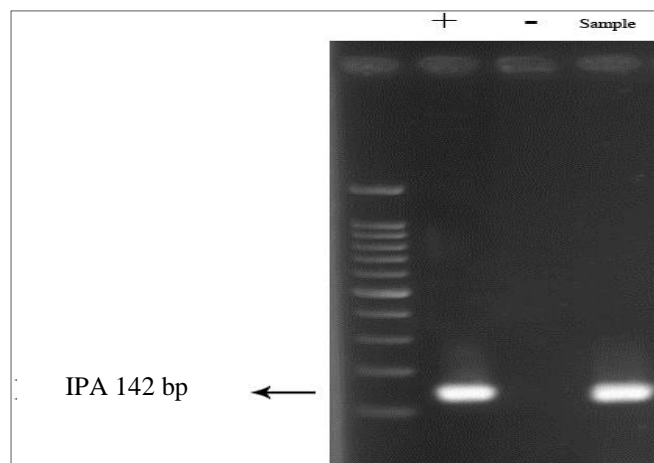


Fig. 1. Electrophoresis of PCR products on a 1% agarose gel. Ladder 100 bp. Positive control. Negative control. Sample of representative *Shigella* isolates that indicated a 142 bp PCR product

The control group showed a rapid growth pattern which followed an ascending trend until the end of the assay. At all probiotic bacteria concentrations, descending trend in bacterial growth was observed so that at the end of the assay (24 h) at 37°C condition, bacterial growth in all treated isolates was nearly zero.

***B. lactis* decreased the expression of *IpaC* gene**

Assessing the expression levels of *IpaC* gene at different concentrations of probiotic bacteria was performed by quantifying numbers of mRNA transcript copy corresponding to gene through RT-PCR. The analysis showed that the best melting temperature for the *IpaC* gene was 86.94°C. Real time assay data profiling showed evidence of differential expression levels between treated and untreated *S. dysenteriae* harboring *IpaC* strains in which the mean expression levels of the *IpaC* gene was significantly lower in treated strains than in untreated ones presenting inhibition of almost all bacteria ($p < 0.001$). According to our results,

probiotic treatment with *Shigella* strain can reduce *ipaC* gene expression by 31.7%.

Discussion

The present study was carried out to investigate the antibacterial properties of probiotic lactic acid bacteria for the treatment of antibiotic resistant strains of *S. dysentery* isolated from patients with bloody diarrhea. To investigate whether probiotic lactic acid bacteria would be able to inhibit the gene expression of *IpaC*, the invasive *S. flexneri* were incubated with *B. lactis*. The results showed that the *ipaC* gene expression in the treated *S. dysentery* with Bifidobacterium was lower which indicates the positive role of Bifidobacter in reducing the expression of the *IpaC* gene expression in *S. dysentery* bacteria.

S. dysenteriae harboring Invasin *IpaC* gene strains were determined using molecular methods such as PCR. Novel methods for the early detection of invasive gastrointestinal pathogens such as genetic analysis, are

being introduced to complement routine microbiological and biochemical tests that often take a few days to get to a definite diagnosis [7]. PCR detection of plasmid-encoded virulence genes represents an excellent diagnostic tool for the detection of invasive shigellosis and IpaC could be used as a marker for molecular identification of *Shigella* strains [3]. Furthermore, comparison of plasmid profiles is a rational approach for evaluating the potential relatedness of individual clinical isolates of a certain bacterial species for molecular epidemiological surveys [23].

With the identification of many virulence-essential factors from different enteric bacteria such as *shigella spp.*, efforts are under way to regulate the expression of invasion plasmid antigen virulence genes. Reduced expression of invasion plasmid antigens leads to a loss of the invasive phenotype and in subsequent virulence properties [6]. *In vitro* adhesion and invasion inhibition of *S. dysenteriae* by treating with human milk proteins [24] and Lactobacilli [25] has previously been documented. Probiotics represent a potential alternative biocontrol agents in the prevention of foodborne diseases, mainly through their antagonistic activity against potentially intestinal pathogenic bacteria [26]. Several studies have shown the ability of bifidobacteria to inhibit enteric pathogen adhesion to intestinal epithelium [27, 28]. In this line, a study by Shu et al. (2000) further indicated that *B. lactis* decreased the risk of *Salmonella Typhimurium* infection in a murine model, and also boosted innate and adaptive immunity [21]. Adherence inhibition of pathogenic bacteria is thought to depend on the

particular probiotics and enteric pathogens [29]. As mentioned, Ipa proteins are the most important factor involved in adhesion and invasion of epithelium in *Shigella spp* and can be potential targets for probiotic properties of *B. lactis* [10].

For the first time, we have been able to document the possible mechanism of adhesion and invasion inhibition of *S. dysenteriae* treated with probiotic by affecting invasion plasmid antigens. Several mechanisms have been proposed to explain decreased *IpaC* gene expression in pathogen treated with probiotic, including intermicrobial competition with shigella for intestinal attachment sites, the production of substances that are directly microbicidal for pathogens [26, 30-32], increased cell membrane permeability by production of metabolites such as quenchers of quorum-sensing system [33], and bacteriocin-like inhibitory substance. IpaC exhibits three distinctive functional domains and the central hydrophobic domain of IpaC is critical in a mechanism of specific attachment to the host plasma membrane [34]. In future research, more functional researches are needed to investigate the association between three distinctive domains of IpaC in pathogen treated with Bifidobacteria.

Conclusion

In summary, our data provide, for the first time, clear evidence supporting the idea that *B. lactis* inhibits invasion phenotype *S. dysenteriae* by suppressing gene expression of invasion plasmid antigens. Treatment of wild type strains of *S. dysenteriae* with probiotics can reduce the

expression of the *IpaC* gene in these strains. Therefore, probiotics reduce the risk of Shigella infection and can be used as a complementary therapeutic agent in the pathogenetic pathogenesis of Shigellosis. Further research is needed to confirm this novel finding.

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Conflict of Interest

None declared.

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