Inhibitory Effects of the Spore-forming Bacillus spp.
on the Expression Levels of eae, luxS, flu, and ctxM
Genes in E. coli Isolates

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Abstract

Background: Escherichia coli infections are one of the major health problems in many countries. The emergence of antibiotic resistance has complicated the treatment of infections caused by this microorganism. Today, researchers are focusing on novel approaches for the treatment of E. coli infections among which is using spore-forming probiotics for their high stability in the harsh gastrointestinal (GIT) environment.

Results: This study screened the potential of B. coagulans and B. subtilis to inhibit the expression of flu, eae, luxS, and ctxM genes in 40 E. coli isolated from a total of 300 fecal samples of patients suffering from diarrhea from August 2018 to January 2019. For this purpose, first the presence of these genes was confirmed in the isolates using the PCR method. Then, the standard strains and broiler-derived B. subtilis and B. coagulans were used to screen their ability to tolerate low pH, bile salts, and gastric enzymes. After confirming their ability to survive under the simulant gastric environment, they were cocultured with E. coli isolates (n=4) harboring all the flu, eae, luxS, and ctxM genes. After reaching the logarithmic growth phase, the expression levels of flu, eae, luxS, and ctxM genes were determined using the real-time PCR methods. According to the results, there was a statistically significant relationship between the exposure of E. coli with commercial and broiler-derived spore-forming probiotics and the reduced expression of these genes.

Conclusion: The broiler-derived isolates had a greater capacity to decrease the expression of these genes compared to the standard strains, proposing their adoption for dietary supplementations. However, more studies are required to study the effects of these spore-forming bacteria on E. coli infections in vivo and their possible influence on lactose tolerance, nutritional absorption, and reduced cholesterol levels.

Background

Escherichia coli normally colonizes the gastrointestinal tract of human infants and over time, forms the normal intestinal microflora. These commensal E. coli strains can lead to infections following compromising the host immune system. In fact, several E. coli clones have evolved by acquiring specific virulence traits, allowing them to cause a wide array of infections, including the enteric disease, urinary tract infections (UTIs), and sepsis [1-3].

In fact, pathogenic E. coli strains harbor various virulence factors among which are Ag43, LuxS, and intimin. The surface protein antigen 43, Ag43, is encoded by the flu gene at 43 min on the E. coli chromosome with the ability of promoting bacterial adhesion and 3D biofilm structures [4]. LuxS has been suggested to be an interspecies signal with crucial role in physiologic functions of bacteria by contributing to quorum sensing [5]. Finally, the intimin surface protein, encoded by the eae gene, contributes to bacterial attachment to the intestinal cells and induces attaching and effacing lesions [6].

The emergence of resistant E. coli strains and the subsequent failure in antibiotic therapy has become a worldwide health concern. Resistance in Gram-negative bacteria is attributed to various mechanisms,
including altered target sites, enzymatic inactivation of antibiotics, and active efflux pumping [7]. However, one of the most frequent resistance mechanisms adopted by *E. coli* strains is the production of Extended-Spectrum Beta-Lactamase enzymes (ESBL). The CTX-M beta lactamase types are currently the most common forms of these enzymes leading to antimicrobial resistance [8].

Antimicrobial resistance has therefore necessitated the development of other therapeutic methods to combat *E. coli* infections. In this regards, one of the safest ways is the exploitation of probiotic bacteria. For many years, *Lactobacilli* and *Bifidobacteria* have been used to treat gastrointestinal tract (GIT) diseases [9]. However, these bacteria are sensitive to physiological conditions, such as pH of the stomach and bile salts. In addition, various conditions of production, storage, and transportation may affect their bioavailability [10]. Therefore, to overcome these hurdles, using spore-forming *Bacillus* spp. as probiotics has recently come to notice. These bacteria have an innate ability to produce a wide number of enzymes and vitamins, and are highly tolerant to harsh environment of GIT. Moreover, *Bacillus* spp. are ideal candidates for probiotics owing to their stability during food processing and storage [11-13].

Considering the merits of *Bacillus* spp. as probiotics, this study aimed to investigate the effects of *B. coagulans* and *B. subtilis* against the expression levels of *eae*, *flu*, *ctxM*, and *luxS* genes to gain a deeper insight into the mechanisms by which spore-forming *Bacillus* spp. can affect *E. coli* infections.

**Results**

**Isolation of *E. coli* from fecal samples and the presence of the studied genes**

Of 300 fecal samples obtained from patients with diarrhea, 40 were positive for *E. coli*. Identification and confirmation of bacterial strains was by cultivation on samples on MacConkey agar and EMB agar in addition to performing complementary biochemical tests including MR/VP, consumption of citrate, production of urease and lysine decarboxylase, and production of ornithine. In order to confirm the presence of *flu*, *luxS*, *eae*, and *ctxM* genes in clinical *E. coli* isolates, specific primers were designed and PCR assay was performed. According to the results of amplification assay, the prevalence of *luxS*, *flu*, *ctxM*, and *eae* genes were 35% (n=14), 62.5% (n=25), 37.5% (n=15), and 17.5% (n=7), respectively. Among these, four *E. coli* isolates carried all the *flu*, *luxS*, *ctxM*, and *eae* genes.

**Isolation of spore-forming *Bacillus* spp. from the intestinal content of broilers**

Spore-forming bacteria were selected by heat or ethanol treatment of the intestinal content of 10 broilers. Treated samples were subsequently identified by API CHB test. Colonies identified as *B. subtilis* and *B. coagulans* were collected. Isolates showing positive catalase test were differentiated from the anaerobic spore-forming *Clostridium* spp. Moreover, isolates showing no hemolysis on 5% sheep blood agar were considered as *Bacillus* spp. *B. subtilis* ATCC 6633 and *B. coagulans* MTCC 5856 were used as controls. Molecular analysis was used to confirmed the characterization of the isolates. According to the results of biochemical and microbiological testings as well as PCR, one *B. subtilis* and one *B. coagulans* was isolated from the intestinal content of broilers.
Acid and bile tolerance of *Bacillus* cells

We evaluated the survival rates of *B. subtilis* and *B. coagulans* cells after exposure to both simulated gastric conditions and bile salts. According to the results of the CFU measurements, both *B. coagulans* and *B. subtilis* were resistant to 1 mg/mL pepsin and 1mg/mL trypsin, as well as 0.2% bile salts and pH=2.

Expression levels of *flu*, *luxS*, *eae*, and *ctxM* genes in *E. coli* after colculture with *B. coagulans* and *B. subtilis* isolates

In order to study the effects of *B. coagulans* and *B. subtilis* as the studied probiotics on the expression levels of genes involved in bacterial attachment, biofilm formation, signaling, and antimicrobial resistance, total RNA of four isolates harboring all *flu*, *luxS*, *eae*, and *ctxM* genes was extracted after the coculture assay at the logarithmic growth phase.

Results of the expression of *flu*, *luxS*, *eae*, and *ctxM* genes revealed that after coculture of *E. coli* with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*, the expression levels of these genes decreased significantly (p-value<0.05). However, the broiler-derived *B. subtilis* showed a greater effect on the expression levels of *eae*, *luxS*, and *ctxM*. *B. coagulans* MTCC 5856 and broiler-derived *B. coagulans* also led to the significant decreased expression levels of all the studied genes (p-value<0.05), while the standard strain had a greater impact on the expression of *ctxM* compared to the broiler-derived strains. Comparing the results of the expression levels of the studied genes, *B. subtilis* showed a more substantial effect on the reduced expression levels of *eae*, *ctxM*, and *flu* compared to *B. coagulans* (Fig.1).

Discussion

*E. coli* strains are considered as harmless commensal bacteria, however, several strains have gained the ability to cause infections in human hosts. These pathogenic variants have obtained a variety of virulence factors which confer environmental adaptations and pathogenicity [14]. Moreover, these pathogenic variants have adopted several mechanisms to resist antibiotic therapy with the production of beta-lactamase enzymes being the most common strategy [15]. Therefore, finding novel therapeutic approaches to fight *E. coli* infections seems necessary. In this regard, using the probiotic properties of various bacteria has become an interesting subject in recent years [16].

Commonly, lactic-acid-producing bacteria are known as probiotics. However, in recent years, researchers have focused on developing drugs based on more resistant bacterial species due to antagonistic environment of the human gastrointestinal tract [17]. Spore-forming *Bacillus* spp. are one of these bacteria with high levels of tolerance to harsh conditions. Moreover, these bacteria are highly stable during processes of food manufacturing and storage, making them suitable candidates for health improvement strategies. In fact, approved spore-forming *Bacillus* spp. are currently being used in dry probiotic products due to their ability of survival in conditions, including high or low temperatures, aridity, and high oxygen levels [18]. This ability is ascribed to the possession of an endospore with a cortex.
replete with peptidoglycan and protein-containing materials [19]. According to several studies, the benefits of using Bacillus spp. for their probiotic properties include the improvement of immunity systems and prevention of GIT disorders, including diarrhea and irritable bowel syndromes [20]. However, limited studies have focused on the effects these probiotic bacteria may have on the virulence factors of gastrointestinal pathogenic bacteria including E. coli. Here, we investigated the effects of the standard strains and broiler-derived B. coagulans and B. subtilis on the expression of various virulence genes in E. coli, including flu (coding for Ag43 protein involved in biofilm formation), eae (coding for a protein involved in intimate attachment of E. coli to the intestinal epithelial cells), and luxS (coding for a protein involved in bacterial quorum sensing). In addition, we investigated the potential effects of these strains on the expression levels of ctxM, a gene commonly associated with antimicrobial resistance in E. coli. In this regard, we isolated 40 E. coli out of 300 fecal samples of patients with diarrhea using cultivation and biochemical methods. According to the results of PCR, the prevalence of flu, eae, luxS, and ctxM genes in these isolates was 62.5% (n=25), 17.5% (n=7), 35% (n=14), and 37.5% (n=15), respectively. Moreover, amplification results showed the presence of all the studied genes (flu, eae, luxS, and ctxM) in four E. coli isolates, indicating their importance in bacterial pathogenicity. To study the effects of spore-forming Bacillus spp., in addition to the standard strains, we isolated one B. subtilis and one B. coagulans from the intestinal contents of 10 broilers and determined their tolerance to simulant GIT environment. We also PCR assay for the molecular confirmation of isolates as B. subtilis and B. coagulans. According to the results, the studied B. subtilis and B. coagulans were tolerant to low pH (pH=2), 0.2% bile salts, and gastric enzymes, including pepsin and trypsin, therefore, suggesting their suitability for probiotic use. Then, the effects of B. coagulans and B. subtilis on the expression levels of the studied genes was studied using the real-time PCR method. According to the results, after coculture of E. coli isolates harboring all the studied genes with each individual B. subtilis ATCC 6633, B. coagulans MTCC 5856, and broiler-derived B. subtilis and B. coagulans strains, the expression levels of these genes decreased significantly. These results reveal the important roles of B. subtilis and B. coagulans isolates in reducing the expression of genes involved in virulence and antimicrobial resistance in E. coli isolates. Moreover, since broiler-derived isolates showed similar or even better effects than the standard strains, in case of unavailability of commercial strains, gut microbiota of broilers can be exploited as suitable sources of these probiotic bacteria.

Furthermore, according to the results, B. subtilis had a greater influence on the expression levels of eae, ctxM, and flu, while B. coagulans strains showed a greater influence on the expression levels of luxS. These results suggest that B. subtilis mostly affects the expression of genes involved in attachment, biofilm formation, and antibiotic resistance, whereas B. coagulans has a greater potential to reduce the expression of genes involved in bacterial quorum sensing, proposing the different capabilities of these probiotic bacteria and different pathways they may adopt to combat bacterial pathogens.

Several studies have shown the probiotic properties of spore-forming Bacillus spp. on E. coli infections. Guo et al. collected a total of 124 intestinal samples from broilers and isolated six spore-forming Bacillus spp. with ability to inhibit E. coli K88 and E. coli K99. Of these isolated Bacillus spp., B. subtilis MA139...
showed a great tolerance to pH=2, 0.3% bile salts and demonstrated the highest activity against *E. coli* strains by coculture method [21].

In another study by Kim et al., dietary supplementation of *B. subtilis DSM 25841* significantly reduced the F18 *E. coli* infection. In overall, they showed the positive influence of this probiotic microorganism on the promotion of health after infecting pigs with pathogenic *E. coli* [22]. Also, Lin et al. revealed the effect of *B. coagulans* on the intestinal microbiota of broilers. In their study, probiotic supplementation with 0.02% and 0.04% *B. coagulans* significantly increased *Lactobacillus* count in duodenum and cecum, while significantly reducing the *E. coli* counts in the aforementioned body sites [23]. The effect of these spore-forming *Bacillus* spp. on the expression of virulence genes and beta-lactamase genes in *E. coli* was not found in the literature. However, Medellin-Pena et al. showed the decreased expression of autoinducer-2 and several genes associated with virulence in *E. coli O157:H7* (EHEC) following exposure to *L. acidophilus*. They suggested the role of *L. acidophilus* as an inhibitor of quorum sensing in EHEC O157 strains [24].

**Conclusion**

The results of the current study obviously indicate the capacity of broiler-derived *B. coagulans* and *B. subtilis* to significantly reduce the expression of genes involved in bacterial attachment, biofilm formation, quorum sensing, and antibiotic resistance in *E. coli* isolates. Standard strains of these spore-forming *Bacillus* spp. also showed the same effects. However, *B. coagulans* strains had a greater potential to reduce the expression of genes involved in quorum sensing while *B. subtilis* showed a comparatively greater ability to reduce the expression of genes involved in attachment, biofilm formation, and antibiotic resistance suggesting the difference in mechanisms adopted by probiotics to combat *E. coli* infections. Moreover, the broiler-derived isolates showed a greater capacity to reduce the expression of these genes compared to the standard strains, proposing their large-scale usage in dietary supplementations. However, more studies are required to study the effects of these spore-forming bacteria on *E. coli* infections *in vivo* and their possible influence on lactose tolerance, nutritional absorption, and reduced cholesterol levels.

**Materials And Methods**

1. *coli* isolation and culture conditions

A total of 300 fecal samples were collected from Imam Khomeini hospital, Tehran, Iran, from August 2018 to January 2019. Clinical samples were at first plated onto 5% sheep blood agar and MacConkey Agar (Biolife Laboratories, Milano, Italy). Samples were then confirmed as *E. coli* based on their morphology, Gram-staining, and routine biochemical tests, including MR/VP, utilization of citrate, presence of lysine decarboxylase and urease enzymes, and the production of ornithine. A single colony was obtained from each sample and maintained in TSB medium (Invitrogen, Paisley, Scotland) with 30% sterile glycerol at −80°C for future experiments.
Primer design and polymerase chain reaction

To confirm the presence or absence of *eae, flu, luxS, and ctx-M* genes, specific primers were designed using Primer-BLAST (Table 1). Total genomic DNA of *E. coli* isolates were extracted using DNA Extraction kit (Roche, Mannheim, Germany) according to the recommended protocol by the manufacturer. To investigate the presence of *eae, flu, luxS, and ctx-M* genes, the PCR assay was performed in a DNA thermal cycler (Bio-Rad, USA) in a volume of 25 μl according to the following reaction conditions: initial denaturation step at 94 °C for 5 min; 33 × denaturation at 94 °C for 30 s; annealing at 60 °C for 30 s; and extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. No template control (NTC) was used as a negative control. Finally, amplicons were observed following gel electrophoresis, and sent for sequencing after purification.

Table 1. Characteristics of the primers used in this study
<table>
<thead>
<tr>
<th>Reference</th>
<th>Product size</th>
<th>Sequence (5’ à 3’)</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>This study</td>
<td>73 bp</td>
<td>F; ACTAACTCCAGTTCC GCCG</td>
<td>eae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; AGTCGCTTTAACCTCA GCCC</td>
<td></td>
</tr>
<tr>
<td>[25]</td>
<td>113 bp</td>
<td>F; ACCGGCCGATAATTGCAG AGAT</td>
<td>ctxM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; TGCTTATCGCTCTCGC TCTG</td>
<td></td>
</tr>
<tr>
<td>[26]</td>
<td>124 bp</td>
<td>F; ACGGTAAATGGCGGA CTGTT</td>
<td>flu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; CACGGATGGTCAGGG TATCG</td>
<td></td>
</tr>
<tr>
<td>[27]</td>
<td>113 bp</td>
<td>F; GTGCCAGTTCTTCTG TGCTG</td>
<td>luxS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; GAACGTCTACCAGTG TGGCA</td>
<td></td>
</tr>
<tr>
<td>[28]</td>
<td>190 bp</td>
<td>F; CATTGACGTTACCC GCAGAAGAAGC</td>
<td>16srRNA E. coli</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; CTCTACGAGACTCAAG CTTGC</td>
<td></td>
</tr>
<tr>
<td>This study</td>
<td>165</td>
<td>F; AAAAGACATTGCCA CCCCCA</td>
<td>16srRNA B. coagulans</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; GGACCGATTTCAAC AACGCC</td>
<td></td>
</tr>
<tr>
<td>This study</td>
<td>108</td>
<td>F; TGTTGATCAGCGGG AAGTGA</td>
<td>16srRNA B. subtilis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R; AATGCCACGACCTT TTTGC</td>
<td></td>
</tr>
</tbody>
</table>

F: Forward; R: Reverse
Isolation of spore-forming probiotics from gastrointestinal tracts of broilers

A total of 10 broilers aged 6-12 months that did not take any antibiotics or probiotics during their life time were chosen. After slaughter in sterile conditions, intestinal contents were collected and diluted 1:1 (wt:vol) in buffered peptone-water (Oxoid) and resuspended by vigorous vortexing until obtaining an evenly distributed suspension. Then, aerobic spore-forming isolates were selected by heat (80 °C) and ethanol treatment. Ethanol treatment was performed by diluting the primary suspension (1:1) in ethanol (final concentration, 50% vol/vol) and incubation at room temperature for 1 h. 0.1-ml aliquots were cultured on nutrient agar plates and incubated at 37°C for 24-48 h. Colonies were picked randomly and purified by re-streaking on Luria-Bertani agar plates. The laboratory strain \textit{B. subtilis} ATCC 6633 was used as a control throughout the experiments. Isolates were identified using the API 50 CHB strips according to the manufacturer's protocols (bioMérieux) and catalase and hemolysis tests were carried out to confirm the identified isolates. Finally, the identified \textit{B. subtilis} and \textit{B. coagulans} were selected for further analysis. Moreover, to confirm the production of spores, \textit{B. subtilis} and \textit{B. coagulans} isolates were grown on Difco sporulation medium (DSM) for 24-48 h. Then, cultures were purified as described by Henriques et al [29] and stored at -80°C in Difco heart-infusion broth (HIB) with 30% glycerol for future use.

Molecular detection of spore-forming probiotics

Total genomic DNA of the isolated spore-forming probiotics was extracted using pepGOLD Bacterial DNA kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. For molecular identification of the isolated spore-forming probiotic bacteria, \textit{16s}rRNA gene was investigated using the specific primers designed in this study by Primer-BLAST (Table 1). PCR assay was performed in a DNA thermal cycler (Bio-Rad, USA) in a volume of 25μl according to the following reaction conditions: initial denaturation step at 94 °C for 5 min; 30× denaturation at 94 °C for 30 s; annealing at 61 °C for 30 s; and extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. No template control (NTC) was used as a negative control. Finally, after observing PCR products following gel electrophoresis, amplicons were sent for sequencing after purification.

**Probiotic characterization of isolated bacterial strains**

Resistance of vegetative \textit{B. subtilis} and \textit{B. coagulans} to bile salts and simulated gastric conditions was determined using the overnight LB cultures of \textit{B. subtilis} and \textit{B. coagulans} isolates and resuspending them in fresh LB, LB supplemented with 0.2% bile salts, or LB acidified to pH=2, and supplemented with 1 mg/mL pepsin and 1 mg/mL trypsin, respectively.

**Bacterial coculture assay**

Coculture of the two \textit{Bacillus} \textit{spp.} strains (\textit{B. subtilis} and \textit{B. coagulans}) with \textit{E.coli} isolates harboring all the studied genes (\textit{luxS}, \textit{flu}, \textit{ctxM}, and \textit{eae}) were performed to determine any changes in expression levels of the studied virulence genes in \textit{E. coli} strains. Briefly, overnight cultures of \textit{B. subtilis} and \textit{B. coagulans} were centrifuged and the obtained supernatant was collected and after filtering, \textit{B. subtilis} and \textit{B. coagulans}.
*B. coagulans* strains were inoculated individually in the tubes containing 5 ml of nutrient broth. Then, overnight cultures of *E. coli* isolates were also inoculated in each tube and once these cultures were set up, the tubes were incubated at 37 °C under microaerophilic conditions. Each strain was also cultured alone as controls. To determine the effects of *B. subtilis* and *B. coagulans* on the expression levels of the studied virulence genes in *E. coli*, samples were withdrawn at the logarithmic growth phase (OD=0.08-0.1). Experiments were carried out three times independently.

**RT-qPCR analysis of transcript levels of the studied virulence genes in *E. coli***

To investigate the expression of the studied virulence genes in *E. coli* after coculture assay, real-time PCR experiment was carried out. Briefly, after at the logarithmic growth phase, 1ml of tubes was collected for RNA extraction using commercially available kits (QIAGEN RNeasy Mini kit). Samples were treated with Turbo DNase (Ambion, Grand Island, NY, USA) to eliminate any genomic DNA whose absence was confirmed using PCR and running samples on a 1% agarose gel. The quality of total RNA was assessed using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthetized using random hexamers (Applied Biosystems, CA, USA) and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), according to the recommended protocols.

Finally, quantitative real-time PCR was performed in a Rotor-Gene thermal cycler (Corbett 6000; Australia) using the SYBR Green method (AccuPower Green Star qPCR Master Mix; Bioneer; Korea). Thermal cycling consisted of an initial cycle of 95 °C for 10 min, 40 cycles of 95 °C for 12 s, 58 °C for 25 s, and 72 °C for 30 s. *16s rRNA* was used as the internal reference gene. After confirming the absence of primer dimers, qRT-PCR results were analyzed by $2^{-\Delta\Delta C(t)}$ method [30]. A P-value less than 0.05 was considered statistically significant.

**Statistical analysis**

In order to evaluate the significant effect of probiotic properties of *B. subtilis* and *B. coagulans* on the expression of *flu, eae, luxS*, and *ctxM* genes in *E. coli* isolates, one sample T test was performed using SPSS v. 24. A p-value<0.05 was considered as significant.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

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Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors’ contributions

MBS designed the study, JGK. ZE carried out the experiments, ZE and MG wrote the manuscript under supervision of MS. All authors have read and approved the manuscript.

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References


Figures

**Figure 1**

Expression levels of flu, eae, luxS, and ctxM genes in E. coli isolates, after co-culture with individual B. subtilis ATCC 6633, B. coagulans MTCC 5856, and broiler-derived B. subtilis and B. coagulans.