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Significance of Biofilm Formation by Enteric *Escherichia Coli* Obtained from Fresh Produce in Increased Antimicrobial Resistance and Persistence

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Abstract:- The risk of enteric pathogen contamination and growth on fresh produce is one of the main safety concerns associated with the increasing number of foodborne outbreaks in recent years. Plants in general are not considered as host for enteric pathogens. However, the increased persistence of pathogenic and commensal *E. coli* in plants has led to the consideration of these fresh produce as a secondary reservoir although factors associated in the increased persistence are not very clear. Biofilms formation by pathogenic *E. coli* and other Enterobacter pose a serious threat to the safety of fresh produce as they can persist and colonize for long periods of time in the food processing environment and thus represent a source of recurrent contamination. Concurrently, increased antibiotic resistance is equally a major concern with respect to biofilm formation as these factors aids in the enhanced levels of virulence, persistence and colonization of the pathogenic *E. coli* in fresh produce. In the present study the biofilm formation, antibiotic resistance and the multicellular behavior by pathogenic/non-pathogenic *E. coli* strains derived from fresh produce has been investigated. The formation of biofilm and its associated role with antibiotic resistance has been addressed unveiling the importance of factors leading to an increased host – microbe interaction. A total number of 33 *E. coli* strains were isolated from ready- to- eat fresh produce. The obtained results confirmed the ability to form biofilm by the *E. coli* strains obtained from fresh produce. Most of the *E. coli* with increased number of cellulose/curli-fimbriae production was able to form stronger biofilm and showed a significant number of antibiotic resistances. Overall, the present study suggests a correlation between biofilm formation and antibiotic resistance, implicating its important role in the food borne intoxications and increased virulence and its potential relevance for the management of food-borne illnesses linked with consumption of fresh produce.

Keywords:- *E. Coli, Fresh Produce, Biofilm, Curli-Fimbriae, Cellulose, Antimicrobial Resistance.*

I. INTRODUCTION

With major advancements in various aspects of biological applications and technology, people around the world are still affected and falling ill from consumption of contaminated food with exerting heavy toll on both human health and nation's economy. This is not only the case in developing countries with lack of proper sanitation and health but also in developed countries [1]. Human food borne infections traditionally are acquired through the ingestion of foods of animal origin. Fresh fruits and vegetables have emerged as new vehicles for the transmission of diseases [2]. Leafy green vegetables (cabbage, spinach, lettuce, carrot and salad leaves of all variety) have been identified as commodity group of highest concern in view of microbiological safety. Pathogenic *E. coli*, Salmonella sp. and Enteric bacteria are involved in food borne outbreaks causing gastroenteritis and chronic infection [3]. *E. coli* infection arising from contaminated food continues to be an immense problem with millions of cases occurring annually throughout the world [4]. In addition to the misery caused, financial loss is enormous.

Fresh vegetables are fundamental components of the human diet and there is considerable evidence of the health and nutritional benefits associated with the consumption of fresh vegetables. This has led to significant rise in the demand of fresh produce, changes in life styles and major shifts in consumption trends [5]. Vegetables can become contaminated with microorganisms capable of causing human diseases while still on the fields. The primary source of transmission of pathogenic *E. coli to* human is through contaminated foods consumption such as raw or undercooked ground meat products, fresh produce and raw milk [6]. Cross contamination during preparations and poor storage facilities along with contamination of water and other foods leads to increased infection.

In India, the burden of food-borne disease is unclear. Most food-borne diseases go unreported, only few are reported by the media, usually those with high morbidity

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and/or occurring in urban areas [7, 8]. In the South-East Asia Region, WHO report shows nearly 150 million people fell ill with food borne diseases in 2010, which led to 175 000 deaths. Of these, 40% of food borne diseases burden was among children under 5 years.

Generally, it is considered that non-hosts environments such as plants do not support the colonization and survival of human enteric pathogens. However, continuous increases in the microbial contamination of agricultural plants indicate that human enteric bacteria are able to colonize and survive in fresh produce during cultivation and post-harvest processing [9, 10]. Further, foodborne bacteria pathogens such as *E. coli* and Salmonella not only colonize or survive on the agricultural plant surface, but grow until the fresh produce is consumed by a new host. The most important strategies used by *E. coli* and Salmonella and other enteric bacteria during colonization or survival in the agricultural plants is the formation of biofilm [11]. A biofilm is an assembled and structural organization of bacterial cells confined within a matrix of the EPS (extracellular polymeric substance) and which adhere to a living or inert surface. Several studies have demonstrated that biofilm formation is significantly associated with *E. coli* and Salmonella strains isolated from different sources such as clinical and animal [12]. Biofilm formation behaviors of many bacteria including *E. coli* is significantly associated to enhance survival in natural environments, resistance to antimicrobial agents and during interaction with hosts. Recently, few studies indicate that *E. coli* and Salmonella and other foodborne bacteria pathogens are able to form biofilm on the surface of plants as well as in inner spaces of the plant tissues. It is also believed that bacterial cells confined in biofilm are more resistant to antibiotics and stress conditions [13, 14]. Curli is a major component of biofilm in many enteric bacteria including *E. coli* and are important for adherence to different biotic and abiotic surfaces [15]. Thus, it is considered that biofilm formation is a survival strategy of *E. coli* and Salmonella to withstand on the surfaces of the agricultural plant under unfavorable conditions. The production of curli fimbriae or thin aggregative fimbriae and cellulose in *E. coli* was found to be important components in the formation of extracellular polymeric matrix, which is essential during biofilm formation and persistence in various surfaces. Previous studies suggest that production of curli fimbriae and cellulose may be associated with the survival and persistence of *E. coli* and *Salmonella* in the food environment. Evidences also suggest that production of cellulose and curli-fimbriae also offers protection to bacterial cells against harsh environmental conditions such as desiccation, osmotic shock, and UV radiation [13, 14]. Conversely, expression of cellulose and curli-fimbriae in *E. coli* associated with their initial attachment in animal hosts or provides a physical barrier against the transmission of antimicrobial agents and compounds of the host immune response. On the basis of the production of curli fimbriae and cellulose, *E. coli* displayed different colony morphotypes; RDAR (red, dry and rough), indicating the production of cellulose and curli; b), PDAR (pink, dry and rough) indicating the production of cellulose only; c), BDAR (brown, dry and rough) indicating the expression of curli-fimbriae only; d), SAW (smooth and white) indicating no production of cellulose and curli-fimbriae. The distinct colony morphotypes such as BDAR and RDAR have been reported in *E. coli* and *Salmonella* strains isolated from clinical and animal sources [14, 15]. The BDAR and RDAR morphotypes of Salmonella has been linked to increase their virulence or tolerance in long-term desiccation and nutrient depletion in biofilm [16]. However, limited information is available on the role of production of curli fimbriae and cellulose and related colony morphotypes in agricultural plant-related *E. coli* and Salmonella.

The present study focuses on the occurrence and formation of biofilm as one of the approaches for colonization and persistence of enteric bacteria in fresh produce with enhanced antimicrobial resistance.

II. MATERIAL AND METHODS

- *Recovery and Isolation of E. Coli from Fresh Produce:*

The ready to eat fresh produce samples (n=170): leafy greens, carrot, radish, lettuce, cucumber and tomato were brought to laboratory from various local markets of New Delhi (India) aseptically in sterile plastic containers. All samples were collected, labelled and transported to the laboratory aseptically. Bacteriological analysis of each collected sample was carried out on the same day.

For the recovery of *E. coli* from the collected samples, previously described methods were used with slight modifications. Presumptive identification of the produce isolated *E. coli* was done by conventional microbiological methods such as Gram's staining and biochemical characterization was performed to further characterize the isolates such as IMViC, oxidase test and catalase test. Additionally, *E. coli* strains using molecular methods were screened and identified for the presence of *uidA* gene, Shiga toxin (*stx*) and intimin gene (*eae*) by PCR assay. STEC were identified using PCR primers targeting *stx1, stx2,* and *eae* genes, as described previously [17].

- *Biofilm Formation Assay:*

The biofilm development ability of the isolated *E. coli* isolates was determined by using the microtiter plate method as previously described [18]. The overnight growth of each bacterial strain with an OD_{600} of ~1.0 was diluted 1: 100 in fresh PBS and 200µl of the culture was added into the triplicate well and incubated at 37^0C for 48 h. Culture was withdrawn, and each well was washed twice with sterile distilled water. The plates were air-dried, heat-fixed (60°C for 2 hours), and stained with crystal violet (0.2%) for 10 min. The wells were washed twice to remove extra stain with distill water, air dried and solubilized in 200µl of 95% ethanol. Absorbance was obtained at OD_{570} . Wells containing only sterile media without bacterial inoculation were taken as blank or negative control.

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➢ *Cellulose and Curli Fimbriae Morphotype Screening:*

The production of cellulose and curli-fimbria by all *E. coli* strains was assessed using colony morphotypes on congo red (CR) agar plates as described previously [13, 15]. Overnight grown fresh bacterial culture was inoculated on CR agar plates prepared with LB agar without salt as a base and supplemented with 40 µg/ml Congo red dye and 20 µg/ml of Coomassie brilliant blue and incubated at 25-28ºC for 72 hrs. Colony morphologies on CR plates were scored as RDAR (Red Dry and Rough, indicates the expression of both curli fimbriae and cellulose); BDAR (Brown Dry and Rough, indicates the expression of curli-fimbriae only); PDAR (Pink Dry and Rough, indicates the expression of cellulose only); SAW (Smooth and White, indicates the no expression of curli-fimbriae nor cellulose). The screening experiment was performed in duplicates and at least 50-100 colonies per isolate was observed of each morphotype for examination.

➢ *Antimicrobial Susceptibility Testing:*

Antimicrobial susceptibility of obtained *E. coli* isolates was determined using the Kirby-Bauer disc diffusion method on MHA medium according to the Clinical and Laboratory Standards Institute (CLSI 2012). The antimicrobial susceptibility was tested against seven classes of drugs comprising of total 16 antibiotics. Reference strains *E. coli* ATCC 25922 was used as control.

III. RESULTS

➢ *Recovery and Isolation of E. Coli from Fresh Produce:*

To understand the factors associated with *E. coli* persistence on plants/fresh produce, a total of 33 (12.2%) isolates of *E. coli* were recovered among the 170 fresh ready to eat vegetable samples (Fig 1) Most isolates were obtained from leafy greens (spinach and parsley) followed by lettuce, carrot and cucumber (Fig 2).

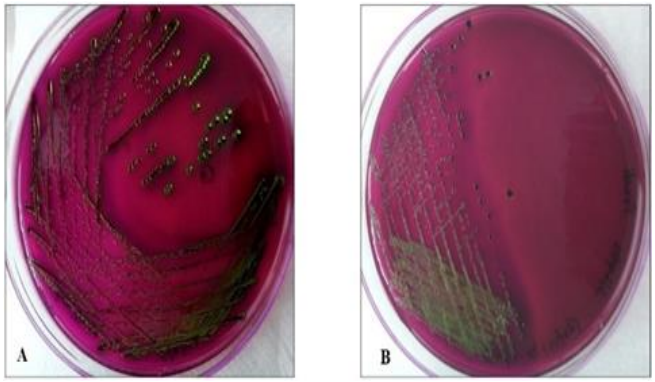

Fig 1 E. Coli Growth on EMB Agar Showing Green Sheen: (A) E. Coli Isolated From Vegetable (Cucumber) And (B) E. Coli 25922 Control

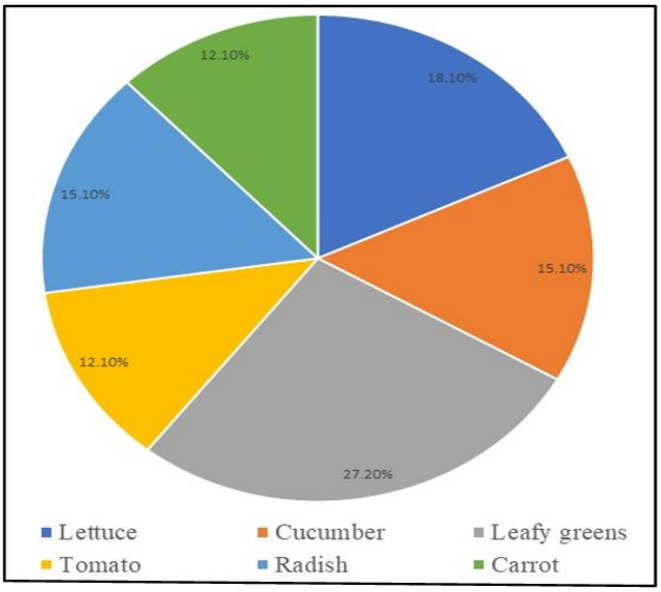

Fig 2 Percentage Recovery of E. Coli Colonize and Persist in Vegetables/Fruits

The PCR analysis of the recovered enteric *E. coli* was performed for the confirmation of the isolated strains. The diagnostic gene *uidA* encoding beta-glucuronidase in most of the *E. coli* were used for analysis. Among the 36 picked isolates of presumptive *E. coli* 83% of the isolates showed the presence of the *uid* gene (Fig 3). The number of isolates obtained by genotypic analysis was less as compared to phenotypic analysis. Hence 33 *E. coli* were identified using both conventional and molecular methods that were used for further analysis.

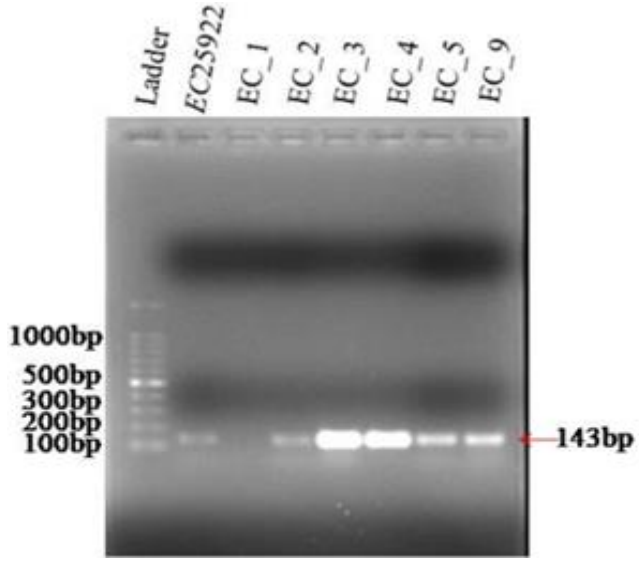

Fig 3 PCR Amplification of 'Uida' (143bp) Genes from Six Representatives E Coil Isolates

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➢ *Biofilm Formation:*

The elucidation of the biofilm formation was analyzed as described by Stepanović *et al*., 2000; the obtained data require the definition of the cut-off value that splits biofilm-producing from non-biofilm-producing strains compared to negative control. According to the biofilm formation assays, most of the produce-origin isolates were forming the strong biofilm. The OD570 for strong biofilm was observed to be in the range of 0.408–0.854 for the *E. coli* isolates. The adherence ability of strains tested was classified into four categories: strong biofilm producer, moderate biofilm producer, weak biofilm producer, and no biofilm producer based upon the OD obtained. On the basis of the values defined, among the 33 isolates of *E. coli*, 55% (n = 18) showed strong, 15% (n = 5) moderate, 18% (n = 6) weak biofilm formation ability, while 12% (n = 4) were not able to form biofilm. Statistically significant difference ($P < 0.05$) was observed between biofilm producers categorized as strong, moderate, weak or none (Fig. 4)

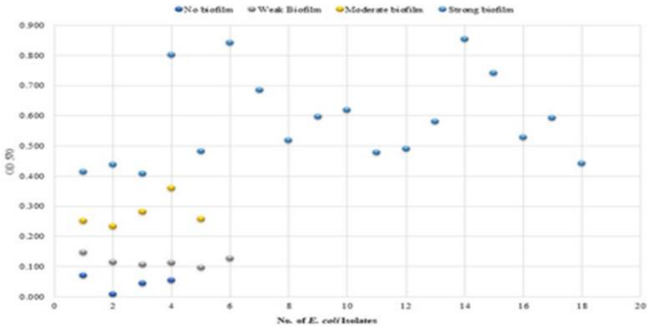

Fig 4 Biofilm Formation Ability of Vegitable/Fruit-Origin Escherichia Coil Isolates (N=33), Strong Biofilm Producer (N=18; 55%), Moderate Biofilm Producer (5; 15%), Weak Biofilm Producer (6; 18%), and No-Biofilm Producer (4;12%)

➢ *Cellulose and Curli Fimbriae Morphotype Screening:*

The *E. coli* isolates screened for cellulose and curli fimbriae showed distinct colony morphotypes distribution. The cellulose and curli fimbriae occurrence are exhibited as RDAR, PDAR, BDAR and SAW. The distribution of *E. coli* showing multicellular behaviors was observed as RDAR (n = 16), PDAR (n = 8), BDAR (n = 7) and SAW (n = 2) as depicted in Fig 5.

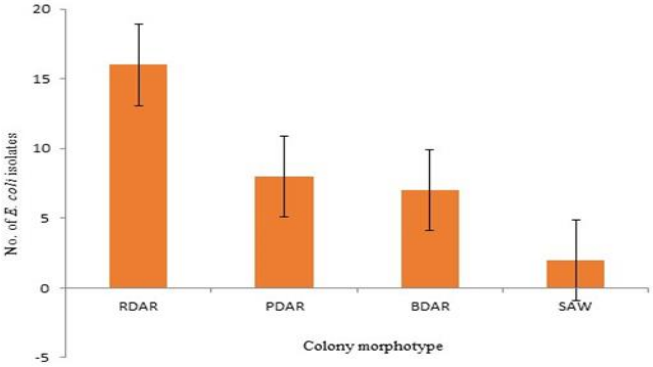

Fig 5 RDAR (red, dry and rough), PDAR (pink, dry and rough), BDAR (brown, dry and rough), SAW (smooth and white). Each bar represents no of E. coli isolates for particular colony morphotype

➢ *Prevalence of antibiotic resistance in Escherichia coli:*

Among each class of antimicrobial drugs used in the study, highest proportion of resistance was observed against quinolones (66.7%) followed by penicillin (36%) and aminoglycosides (28%). Carbapenem, macrolids and cephalosporins showed moderate resistance 15%, 18% and 9% respectively. However, phenicol were 100% susceptible for the *E. coli* isolates. The drug resistance prevalence pattern of *E. coli* is represented, in Fig. 6. To summarize, *E. coli*, recovered isolates exhibited MDR pattern against majority of the critically and highly important antimicrobial drugs (Fig. 7). It was observed that majority of the isolates showing MDR pattern were able to form strong biofilm.

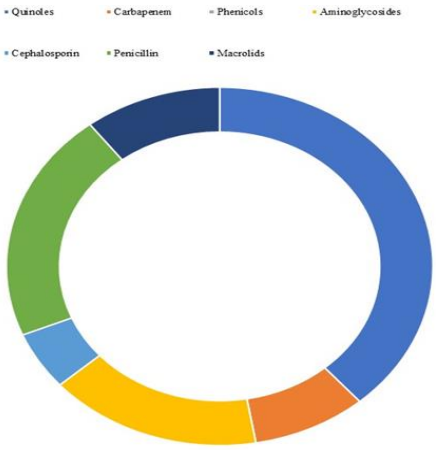

Fig 6 Percentage Prevalence of Antimicrobial Resistance of E. Coli Against Clinically Important Antibiotic Classes

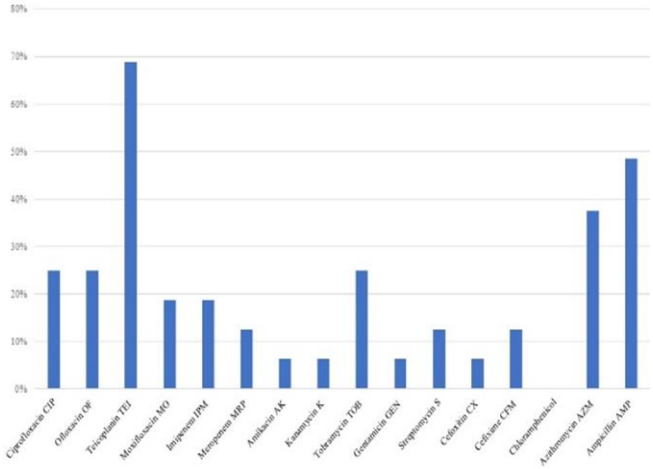

Fig 7 Percentage Resistant E. Coli Isolates Against Individual Antibiotic

IV. DISCUSSION

Survival of human enteric pathogens in plants and formation of biofilm has led to increase in the number of food borne illnesses. The main question arises is how these human pathogens invade the fresh produce and after invading not only survive inside the plant, vegetable or fruits also behaves as a carrier of the particular pathogens to human. In the present study, we observed the survival strategies adopted by the human enteric pathogens to overcome the environmental adverse conditions to colonize and persist in the plant's habitat. The isolated *E. coli*

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displayed multicellular behavior showing RDAR, BDAR, PDAR morphotype, which indicates the expression/production of both or either of cellulose or curli-fimbriae [12, 13]. Mechanisms involved in surface attachment of human pathogens to plants are biofilm formation, fimbriae, extracellular polysaccharide and presence of flagella. Curli fimbriae and cellulose are the extracellular polysaccharide supposed to be linked with biofilm formation, majorly found to be determinant of cell-cell interactions and adherence to biotic and abiotic surfaces. The occurrence of high number of RDAR morphotype in our studies signifies the importance of cell adhesion to the plant surface. This is in agreement with the previous studies which showed that cellulose and thin aggregative fimbriae are important for *E. coli* interaction with plants [19, 20]. The distinct colony morphotypes such as RDAR and BDAR have also been reported in *E. coli* strains isolated from clinical and animal sources. Studies have shown increased survivability of the *E. coli* possessing RDAR morphotype. Interestingly, *E. coli* isolates in our collection majorly displayed RDAR and BDAR morphotypes, which suggests of their survival in the plant environment with virulence potential. A similar significant finding has been reported in *E. coli* isolated from human gastrointestinal tract [12, 13]. Moreover, it has also been shown that on plant surface the biofilm formation is facilitated by the expression/production of cellulose and curli-fimbriae by *E. coli* [12, 20]. Biofilms have a significant value associated with public health as it aids in high resistance towards antibiotics [21]. Persistence of antimicrobial resistance in human pathogens associated in agricultural produce has an important role in human health as well as transfer of these antimicrobial resistance to other pathogens or local plant microbiota through horizontal gene transfer [22]. In our study we determined human enteric pathogens forming strong biofilms, 55% of *E. coli* isolates were having strong biofilm forming ability. Biofilm formation in food industry is a serious concern as this attachment of the pathogens to the vegetables and fruits are quite difficult and leading to illness. Our results of correlation between colony morphotypes, biofilm formation and antibiotic resistance also suggest that the expression/ production of cellulose and curli-fimbriae are implicated in biofilm formation and strong biofilm formation facilitates enhanced antibiotic resistance. Studies have shown that antibiotic resistance is significantly associated with enhanced biofilm formation [23]. The EPS production during biofilm formation prevent the exposure of antibiotics to the *E. coli* isolates and further increasing the possibility of genetic exchange among the consortium in the biofilm formed environment along with pathogenic and non-pathogenic bacterial isolates. The bacteria form biofilm along with the other bacterial communities and create a favorable niche that protects it from the adverse environment conditions. Various material such as cellulose and curli fimbriae helps in the attachment to the plant surface. These molecules present in bacteria helps in easy attachment and mask the molecules of the plant. Taken together, our results strongly suggest that biofilm formation along with expression/production of cellulose and curli fimbriae are involved in the colonization and survival of *E. coli* in plants with significantly increased antibiotic resistance.

V. CONCLUSION

In conclusion, the present study suggests that fresh produce (leafy greens, ready-to-eat produce) are potential reservoirs or sources of multidrug-resistant *E. coli* strains. Further, this study reports the prevalence and pattern of correlation of Biofilm formation and MDR in *E. coli* originating from fresh produce. The major limitation of the present study is that the analysis relied on phenotypic resistance data. Therefore, we did not have the opportunity to account for the multiple genetic determinants that underlie drug resistance. Despite this limitation, our study represents an important contribution to providing a large data set to identify *E. coli*, in particular to characterize the level and dynamics of biofilm formation and antimicrobial resistance in these pathogens present in leafy greens/fresh produce.

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REFERENCES

[1]. Callejón, R.M., Rodríguez-Naranjo, M.I., Ubeda, C., Hornedo-Ortega, R., Garcia-Parrilla, M.C. and Troncoso, A.M., 2015. Reported foodborne outbreaks due to fresh produce in the United States and European Union: trends and causes. *Foodborne pathogens and disease*, *12*(1), pp.32-38.

[2]. Brandl, M.T., 2006. Fitness of human enteric pathogens on plants and implications for food safety. *Annu. Rev. Phytopathol.*, *44*, pp.367-392.

[3]. Holden, N., Pritchard, L. and Toth, I., 2009. Colonization outwith the colon: plants as an alternative environmental reservoir for human pathogenic enterobacteria. *FEMS Microbiology Reviews*, *33*(4), pp.689-703.

[4]. Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R.V., Braden, C.R., Angulo, F.J. and Griffin, P.M., 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. *Emerging infectious diseases*, *19*(3), p.407.

[5]. Slavin, J.L. and Lloyd, B., Health benefits of fruits and vegetables. Adv Nutr 2012; 3 (4): 506-516.

[6]. Yang, S.C., Lin, C.H., Aljuffali, I.A. and Fang, J.Y., 2017. Current pathogenic Escherichia coli foodborne outbreak cases and therapy development. *Archives of microbiology*, *199*, pp.811-825.

[7]. Pande, T.K., Khan, A.H., Pipersania, R., Sethi, S.K. and Rath, Y., 2002. Watermelon poisoning. *Postgraduate Medical Journal*, *78*(916), pp.124-125.

[8]. Kunwar, R., Singh, H., Mangla, V. and Hiremath, R., 2013. Outbreak investigation: Salmonella food poisoning. *medical journal armed forces india*, *69*(4), pp.388-391.

[9]. Kisluk, G. and Yaron, S., 2012. Presence and persistence of Salmonella enterica serotype Typhimurium in the phyllosphere and rhizosphere of spray-irrigated parsley. *Applied and environmental microbiology*, *78*(11), pp.4030-4036.

[10]. Olaimat, A.N. and Holley, R.A., 2012. Factors influencing the microbial safety of fresh produce: a review. *Food microbiology*, *32*(1), pp.1-19.

[11]. Mann, R., Holmes, A., McNeilly, O., Cavaliere, R., Sotiriou, G.A., Rice, S.A. and Gunawan, C., 2021. Evolution of biofilm-forming pathogenic bacteria in the presence of nanoparticles and antibiotic: adaptation phenomena and cross-resistance. *Journal of nanobiotechnology*, *19*(1), pp.1-17.

[12]. Solomon, E.B., Niemira, B.A., Sapers, G.M. and Annous, B.A., 2005. Biofilm formation, cellulose production, and curli biosynthesis by Salmonella originating from produce, animal, and clinical sources. *Journal of food protection*, *68*(5), pp.906-912.

[13]. Yaron, S. and Römling, U., 2014. Biofilm formation by enteric pathogens and its role in plant colonization and persistence. *Microbial biotechnology*, *7*(6), pp.496-516.

[14]. Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A. and Kjelleberg, S., 2016. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, *14*(9), pp.563-575.

[15]. Bokranz, W., Wang, X., Tschäpe, H. and Römling, U., 2005. Expression of cellulose and curli fimbriae by Escherichia coli isolated from the gastrointestinal tract. *Journal of Medical Microbiology*, *54*(12), pp.1171-1182..

[16]. Vestby, L.K., Møretrø, T., Ballance, S., Langsrud, S. and Nesse, L.L., 2009. Survival potential of wild type cellulose deficient Salmonella from the feed industry. *BMC veterinary research*, *5*, pp.1-10.

[17]. Ibekwe, A.M. and Grieve, C.M., 2003. Detection and quantification of Escherichia coli O157: H7 in environmental samples by real-time PCR. *Journal of applied microbiology*, *94*(3), pp.421-431.

[18]. Stepanović, S., Vuković, D., Dakić, I., Savić, B. and Švabić-Vlahović, M., 2000. A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of microbiological methods*, *40*(2), pp.175-179.

[19]. Jain, S. and Chen, J., 2007. Attachment and biofilm formation by various serotypes of Salmonella as influenced by cellulose production and thin aggregative fimbriae biosynthesis. *Journal of food protection*, *70*(11), pp.2473-2479.

[20]. Méric, G., Kemsley, E.K., Falush, D., Saggers, E.J. and Lucchini, S., 2013. Phylogenetic distribution of traits associated with plant colonization in E scherichia coli. *Environmental microbiology*, *15*(2), pp.487-501.

[21]. Madani, A., Esfandiari, Z., Shoaei, P. and Ataei, B., 2022. Evaluation of Virulence Factors, Antibiotic Resistance, and Biofilm Formation of Escherichia coli Isolated from Milk and Dairy Products in Isfahan, Iran. *Foods*, *11*(7), p.960..

[22]. Thanner, S., Drissner, D. and Walsh, F., 2016. Antimicrobial resistance in agriculture. *MBio*, *7*(2), pp.e02227-15.

[23]. Bhardwaj, D.K., Taneja, N.K., Shivaprasad, D.P., Chakotiya, A., Patel, P., Taneja, P., Sachdev, D., Gupta, S. and Sanal, M.G., 2021. Phenotypic and genotypic characterization of biofilm forming, antimicrobial resistant, pathogenic Escherichia coli isolated from Indian dairy and meat products. *International Journal of Food Microbiology*, *336*, p.108899.