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Evaluation of Expression Levels of Virulence Associated *luxS*, and *ctxM* Genes in *Escherichia coli* Isolated from Dairy Products Co-cultured with *Bacillus coagulans*

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Abstract

The purpose of this study was the evaluation of expression levels of virulence associated *luxS*, and *ctxM* genes in *Escherichia coli* co-cultured with domestic probiotics. *E. coli* was isolated from 20 samples of dairy products (raw milk and cheeses). Then probiotic characteristics of *Bacillus* spp. isolated from intestinal tracts of poultry were assessed for ability to survive at acidic pH, bile salts, simulated gastric juice, production of bioactive compound and proteolytic activity. The isolate exhibited favorable characters, was identified by molecular method using 16SrDNA gene sequencing. Then expression levels of *luxS*, and *ctxM* genes in *E. coli* isolates in co-cultured with the probiotic isolates was evaluated by Real time-PCR method. The results obtained indicated that out of 16 *E. coli* isolates, two strains carried the genes. In addition, out of 12 *Bacillus* spp. isolates, one strain showed probiotic characters. The results on molecular identification verified the isolation of two strains of *E. coli* (carried *luxS*, and *ctxM* genes) and one strain of *Bacillus coagulans*. The results on expression levels of *luxS*, and *ctxM* genes in *E. coli* in co-cultured with *Bacillus coagulans* isolates indicated that expression levels of the genes diminished significantly (*P*-value<0.05). Hence, it can be interpreted that native spore-forming probiotic can greatly decrease virulence association gene expression in *E. coli*. Therefore, based on our finding it can be interpreted that the consumption of native probiotics as food supplement can greatly eliminate gastrointestinal diseases caused by *E. coli*. Hence, consumption of native probiotics potentially improves gut health. However, it needs more evaluation.

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Introduction

Escherichia coli generally lives in intestinal tracts of human and animals. Most strains are harmless; however, a few strains cause gastrointestinal disease. Although some pathogenic *E. coli* strains

are extracellular, Enteroinvasive *E. coli* is an intracellular pathogen with the capability to replicate inside of macrophages and epithelial cells. Internalization is possible at a low level, but no evidence exists regarding replication of *E. coli* inside of the cells (Köhler & Dobrindt, 2011).

It has been proved that many bacteria including *E. coli* acquired antibiotic resistance genes. In addition, potential of the bacteria for biofilm formation can be associated with pathogenicity. Therefore, potent activity in biofilm formation and antibiotic resistant character can contribute for extensive dissemination of multi-drug resistant (Ur Rahman *et al*., 2018).

As mentioned above, biofilm formation potential as well as expression of antibiotic resistant genes cooperate the potential for causing the serious infections. In this regard, *ctxM* and *luxS* genes are virulence associated gene in *E. coli*. *ctxM* gene is related to beta lactamase enzyme production and *luxS* gene play the major role in biofilm formation (Kaur, Capalash, & Sharma, 2020; Medellin-Peña, Wang, Johnson, Anand, & Griffiths, 2007). Based on foregoing evidence many ideas including probiotics consumption have been explained to prevent the human infections. Of all probiotic bacteria, spore forming bacteria are noticeable because of their resistance character in harsh environments (such as gastrointestinal environment). Therefore, the present study was undertaken to investigate the effect of spore forming probiotic bacteria on expression levels of virulence associated *ctxM* and *luxS* genes in *E. coli* isolates.

Materials and methods

Sample collection

20 samples were collected from dairy products such as raw milk and cheeses. The samples were collected using sterile tubes and transferred to the laboratory within 2 h of sampling and subjected to detection of *E. coli* by MacConkey agar and presumptive colonies were verified by biochemical tests such as catalase, oxidase, IMViC, TSI and urease tests. The isolates inoculated in Luria-bertani broth with 20% glycerol and kept at -20 °C in freezer (Croxen *et al*., 2013).

Isolation of spore-forming bacteria and their evaluation for probiotic properties

A total of 20 intestinal tracts of chickens were collected: diets of chickens were without probiotics and antibiotics). Intestinal contents of each sample were collected and diluted in ratio of 1:1 (w:v) in buffered peptone water. The samples were kept in water bath at 80 °C for 15 min, and a loopful of each suspension was taken and cultivated on blood agar and incubated at 37 °C for 24-48 h. Different colonies grew on the media were picked up and subjected to phenotypic identification using microscopy examination and biochemical tests such as catalase, sugars and hemolysis assay (Barbosa, Serra, La Ragione, Woodward, & Henriques, 2005).

Screening of spore forming probiotic

Resistance to acidic pH, bile salts and simulated Gastric fluid of the isolates were carried in the present study. Briefly, acid tolerance property of spore forming bacteria was assessed after adjusting pH of BHI broth at 2.5 and 7 (control) and inoculation of the overnight culture into BHI broth and incubated for 3 h at 37 °C. In addition, the bile salt tolerance property of the isolates was evaluated by growth in BHI broth plus 0.2% bile salts and incubated at 37 °C for 3 h. To perform resistance to simulated Gastric, 20 mL simulated gastric juice NaCl, 125 $mmol/L^{-1}$; KCl 7 $mmol/L^{-1}$; $NaHCO_3$, 45 $mmol/L^{-1}$ and pepsin, 1 mg/mL^{-1} (pH, 2) was prepared and the spore forming bacteria separately inoculated into the

tubes and incubated in incubator at 37 °C for 3 h.

Aliquot from each experiment was taken for enumeration of viable colonies using the formula below (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017; Lin, Hung, & Lu, 2011):

$$Survivability = \frac{Treatment\ CFU/mL}{Control\ CFU/mL} \quad (1)$$

Production of bioactive compound and proteolytic activity

The isolates separately were inoculated into Trypticase soy broth and incubated at 37 °C for 48 h. The suspensions were centrifuged at 3000 rpm for 5 min, then 100 μL of each supernatant was added into the well in seeded Mueller Hinton agar with *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Staphylococcus aureus.* The plates incubated at 37 °C for 24 h and observation of inhibition zone considered the production of bioactive compound. In addition, filter disk placed on center of casein agar and 50 μL of culture suspension (No, 0.5 McFarland tube (1.5×10^8 CFU/mL)) was added onto the filter disc and the plates were incubated at 30 °C for 48 h. Clear zone around each filter disk considered as protease activity (Baserisalehi & Bahador, 2013).

Phylogenetic analysis

The 16SrDNA sequence of *Bacillus coagulans* was aligned using the *W* program against corresponding nucleotide sequences of *Bacillus* spp. available in GenBank databases by Blastn search. Some of the strains were probiotic. Phylogenetic tree was constructed by the neighbor-joining method of MEGA-X program package (Saitou & Nei, 1987).

Detection of genes in *E. coli* isolates

To detect *luxS*, and *ctxM* genes in *E. coli* isolates, specific primers were used (Table 1). DNA of the isolates was extracted using kit (Roche, Mannheim, Germany) and PCR assay was carried out by thermal cycler (Bio-Rad, USA). Thermal programs used in the present study were 94 °C (5 min), then 35 cycles of 94 °C (30 s), 65 °C (30 s), 72 °C (30 s) and final extension at 72 °C (5 min). Finally, all amplicons were verified by gel electrophoresis and sent for sequencing to Macrogen company (Nakayama, Kawahara, Kumeda, & Yamamoto, 2018).

Authentication of the isolates

One strain of spore forming bacterium with probiotic character and two strains of *E. coli* isolates (carried *luxS*, and *ctxM* genes) were subjected to molecular identification using 16SrDNA gene (Table 1). PCR assay was performed using thermal cycler (Bio-Rad, USA). Thermal programs used in the present study were 94 °C (5 min), then 35 cycles of 94 °C (30 s), annealing at 61 °C (30 s), extension at 72 °C (30 s) and final extension at 72 °C (7 min). The PCR products were sent for sequencing to Macrogen company followed by gel electrophoresis.

Table 1. The primers used in the present study

Target gene	Sequences	product size	references
Universal 16srDNA	27F5′-AGAGTTTGATCCTGGCTCAG-3′ 1492R 5′-TACGGYTACCTTGTTACGACTT-3′	996 bp	(Chelius & Triplett, 2001)
ctxM	F; ACCGCCGATAATTCGCAGAT R; TGCTTATCGCTCTCGCTCTG	113 bp	(Shen *et al*., 2017)
luxS	F; GTGCCAGTTCTTCGTTGCTG R; GAACGTCTACCAGTGTGGCA	113 bp	(Wang *et al*., 2016)

F: Forward; R: Reverse

Bacterial co-culture assay

Co-culture of the *Bacillus coagulans* with *E. coli* isolates (harboring *luxS*, and *ctxM* genes) was performed to determine expression levels of the genes in *E. coli* strains. Overnight cultures of *B. coagulans* were centrifuged and the cells were inoculated into tubes containing 5 mL of nutrient broth. In addition, overnight cultures of two *E. coli* isolates were inoculated into the tubes and *B. coagulans* inoculated into each tube (two *E. coli* culture tubes were used as control). The tubes incubated at 37 ºC for 12-18 h and the effects of *B. coagulans* on the expression of the virulence genes in *E. coli* was determined after picking up the samples and their subjection to Real time-PCR (RT-PCR) experiment (Fooks & Gibson, 2002).

Real time-PCR analysis

To perform experiment, one set was *E. coli* isolates and the second set was *E. coli* isolates co-culture with *B. coagulans.* To perform the experiment RNA extraction was carried out on 1 mL of each set using commercially kits (QIAGEN RNeasy Mini kit). Then, the samples were treated with DNase and quality of total RNA was evaluated using the NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA was synthetized using random hexamers and SuperScript II Reverse Transcriptase according to the recommended protocols of Invitrogen, Carlsbad, CA, USA. Finally, quantitative RT-PCR was prepared by the SYBR Green method. Thermal programs were 95 °C (10 min) and 40 cycles of 95 (12 s), 58 (25 s) and 72 °C (30 s). RT-PCR results were analyzed by the $2^{-(\Delta\Delta Ct)}$ method folowed by confirming the absence of primer dimers. A *P*-value less than 0.05 was considered statistically significant (Livak & Schmittgen, 2001).

Statistical analysis

Statistical analysis of data obtained in the study was performed using one sample t-test at a significe level of *P*-value<0.05.

Results and discussion

In total, 16 strains of *E. coli* were isolated from all samples. *LuxS* and *ctxM* genes existed in them with frequency of 60% (n=12) and 50% (n=10) respectively. Two of the isolated strains carried both *luxS* and *ctxM* genes (Figs. 1 and 2). In addition, of 12 strains of *Bacillus* spp., one strain exhibited probiotic characters. This strain remained alive in BHI (pH, 2.5), BHI with 0.2% bile salt and simulated gastric juice for 3 h. In addition, the isolate grew well at 55 °C with proteolytic activity. In addition, the *Bacillus* isolate showed potent activity in the production of bioactive compounds. *Staphylococcus aureus* and *Bacillus cereus* were sensitive and, *E. coli* and *Pseudomonas aeruginosa* were resistant to the compound. Molecular identification of the isolates indicated that the isolate was *Bacillus coagulans* strain HM-08. Phylogenic analysis of *Bacillus coagulans* isolates by the Neighbor joining indicated that the isolate fall into the clade along with a spore forming probiotic retrieved in GenBank (Fig. 3).

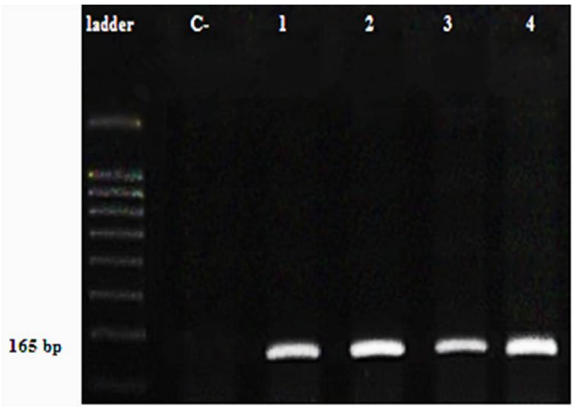

Fig. 1. Gel electrophoresis of PCR product of *luxS*, gene in *E. coli* isolates
Lanes 1, 2, 3 and 4: samples; Lane C-:negative control, and Ladder: 100bp

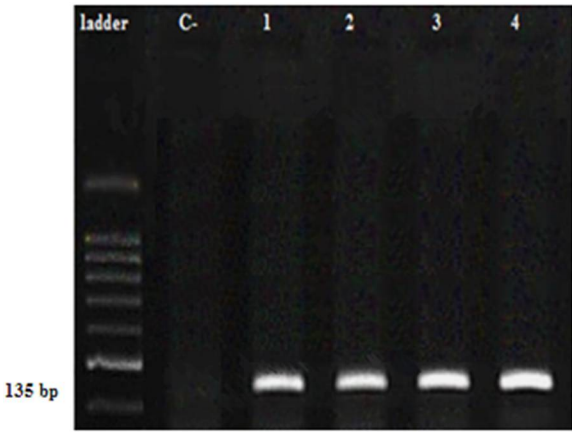

Fig. 2. Gel electrophoresis of PCR product of *ctxM* gene in *E. coli* isolates
Lanes 1, 2, 3 and 4: samples; Lane C-: negative control, and Ladder: 100bp

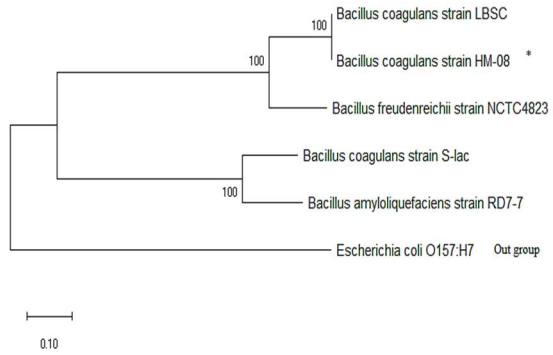

Fig. 3. Phylogenetic tree of *Bacillus coagulans* isolates based on 16SrDNA gene sequence data compare with other *Bacillus* spp.

In addition, 16SrDNA gene sequencing verified detection of *E. coli* from dairy products (Table 2). The results on *luxS*, and *ctxM* genes expression in *E. coli* isolates co-culture with *Bacillus coagulans* indicated that expression of the genes in two strains of *E. coli* isolates relatively decrease. As shown in Table (2) average reduction was 3.6 and 1.5 folds for *luxS* and *ctxM* genes respectively. To interpret the results it must be noted that some strains of *E. coli* cause human infections. These strains have numerous virulence factors (Djuikoue *et al*., 2016) as well as several antibiotic resistant mechanisms such as Efflux pump activity and β-lactamase production (Piri, Tajabadi Ebrahimi, & Amini, 2019). Hence, to eliminate *E. coli* infections, recently researchers have investigated on the new remedies to find active drugs in human gastrointestinal tracts. In this regard, consumption of probiotics is an interesting suggestion (Köhler & Dobrindt, 2011). Fijan (2014) reported that *Bacillus* spp. prevent gastrointestinal tracts disorders and improve immunity systems. Guo, Li, Lu, Piao, & Chen (2006) stated that spore-forming *Bacillus* spp. inhibit *E. coli* K88 and K99. Kim *et al*. (2019) showed that *Bacillus subtilis* reduced the *E. coli* F18 infection. In the present study, two *E. coli* strains carried the virulence *ctxM* and *luxS* genes isolated from dairy products and expression of the genes was evaluated in co-culture with *B. coagulans* isolates. Our findings showed reduction of the *luxS,* and *ctxM* gene expression in *E. coli* isolates 3.6 and 1.5 fold respectively (Table 3). Medellin-Peña *et al*. (2007) and Kaur *et al*. (2020) designed their investigation similar to our experiment and their results were supported our finding. However, it must be noted that their probiotic and *E. coli* strains were varied to our strains, but their results were similar to our findings.

Table 2. Authentication of the bacterial isolates by 16SrDNA gene sequencing

Spore forming probiotic	**Accession Number**
Bacillus coagulans strain HM-08	CP010525.1
Bacterial strains	**Accession Number**
Carried 2 virulence genes	
Escherichia coli strain 13479	MF372553.1
Escherichia coli strain NW_A26	MG543839.1

Table 3. Statistical analysis for gene expression using one sample t-test (df=19, *P*-value<0.05)

Co-culture of	N	Mean	Std. Deviation	Result
ctxM gene				
E.coli. B.coagulans	20	-1.5800	0.93505	T= -12.34
luxS gene				
E.coli. B.coagulans	20	-3.6800	0.77500	T= -27.00

The data represented in the table obtained from data analysis by the $2^{-(\Delta\Delta Ct)}$ method.

Conclusions

Based on foregoing evidence it can be interpreted that native probiotic bacteria such as *Bacillus coagulans* can greatly decrease the expression levels of virulence associated *ctxM* and *luxS* genes in *E. coli*. Therefore, consumption of probiotics as food supplement can reduce frequency of occurrence of antibiotic resistant and biofilm formation in pathogenic *E. coli*. This finding verified the beneficial effect of native probiotics. However, it requires more investigation.

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ارزیابی بیان ژن‌های مشارکت‌کننده در بیماری‌زایی *luxS* و *ctxM* *اشریشیاکلی* جداشده از نمونه‌های لبنی در کشت هم‌زمان با *باسیلوس کواگولانس*

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چکیده

هدف از این مطالعه ارزیابی بیان بعضی ژن‌های مشارکت‌کننده در بیماری‌زایی (*luxS* و *ctxM*) *اشریشیاکلی* در کشت هم‌زمان با پروبیوتیک‌های بومی می‌باشد. برای انجام این تحقیق باکتری *اشریشیاکلی* از 20 نمونه محصولات لبنی (شیر و پنیر) جدا گردید. سپس ویژگی پروبیوتیکی باسیلوس‌های جداشده از دستگاه گوارشی طیور با استفاده از قابلیت بقای آنها در pH اسیدی، نمک‌های صفراوی، مایع معدهٔ تحریک‌شده، تولید ترکیبات فعال‌زیستی و فعالیت پروتولیتیکی موردارزیابی قرار گرفت. جدایه‌هایی که خصوصیت‌های مطلوب پروبیوتیکی داشتند با استفاده از روش مولکولی توالی ژن 16SrDNA شناسایی شدند و میزان بیان ژن‌های *luxS* و *ctxM* در جدایه‌های *اشریشیاکلی* در کشت هم‌زمان با آنها با استفاده از روش ریل‌تایم پی‌سی‌آر ارزیابی گردید. نتایج به‌دست‌آمده نشان داد که از 16 سویهٔ باکتری *اشریشیاکلی*، دو سویه حاوی هر دو ژن و از 12 سویهٔ باسیلوس، یک سویه دارای ویژگی پروبیوتیکی بودند. نتایج شناسایی مولکولی جداسازی دو سویهٔ *اشریشیاکلی* و یک سویهٔ *باسیلوس کواگولانس* را تأیید نمود. ازطرف‌دیگر میزان بیان ژن‌های فوق در جدایه‌های *اشریشیاکلی* در کشت هم‌زمان با جدایهٔ *باسیلوس کواگولانس* به‌صورت معنی‌داری (*P*-value<0/05) کاهش یافته است. بنابراین براساس یافته‌های این تحقیق نتیجه‌گیری می‌شود که استفاده از پروبیوتیک‌های بومی به‌عنوان مکمل غذایی قادر است بیماری‌های گوارشی ایجادشده توسط باکتری *اشریشیاکلی* را محدود نماید، اگرچه مطالعهٔ بیشتری برای تأیید این یافته باید انجام شود.

واژه‌های کلیدی: *باسیلوس کواگولانس*، بیان ژن، ژن‌های بیماری‌زا، *ctxM* و *luxS*