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Biofilm formation, antibiotic resistance, and virulence genes in *Escherichia coli* isolated from raw milk, traditional cheese, and butter

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Abstract

Escherichia coli is one of the most important pathogens with a multi-drug resistance pattern in animal-origin food, especially dairy products. This study aimed to assess the prevalence of *E. coli* in raw milk and traditional dairy products, including 60 raw milk, 60 traditional fresh cheese, and 60 traditional butter, and to characterize the antibiotic resistance profiles, biofilm-forming capacity, and virulence genes of the isolates. *Escherichia coli* was isolated from 21.12% of all samples, including 26.67% of raw milk, 16.67% of cheese, and 20.00% of butter. There was no statistically significant difference between the groups. All isolates were resistant to ampicillin, and none were resistant to aztreonam, with 36.84% of isolates identified as the multi-drug resistant. In addition, 60.52, 26.31, and 13.15% of isolates produced strong, moderate, and weak biofilm, respectively. The frequencies of *stx1*, *stx2*, *csgA*, and *rfbE* genes were 13.15, 18.42, 71.05, and 68.42%, respectively, and the differences between the frequencies were significant. Raw milk and traditional dairy products are potential sources of pathogenic *E. coli*, and continuous monitoring of these food products is required to ensure public health.

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Introduction

Milk and dairy products provide essential nutrients, including proteins, fatty acids, vitamins, and minerals, making them a complete food source across all age groups. However, these products are also potential sources of food-borne pathogens and can cause foodborne diseases in consumers. Generally, pasteurization of milk inactivates food-borne pathogens; so, they do not pose a health threat if they are packaged properly and stored under appropriate conditions. A key issue is the increasing consumer preference for traditional products, especially dairy products. Traditional dairy products are often prepared under inadequate hygienic conditions, using unregulated raw milk, without sufficient heat treatment or proper packaging, increasing the risk of foodborne diseases.[1] *Salmonella*, *Campylobacter*, Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, and enterotoxin-producing *Staphylococcus aureus* are the most common organisms implicated in the outbreak of dairy-borne diseases. The *E. coli* is one of the most significant foodborne pathogens, transmitted to humans primarily through the consumption of contaminated food, mainly animal-derived products, such as milk and dairy items.[2]

Hemolytic uremic syndrome and bloody diarrhea are known complications of *E. coli O157:H7*, threatening the health of consumers, especially people with suppressed immune systems. According to the literature, hemolytic uremic syndrome usually occurs in the presence of *E. coli O157*-specific *rfbE* gene and STEC virulence genes, including *stx1* and *stx2*.[3,4]

Recently, the global rise in multi-drug resistant (MDR) *E. coli* strains, alongside their prevalence and pathogenic potential, has raised serious concerns about the threat posed by foodborne pathogens.[5,6] These strains complicate patient treatment, extend the duration of illness, and can contribute to the spread of infection and, in some cases, death.[1,5,6]

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Furthermore, foodborne pathogenic bacteria, including *E. coli*, can form biofilms, facilitating persistent contamination by serving as a nutrient source supporting bacterial growth and survival. Biofilm formation also significantly increases resistance to disinfectants, making cleaning and sanitation processes less effective.[7,8] In addition to contributing to the transmission of foodborne diseases, biofilms pose a major challenge to the food industry due to the damage they can cause to processing equipment.[9] Several factors influence biofilm formation, among which the *csgA* gene plays a critical role. This gene is involved in the production of curli fimbriae, facilitating bacterial adhesion to the eukaryotic cells and being recognized as important virulence factors.[10,11]

Although several studies have investigated the prevalence of *E. coli* in raw milk and traditional dairy products,[2,12-14] limited information is available about the phenotypic ability of isolates to form biofilm and biofilm-producing genes and their relationship with antibiotic resistance. Since understanding the characteristics of food-borne pathogenic bacteria and their sources can effectively lead to controlling the spread of bacteria and improving public health, the present study investigated their phenotypic biofilm production ability, the abundance of biofilm-producing (*csgA*) and virulence genes (*stx1*, *stx2*, and *rfbE*), and the multi-drug antibiotic resistance pattern of *E. coli* isolated from raw cow's milk and traditional dairy products collected from Qazvin, Iran.

Materials and Methods

Materials. The culture media used in this study included Peptone Water, Tryptic Soy Broth (TSB), Plate Count Agar, Eosin Methylene Blue , Mueller-Hinton Agar, Tryptone Phosphate, and MacConkey Agar, all obtained from Mirmedia Co. (Khorramshahr, Iran). Additional materials, such as phosphate-buffered saline, crystal violet, Simmons citrate agar, triple sugar iron agar, and glycerol were sourced from Merck (Darmstadt, Germany), while antibiotic discs were purchased from Padtan Tab Co. (Tehran, Iran). The molecular analysis materials included a DNA extraction kit (SambioT, Gimpo-si, South Korea), Red Master Mix (Ampliqon, Odense, Denmark), primers (Pishgam Co., Tehran, Iran), 100 bp DNA ladder (Qiagen Co., Hilden, Germany), agarose gel (Merck), Safe Stain (CinnaGen, Tehran, Iran), and loading dye (Pishgam Co.).

Sample collection. This cross-sectional laboratory-based study was conducted in Qazvin (N, 50.00° E 36.27°), Iran, between January and June 2024. For sampling purposes, the city was divided into four geographical sectors (north, south, east, and west), and traditional dairy distribution centers were randomly selected within each sector. A total of 180 dairy samples were collected, including raw cow's milk (n = 60), traditional fresh cheese (n = 60), and traditional butter (n = 60). Each sample (100 g) was aseptically collected in sterile zip-lock bags and transported to the laboratory within 2 hr under refrigerated conditions to preserve the cold chain. Before microbiological analyses, samples were examined for physicochemical characteristics, including color, odor, texture, and pH. Samples complying with the relevant quality standards were selected for further investigation.

Isolation and identification of *E. coli*. According to the Food and Drug Administration's Bacteriological Analytical Manual, approximately 25.00 mL of each sample was added to the 225 mL of TSB and incubated at 35.00 ˚C for 3 hr. Then, 25.00 mL of the suspension was transferred to 225 mL of double-strength Tryptone Phosphate broth and incubated at 44.00 ˚C for 20 hr. Afterwards, one loopful from the suspensions was streaked onto both MacConkey/ Eosin Methylene Blue agar plates and incubated at 37.00 ˚C for 24 hr. Subsequently, colonies suspected of being *E. coli* (with metallic sheen on Eosin Methylene Blue or pink on MacConkey) were selected and confirmed by biochemical tests of indole, methyl red, Voges-Proskauer, Simmons citrate, and Triple Sugar Iron, using specific culture media provided from Merck. Finally, positive colonies were preserved in TSB, containing 20.00% glycerol, and stored at − 20.00 ˚C short-term preservation until completion of the experiments.[11,15]

Anti-microbial resistance pattern. According to Clinical and Laboratory Standards Institute guidelines, antibiotic resistance pattern of the isolates was evaluated using disk diffusion method with 12 commercial antibiotic disks, including ciprofloxacin (5.00 µg), amoxicillin/ clavulanic acid (20.00/10.00 µg), sulphamethoxazole/ trimethoprim (1.25/23.75 µg), tetracycline (30.00 µg), chloramphenicol (30.00 µg), gentamycin (10.00 µg), cefotaxime (30.00 µg), imipenem (10.00 µg), nalidixic acid (30.00 µg), ampicillin (10.00 µg), aztreonam (30.00 µg), and ceftazidime (30.00 µg). After overnight cultivation of the bacteria, the optical density (OD) was adjusted to 0.10 ± 0.02, using a spectrophoto-meter (HACH, Dusseldorf, Germany) at 600 nm and then, diluted up to 1.00×10^6 colony-forming unit mL^{-1}. After that, 100 µL of the suspension was spread onto the Mueller-Hinton Agar plates, and the discs were placed on the plates. After 24 hr of incubation at 37.00 ˚C, the diameter of the inhibition zone was measured, using a caliper, and interpreted according to the guideline. In parallel, to ensure the accuracy of the results, the test was conducted using a standard strain, *E. coli* ATCC 25922, as a reference strain.[16]

Biofilm formation capacity. Biofilm assays were performed using the crystal violet staining method, as described by Manafi *et al.*[1] First, a 1:10 ratio of the isolate (1.00×10^6 colony-forming unit mL^{-1}) and TSB was created in several wells of a 96-well microplate (three wells for each isolate) in a final volume of 200 µL. Only TSB and *Pseudomonas aeruginosa* ATCC 27853 with TSB (1:10 ratio) were used as a negative and positive control,

respectively. After 72 hr incubation at 37.00 ˚C, the microplate was inverted to drain the suspension and then washed with phosphate-buffered saline (three times). The microplate was dried, and the wells were filled with methanol (200 µL) and kept at room temperature for 15 min. After draining, the wells were stained with 200 µL of crystal violet (1.00%) for 30 min. Then, the stain was discarded, and the microplate was washed three times with phosphate-buffered saline and kept at room temperature for 60 min to dry completely. Subsequently, the wells were filled with 200 µL of absolute ethanol, and the absorbance was measured at a wavelength of 570 nm, using a microplate reader system (DA3200; DANA, Tehran, Iran). Finally, based on the adsorption rate, the isolates' ability to form a biofilm was classified into four groups, including no biofilm (OD ≤ control OD [ODC]), weak (ODC < OD ≤ [2 × ODC]), moderate ([2 × ODC] < OD ≤ [4 × ODC]), and strong (OD ≥ [4 × ODC]).[1]

Detection of target genes. For molecular identification of target genes, a single colony of each isolate was incubated in a TSB medium overnight at 37.00 ˚C. Genomic DNA was extracted using a commercial kit (SanBio Co., Tokyo, Japan) according to the instructions. Then, the quantity and quality of extracted DNA were analyzed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and the 0.70% agarose gel, respectively. Consequently, multiplex PCR was used to identify virulence genes (*stx1* and *stx2*). First, the reaction materials, including 10.00 µL of Master Mix, 1.00 µL of the forward and reverse primers, and 50.00 ng μL^{-1} of DNA, were mixed, and distilled water was added to the above contents to bring the final reaction volume to 20.00 µL. The samples were then amplified for 35 cycles, with the following conditions: Cycles 1 - 10: Denaturation at 95.00 ˚C for 1 min, annealing at 65.00 ˚C for 2 min, and extension at 72.00 ˚C for 1.50 min, cycles 11-25: Denaturation at 95.00 ˚C for 1 min and annealing temperature was decreased to 60.00 ˚C for 2 min, followed by extension at 72.00 ˚C for 1.50 min, and cycles 25 - 35: Denaturation at 95.00 ˚C for 1 min, annealing at 60.00 ˚C for 2 min, and extension at 72.00 ˚C for 2.50 min.[17] Also, conventional PCR was used to detect *csgA* and *rfbE* genes by preparing a 20.00 µL mixture (separately for each gene) as described above. Next, the amplification was performed on a thermal cycler (Veriti; ABI, Foster City, USA) with an initial denaturation for 5 min at 95.00 ˚C (1 cycle), followed by denaturation at 95.00 ˚C for 30 sec, annealing at 59.00 ˚C (*rfbE*)/60.00 ˚C (*csgA*) for 40 sec, and extension at 72.00 ˚C for 1 min (35 cycles), and final extension at 72.00 ˚C for 5 min (1 cycle).[18] The sequences of the primers and their amplicon sizes are available in Table 1. Finally, the products were analyzed by electrophoresis on a 2.00% (w/v) agarose gel, containing Safe Stain, using a 100 bp DNA ladder. More precisely, 2.00 g of agarose was dissolved in 100 mL of 1.00X Tris-Borate-EDTA (TBE) buffer. After complete dissolution and cooling, 5.00 µL of DNA Safe Stain was added to the gel before casting. For each sample, 5.00 µL of PCR product was loaded into the gel wells. A 100-bp DNA ladder (5.00 µL) was included as a molecular size marker. The electrophoresis was conducted in 1.00X TBE running buffer at 100 V for 50 min, and the results were visualized using an ultraviolet trans-illuminator system (TM-26; Labnet, Edison, USA). The *E. coli* O157:H7 ATCC 43895 was included as a positive control to verify the accuracy of the results. Water was used as a negative control.

Statistical analysis. All tests were performed in triplicate and the mean was reported. The study data were analyzed with SPSS (version 18.0; IBM Corp., Armonk, USA), using chi-square to express the prevalence difference of *E. coli* between samples, and one-way ANOVA to express the difference in the biofilm formation ability of the isolates. The confidence level was set at 95.00%. In addition, the relationship between biofilm formation and the MDR characteristics of the isolates was determined by the chi-square test.

Results

Prevalence of *E. coli.* In the present study, *E. coli* was isolated from traditional dairy products (raw milk, cheese, and butter), using a culture-based method and confirmed by biochemical tests. Overall, *E. coli* was detected in 38 of 180 samples (21.12%). Among the examined products, *E. coli* was identified in 16 of 60 raw milk samples (26.67%), 10 of 60 cheese samples (16.67%), and 12 of 60 butter samples (20.00%). The number of positive samples for each product is presented in Figure 1. No statistically significant difference was observed in the prevalence of *E. coli* among the different sample types ($p > 0.05$).

Table 1. Target genes, primer sequences, and their amplicon size.

Genes	Primer sequences (5′ to 3′)	Amplicon sizes (bp)	References
stx1	F: ATAAATCGCCATTCGTTGACTAC R: AGAACGCCCACTGAGATCATC	180	17
stx2	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	255	17
rfbE	F: CGGACATCCATGTGATATGG R: TTGCCTATGTACAGCTAATCC	259	17
csgA	F: AGATGTTGGTCAGGGCTCAG R: CGTTGTTACCAAAGCCAACC	200	18

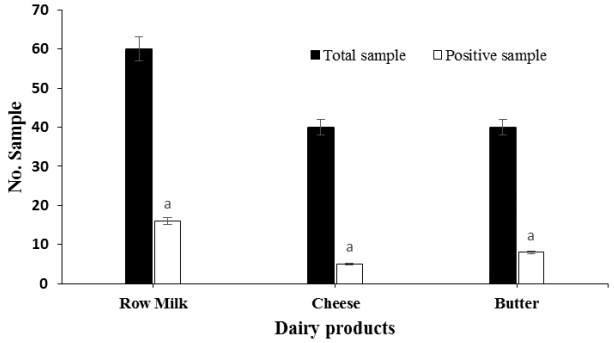


Fig. 1. The number of positive samples for *Escherichia coli* in the examined dairy products. Similar lowercase letters indicate no significant difference between the prevalence of *E. coli* in different sources ($p > 0.05$).

Anti-microbial resistance pattern. All isolates (100%) were resistant to AMP. High resistance rates were also observed for tetracycline (84.21%), imipenem (65.79%), amoxicillin/clavulanic acid (63.16%), sulphamethoxazole/trimethoprim (57.89%), ciprofloxacin (44.74%), cefotaxime (42.11%), nalidixic acid (42.11%), ceftazidime (34.21%), and chloramphenicol (31.58%). In contrast, resistance to gentamicin was detected in only 2.63% of isolates, while no resistance to aztreonam was observed (Fig. 2).

According to the Table 2, 14 of 38 isolates (36.84%) were resistant to at least one anti-microbial agent in three or more anti-microbial classes and therefore classified as MDR. Furthermore, the frequency of resistant isolates did not differ significantly among the different sample sources ($p > 0.05$).

Biofilm assay. Based on the comparison of absorbance values between isolates and controls, all isolates were capable of forming biofilms. Of these, 60.52% formed strong biofilms, 26.31% formed medium biofilms, and 13.15% formed weak biofilms. Table 3 provides quantitative and qualitative analyses of the biofilm formation by each isolation categorized by origin. The *E. coli* isolates from raw milk demonstrated stronger biofilm formation (OD_{570} = 1.349 ± 0.629) compared to those from cheese (OD_{570} = 0.846 ± 0.231) and butter (OD_{570} = 1.033 ± 0.447), although the differences were not statistically significant ($p > 0.05$). However, a statistically significant association was found between biofilm formation and the MDR characteristics of the isolates, with most strong biofilm producers also exhibiting MDR properties ($p < 0.05$).

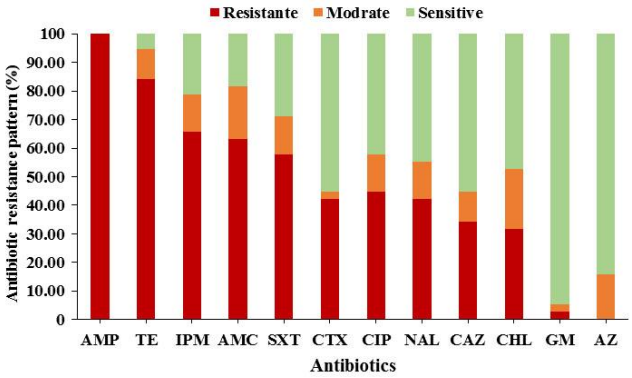


Fig. 2. Phenotypic antibiotic resistance pattern of isolates against target antibiotics by disk diffusion method. AMP: ampicillin; TE: tetracycline; IPM: imipenem; AMC: amoxicillin/clavulanic acid; SXT: sulphamethoxazole/trimethoprim; CTX: cefotaxime; CIP: ciprofloxacin; NAL: nalidixic acid; CAZ: ceftazidime; CHL: chloramphenicol; GM: gentamycin; AZ: aztronam.

Detection of target genes. Figures 3 and 4 show the agarose gel electrophoresis results of multiplex and conventional PCR products, respectively. A total of 38 confirmed *E. coli* isolates were subjected to the molecular analysis. The *stx1*, *stx2*, *csgA*, and *rfbE* genes were detected in 5 (13.16%), 7 (18.42%), 27 (71.05%), and 26 (68.42%) isolates, respectively. Furthermore, two isolates (5.26%) simultaneously carried all four target genes, whereas five isolates (13.16%) lacked all investigated genes. The distribution of gene combinations among the isolates is presented in Table 4.

Regardless of the isolate origin, the frequencies of *rfbE* and *csgA* genes were significantly ($p < 0.05$) higher than those of the Shiga toxin genes (*stx1* and *stx2*). However, according to the Figure 5, no statistically significant differences were observed in gene frequencies among isolates recovered from different sample types ($p > 0.05$).

Table 2. Multidrug resistance of *Escherichia coli* isolates from raw milk and traditional dairy products.

Antibiotics	Number of isolates (%)	Raw milk	Cheese	Butter
CIP, AMC, SXT, TE, CHL, GM, CTX, and IPM	3 (7.89)	2	-	1
NAL, AMC, TE, CIP, CHL, and IPM	1 (3.44)	1	-	-
CTX, AMP, SXT, TE, and AMC	2 (6.88)	-	2	-
GM, AMP, SXT, TE, and AM	1 (3.44)	-	1	-
AMP, TE, CTX, and CHL	2 (5.26)	1	1	-
AMP, SXT, TE, and AMC	2 (6.88)	-	2	-
AMP, SXT, TE, and IPM	1 (3.44)	-	-	1
AMP, TE, and AMC	2 (5.26)	-	-	2
Total	14 (36.84)	4	6	4

CIP: ciprofloxacin; AMC: amoxicillin/clavulanic acid; SXT: sulphamethoxazole/trimethoprim; TE: tetracycline; CHL: chloramphenicol; GM: gentamycin; CTX: cefotaxime; IPM: imipenem; NAL: nalidixic acid; AMP: ampicillin.

Table 3. Biofilm formation profile of *Escherichia coli* isolates.

Isolates	Type of sample	Biofilm formation (optical density: 570 nm)	Biofilm formation category
M1	Raw milk	2.875	Strong
M2		2.110	Strong
M3		0.632	Moderate
M4		1.124	Strong
M5		1.752	Strong
M6		1.184	Strong
M7		1.574	Strong
M8		0.785	Moderate
M9		2.089	Strong
M10		1.266	Strong
M11		1.526	Strong
M12		1.040	Strong
M13		0.315	Weak
M14		1.159	Strong
M15		1.514	Strong
M16		0.644	Moderate
B17	Butter	0.999	Strong
B18		1.250	Strong
B19		0.436	Weak
B20		0.695	Moderate
B21		1.727	Strong
B22		1.386	Strong
B23		0.691	Moderate
B24		0.392	Weak
B25		1.241	Strong
B26		1.621	Strong
B27		0.598	Moderate
B28		1.364	Strong
Ch29	Cheese	0.571	Moderate
Ch30		0.619	Moderate
Ch31		1.178	Strong
Ch32		0.737	Moderate
Ch33		0.559	Moderate
Ch34		0.425	Weak
Ch35		0.732	Strong
Ch36		1.614	Strong
Ch37		1.635	Strong
Ch38		0.398	Weak
Negative control	Tryptic soy broth	0.265	-
Positive control	*Pseudomonas aeruginosa*	3.351	Strong

Table 4. Frequency of target genes in *Escherichia coli* isolates from row milk and traditional dairy products.

Isolates	Type of sample	*stx1*	*stx2*	*rfbE*	*csgA*
M1	Raw milk	-	+	+	+
M2		+	-	+	+
M3		-	-	+	+
M4		-	-	+	+
M5		-	-	+	-
M6		+	+	+	+
M7		-	-	-	+
M8		-	-	-	-
M9		-	-	+	-
M10		-	-	-	+
M11		-	-	+	+
M12		-	-	+	+
M13		-	-	+	-

Table 4. Continued.

Isolates	Type of sample	*stx1*	*stx2*	*rfbE*	*csgA*
M14	Raw milk	-	+	-	+
M15		-	-	-	+
M16		-	-	+	-
B17	Butter	-	-	+	+
B18		+	+	+	+
B19		-	-	-	+
B20		-	-	+	+
B21		-	+	+	+
B22		-	-	-	-
B23		-	-	+	+
B24		-	-	-	+
B25		-	-	+	-
B26		-	-	-	+
B27		-	-	+	-
B28		-	-	+	+
Ch29	Cheese	-	-	-	-
Ch30		-	-	+	+
Ch31		-	+	+	+
Ch32		-	-	-	-
Ch33		-	-	+	+
Ch34		+	-	+	+
Ch35		-	-	-	-
Ch36		-	-	+	+
Ch37		-	+	+	+
Ch38		+	-	+	+
Total (%)		5 (13.15)[a]	7 (18.42)[a]	26 (68.42)[b]	27 (71.05)[b]

[ab] Different letters indicate that there is a difference between the frequencies of different genes ($p < .05$).

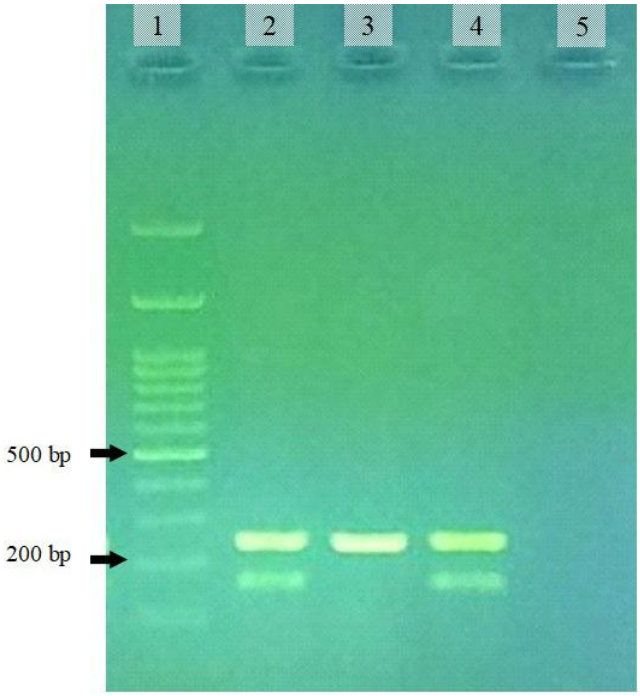


Fig. 3. Agarose gel electrophoresis of multiplex PCR product for *stx1* (180 bp) and *stx2* (255 bp) genes. Line 1: DNA ladder 100 bp; Line 2: positive control for *stx1* and *stx2*; Line 3: positive *Escherichia coli* isolates for *stx2*; Line 4: positive *E. coli* isolates for *stx1*/*stx2*; Line 5: negative controls.

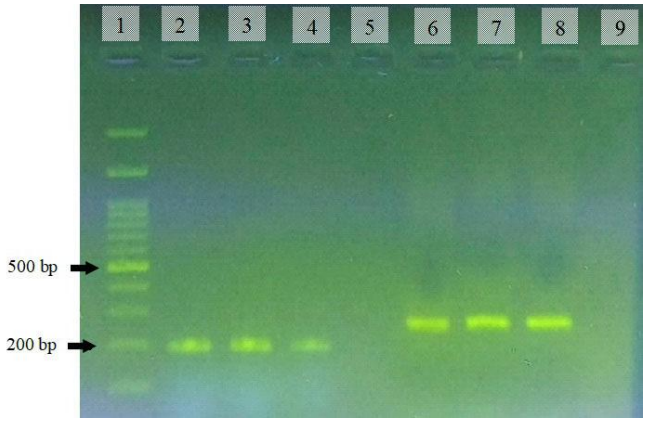


Fig. 4. Agarose gel electrophoresis of PCR product for *csgA* (200 bp) and *rfbE* (259 bp) genes. Line 1: DNA ladder 100 bp; Line 2: positive control for *csgA*; Lines 3 - 4: positive *Escherichia* coli isolates for *csgA*; Line 5: negative control for *csgA*; Line 6: positive control for *rfbE*; Lines 7-8: positive *E. coli* isolates for *rfbE*; Line 9: negative control for *rfbE*.

Discussion

The *E. coli* is a food-borne pathogen transmitted to consumers through contaminated foods, particularly foods being not pasteurized or heated before consumption, causing numerous digestive diseases, especially in individuals with weakened immune systems.[12,19]

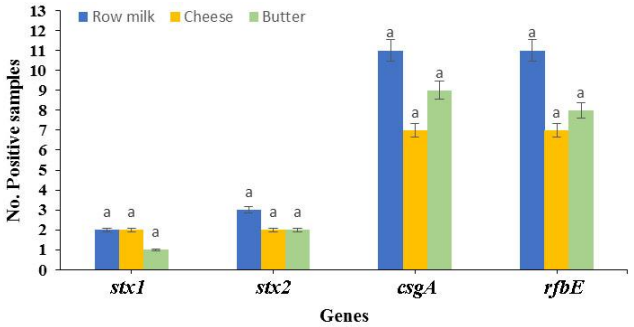


Fig. 5. The frequencies of target genes in isolates from different origins. Similar lowercase letters indicate no significant difference between gene frequencies in isolates from different sources ($p > 0.05$).

Therefore, the detection of susceptible food products can significantly improve public health in many regions. In the present study, *E. coli* was detected in 21.12% of traditional dairy products, being lower than the prevalence reported in Indonesia (70.40%) and Egypt (69.64%), but higher than those reported in the United States (4.30%) and some regions of Iran (8.33%).[7,10,11,20,21] In another study in Iran, Ranjbar *et al.* reported prevalence rates of 80.00, 40.00, and 42.85% in traditional cheese, traditional butter, and raw bovine milk, respectively.[2,3] Furthermore, previous studies have demonstrated a wide variation in the prevalence of *E. coli* in raw milk and traditional dairy products, ranging from 1.00 to 27.00%, across different regions of the world.[1,7,8,22-24] The prevalence of *E. coli* in dairy products is affected by a range of factors, including milking practices, storage conditions, the use of unpasteurized milk, personnel hygiene, climatic conditions, sampling season, laboratory detection methods, sample size, and hygiene management throughout the production and distribution chain, collectively contributing to the regional variations in contamination rates.[1,2]

Similarly, previous studies conducted in Iran and Egypt reported that all *E. coli* isolates were resistant to ampicillin.[2,3,10] Conversely, a recent report from Brazil revealed only 20.00% of *E. coli* isolates from cheese made from raw milk were resistant to the ampicillin.[25] High resistance to the antibiotic tetracycline was also observed here, although the rate (79.31%) was relatively lower than the resistance reported from Kashan, Iran (100%).[26] Dehkordi *et al.* also reported high resistance to tetracycline (84.00%) in *E. coli* isolates from fermented dairy products, almost similar to the present results.[11] Likewise, Bangladesh (61.90%), Indonesia (57.50%), and Nigeria (57.10%) demonstrated high resistance of *E. coli* isolates to tetracycline.[27-29] The widespread resistance to ampicillin and tetracycline may be associated with the extensive use of these anti-microbials in veterinary medicine and livestock production.[10,12,26,30] Moreover, in developing countries, tetracycline, amoxicillin, and sulfamethoxazole/ trimethoprim are widely used to treat diarrhea in livestock due to their cheapness and availability, thus increasing bacterial resistance. Unexpectedly, a high level of resistance to imipenem was observed, which may indicate the emergence of carbapenem-resistant strains and warrants further molecular investigation Alarmingly, the persistence of chloramphenicol resistance despite its restricted use may be due to the maintenance of resistance genes and co-selection by other anti-microbial agents.[6,23,27,28,31] Notably, all isolates were susceptible to aztreonam, indicating that this anti-microbial may still be effective against *E. coli* strains isolated from traditional dairy products.

In the present study, only 3.40% of the isolates exhibited resistance to gentamicin, whereas substantially higher resistance rates have been reported in studies conducted in Egypt (73.33%) and Nigeria (89.30%).[10,28] Conversely, Bangladesh found that 85.00% of *E. coli* isolates were susceptible to gentamicin. Additionally, about one-third of the isolates in this study were MDR, a trend that has been observed in several previous studies on *E. coli* in milk and dairy products.[26,31-33] For example, studies from Nepal, Egypt (2020), Egypt (2023), and Brazil reported MDR rates of 86.70, 86.11, 8.33, and 6.00%, respectively, in *E. coli* isolates from milk and dairy products.[4,10,17,27,29,32] These differences in resistance patterns may be attributed to the diverse mechanisms and abilities of *E. coli* isolates to resist antibiotics.[20,26] Other contributing factors include variations in antibiotic pricing, availability, and national regulations and their enforcement, as well as veterinary practices regarding antibiotic prescriptions, including illegal and inappropriate use across different regions.[2,3,10]

In a similar study performed in Iran, 68.42% of isolates were capable of producing biofilm, and 28.90%, 10.50%, and 28.90% of these isolates had high, moderate, and weak biofilm-forming abilities, respectively.[1,21] In a further study, *E. coli* isolates from raw milk were identified as strong (40.00%), moderate (13.30%), and weak (26.70%) biofilm producers.[14,21,31] In contrast, another report found that while all isolates were capable of biofilm formation, the proportion of strong biofilm producers was notably lower than the present study. Only 8.00% of isolates produced strong biofilms, while 76.00 and 16.00% formed moderate and weak biofilms, respectively.[25] In a study by de Campos *et al.*, it was found that only 10.30% of *E. coli* isolates from cheese made with raw cow's milk produced moderate biofilms, while the remaining isolates did not form biofilms.[30] Furthermore, the biofilm-forming ability of *E. coli* isolates from raw milk was assessed on various surfaces commonly used in the dairy production chain. The results revealed that isolates had a lower tendency to adhere and form biofilms on steel surfaces compared to the polystyrene and silicone, with 21.48, 42.28, and 56.38% of isolates forming biofilms on these surfaces,

respectively.[8,34] The development of biofilms on different surfaces in the dairy industry presents a significant risk, as it can lead to recontamination, spoilage, and reduced shelf life, and pose a threat to food safety and public health.[8,14,34] Importantly, even pathogenic *E. coli* serotypes forming weak biofilms can stabilize, replicate, and potentially re-contaminate food at lower doses. Consequently, the findings of this study, demonstrating the biofilm-producing potential of all isolates, are concerning. As highlighted by previous research, various environmental factors, such as nutrient availability, temperature, pH, surface adhesion ability, surface characteristics, inadequate equipment hygiene, and other properties play a significant role in bacterial biofilm formation.[4,14,34-36]

Compared to the present results, a higher prevalence of Shiga toxin genes has been reported in *E. coli* isolated from milk and dairy products, with 50.00% of isolates containing both *stx1*/*stx2* genes, 77.27% containing *stx1* gene, and 66.16% containing *stx2* gene. Similarly, another study found that the frequency of *Stx1* and *stx2* genes in *E. coli* strains associated with dairy products was 44.00% and 30.00%, respectively.[6,14] These genes were also found in 50.00% and 25.00% of *E. coli* isolates from raw milk in Pakistan, respectively.[18,29] In addition, *stx1* and *stx2* genes have been identified in most STEC isolated from cheese in France,[4,37] and *stx* gene was positive in 9.40% of isolates from cheese in Switzerland.[24] Molecular results also showed that *E. coli* isolates obtained from raw milk in Spain carried both Shiga toxin genes (*stx1*/*stx2*).[9,23,38] A similar study was conducted in Nigeria on dairy products, and 66.70% of isolates had the *rfbE* gene, consistent with the current results.[8,34] However, in another study, the *rfbE* gene was detected in only 8.33% of *E. coli* isolates from milk and dairy products in Egypt.[20] Similar reports have also been presented regarding the *csgA* gene. For example, Cruz-Soto *et al*., identified the *csgA* gene in 60.00 and 95.00% of *E. coli* isolated from dairy products, respectively, being very close to the frequency observed in the present study (71.51%).[5,10,15,39,40] These results highlight the importance of monitoring virulence genes in raw milk and dairy products to reduce food safety hazards associated with *E. coli*. Besides, Shiga toxin genes and the high prevalence of *csgA* and *rfbE* among isolates indicate the potential public health risk associated with consumption of unpasteurized dairy products. These genes play various roles in the bacterial pathogenicity process and can lead to severe infections in humans. The *rfbE* gene is involved in the biosynthesis of the *O157* antigen and is commonly used as a molecular marker for the identification of *E. coli O157* strains. Generally, it helps the organism escape detection and destruction by the host's immune system. The *stx1* and *stx2* genes are characteristic virulence markers of STEC. Among them, *stx2* is more frequently associated with severe clinical outcomes, particularly hemolytic uremic syndrome, whereas *stx1* is generally linked to the milder disease manifestations. The *csgA* gene is another *E. coli* virulence-enhancing factor playing a role in the production of proteins (curli) responsible for the production of curli fimbriae contributing to the adhesion and biofilm formation.

The current study demonstrated the occurrence of *E. coli* in raw milk, butter, and fresh cheese. A significant proportion of isolates showed resistance to the target antibiotics, with one-third exhibiting MDR characteristics. Additionally, all isolates demonstrated the ability to produce biofilms, with more than half forming strong biofilms. Molecular analysis indicated that approximately 86.00% of the isolates carried at least one of the virulence genes, including *stx1, stx2, csgA*, and *rfbE*. These findings suggest that raw milk, cheese, and traditional butter may serve as reservoirs of *E. coli* strains carrying virulence-associated genes and anti-microbial resistance traits, thereby representing a potential public health concern. The patterns of antibiotic resistance particularly multidrug resistance along with the strong biofilm-forming ability of the isolates and the presence of multiple virulence genes, raise significant concerns about the safety of raw milk and traditional dairy products, especially for vulnerable individuals. Therefore, it is crucial to establish an effective monitoring system for traditional dairy products to control and prevent the spread of pathogenic bacteria and safeguard public health. Nevertheless, the findings of the present study should be interpreted in light of certain limitations, including the relatively limited sample size, sampling from a single geographical region, and investigation of only selected virulence genes. Future studies involving larger sample sizes, broader geographical coverage, and comprehensive molecular characterization are warranted.

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Conflict of interest

The authors declare no known competing interest.

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