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Molecular Characterization and Biofilm Formation of *Escherichia coli* from Vegetables

Fatma ÖZDEMİR*[1], Seza ARSLAN[1]

Abstract

Escherichia coli can cause diarrheal and extraintestinal illnesses in humans. Diarrheagenic *E. coli* can be transmit to human through consumption of contaminated food, including vegetables. Biofilm produced by *E. coli* during food processing plays a role in development of foodborne illnesses. Vegetables have often been involved in diarrheal *E. coli* infections. A total of 40 *E. coli* isolates from vegetables were tested to determine biofilm formation at 12°C, 25°C and 37°C by the crystal violet and MTT assays. All isolates were performed for the production of curli fimbriae and cellulose associated with biofilm formation on Congo red agar. Biofilm formation by the crystal violet assay at 37°C, 25°C and 12°C was detected in 87.5%, 70% and 70% of the isolates, respectively. The biomass and viability of *E. coli* biofilms were similar according to the results of crystal violet and MTT assays. Biofilm formation among the *E. coli* isolates using the crystal violet and MTT assays showed a statistically significant difference between 12°C and 25°C as well as 12°C and 37°C ($p < 0.05$). However, no significant difference between 25 and 37°C ($p > 0.05$) was obtained. Three different morhotypes (bdar, pdar and saw) were identified based on the expression of curli fimbriae and cellulose. The incidence of the bdar morhotype was 27.5% and 50% at 25°C and 37°C, respectively. Prevalence of the pdar morphotype was 50% and 70% at 25°C and 37°C, respectively. At 25°C, only one isolate (2.5%) showed the saw morphotype. All isolates tested expressed curli fimbriae or cellulose, only three of which were non-biofilm producer using the crystal violet assay. This study demonstrated that the presence of biofilm forming *E. coli* isolates in vegetables may cause a risk to human health and food safety.

Keywords: *Escherichia coli*, vegetables, biofilm formation, curli fimbriae, cellulose

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1. INTRODUCTION

Escherichia coli is a non-spore forming, Gram-negative and facultatively anaerobic bacterium. It is a widespread human and animal pathogen commonly found in the healthy human intestinal tract. Most strains of *E. coli* are harmless commensals, but some pathogenic strains are able to cause a variety of diseases such as gastrointestinal illness, urinary tract infections, pericarditis, septicemia, pneumonia, and meningitis [1]. *E. coli* strains linked to gastrointestinal diseases are classified into six pathotypes, including enteropathogenic, shiga toxin-producing, enterotoxigenic, enteroaggregative, enteroinvasive, and diffusely adhering *E. coli* [2]. These pathogenic *E. coli* strains causing human diarrhea of varying severity are well-known foodborne pathogens and pose human health problems worldwide. Transmission of diarrhea-associated *E. coli* strains to human often occurs through eating contaminated food, including raw milk, dairy products, raw or undercooked ground beef, raw fruits and vegetables [3]. Vegetables are important in human nutrition as sources of many nutrients. A diet rich in fresh vegetables can reduce the risk of long-term diseases such as cardiovascular diseases, cancer, diabetes, hypertension, and obesity including certain nutrient deficiencies [4]. The consumption of vegetables has increased substantially due to consumer demand for health promotion in the world. Vegetables can be a potential source of various foodborne infections and outbreaks as they are eaten raw or lightly cooked [4, 5]. Foodborne outbreaks of pathogenic *E. coli* which are caused by eating contaminated vegetables such as spinach [6], lettuce [7-9], cabbage [10] and fresh leafy vegetables [11, 12] have been increasingly reported.

A variety of bacterial pathogens are able to adhere, gather, and produce biofilms on both abiotic and biotic surfaces. Bacteria in biofilms are more resistant to pH and temperature changes, nutrient deprivation, disinfectants, antimicrobials and oxygen radicals better than planktonic organisms. Thus, it is extremely difficult to remove an established biofilm in food environment [13]. Biofilms in food processing environment might contribute the persistence of spoilage and pathogenic bacteria and contamination of foods. They are of important concern in food hygiene and create public health risks and economic losses [2, 13]. Several factors, including environmental conditions such as incubation temperature, growth medium, and surface material, strain origin and serovar effect the biofilm forming ability in *E. coli* strains [14, 15].

Curli fimbriae and cellulose are important biofilm matrix components in *E. coli* and other *Enterobacteriaceae* [16, 17]. Curli fimbria is a fibrous surface protein mainly associated with bacterial attachment, cell accumulation, and biofilm formation. The existence of cellulose in the biofilm matrix confers mechanical, chemical, and physiological protection and promotes bacterial adhesion to abiotic surfaces [1, 18]. Many researchers have been studied the expression of curli fimbriae and cellulose in *E. coli* strains [14, 17, 19].

Vegetables has been consumed increasingly worldwide due to healthy lifestyle recommendations. They can be contaminated with *E. coli* at any stage from production to consumption. *E. coli* presence and its biofilm formation ability in vegetables are important in terms of food safety and hygiene. Therefore, the aim of the present study was to determine the biofilm formation of the *E. coli* isolates originated from vegetables at different temperatures using the microtiter plate and MTT assays and detect the expression of biofilm matrix components (curli fimriae and cellulose) using the Congo red agar assay.

2. MATERIALS AND METHODS

2.1. Bacterial isolates

A total of 40 *E. coli* isolates from vegetables including spinach (n=15), lettuce (n=12), arugula (n=9), black cabbage (n=3) and lamb's lettuce (n=1) were used in this study. Vegetables collected from various supermarkets and public bazaars were fresh and not precooked or frozen. The isolates were maintained at −20°C in Brain Heart Infusion broth (Merck) with 20% (vol/vol) glycerol until use.

2.2. Phenotypic identification of *E. coli*

Colonies on Eosin Methylene Blue agar (Merck) seen as blue-black colonies, often with a green metallic sheen were counted as suspected *E. coli*. Phenotypic identification was performed using the conventional methods. For this, the following biochemical tests are used: Gram staining, catalase test, indole and H_2S production, citrate utilization, motility, Methyl red and Voges Proskauer test, urease test, and carbohydrate fermentation tests [1].

2.3. Molecular characterization

Genomic DNA extraction of the *E. coli* isolates was carried out using the cetyl trimethyl ammonium bromide (CTAB) method for PCR analysis according to Ausubel et al. [20]. The DNA was dissolved in Tris-EDTA (TE) buffer and stored at -20°C. Molecular characterization of the *E. coli* isolates was performed by amplification of the *E. coli*-specific universal stress protein A (*uspA*) gene. The *uspA* primers were F-5'-CCG ATA CGC TGC CAA TCA GT-3' and R-5'-ACG CAG ACC GTA GGC CAG AT-3', which were predicted to yield an 884 bp product [21]. PCR experiments were carried out using a thermal cycler (Bio-Rad T100). The PCR reaction mix (50 µl) contained 5 µl of 10X PCR buffer (Vivantis), 4 mM $MgCl_2$ (Vivantis), 0.2 mM dNTP mix (Thermo Fisher), 0.4 µM primer, 1.5 U Taq DNA polymerase (Vivantis), 4 µl (50 ng) extracted DNA and 31.7 µl nuclease free water (AppliChem). The cycling conditions were carried out with the following setup: 94°C for 5 min and 30 cycles of denaturation (2 min, 94°C), annealing (1 min, 60°C), extension (1 min, 72 °C), and final extension (5 min, 72°C). PCR products were analyzed by electrophoresis (Bio-Rad) in 1% agarose gels and visualized with UV transilluminator (DNR Minilumi Bioimaging Systems). *E. coli* ATCC 8739 was used as a reference strain in this study. Representative agarose gel photograph of the PCR products of the *uspA* gene is presented in Figure 1.

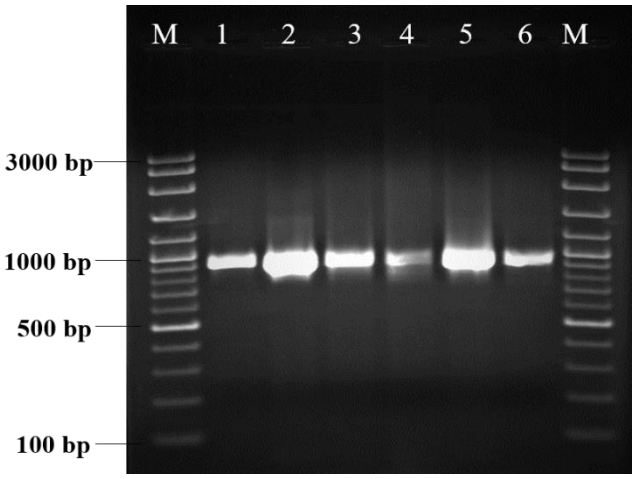

Figure 1. Gel electrophoresis of PCR product of the *uspA* (884 bp). M: 100 bp DNA ladder. Lane 1: positive control (*E. coli* ATCC 8739). Lanes 2-6: The *uspA* positive isolates from the spinach, lettuce, arugula, black cabbage, and lamb's lettuce samples, respectively.

2.4. Detection of biofilm by the microtiter plate assay

Biofilm formation ability of the *E. coli* isolates on polystyrene plates was performed by the microtiter plate assay as described previously with some modifications [22, 23]. In brief, the turbidity of each isolate grown overnight in Tryptic Soy Broth (TSB) was adjusted to 0.5 McFarland. An aliquot of 200 µl of this suspension was inoculated into wells of a 96-well flat-bottom microplate and incubated for 24h at 12, 25 and 37°C. Following incubation, the plates were washed with sterile phosphate-buffered saline three times and fixed with 200 µl methanol (99%) (Merck) for 15 min. The wells were decanted to dry in the air and stained with 0.1% crystal violet solution for 15 min. The plates were washed with sterile distilled water, air-dried and then the adherent cells were resuspended in 160 µl of 33% (v/v) glacial acetic acid (Merck) per well. The optical density (OD) of each well was measured at OD_{570} nm using spectrophotometer. The experiments were made in triplicates. Sterile TSB was a negative control. The isolates were categorized as non-biofilm, weak, moderate and strong biofilm producers based on the OD values [22].

2.5. Detection of viability of cells in biofilm by the MTT assay

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium assay to assess the metabolic activity of biofilm established by the *E. coli* isolates was performed as described in previous studies [24, 25]. All isolates of *E. coli* were grown overnight in TSB. Then, 200 µl of the adjusted suspension to 0.5 McFarland were added to each well in microtiter plate. After 24 h incubation at 37°C, the culture medium was removed and plate was washed with sterile phosphate-buffered saline (PBS) three times (200 µl per each well). After, 20 µl of pre-filter sterilized MTT solution (5 mg/ml in PBS) and 180 µl of TSB were added to individual well and incubated for 3 h at 37°C. The suspension was discarded and 150 µl of dimethyl sulfoxide (Sigma-Aldrich) were added to solubilize the formed formazan crystals. Viability of the cells was determined by measuring the optical density at OD_{570} nm using a microplate reader (Thermo Electron Corporation, Finland).

2.6. Detection of biofilm morphotypes

Biofilm morphotypes of the *E. coli* isolates were tested on Congo red agar (CRA) according to the method the previously described by Romling et al. [16] and Bokranz et al. [17]. CRA medium was prepared using Luria-Bertani agar without salt supplemented with Congo Red (40 µg/ml) and Coomassie brilliant blue (20 µg/ml). After overnight growing, 10µl of the culture were inoculated on the CRA plates and incubated at 25ºC and 37ºC for 96 h. The colonies were visualized and classified according to previous described four morphotypes: red, dry, and rough (rdar), which produce curli fimbriae and cellulose; pink, dry, and rough (pdar), which produce cellulose; brown, dry and rough (bdar), which produce curli fimbriae; smooth and white (saw), which do not produce both curli and cellulose [16, 17].

2.7. Statistical analysis

Statistical analysis were performed using the SigmaPlot 12.3 (Systat Software Inc.). The one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used to compare the results of biofilm formation at different temperatures. $p \leq 0.05$ was accepted to be significant.

3. RESULTS

3.1. Biofilm formation

This study investigated the ability of *E. coli* isolated from vegetables to form biofilm at three different temperatures. The results of biofilm formation of the *E. coli* isolates from vegetables are shown in Figure 2. Biofilm formation at 37°C, 25°C and 12°C was detected in 87.5%, 70% and 70% of the isolates, respectively (Tables 1 and 2). Under 37ºC condition, 11 (27.5%), 14 (35%), 10 (25%) and 5 (12.5%) of the isolates were strong, moderate, weak and non-biofilm producers, respectively. At 25ºC, 9 (22.5%) isolates were strong biofilm formers, 11 (27.5%) moderate, 8 (20%) weak, and 12 (30%) non-biofilm producers. At 12ºC, 2 (5%) isolates were classified as strong biofilm producers, 6 (15%) isolates as moderate producers, 20 (50%) isolates as weak biofilm producers, whereas 12 (30%) isolates did not produce biofilm. The findings showed that there was a statistically significant difference in biofilm formation using crystal violet staining at 12°C and 25°C ($p < 0.05$) as well as at 12°C and 37°C ($p < 0.05$). However, there was no significant difference between 25°C and 37°C ($p > 0.05$). We also assessed the viability of the *E. coli* cells in biofilm using the MTT assay (Figure 3). The results for the metabolic activity of *E. coli* at 12°C and 25°C ($p < 0.05$) as well as at 12°C and 37°C were statistically significant ($p < 0.05$), but there was no significant difference between 25 and 37°C ($p > 0.05$).

Table 1. Biofilm formation and morphotypes of *E. coli* isolates from vegetables

			Biofilm formation			Colony morphotypes	
No	Isolate	Origin	12°C	25°C	37°C	25°C	37°C
1	V1	Spinach	No biofilm	Weak	No biofilm	pdar	pdar
2	V2	Spinach	No biofilm	No biofilm	No biofilm	pdar	pdar
3	V3	Spinach	No biofilm	No biofilm	Moderate	pdar	pdar
4	V4	Spinach	No biofilm	No biofilm	No biofilm	pdar	pdar
5	V5	Spinach	Weak	No biofilm	No biofilm	pdar	pdar
16	V6	Spinach	Weak	Strong	Moderate	pdar	pdar
17	V7	Spinach	Weak	Strong	Weak	pdar	pdar
18	V8	Spinach	Weak	No biofilm	Weak	bdar	bdar
19	V9	Spinach	No biofilm	No biofilm	No biofilm	bdar	bdar
20	V10	Spinach	No biofilm	Weak	Moderate	pdar	bdar
21	V11	Spinach	Weak	Weak	Moderate	pdar	bdar
22	V12	Spinach	No biofilm	Moderate	Moderate	pdar	bdar
23	V13	Spinach	Weak	Strong	Strong	pdar	bdar
24	V14	Spinach	Weak	Moderate	Strong	pdar	bdar
25	V15	Spinach	Weak	Strong	Strong	pdar	bdar
6	V16	Lettuce	Weak	No biofilm	Weak	pdar	bdar
7	V17	Lettuce	Strong	Moderate	Strong	saw	bdar
8	V18	Lettuce	No biofilm	No biofilm	Weak	pdar	pdar
9	V19	Lettuce	Weak	No biofilm	Weak	bdar	bdar
10	V20	Lettuce	No biofilm	No biofilm	Weak	bdar	bdar
26	V21	Lettuce	Weak	Moderate	Weak	bdar	bdar
27	V22	Lettuce	Moderate	Strong	Strong	pdar	pdar
28	V23	Lettuce	Weak	Moderate	Strong	pdar	pdar
29	V24	Lettuce	Weak	Weak	Moderate	pdar	pdar
34	V25	Lettuce	Weak	Moderate	Weak	bdar	bdar
35	V26	Lettuce	No biofilm	Weak	Weak	bdar	bdar
36	V27	Lettuce	No biofilm	Weak	Weak	bdar	bdar
11	V28	Arugula	Weak	Moderate	Moderate	pdar	pdar
12	V29	Arugula	Moderate	Moderate	Moderate	pdar	pdar
13	V30	Arugula	Moderate	Moderate	Strong	pdar	pdar
14	V31	Arugula	Weak	Moderate	Moderate	pdar	pdar
15	V32	Arugula	Moderate	Weak	Moderate	pdar	pdar
30	V33	Arugula	No biofilm	No biofilm	Moderate	pdar	bdar
31	V34	Arugula	Moderate	Weak	Moderate	bdar	bdar
31	V35	Arugula	Moderate	No biofilm	Strong	pdar	pdar
33	V36	Arugula	Weak	Moderate	Moderate	bdar	bdar
37	V37	Black cabbage	Weak	Strong	Moderate	pdar	pdar
38	V38	Black cabbage	Strong	Strong	Strong	bdar	bdar
39	V39	Black cabbage	Weak	Strong	Strong	pdar	pdar
40	V40	Lamb's lettuce	Weak	Strong	Strong	pdar	pdar

Table 2. Biofilm formation categories of the *E. coli* isolates from vegetables at 12°C, 25°C and 37°C

Biofilm formation ability	12°C		25°C		37°C	
	No. (%)	OD_{570}[1]	No. (%)	OD_{570}	No. (%)	OD_{570}
Strong	2 (5)	0.257 ± 0.027	9 (22.5)	0.641 ± 0.086	11 (27.5)	0.332 ± 0.092
Moderate	6 (15)	0.101 ± 0.021	11 (27.5)	0.448 ± 0.084	14 (35)	0.200 ± 0.050
Weak	20 (50)	0.071 ± 0.007	8 (20)	0.169 ± 0.026	10 (25)	0.126 ± 0.018
Non-biofilm	12 (30)	0.053 ± 0.003	12 (30)	0.132 ± 0.017	5 (12.5)	0.065 ± 0.005

[1]OD_{570}: Optical density; values area expressed as mean ± standard deviation

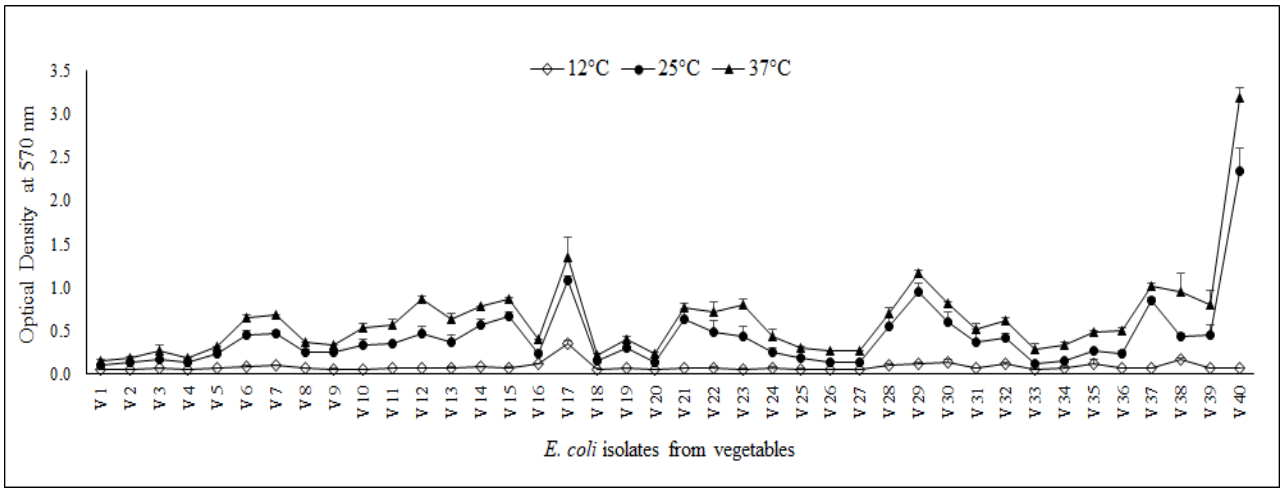

Figure 2. Biofilm formation of the 40 *E. coli* isolates from vegetables at 12ºC, 25ºC, and 37ºC by the crystal violet assay

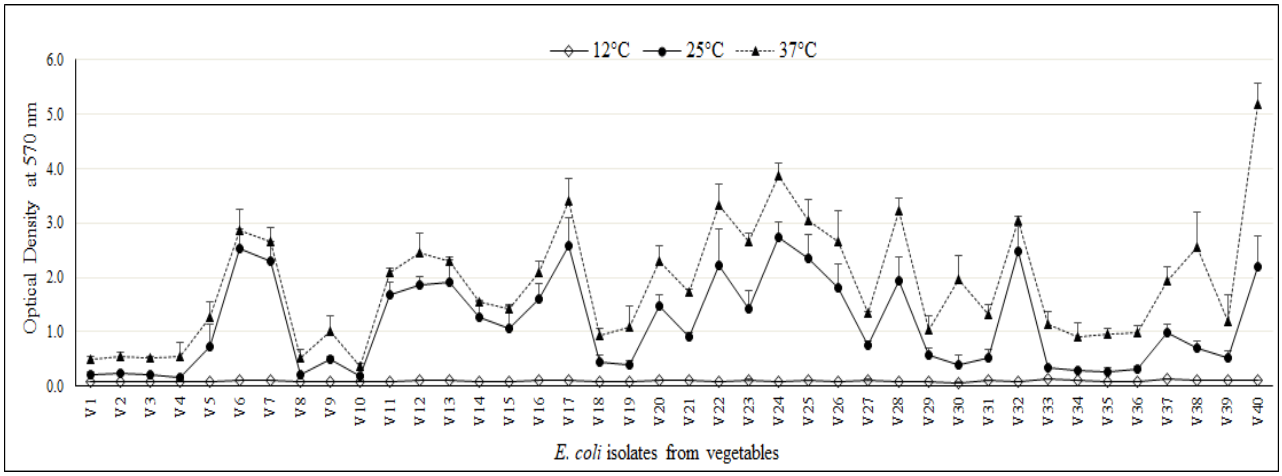

Figure 3. The viability of the 40 *E. coli* isolates from vegetables at 12ºC, 25ºC, and 37ºC by the MTT assay

3.2. Biofilm morphotypes

In this study, representative morphotypes expressed at 37°C and 25°C by the *E. coli* isolates are presented in Figure 4. Three different morphotypes (bdar, pdar and saw) were detected (Table 1, Figure 4). However, colony morphotype at 12°C was not detected in none of the isolates. The occurrence of the bdar and pdar morphotypes at 37°C was 50%, but none of the isolates displayed saw morphology. Prevalence of the bdar and pdar morphotypes at 25°C was 27.5% and 70%, respectively. Under the same temperature, only one isolate (2.5%) showed the saw morphotype, indicating a lack of synthesis of both important biofilm components. All isolates were rdar negative at both temperature. The findings of this study revealed that there was no apparent association between the biofilm forming capabilities and morphotypes of the *E. coli* isolates from vegetables (Table 1).

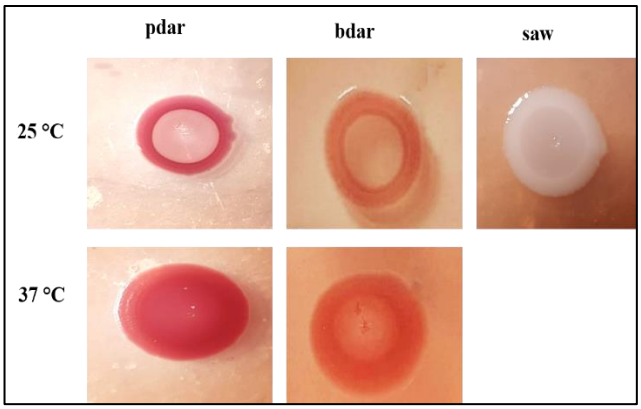

Figure 4. The identified morphotypes in the *E. coli* isolates from vegetables at 25ºC and 37ºC
pdar (pink, dry, and rough), bdar (brown, dry and rough), saw (smooth and white)

4. DISCUSSION

Biofilm produced by foodborne pathogens, including *E. coli* during food processing is involved in development of foodborne outbreaks [2, 13]. Leafy vegetables have often been implicated in *E. coli* outbreaks [4]. We investigated the ability of biofilm formation of the *E. coli* isolates from vegetables. It was detected that more than 70% of the isolates produced biofilm using crystal violet staining at three different temperatures. There was a statistically significant difference in biofilm formation at 12°C and 25°C ($p < 0.05$) as well as at 12°C and 37°C ($p < 0.05$). However, there was no significant difference between 25°C and 37°C ($p > 0.05$). The incidence of biofilm formation by *E. coli* isolates varied at different conditions [15, 19]. The ability of biofilm production was influenced by several factors such as incubation temperature and growth medium [15, 26, 27]. Nesse et al. [15] indicated that the *E. coli* isolates produced more biofilm at 25°C and 37°C than at 12°C, as we reported. Marti et al. [27] tested biofilm formation of *E. coli* isolates at 12, 28, and 37°C in different media. They reported that *E. coli* isolates showed the highest biofilm formation capacity at 28°C. The cultivation of *E. coli* isolates in nutrient rich medium (Tryptic Soy Broth) at lower temperature (20°C) exhibited a positive effect on biofilm formation [26].

In this study, three different morhotypes (bdar, pdar and saw) were identified based on curli fimbriae and cellulose production which are involved in attachment to surfaces and biofilm formation. As shown in Table 1, 27.5% and 50% of the isolates had the bdar morphotype, showing only curli fimbriae at 25°C and 37°C, respectively. Similarly, Schiebel et al. [19] found that the bdar morphotype was observed in 32.6% of the isolates at 28°C and in 38.5% of the isolates at 37°C. In other study, 83.3% and 77.7% of the isolates produced only curli fimbriae at 28°C and 37°C, respectively [14]. In contrast to our study, Dubravka et al. [26] documented that curli fimbriae were found in all *E. coli* isolates at 37°C. Cellulose, the second component of extracellular matrix, was expressed alone by some *E. coli* strains [18]. Silva et al. [14] documented the production of cellulose at 28°C (52.8%) and at 37°C (25%), similar to our results. At 25°C, most of our isolates (70%) from vegetables produced cellulose. In contrast, none of *E. coli* strains from mastitis milk samples produced cellulose [26]. In this study, the incidence of the saw morphotype (2.5%) was considerably lower than that (36%) obtained by Dubravka et al. [26]. Besides, we did not detect the rdar morhotype (expresses curli fimbria and cellulose) among the isolates. The production of both components at different

temperatures was observed in dairy isolates [27] and fecal isolates of *E. coli* [17]. The influence of environmental factors including low temperature, low osmolarity and poor carbon sources on expression of morphotypes has been reported [28].

In the present study, all isolates tested expressed curli fimbriae or cellulose, only three of which were not positive for biofilm formation using the crystal violet assay. Thus, this result may show a possible relationship between biofilm production and presence of curli fimbria and cellulose. A positive correlation was detected between cellulose production and biofilm production [27]. However, some studies demonstrated that there was no apparent association between the morphotype and biofilm formation [14, 17, 19].

5. CONCLUSION

The present study showed that most of the *E. coli* isolates from vegetables had biofilm formation ability at 12, 25, and 37°C. Three different morhotypes (bdar, pdar and saw) were identified based on the production of curli fimbriae and cellulose involved in biofilm formation. All isolates tested expressed curli fimbriae or cellulose, only three of which were not positive for biofilm formation using the crystal violet assay. In conclusion, biofilm formation by *E. coli* in leafy vegetables may be a primary source of food contamination and may cause a risk to human health and food safety.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

All authors have contributed in experimental study and writing of the manuscript equally.

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