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

Fatma ÖZDEMİR¹, Seza ARSLAN¹

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 morhotype was 50% and 70% at 25°C and 37°C, respectively. At 25°C, only one isolate (2.5%) showed the saw morhotype. 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
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 have 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 fimbriae 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 H₂S 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'-CGG 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 MgCl₂ (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.

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 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 OD₅₇₀ 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\textsubscript{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 25°C and 37°C 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 ≤ 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 37°C 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 25°C, 9 (22.5%) isolates were strong biofilm formers, 11 (27.5%) moderate, 8 (20%) weak, and 12 (30%) non-biofilm producers. At 12°C, 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

| No | Isolate | Origin  | Biofilm formation | Colony morphotypes |
|----|---------|---------|-------------------|--------------------|
|    |         |         | 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 |
| 6  | V6      | Spinach | Weak | Strong | Moderate | pdar | pdar |
| 7  | V7      | Spinach | Weak | Strong | Weak | pdar | pdar |
| 8  | V8      | Spinach | Weak | No biofilm | Weak | bdar | bdar |
| 9  | V9      | Spinach | No biofilm | No biofilm | No biofilm | bdar | bdar |
| 10 | V10     | Spinach | No biofilm | Weak | Moderate | pdar | bdar |
| 11 | V11     | Spinach | Weak | Weak | Moderate | pdar | bdar |
| 12 | V12     | Spinach | No biofilm | Moderate | Moderate | pdar | bdar |
| 13 | V13     | Spinach | Weak | Strong | Strong | pdar | bdar |
| 14 | V14     | Spinach | Weak | Moderate | Strong | pdar | bdar |
| 15 | V15     | Spinach | Weak | Strong | Strong | pdar | bdar |
| 16 | V16     | Lettuce | Weak | No biofilm | Weak | pdar | bdar |
| 17 | V17     | Lettuce | Strong | Moderate | Strong | saw | bdar |
| 18 | V18     | Lettuce | No biofilm | No biofilm | Weak | pdar | pdar |
| 19 | V19     | Lettuce | Weak | No biofilm | Weak | bdar | bdar |
| 20 | V20     | Lettuce | No biofilm | No biofilm | Weak | bdar | bdar |
| 21 | V21     | Lettuce | Weak | Moderate | Weak | bdar | bdar |
| 22 | V22     | Lettuce | Moderate | Strong | Strong | pdar | pdar |
| 23 | V23     | Lettuce | Weak | Moderate | Strong | pdar | pdar |
| 24 | V24     | Lettuce | Weak | Weak | Moderate | pdar | pdar |
| 25 | V25     | Lettuce | Weak | Moderate | Weak | bdar | bdar |
| 26 | V26     | Lettuce | No biofilm | Weak | Weak | bdar | bdar |
| 27 | V27     | Lettuce | No biofilm | Weak | Weak | bdar | bdar |
| 28 | V28     | Arugula | Weak | Moderate | Moderate | pdar | pdar |
| 29 | V29     | Arugula | Moderate | Moderate | Moderate | pdar | pdar |
| 30 | V30     | Arugula | Moderate | Moderate | Strong | pdar | pdar |
| 31 | V31     | Arugula | Weak | Moderate | Moderate | pdar | pdar |
| 32 | V32     | Arugula | Moderate | Weak | Moderate | pdar | pdar |
| 33 | V33     | Arugula | No biofilm | No biofilm | Moderate | pdar | bdar |
| 34 | V34     | Arugula | Moderate | Weak | Moderate | bdar | bdar |
| 35 | V35     | Arugula | Moderate | No biofilm | Strong | pdar | pdar |
| 36 | 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 \textit{E. coli} isolates from vegetables at 12°C, 25°C and 37°C

| Biofilm formation ability | 12°C       | 25°C       | 37°C       |
|--------------------------|------------|------------|------------|
|                          | No. (%)    | OD$_{570}$ | 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 \textit{E. coli} isolates from vegetables at 12°C, 25°C, and 37°C by the crystal violet assay

Figure 3. The viability of the 40 \textit{E. coli} isolates from vegetables at 12°C, 25°C, and 37°C 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 morphology, 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 25°C and 37°C](image)

**Figure 4.** The identified morphotypes in the E. coli isolates from vegetables at 25°C and 37°C

- 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 morphotypes (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].
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 morphotypes (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**

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**Authors' Contribution**

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

**The Declaration of Ethics Committee Approval**

The authors declare that this document does not require an ethics committee approval or any special permission.

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**REFERENCES**

[1] F. Scheutz and N. A. Strockbine, “Genus *I Escherichia*”. In: Garrity, GM, Brenner DJ, Krieg NR, Staley JT. (Eds.), Bergey’s manual of systematic bacteriology 2 (Part B). Springer, New York, pp. 607–623, 2005.

[2] A.K. Bhunia, “Foodborne microbial pathogens: mechanisms and pathogenesis,” Springer, New York, 2008.

[3] WHO, “Home/Newsroom/Fact sheets/Detail/E. coli, 7 February 2018,”https://www.who.int/news-room/fact-sheets/detail/e-coli2018, 2018.

[4] G. I. Balali, D. Dekugmen, V. G. A. Dela, and P. Adjei-Kusi, “Microbial contamination, an increasing threat to the consumption of fresh fruits and vegetables in Today’s world,” International Journal of Microbiology, Article ID 3029295, 2020.

[5] FAO, “Increasing fruit and vegetable consumption becomes a global priority,” http://www.fao.org/english/newsroom/food-priority/fruitveg1.htm, 2003.

[6] M. T. Jay, M. Cooley, D. Carychao, G. W. Wiscomb, R. A. Sweitzer, and L. Crawford-
Miksza, “*Escherichia coli* O157:H7 in feral swine near spinach fields and cattle, central California coast,” Emerging Infectious Diseases, vol. 13, pp. 1908–1911, 2007.

[7] I. Friesema, G. Sigmundsdottir, K. van der Zwaluw, A. Heuvelink, B. Schimmer, C. de Jager, B. Rump, H. Briem, H. Hardardottir, A. Atladottir, E. Gudmundsdottir, and W. van Pelt, “An international outbreak of shiga toxin-producing *Escherichia coli* O157 infection due to lettuce, September–October 2007” Surveillance and outbreak reports, vol. 13, no. 50, pp. 1–5, 2008.

[8] R. B. Slayton, G. Turabelidze, S. D. Bennett, C. A. Schwensohn, F. Khan, C. Butler, E. Trees, T. L. Ayers, M. L. Davis, A. S. Laufer, S. Gladbach, I. Williams, and L. B. Gieraltowski, “Outbreak of shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011,” PLoS One vol. 8, no.2, e55300, 2013.

[9] CDC, “Outbreak of *E. coli* Infections Linked to Romaine Lettuce Final Update,” Available: https://www.cdc.gov/ecoli/2019/o157h7-11-19/index.html, 2020.

[10] CDC, “Multistate outbreak of shiga toxin-producing *Escherichia coli* O121 infections linked to raw clover sprouts (Final Update),” Available: https://www.cdc.gov/ecoli/2014/o121-05-14/index.html, 2014.

[11] K.M. Herman, A.J. Hall, and L. H. Gould, “Outbreaks attributed to fresh leafy vegetables, United States, 1973–2012,” Epidemiology and Infection, vol. 20, pp. 1–11, 2015.

[12] CDC, “Outbreak of *E. coli* Infections Linked to Romaine Lettuce,” Available: https://www.cdc.gov/ecoli/2018/o157h7-11-18/index.html, 2018.

[13] E. Giaouris, E. Heir, M. Desvaux, M. Hebraud, T. Moretro, S. Langsrud, A. Doulgeraki, G.-J. Nychas, M. Kacaniova, K. Czaczyk, H. Olmez, and M. Simoes, “Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens,” Frontiers in Microbiology, vol. 6, no. 841, 2015.

[14] G. I. Balali, D. Dekugmen, V. G. A. Dela, and P. Adjei-Kusi, “Microbial contamination, an increasing threat to the consumption of fresh fruits and vegetables in Today’s world,” International Journal of Microbiology, Article ID 3029295, 2020.

[15] L. L. Nesse, C. Sekse, K. Berg, K.C. Johannesen, H. Solheim, L. K. Vestby, and A. M. Urdaib, “Potentially pathogenic *Escherichia coli* can form a biofilm under conditions relevant to the food production chain,” Applied and Environmental Microbiology, vol. 80, no. 7, pp. 2042–9, 2014.

[16] U. Romling, W. D. Sierralta, K. Eriksson, and S. Normark, “Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter,” Molecular Microbiology, vol. 28, pp. 249–264, 1998.

[17] W. Bokranz, X. Wang, H. Tschape, and U. Römling, “Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract,” Journal of Medical Microbiology, vol. 54, pp. 1171–1182, 2005.

[18] X. Zogaj, M. Nimtz, M. Rohde, W. Bokranz, and U. Romling, “The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix,” Molecular Microbiology, vol. 39, pp. 1452–1463, 2001.

[19] J. Schiebel, A. Bohm, J. Nitschke, M. Burdakiewicz, J. Weinreich, A. Ali, D. Roggenbuck, S. Rödiger, and P. Schieracka, “Genotypic and phenotypic characteristics associated with biofilm formation by human clinical *Escherichia coli* isolates of different pathotypes,” Applied and Environmental Microbiology, vol. 83, no. 24, e01660-17, 2017.
Struhl, “Current protocols in molecular biology,” Greene Publishing Associates & Wiley Interscience, New York, 1991.

[21] J. Chen and M.W. Griffiths, “PCR differentiation of *Escherichia coli* from other Gram negative bacteria using primers derived from the nucleotide sequences flanking the gene encoding the universal stress protein,” Letters in Applied Microbiology, vol. 27, pp. 369–371, 1998.

[22] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, and M. Svabic-Vlahovic, “A modified microtiter-plate test for quantification of Staphylococcal biofilm formation,” Journal of Microbiological Methods, vol. 40, pp. 175–179, 2000.

[23] S. Stepanovic, D. Vukovic, V. Hola, G. D. Bonaventura, S. Djukic, I. Cirkovic, and F. Ruzicka, “Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci,” APMIS, vol. 115, pp. 891–899, 2007.

[24] E. Walencka, S. Rozalska, B. Sadowska, and B. Rozalska, “The influence of *Lactobacillus acidophilus*-derived surfactants on Staphylococcal adhesion and biofilm formation,” Folia Microbiology, vol. 53, pp. 61–66, 2008.

[25] Y.T Wu, H. Zhu, M. Willcox, and F. Stapleton, F, “Removal of biofilm from contact lens storage cases,” Investigative Ophthalmology and Visual Science, vol. 51, pp. 6329-6333, 2010.

[26] M. Dubravka, P. Bojana, V. Maja, and T. Dalibor, and P. Vladimir, “Investigation of biofilm formation and phylogenetic typing of *Escherichia coli* strains isolated from milk of cows with mastitis,” Acta Veterinaria-Beograd, vol. 65, no. 2, pp. 202–216, 2015.

[27] R. Marti, M. Schmid, S. Kulli, K. Schneeberger, J. Naskova, S. Knochel, C. H. Ahrens, J. Hummerjohanna, “Biofilm formation potential of heat-resistant *Escherichia coli* dairy isolates and the complete genome of multidrug-resistant, heat-resistant strain fam21845,” Applied and Environmental Microbiology, vol. 83, no. 15, e00628-17, 2017.

[28] L.D. Sante, A. Pugnalonib, F. Biavascoa, E. Giovanetta, and C. Vignarolia, “Multicellular behavior of environmental *Escherichia coli* isolates grown under nutrient-poor and low-temperature conditions,” Microbiological Research, vol. 210, pp. 43–50, 2018.