

Study of intestinal microbial flora of local chickens to investigate the effect of probiotics *Bacillus subtilis* and *Bacillus coagulans* on the expression of *ctxM* and *luxS* pathogenic genes in isolates of *Escherichia coli*

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Abstract

Background and objective: Antibiotics are widely used worldwide. However, due to the emergence of antibiotic resistance in a wide range of microorganisms, their use worldwide has failed. Probiotics are suggested as complementary and alternative medicine. The present study aimed to investigate the effects of probiotics isolated from local chickens on the expression of *luxS* and *ctxM* genes in resistant *Escherichia coli*.

Materials and methods: 300 fecal samples were taken from patients referring to Imam Khomeini Hospital in Tehran during May–September 2016 and *Escherichia coli* samples were isolated using specific culture media and biochemical tests and then the presence of *luxS* and *ctxM* genes were identified using PCR with specific primers. In order to extract the probiotics forming spores, the intestinal contents of 10 poultry that had not used any antibiotics and probiotics were cultured, isolated, and identified using biochemical and PCR methods. Commercial strains of *Bacillus subtilis* and *Bacillus coagulans* were purchased to compare their effects with native bacteria. These strains were then co-cultured with resistant *Escherichia coli* strains containing *ctxM* and *luxS* genes. Real-time PCR was used to evaluate the effect of these probiotics on gene expression

Results: The results indicated that 40 isolates (7.5%) of *Escherichia coli* were obtained from the 300 fecal samples. Thirteen samples (32.5%) were outpatients and 27 (67.5%) were inpatients. All isolates were isolated from men and women aged 21–62. Four *Escherichia coli* strains were isolated from patients carrying *ctxM* and *luxS* genes. Isolation of *Bacillus coagulans* and *Bacillus subtilis* from samples was confirmed by biochemical and molecular experiments. The commercial and native strains of *Bacillus coagulans* reduced the expression of the *luxS* and *ctxM* genes by 3.60, 3.30, 1.58, and 2.70 times respectively. Also, the commercial and native strains of *Bacillus subtilis* decreased the expression of the *luxS* and *ctxM* genes by 1.37, 1.10, 2.20, and 2.80 times respectively. The results of statistical analysis showed a significant relationship between the presence of native and commercial probiotics in culture and reduced expression of *ctxM* and *luxS* genes.

Conclusion: According to the results, supplements of *Bacillus coagulans* and *Bacillus subtilis* increase the effect of antibiotics resistance in *Escherichia coli* by reducing the expression of resistance genes.

Keywords

Bacillus coagulans, *Bacillus subtilis cthM*, *Escherichia coli*, *luxS*, probiotics

Introduction

The use of antibiotics is the easiest and the most accessible way to fight infections (Wang et al. 2021). In 2016, the consumption of antibiotics in Iran was reported to be 1178.1 tons per year, ranking Iran third in the world after Brazil and Turkey (Mirzaei et al. 2019). Prospective studies predict that antibiotic-resistant infections will cause more deaths than cancers by 2050, and will cost health systems about 100 trillion dollars, reducing the revenue of the countries by 2.5–3.5 percent (Goel et al. 2021).

Many medical techniques, such as cesarean section, chemotherapy, joint implants, and organ transplants, are heavily dependent on antibiotics, and a decrease in antibiotic efficacy will result in an increase in the vulnerability of these treatment methods (Moini et al. 2020). Infections such as tuberculosis, malaria, and *Escherichia coli* are likely to be the future health problems of the world and will kill more people than different types of cancer (Melnikov et al. 2020).

The search for new and alternative antibiotics is not the priority of pharmaceutical companies due to its slow and challenging trend, and investing in this field is facing serious problems (Laxminarayan et al. 2013). The loss of efficacy of existing antibiotics has directed the attentions to complementary and alternative methods of antibiotic treatment (Lezotre 2014). In recent years, many studies are conducted on substances with antibiotic effects, including nanoparticles, ionic liquids, various extracts, and probiotics (Czarnik and Mei 2007). Probiotics have always been an integral part of human food, and many of these strains coexist in the body of various organisms and therefore have fewer side effects for the host than synthetic compounds such as nanoparticles (Federation 2015). Probiotics improve the health of the host by modulating intestinal microbes, eliminating pathogens, affecting the expression of certain genes, and regulating the immune system (El-Saadony et al. 2021). Probiotics must be non-pathogenic, tolerate gastrointestinal conditions; acid and bile, be able to adhere to the gastrointestinal mucosa, and have antimicrobial activity against gastrointestinal pathogens to be a probiotic strain (Asaithambi et al. 2021).

The combination of pathogenic genes with antibiotic resistance has greatly increased the duration of treatment and mortality rate of patients with infection (Ahmad and Khan 2019). In recent years, recurrent resistant *Escherichia coli* infections are reported in various countries (Wu et al. 2003). Molecular studies of these infections have shown that pathogenic genes such as *luxS*, *eae*, and *flu* in these pathogens are combined with antibiotic-resistant genes such as *ctxM*, *SHV*, and *TEM* (Percival and Williams 2014).

Biofilm formation is one of the main factors in increasing pathogenicity and resistance to treatment in

Escherichia coli. One of the main factors in biofilm formation in *Escherichia coli* is the *luxS* gene, which causes bacterial accumulation and biofilm formation by the Quorum sensing process (Ling et al. 2010). The extended-spectrum β -lactamase genes of the *CTX-M* group are also known as the important mechanism of resistance to cephalosporin in *Escherichia coli* and are increasing rapidly, which has led to serious resistance to treatment in these bacteria (Rehman et al. 2021). The combination of *luxS* and *CTX-M* genes has increased pathogenicity and resistance to *Escherichia coli* (Percival and Williams 2014). The present study aimed to screen Spore-forming probiotics with the potential to inhibit the expression of *ctxM* and *luxS* pathogenic genes in *Escherichia coli* isolated from gastrointestinal infections, as antibiotics are not effective against this type of bacteria.

Materials and methods

Having obtained the necessary licenses and code of ethics from the Islamic Azad University of Shiraz, 300 patients who referred to Imam Khomeini Hospital in Tehran during May–September 2016 due to gastrointestinal problems were sampled after obtaining consent and filling out a demographic questionnaire. Blood agar and MacConkey's agar were cultured on two basic media. After incubation at 37 °C for 24 hours, 5 colonies of lactose positive and 2 colonies of lactose negative were selected from MacConkey agar medium and cultured separately in TSI medium (Fu et al. 2021; Li et al. 2021) SIM, MR / VP, TSI urea, and simon citrate and lysine decarboxylase, urease, arabinose, and ornithine tests were performed and the results were evaluated after incubating *Escherichia coli* isolation for 24 hours at 37 °C (Li et al. 2021). DNA of the samples was extracted using a kit. Multiplex PCR was performed and primers introduced in Table 1 were designed using Oligo 7 software and evaluated with NCBI Database Primer Blast and synthesized by South Korea Macrogen Company.

Electrophoresis of PCR products was performed on 1% agarose and related genes were recorded and analyzed ba-

Table 1. Primers used in the present study.

Bacteria	Gene	Sequence	Product size
<i>Escherichia coli</i>	<i>flu</i>	F: ACGGTAATGGCCGACTGTT R: CACGGATGGTCAGGGTATCG	124 bp
	<i>luxS</i>	F: GTGCCAGTTCTTCGTTGCTG R: GAACGTCTACCAAGTGTGGCA	113 bp
	<i>16 srRNA</i>	F: CATTGACGTTACCCGAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	190 bp
<i>Bacillus coagulans</i>	<i>16 srRNA</i>	AAAAGACATTGCCACCCCA R: GGACCGATTTCAACAACGCC	165 bp
<i>Bacillus subtilis</i>	<i>16 srRNA</i>	TGTTGATCACGCGGAAGTGA RAATGCCACGACCTTTTTCGC	108 bp

sed on the presence of a specific weight band and samples containing both *luxS* and *ctxM* genes were frozen and stored for further experiments.

In the next stage, 10 birds aged 6–18 months were purchased from rural areas of Nowshahr, Mazandaran province, Iran in September 2019. The chicks were raised in a rural backyard without the use of antibiotics. After euthanizing under sterile conditions and according to ethical principles, the contents of the intestine were collected in sterile Falcons. Then, the contents were diluted in buffered peptone water and the Non-spore-forming bacteria were killed at 80 °C and the spores in the samples were collected. 0.1 ml of each suspension was cultured on a nutrient agar medium at 37 °C and the resulting colonies were re-cultured linearly.

After bacterial identification by API20e microbial kit and confirmation under aerobic conditions, these bacilli were grown in LB or DSM agar at 37 °C. Catalase, arginine hydrolysis, hemolysis, bile acid resistance, and acid tolerance tests were performed on isolates to measure probiotic capability, and probiotic isolates were selected and their DNA was extracted. 16SrRNA primers (Table 1) were used for PCR and electrophoresis of PCR products in 2% agarose gel was analyzed and finally, the samples were sent to the South Korean Macrogen Company for sequencing. The results of sequencing were analyzed in the GenBank database by the BLAST algorithm.

In this experiment, *enterohemorrhagic Escherichia coli* bacteria O157: H7 and *Bacillus subtilis* ATCC: 6633, purchased from Pasteur Institute of Iran, Tehran, were used for positive control samples.

To study the expression of *ctxM* and *luxS* genes, *Escherichia coli* and probiotics were co-cultured. *Escherichia coli* and commercial and native spore-forming probiotics were cultured on Müller-Hinton agar medium and incubated at 37 °C (Redweik et al. 2020). After 24 hours, new probiotic colonies were transferred into the nutrient medium. Then, 24 hours later, the bacterial suspension was centrifuged and the desired bacteria supernatant was removed and after filtration, the broth was added to the culture medium. *Escherichia coli* strains were then cultured in tubes containing culture medium and supernatant (Darvishi et al. 2021). Real-time PCR was used to study the gene expression and bacterial RNA was extracted from *Escherichia coli* bacteria in the logarithmic phase of growth by light absorption of 0.08–0.1. The 16SrRNA gene was also used as an internal control. Rotor-Gene was used to measure the expression ratio of *ctxM* and *luxS* genes to 16SrRNA control gene in samples treated with native probiotics and samples treated with commercial probiotics.

The statistical significance level in all stages was considered 0.05 and REST software (Relative expression software tool 2009) was used for comparing the groups.

Results

From 300 fecal taken samples, 40 isolates (7.5%) of pure *Escherichia coli* were obtained, which were confirmed by biochemical and molecular tests. Examination of demographic

data showed that 13 samples (32.5%) were outpatients and 27 (67.5%) were hospitalized. All isolates were taken from men and women aged 21–62.

PCR results of *luxS* and *ctxM* genes showed that 4 *Escherichia coli* strains (10% of isolates) carried *ctxM* and *luxS* genes.

The results of biochemical and API experiments indicated that the bacteria isolated from the intestinal contents were native chickens of *Bacillus subtilis* and *Bacillus coagulans*. In addition, the results showed that both detected *Bacillus* were catalase-positive and could tolerate acid and bile. Isolates were not able to hydrolyze arginine and hemolysis was negative.

The expression of *luxS* and *ctxM* genes is shown in Figs 1, 2.

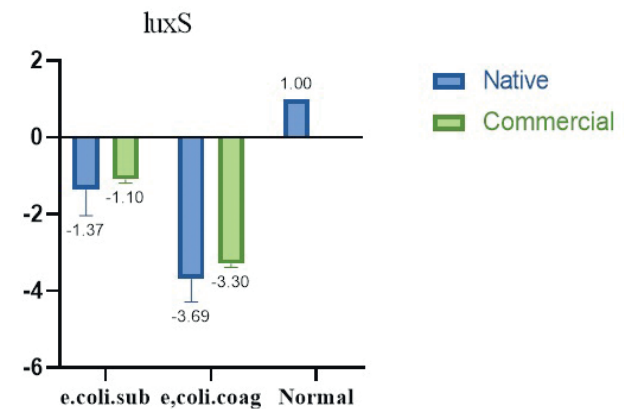


Figure 1. Comparison of *luxS* Gene Expression in Control and Probiotic-Treated Strains.

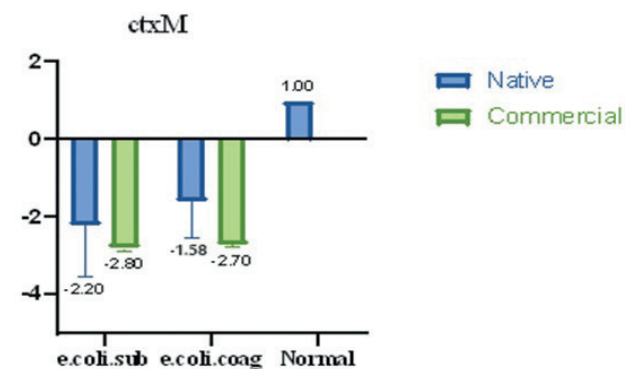


Figure 2. Comparison of *ctxM* Gene Expression in Control and Probiotic-Treated Strains.

As Fig. 1 shows, the commercial strain of *Bacillus coagulans* reduced the expression of *luxS* gene in *Escherichia coli* strains isolated from patients by 3.3 times compared to the control group ($P = 0.018$), but its native strain decreased gene expression in *Escherichia coli* strains isolated from patients by 3.6 times compared to the control group ($P = 0.021$). The commercial strain of *Bacillus subtilis* reduced the expression of *luxS* gene in *Escherichia coli* strains isolated from patients by 1.1 times compared to the control group ($P = 0.025$) and its native strain decreased the expression of this gene in *Escherichia coli* strains isolated from patients by 1.37 times compared to the control group ($P = 0.028$).

The commercial strain of *Bacillus coagulans* decreased the expression of *ctxM* gene in *Escherichia coli* strains isolated from patients by 2.7 times compared to the control group ($P = 0.026$) but the native strain of expression of this gene in *Escherichia coli* strains isolated from patients was decreased by 1.5 times compared to control group ($P = 0.02$). The commercial strain of *Bacillus subtilis* decreased *ctxM* gene expression in *Escherichia coli* strains isolated from patients by 2.8 times compared to the control group ($P = 0.031$) and the native strain of this gene in *Escherichia coli* strains isolated from patients was decreased by 2.2 times compared to the control group ($P = 0.014$).

It was also observed that *Bacillus subtilis* decreased *ctxM* gene expression more than *Bacillus coagulans* ($P = 0.031$), but *Bacillus coagulans* had a greater reduction effect on *luxS* gene expression ($P = 0.041$). The results showed a better effect of both native strains on reducing *luxS* gene expression than commercial strains ($P < 0.05$), but the commercial strain had a better effect on reducing *ctxM* gene expression ($P = 0.031$).

Discussion and conclusion

Recent studies have revealed the potential of *Escherichia coli* to combine resistance and pathogen genes and increase immunity to treatment along with increasing pathogenicity (Percival and Williams 2014). One way to combat such bacteria is to use alternative or complementary methods to antibiotic therapy. This study aimed to screen spore-forming probiotics with the potential to inhibit the expression of the pathogenic genes *ctxM*, *luxS* in *Escherichia coli* isolated from gastrointestinal infections. The results showed that *Escherichia coli* was one of the most important causes of gastrointestinal disease leading to hospitalization in Imam Khomeini Hospital in Tehran and 10% of *Escherichia coli* isolates had *luxS* genes. The results of the study conducted by Fu et al. (2019) showed that isolated strains of *Escherichia coli* were resistant to almost all available antibiotics and contained many pathogenic genes (Fu et al. 2019). In another study, Seo et al. (2010)

reported the ratio of *ctxM* and *luxS* genes in *Escherichia coli* isolates at 4% (Seo et al. 2010). Research by Shawa et al. (2021) showed that 7% of isolated *Escherichia coli* strains carried both *ctxM* and *luxS* genes (Shawa et al. 2021).

The present study also showed that bacteria in the intestines of local chickens protect these omnivorous and highly susceptible organisms and these agents can be isolated and used. Alton and Erginkaya (2021) isolated the isolates of *Bacillus subtilis* and *Bacillus coagulans* from environmental sources and proved their probiotic effects (Altun and Erginkaya 2021).

The results of the present study showed that probiotics co-cultured with *Escherichia coli* reduce the expression of *luxS* and *ctxM* genes compared to untreated samples. Tamtaji et al. (2017) studied the comparative effect of native probiotics isolated from local yogurt with commercial strains on growth inhibition and expression of *Escherichia coli* genes. The results showed that the presence of probiotics was associated with reduced expression of the studied genes (Tamtaji et al. 2017). Jiang et al. concluded that probiotics reduce the expression of Quorum sensing genes in *Pseudomonas aeruginosa*, which plays an important role in regulating the disease pathogenicity of this bacterium (Jiang et al. 2021).

Vincenzi et al. (2021) reported that lactobacilli can affect the expression of some genes by secreting several substances (Vincenzi et al. 2021). They can also control inflammatory responses (Adjei-Fremah et al. 2018).

Kim et al. (2015) reported that lactobacilli were not able to reduce the expression of pathogen genes in *Salmonella typhimurium*, which is inconsistent with the present study (Kim et al. 2015).

The present study showed the important role of two probiotics, *Bacillus subtilis* and *Bacillus coagulans* in reducing the expression of genes involved in bacterial binding and antibiotic resistance in *Escherichia coli*. Considering that the native strains used in this study had similar and better effects than the commercial strains, further study of environmental and local sources can result in the production of probiotics that reduce the pathogenic effects of antibiotic-resistant bacteria by reducing the expression of pathogen genes.

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