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

majid baserisalehi (✉ majidbaseri@hotmail.com)

Islamic Azad University Kazerun Branch

Masood Ghane

Islamic Azad University Tonekabon Branch

zahra elahian

Islamic Azad University Miyaneh Branch

Research article

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

Posted Date: June 17th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-33946/v1>

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

[Read Full License](#)

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 coculture 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 *eeae*, *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 *eeae*, *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

Reference	Product size	Sequence (5' à 3')	Target gene
This study	73 bp	F; ACTAACTTCCAGTTCCGCCG R; AGTCGCTTTAACCTCAGCCC	<i>eeae</i>
[25]	113 bp	F; ACCGCCGATAATTCGCAGAT R; TGCTTATCGCTCTCGCTCTG	<i>ctxM</i>
[26]	124 bp	F; ACGGTAAATGGCGGACTGTT R; CACGGATGGTCAGGGTATCG	<i>flu</i>
[27]	113 bp	F; GTGCCAGTTCTTCGTTGCTG R; GAACGTCTACCAGTGTGGCA	<i>luxS</i>
[28]	190 bp	F; CATTGACGTTACCCGCAGAAGAAGC R; CTCTACGAGACTCAAGCTTGC	<i>16srRNA E. coli</i>
This study	165	F; AAAAGACATTGCCACCCCCA R; GGACCGATTTCAACAACGCC	<i>16srRNA B. coagulans</i>
This study	108	F; TGTTGATCACGCGGAAGTGA R; AATGCCACGACCTTTTTCGC	<i>16srRNA B. subtilis</i>

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 *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 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, *16srRNA* 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 *B. subtilis* and *B. coagulans* to bile salts and simulated gastric conditions was determined using the overnight LB cultures of *B. subtilis* and *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 *Bacillus spp.* strains (*B. subtilis* and *B. coagulans*) with *E.coli* isolates harboring all the studied genes (*luxS*, *flu*, *ctxM*, and *eae*) were performed to determine any changes in expression levels of the studied virulence genes in *E. coli* strains. Briefly, overnight cultures of *B. subtilis* and *B. coagulans* were centrifuged and the obtained supernatant was collected and after filtering, *B. subtilis* and *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 synthesized 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

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.

Acknowledgements

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. Sonnenberg 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. Elshaghabe 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.

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*.