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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Research article

Keywords: probiotics, spore-forming Bacillus spp., Gene Expression

Posted Date: October 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-33946/v3

Abstract

Background

Escherichia coli antibiotic resistance is one of the major health problems in many countries. Nowadays, 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.

Methods

Initially, 300 stool samples were collected from patients with gastrointestinal infections admitted to Imam Khomeini Hospital in Tehran. Then, diagnostic tests were performed to detect *E. coli* isolates on the samples. A DNA test was applied to examine the presence of *ctxM, luxS, eae* and *flu* in the samples. Afterward, the effect of native and commercial probiotics of *Bacillus subtilis* and *Bacillus coagulant* was investigated on the expression of the studied genes.

Results

Genes of *flu, eae, luxS,* and *ctxM* which are involved in bacterial attachment, biofilm formation, signaling, and antimicrobial resistance were existing in four out of 40 *E. coli* isolated among patients suffering from diarrhea. The expression levels of *flu, luxS, eae,* and *ctxM* genes decreased significantly (p-value < 0.05) after co-culture of *E. coli* with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*. Both broiler-derived *B. subtilis* and broiler-derived *B. coagulans* have a significant effect on all the studied genes.

Conclusions

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.

Background

Escherichia coli normally colonize the gastrointestinal tract of human infants and form 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 [1,2].

Similar to other mucosal pathogens, *E. coli* strains follow a multi-step pathogenetic process, starting with the colonization of a mucosal site and continue with host defense evasions, multiplication, and host injury. Although pathogenic *E. coli* strains often remain extracellular, EIEC is an intracellular pathogen with the ability to invade and replicate inside macrophages and epithelial cells. In terms of other *E. coli* strains, internalization by epithelial cells is possible at a low level, but no evidence exists regarding their intracellular replication [3].

The pathogens studied in this research include a pathogenicity island called locus of enterocyte effacement (LEE), which contains all the essential genes that cause attachment-effacing (A/E) lesions on intestinal epithelial surfaces by activating a type III secretory system [4]. A/E lesions are characterized by loss of microvilli and accumulation of cytoskeletal compounds to form pedestal structures on the host cell that act as an anchor for bacteria [5]. The *eae* gene on the LEE encodes an adhesin that is a surface protein called intimin, which is responsible for attaching bacteria to the gut and causing specific damage called effacing/attaching and cup-shaped structures in the intestinal epithelial cells [6].As an antigen encoded by the *flu* gene, Arg43 is a surface autotransporter protein with the ability to enhance cell-to-cell adhesion and is involved in the formation of the three-dimensional structure of biofilms. Numerous studies have shown the correlation between increased levels of *flu* gene expression and the formation of *E. coli* biofilm on abiotic levels [7,8]. CTX-M, SHV, VEB, TEM, and GES enzymes are the main ESBLs of class A, among which the CTX-M family had the highest number of variants described in recent years [9]. The term “CTX-M pandemic” has been used to describe this explosive dissemination of CTX-Ms worldwide [10]. *lux*S gene plays a role in the development of *E. coli* biofilm independently of autoinducer-2 and helps the adaptation to various situations. *LuxS* has been suggested to be an interspecies signal with a crucial role in the physiologic functions of bacteria by contributing to quorum sensing (QS) [11].

The production of Extended–Spectrum Beta-Lactamase enzymes (ESBL) is one of the most frequent resistance mechanisms adopted by *E. coli* strains [12]. 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 [13]. 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 [14]. To overcome these hurdles using spore-forming *Bacillus* spp. as probiotics have recently come to notice. These bacteria have an innate ability to produce a wide number of enzymes and vitamins and are highly tolerant of the harsh environment of GIT. Moreover, *Bacillus* spp. are ideal candidates for probiotics owing to their stability during food processing and storage [15]. 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.

Methods

Sample collection

The present study conducted on 300 patients with *E. coli*-related gastrointestinal diseases who were admitted to Imam Khomeini Hospital in Tehran. The stool samples of the patients were transferred to the laboratory under sterile conditions and subjected to microbiological analysis. Considering the value of P = 0.91 in this study, the sample size is calculated with 95% accuracy. In order to perform microbiological testing in hospital laboratories, clinical specimens were first cultured on two environments: blood agar

base and McKenzie agar base. After incubation at 37° C for 24 hours, a total of 5 lactose-positive and 2 lactose-negative colonies were selected from McKenzie base and cultured separately in TSI medium and incubated at 37 ° C for 24 hours [16].

Detection of *E. coli* bacteria

The detection of *E. coli* bacteria was performed using biochemical tests of glucose degradation in various medium including TSI medium, urease medium, SIM medium to evaluate H2S production, presence of tryptophanase enzyme in bacteria, and reduction of tryptophan to indole amino acid, Simon Citrate Agar medium, Lysine Decarboxylase base, and Methyl Red-Voges Proskauer medium. In the Methyl Red-Voges Proskauer base, both MR and VP tests can be performed for either strain. In order to store *E. coli* strains for the study tests, the bacteria were first cultured in vials containing tryptic soy broth (TSB) medium. After incubation at 37 °C, in the case of bacteria grow, verified *E. coli* strains were stored in Tripticase soy Broth with glycerol in a freezer at -20 ° C for later testing [5].

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 used in this study			
Reference	Product size	Sequence (5’ à 3’)	Target gene
This study	73 bp	F; ACTAACTTCCAGTTCCGCCG R; AGTCGCTTTAACCTCAGCCC	*eae*
[17]	113 bp	F; ACCGCCGATAATTCGCAGAT R; TGCTTATCGCTCTCGCTCTG	*ctxM*
[18]	124 bp	F; ACGGTAAATGGCGGACTGTT R; CACGGATGGTCAGGGTATCG	*flu*
[19]	113 bp	F; GTGCCAGTTCTTCGTTGCTG R; GAACGTCTACCAGTGTGGCA	*luxS*
[20]	190 bp	F;CATTGACGTTACCCGCAGAAGAAGC R; CTCTACGAGACTCAAGCTTGC	*16srRNA E. coli*
This study	165	F;AAAAGACATTGCCACCCCCA R;GGACCGATTTCAACAACGCC	*16srRNA B. coagulans*
This study	108	F;TGTTGATCACGCGGAAGTGA R;AATGCCACGACCTTTTTCGC	*16srRNA B. subtilis*
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 the 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*. [21] 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.*[22] with some modifications. Briefly, about 108-109 spores per ml were suspended in an isotonic buffer containing 0.76% H2PO4, 0.6% [NH4]2SO4, 0.1% trisodium citrate, and 1.24% K2HPO4, 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.

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.

Real time-PCR analysis

Real time-PCR (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). Complementary DNA (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 PCR 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, RT-PCR results were analyzed by the $2^{-(\Delta\Delta C(t))}$ method [23]. 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.

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 (Fig. 1). 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

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. Isolates showing biochemical catalase production, gelatin hydrolysis, motility, MR-/VP+, no urease production, citrate consumption, and fermentation of arabinose, cellobiose, fructose, glucose, glycerol, glycogen, inositol, maltose, mannitol, mannose, starch, sucrose, xylose, and trehalose were confirmed biochemically as *Bacillus* spp. *B. subtilis* ATCC 6633 and *B. coagulans* MTCC 5856 were used as

controls. 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 (Fig. 2).

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* [CFU of 8.8×10^8 (pH 2) compared to CFU of 5.5×10^8 (pH 7)] and *B. subtilis* [CFU of 7.4×10^6 (pH 2) compared to CFU of 6.3×10^6 (pH 7)] were resistant to 1 mg/mL of pepsin, 1 mg/mL of trypsin, 0.2% bile salts, and pH 2.

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

After co-culture of *E. coli* with *B. coagulans* MTCC 5856 and broiler-derived *B. coagulans*, *flu* expression showed 1.4 and 2 fold decrease, respectively, while co-culture of *E. coli* with *B. subtilis* ATCC 6633 and broiler derived *B. subtilis* led to 2.7 and 2.1 fold decrease in *flu* gene expression, respectively. In case of *luxS* gene, co-culture of *E. coli* with *B. coagulans* MTCC 5856 and broiler-derived *B. coagulans* as well as with *B. subtilis* ATCC 663 and broiler-derived *B. subtilis* resulted in 3.3, 3.6, 1.1, and 1.4 fold decreased expression. Similar to *luxS* and *flu* genes, decreased expression of *eae* gene was observed following co-culture of *E. coli* with either *B. coagulans* or *B. subtilis*. In fact, after separate co-culture of *E.coli* with either of the *B. coagulans* MTCC 585, broiler-derived *B. coagulans*, with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*, *luxS* expression showed 1.8, 1.5, 3.3, and 3.8 fold decrease. *ctxM* expression in E. coli after inoculation with *B. coagulans* MTCC 585 and broiler-derived *B. coagulans* reduced 2.7 and 1.7 fold, while its expression showed 2.8 and 2.2 fold decrease following co-culture with *B. subtilis* ATCC 6633 and broiler-derived *B. subtilis*, respectively (Fig. 3).

The expression of *flu*, *luxS*, *eae*, and *ctxM* 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* and *luxS*. *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* and eae 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, flu,* and *ctxM* than *B. coagulans (Table 2).*

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 [24]. Moreover, these pathogenic variants have adopted several mechanisms to resist antibiotic therapy with the production of

beta-lactamase enzymes being the most common strategy [25]. 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 [26]. Recently, researchers have focused on developing drugs based on more resistant bacterial species due to the antagonistic environment of the human gastrointestinal tract [27]. Spore-forming *Bacillus* spp. are one of these bacteria with high levels of tolerance to harsh conditions that are currently being used in dry probiotic products due to their ability to survive in conditions of high or low temperatures, aridity, and high oxygen levels [28].

Based on the studies carried out by Fijan [29] because of the probiotic properties of *Bacillus* spp such as improvement of immunity systems and the prevention of GIT disorders, it's used for the treatment of diarrhea and irritable bowel syndromes [30]. It's while limited studies have focused on the effects these probiotic bacteria may have on the virulence factors of gastrointestinal pathogenic bacteria including *E. coli*. In accordance with the prevalence rates of *flu* (62.5%), *eae* (17.5%), *luxS* (35%), and *ctxM* (37.5%) genes in four *E. coli* isolates, it could be noted that they are very critical in bacterial pathogenicity. After determining the tolerance of *B. subtilis* and *B. coagulans* 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. As a result of examining the effects of *B. coagulans* and *B. subtilis* on the expression levels using the RT-PCR method, it was observed that, 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 [11].

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.*[31] 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 this isolated *Bacillus spp.*, *B. subtilis* MA139 showed great tolerance to pH2 and 0.3% bile salts, with the highest activity against *E. coli* strains by co-culture method. In another study by Kim *et al.* [32] 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* . 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 [33]. The effects of 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 [34].

In conclusion, the current study 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.

Declarations

- Ethics approval and consent to participate

I understand that in any report on the results of this research my identity will remain anonymous.

- Consent to publish

All authors are giving permission to publisher to publish this research.

- Availability of data and materials

The anonymized patient-level data used for this project cannot be shared for reasons of information governance.

- Competing interests

I declare that I have no significant competing financial, professional, or personal interests that might have influenced the performance or presentation of the work described in this manuscript.

- Funding

We will provide funding for publication processing charges.

- Authors' Contributions

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

- Acknowledgements

The authors 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.

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Figures

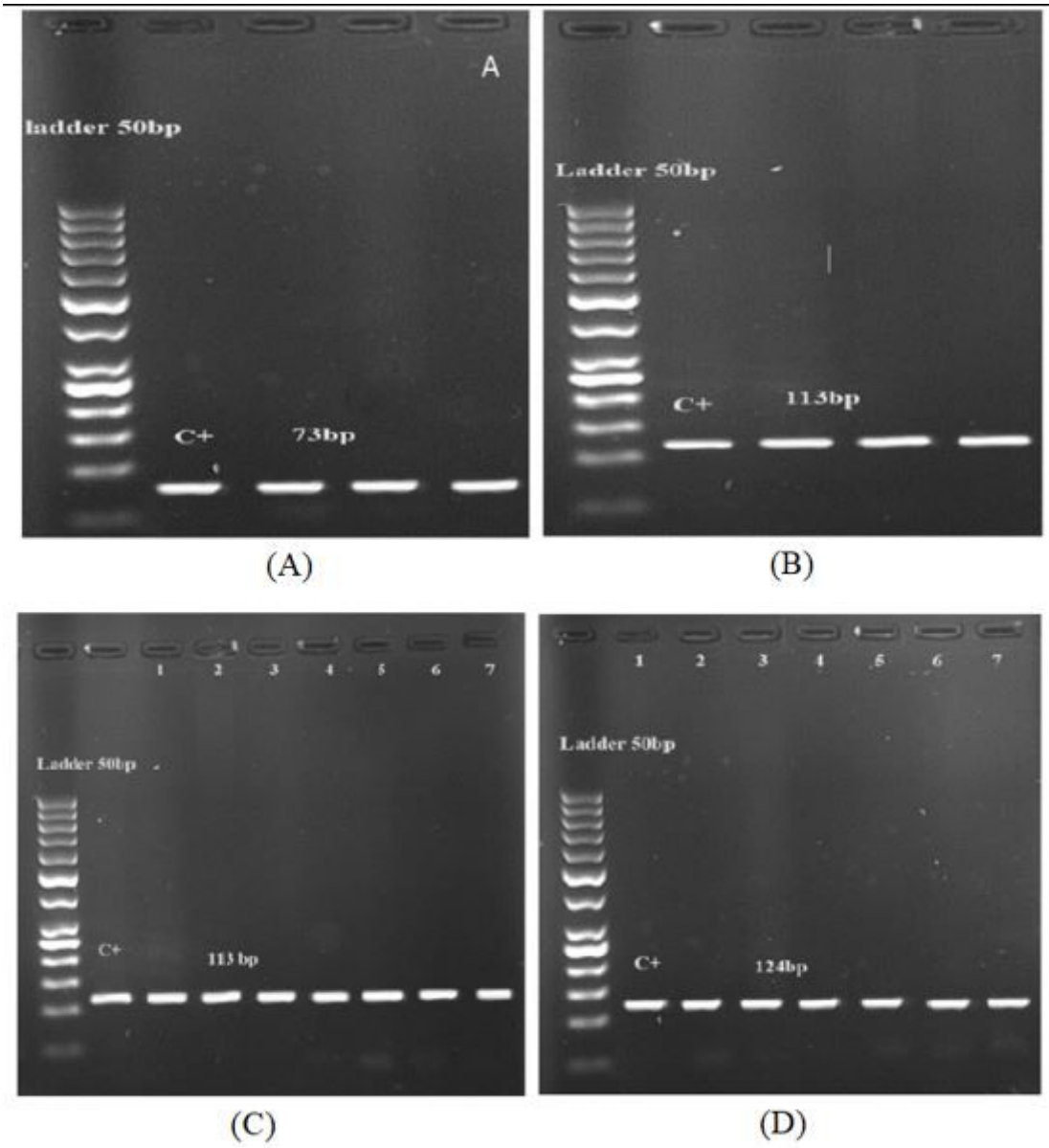

Figure 1

Agarose gel electrophoresis: A) eae, B) ctxM, C) luxS, D) flu genes by PCR. C+: Positive control.

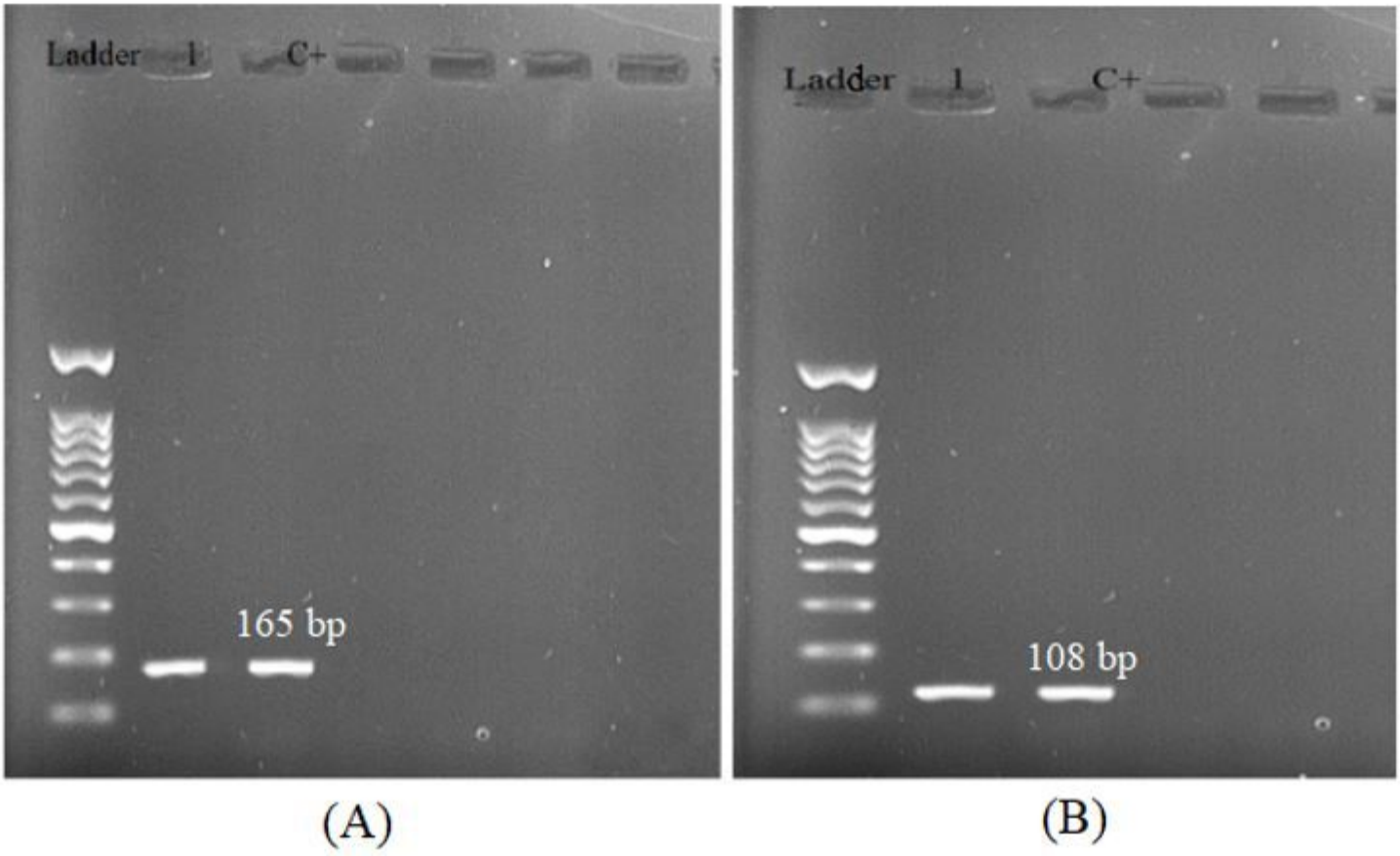

Figure 2

Using molecular analysis for confirming the characterization of the isolates through targeting the 16S rRNA gene. Gel electrophoresis of: A) B. coagulans, B) B. subtilis. Ladder: DNA marker 100 bp; C+: Control positive

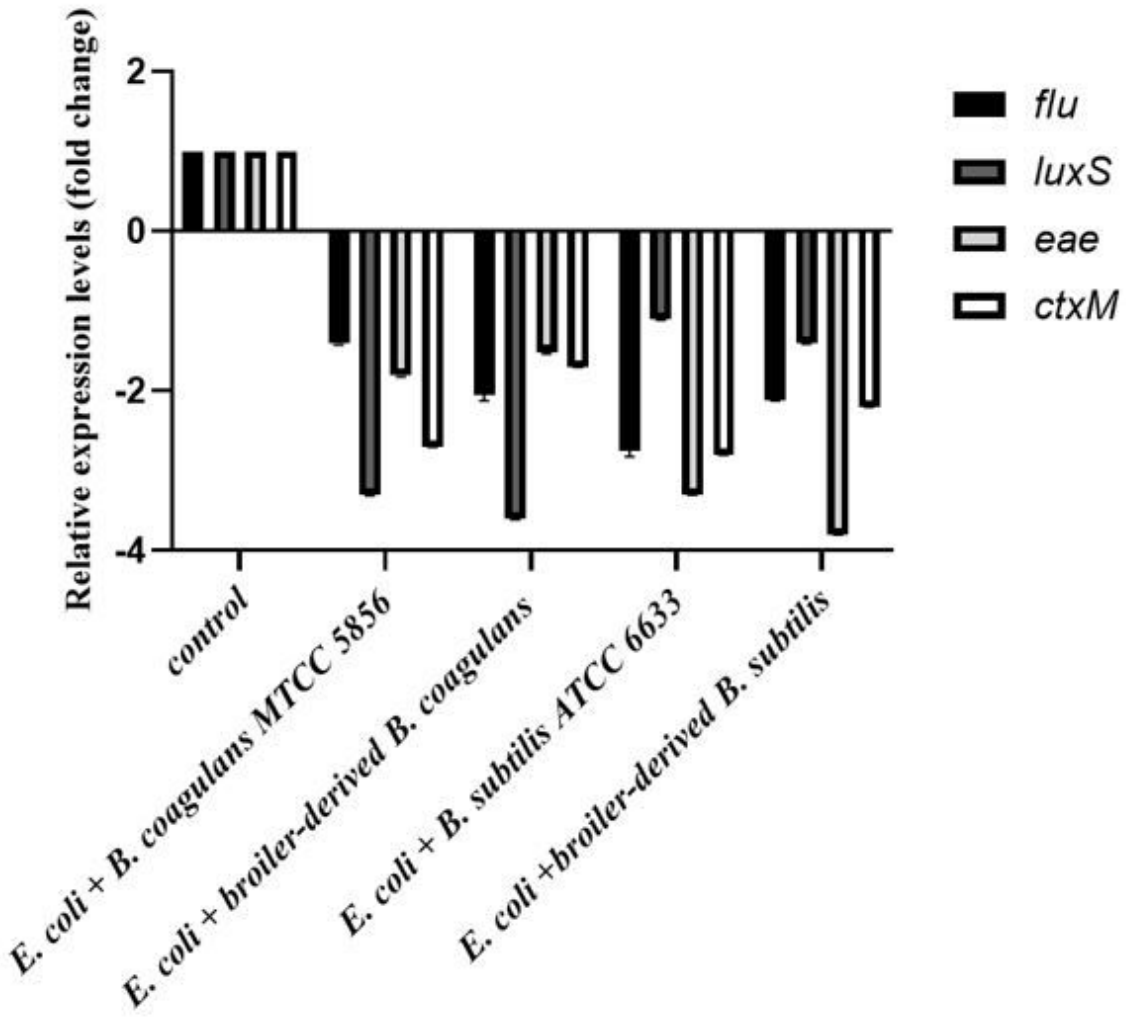

Figure 3

Relative expression levels of flu, luxS, eae, and ctxM genes. Graph data are shown as the means±SD of three independent replicates.