Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

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

**Zahra Elahianfiroz**
islamic azad university, shiraz branch
**majid baserisalehi** ( majidbaseri@hotmail.com )
Islamic Azad University Kazerun Branch
**masood Ghane**
Islamic Azad University Tonekabon Branch

**Research article**

**Keywords:** Escherichia coli; probiotics; spore-forming Bacillus spp.; Gene Expression

**Posted Date:** July 7th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-33946/v2

**License:** This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

# Abstract

**Background:** *Escherichia coli* antibiotic resistance are one of the major health problems in many countries. Today, researchers are focusing on novel approaches for the treatment of *E. coli* infections, including the use of spore-forming probiotics for their high stability in the harsh gastrointestinal (GIT) environment.

**Results:** The presence of resistance genes (*flu*, *eae*, *luxS*, and *ctxM* ) was confirmed by PCR in four out of 40 *E. coli* isolated from 300 fecal samples of patients suffering from diarrhea. Following confirming the ability of broiler-derived *Bacillus coagulans* and *Bacillus subtilis* to survive under the simulant gastric environment, they were co-cultured with the four *E. coli* isolates harboring the studied resistance genes. Results of the expression of *flu*, *luxS*, *eae*, and *ctxM* genes revealed that the expression levels of these genes decreased significantly (p-value < 0.05) after co-culture of *E. coli* with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*. 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 significant decreases in expression levels of all the studied genes (p-value < 0.05), while the standard strain had a greater impact on the expression of *ctxM* than the broiler-derived strains.

**Conclusion:** The broiler-derived isolates had a greater capacity to decrease the expression of these genes than the standard strains, proposing their adoption for dietary supplementations. However, further 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 forms the normal intestinal microflora over time. 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, including Ag43, LuxS, and intimin. The surface protein antigen 43, Ag43, is encoded by the *flu* gene in 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 a 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 have 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 regard, 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]. To overcome these hurdles, therefore, 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 the harsh environment of GIT. Moreover, *Bacillus* spp. are ideal candidates for probiotics owing to their stability during food processing and storage [11-12].

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 achieve 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*. The bacterial strains were identified and confirmed by cultivation of 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. The presence of *flu*, *luxS, eae*, and *ctxM* genes in clinical *E. coli* isolates was confirmed through designing specific primers and PCR assay. According to the results of amplification assay, the prevalence rates 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 the API CHB test. Collected colonies consisted of those identified as *B. subtilis* and *B. coagulans*. 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 confirm the characterization of the isolates. According to the results of biochemical and microbiological tests and PCR, one *B. subtilis* and one *B. coagulans* were isolated from the intestinal content of broilers.

### Acid and bile tolerance of *Bacillus* cells

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

### Expression levels of *flu*, *luxS*, *eae*, and *ctxM* genes in *E. coli* after co-culture 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 co-culture assay at the logarithmic growth phase.

Results of the expression of *flu*, *luxS*, *eae*, and *ctxM* genes revealed that the expression levels of these genes decreased significantly (p-value < 0.05) after co-culture of *E. coli* with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*. 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 significant decreases in expression levels of all the studied genes (p-value < 0.05), while the standard strain had a greater impact on the expression of *ctxM* than the broiler-derived strains. A comparison on the results of the expression levels of the studied genes indicated that *B. subtilis* had a more substantial effect on the reduced expression levels of *eae*, *ctxM*, and *flu* than *B. coagulans* .

## 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 that confer environmental adaptations and pathogenicity[13-14]. 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 to be necessary. In this regard, using the probiotic properties of various bacteria has become an interesting subject in recent years [16].

Lactic acid-producing bacteria are commonly known as probiotics. In recent years, however, researchers have focused on developing drugs based on more resistant bacterial species due to the 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 the food manufacturing and storage processes, 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 of 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, the effects of the standard strains and broiler-derived *B. coagulans* and *B. subtilis* were investigated 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, the potential effects of these strains were investigated on the expression levels of *ctxM*, a gene commonly associated with antimicrobial resistance in *E. coli*. To this end, 40 *E. coli* out of 300 fecal samples of patients with diarrhea were isolated using cultivation and biochemical methods. According to results of PCR, the prevalence rates of *flu*, *eae*, *luxS*, and *ctxM* genes in these isolates were 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. In addition to the standard strains, one *B. subtilis* and one *B. coagulans* were isolated from the intestinal contents of 10 broilers to examine the effects of spore-forming *Bacillus* spp. and determine their tolerance to the simulant GIT environment. PCR assay was utilized for the molecular confirmation of *B. subtilis* and *B. coagulans* isolates. According to the results, the studied *B. subtilis* and *B. coagulans* were tolerant to low pH [2], 0.2% bile salts, and gastric enzymes, including pepsin and trypsin, suggesting their suitability for probiotic use. Then, the effects of *B. coagulans* and *B. subtilis* on the expression levels of the studied genes were studied using the real-time PCR (RT-PCR) method. According to the results, the expression levels of all the studied genes decreased significantly after co-culture of *E. coli* isolates harboring these genes with each of *B. subtilis* ATCC 6633 and *B. coagulans* MTCC 5856, as well as broiler-derived *B. subtilis* and *B. coagulans* strains. These results reveal the important roles of *B. subtilis* and *B. coagulans* isolates in reducing the expression of genes involved in the virulence and antimicrobial resistance in *E. coli* isolates. Moreover, since broiler-derived isolates showed similar to or even better effects than the standard strains, gut microbiota of broilers can be exploited as suitable sources of these probiotic bacteria in case of unavailability of commercial strains.

Furthermore, the results indicated that *B. subtilis* had a greater influence on the expression levels of *eae*, *ctxM*, and *flu*, while *B. coagulans* 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 the 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 and 0.3% bile salts, with the highest activity against *E. coli* strains by co-culture method [21].

In another study by Kim et al., dietary supplementation of *B. subtilis* DSM 25841 reduced the *E. coli* F18 infection significantly. Overall, they showed a 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* led to a significant increase in *Lactobacillus* counts and a significant reduction in *E. coli* counts of duodenum and cecum [23]. The effects of these spore-forming *Bacillus* spp. on the expression of virulence genes and beta-lactamase genes in *E. coli* were not found in the literature. However, Medellin-Pena et al. showed 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 different 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 than the standard strains, proposing their large-scale usage in dietary supplementations. However, further studies are required to clarify the effects of these spore-forming bacteria on *E. coli* infections *in vivo* and their possible influences on lactose tolerance, nutritional absorption, and reduced cholesterol levels.

# Methods

### *E. 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 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 (PCR) assay

To confirm the presence or absence of *eae, flu, luxS, and ctx-M* genes, specific primers were designed using the Primer-BLAST software (Table 1). Total genomic DNA of *E. coli* isolates was extracted using a DNA Extraction kit (Roche, Mannheim, Germany) according to the protocol recommended by the manufacturer. To investigate the presence of *eae, flu, luxS, and ctx-M* genes, the PCR assay was performed in a volume of 25 µl in a DNA thermal cycler (Bio-Rad, USA) according to the following reaction conditions: initial denaturation at 94 °C for 5 min, 33 × denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, 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

| arget gene | Sequence (5’ à 3’) | Product size | Reference |
|---|---|---|---|
| *ae* | F; ACTAACTTCCAGTTCCGCCG<br>R; AGTCGCTTTAACCTCAGCCC | 73 bp | This study |
| *txM* | F; ACCGCCGATAATTCGCAGAT<br>R; TGCTTATCGCTCTCGCTCTG | 113 bp | [25] |
| *u* | F; ACGGTAAATGGCGGACTGTT<br>R; CACGGATGGTCAGGGTATCG | 124 bp | [26] |
| *xS* | F; GTGCCAGTTCTTCGTTGCTG<br>R; GAACGTCTACCAGTGTGGCA | 113 bp | [27] |
| *6srRNA E. coli* | F;CATTGACGTTACCCGCAGAAGAAGC<br>R; CTCTACGAGACTCAAGCTTGC | 190 bp | [28] |
| *6srRNA B. coagulans* | F;AAAAGACATTGCCACCCCCA<br>R;GGACCGATTTCAACAACGCC | 165 | This study |
| *6srRNA B. subtilis* | F;TGTTGATCACGCGGAAGTGA<br>R;AATGCCACGACCTTTTTCGC | 108 | This study |

F: Forward; R: Reverse

## Isolation of spore-forming probiotics from gastrointestinal tracts of broilers

A total of 10 broilers aged 6-12 months were chosen that did not take antibiotics or probiotics during their lifetime. After slaughter of birds 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 treatments. Ethanol treatment was performed by diluting the primary suspension (1:1) in ethanol (final concentration of 50% vol/vol) and incubation at room temperature for 1 h. Aliquots (0.1 ml) 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 *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 *B. subtilis* and *B. coagulans* were selected for further analysis. Moreover, to confirm the production of spores, *B. subtilis* and *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 in Difco heart-infusion broth (HIB) with 30% glycerol at -80°C for future use.

## Molecular detection of spore-forming probiotics

Total genomic DNA of the isolated spore-forming probiotics was extracted using the pepGOLD Bacterial DNA kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. For molecular identification of the isolated spore-forming probiotic bacteria, *16srRNA* gene was investigated using the specific primers designed by the Primer-BLAST software (Table 1). 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, 30 × denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, 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. After observing PCR products following gel electrophoresis, amplicons were finally sent for sequencing after purification.

## Probiotic characterization of isolated bacterial strains

Resistance of vegetative *B. subtilis* and *B. coagulans* to bile salts and simulated gastric conditions was determined using overnight LB cultures of *B. subtilis* and *B. coagulans* isolates. To this end, he tolerance of *Bacillus* spores to bile salts and simulated gastric conditions was tested as previously explained by Duc et al. [30] with some modifications. Briefly, about $10^8$-$10^9$ spores per ml were suspended in an isotonic buffer containing 0.76% $H_2PO_4$, 0.6% $[NH_4]_2SO_4$, 0.1% trisodium citrate, and 1.24% $K_2HPO_4$, also known as Bott and Wilson salts (pH 6.7) as well as 0.2% bile salts consisting of 50% sodium deoxycholate and 50% sodium cholate or in 0.85% NaCl (pH 2), containing 1 m pepsin (Sigma) and incubated at 37°C with shaking. Aliquots were collected immediately and following 30 min-1 h for assessing acid tolerance and 1-3 h for assessing bile tolerance. After direct plating of adequate dilutions in isotonic buffer onto LB plates CFU was determined following incubation at 37°C for 24 h. Control samples were suspended only in the isotonic buffer or 0.85% NaCl.

Similar assay was used to determine the resistance of vegetative cells to bile salts and simulated gastric conditions by resuspending overnight LB cultures of *Bacillus* isolates in fresh LB, LB supplemented with 0.2% bile salts, or acidified LB (pH 2) with HCl supplemented with 1 mg/ml pepsin.

### Bacterial co-culture assay

Co-culture of the two *Bacillus* spp. strains with *E.coli* isolates harboring all the studied genes (*luxS, flu, ctxM*, and *eae*) were performed to determine changes in expression levels of the studied virulence genes in *E. coli* strains. Briefly, overnight cultures of *B. subtilis and B. coagulans* were centrifuged, the supernatant was collected, and after filtering, the two 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 ºC under microaerophilic conditions. Each strain was also cultured alone as a control. 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*

RT-PCR experiment was carried out to investigate the expression of the studied virulence genes in *E. coli* after the co-culture assay . Briefly, after the logarithmic growth phase, 1 ml of tube content 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 remaining 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 RT-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 and 40 cycles of  95, 58 , and 72 °C for 12 s, 25 s, and 30 s, respectively. *16s rRNA* was used as the internal reference gene. After confirming the absence of primer dimers, qRT-PCR results were analyzed by the $2^{-(\Delta\Delta C(t))}$ method [31]. A P-value less than 0.05 was considered statistically significant.

### Statistical analysis

In order to evaluate the significant probiotic effects of 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 at a significe level of p-value < 0.05.

# Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

Not applicable

### Availability of data and materials

### Competing interests

The authors declare that they have no competing interests.

### Funding

Not applicable.

### Authors' contributions

Majid Baserisalehi designed the study, Zahra Elahianfiroz and Masood Ghane carried out the experiments, and Zahra Elahianfiroz wrote the manuscript under supervision of Majid Baserisalehi..

All authors read and approved the manuscript.

### Acknowledgements

The authors would like to thank all who helped us at the Department of Microbiology, Fars Science and Research Branch, Islamic Azad University, Fars, Iran, and Department of Microbiology, Shiraz Branch, Islamic Azad University, Shiraz, Iran.

## References

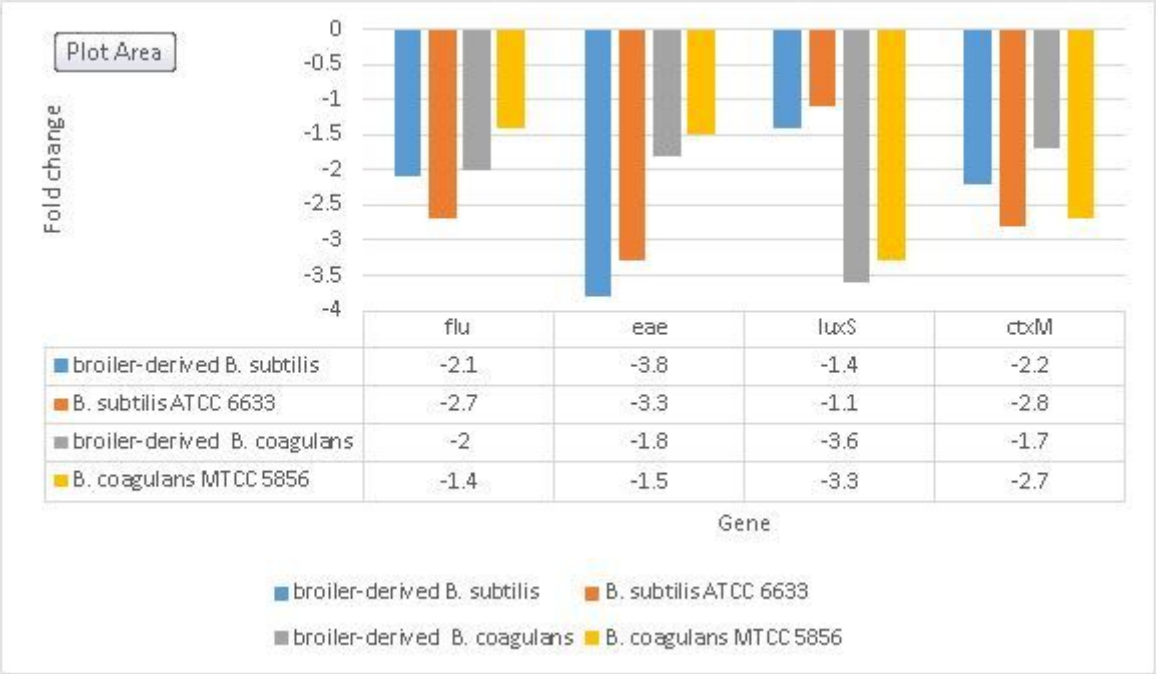
1. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic Escherichia coli. Clinical microbiology reviews. 2013;26(4):822-80.
2. Köhler C-D, Dobrindt U. What defines extraintestinal pathogenic Escherichia coli? International Journal of Medical Microbiology. 2011;301(8):642-7.
3. Pitout J. Extraintestinal pathogenic Escherichia coli: a combination of virulence with antibiotic resistance. Frontiers in microbiology. 2012;3:9.
4. De Luna MdG, Scott-Tucker A, Desvaux M, Ferguson P, Morin NP, Dudley EG, et al. The Escherichia coli biofilm-promoting protein Antigen 43 does not contribute to intestinal colonization. FEMS microbiology letters. 2008;284(2):237-46.
5. Han X, Bai H, Liu L, Dong H, Liu R, Song J, et al. The luxS gene functions in the pathogenesis of avian pathogenic Escherichia coli. Microbial pathogenesis. 2013;55:21-7.

6. Donnenberg MS, Tzipori S, McKee ML, O'Brien AD, Alroy J, Kaper JB. The role of the eae gene of enterohemorrhagic Escherichia coli in intimate attachment in vitro and in a porcine model. The Journal of clinical investigation. 1993;92(3):1418-24.
7. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417-33.
8. Ali T, Ali I, Khan NA, Han B, Gao J. The growing genetic and functional diversity of extended spectrum beta-lactamases. BioMed research international. 2018;2018.
9. Verna EC, Lucak S. Use of probiotics in gastrointestinal disorders: what to recommend? Therapeutic advances in gastroenterology. 2010;3(5):307-19.
10. Corona-Hernandez RI, Álvarez-Parrilla E, Lizardi-Mendoza J, Islas-Rubio AR, de la Rosa LA, Wall-Medrano A. Structural stability and viability of microencapsulated probiotic bacteria: a review. Comprehensive Reviews in Food Science and Food Safety. 2013;12(6):614-28.
11. Shahcheraghi SH, Ayatollahi J, Lotfi M. Applications of Bacillus subtilis as an important bacterium in medical sciences and human life. Tropical Journal of Medical Research. 2015;18(1):1.
12. Duc LH, Hong HA, Barbosa TM, Henriques AO, Cutting SM. Characterization of Bacillus probiotics available for human use. Appl Environ Microbiol. 2004;70(4):2161-71.
13. Talebi S, Makhdoumi A, Bahreini M, Matin M, Moradi H. Three novel Bacillus strains from a traditional lacto-fermented pickle as potential probiotics. Journal of applied microbiology. 2018;125(3):888-96.
14. Donnenberg MS, Whittam TS. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic Escherichia coli. The Journal of clinical investigation. 2001;107(5):539-48.
15. Djuikoue IC, Woerther P-L, Toukam M, Burdet C, Ruppé E, Gonsu KH, et al. Intestinal carriage of Extended Spectrum Beta-Lactamase producing E. coli in women with urinary tract infections, Cameroon. The Journal of Infection in Developing Countries. 2016;10(10):1135-9.
16. Gómez NC, Ramiro JM, Quecan BX, de Melo Franco BD. Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes, Salmonella Typhimurium, and Escherichia coli O157: H7 biofilms formation. Frontiers in microbiology. 2016;7:863.
17. Elshaghabee FM, Rokana N, Gulhane RD, Sharma C, Panwar H. Bacillus as potential probiotics: status, concerns, and future perspectives. Frontiers in microbiology. 2017;8:1490.
18. Konuray G, Erginkaya Z. Potential use of Bacillus coagulans in the food industry. Foods. 2018;7(6):92.
19. Spinosa MR, Braccini T, Ricca E, De Felice M, Morelli L, Pozzi G, et al. On the fate of ingested Bacillus spores. Research in microbiology. 2000;151(5):361-8.
20. Fijan S. Microorganisms with claimed probiotic properties: an overview of recent literature. International journal of environmental research and public health. 2014;11(5):4745-67.
21. Guo X, Li D, Lu W, Piao X, Chen X. Screening of Bacillus strains as potential probiotics and subsequent confirmation of the in vivo effectiveness of Bacillus subtilis MA139 in pigs. Antonie Van

Leeuwenhoek. 2006;90(2):139-46.

22. Kim K, He Y, Xiong X, Ehrlich A, Li X, Raybould H, et al. Dietary supplementation of Bacillus subtilis influenced intestinal health of weaned pigs experimentally infected with a pathogenic E. coli. Journal of Animal Science and Biotechnology. 2019;10(1):52.
23. Lin S, Hung A, Lu J. Effects of supplement with different level of Bacillus coagulans as probiotics on growth performance and intestinal microflora populations of broiler chickens. Journal of Animal and Veterinary Advances. 2011;10(1):111-4.
24. Medellin-Peña MJ, Wang H, Johnson R, Anand S, Griffiths MW. Probiotics affect virulence-related gene expression in Escherichia coli O157: H7. Appl Environ Microbiol. 2007;73(13):4259-67.
25. Tabar MM, Mirkalantari S, Amoli RI. Detection of ctx-M gene in ESBL-producing E. coli strains isolated from urinary tract infection in Semnan, Iran. Electronic physician. 2016;8(7):2686.
26. Roche AJ, McFadden JP, Owen P. Antigen 43, the major phase-variable protein of the Escherichia coli outer membrane, can exist as a family of proteins encoded by multiple alleles. Microbiology. 2001;147(1):161-9.
27. Lu S-Y, Zhao Z, Avillan JJ, Liu J, Call DR. Autoinducer-2 quorum sensing contributes to regulation of microcin PDI in Escherichia coli. Frontiers in microbiology. 2017;8:2570.
28. Bartosch S, Fite A, Macfarlane GT, McMurdo ME. Characterization of bacterial communities in feces from healthy elderly volunteers and hospitalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. Appl Environ Microbiol. 2004;70(6):3575-81.
29. Henriques AO, Beall BW, Roland K, Moran C. Characterization of cotJ, a sigma E-controlled operon affecting the polypeptide composition of the coat of Bacillus subtilis spores. Journal of Bacteriology. 1995;177(12):3394-406.
30. Duc LH, Hong HA, Barbosa TM, Henriques AO, Cutting SM. Characterization of Bacillus probiotics available for human use. Applied and environmental microbiology. 2004;70(4):2161-71.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. methods. 2001;25(4):402-8.

# 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.