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FULL PAPER

Genotypic and phenotypic characteristics associated with biofilm formation in *Escherichia coli* and *Salmonella* spp. isolated from *ulam* in Terengganu

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Abstract

Nowadays, the foodborne outbreaks associated with fresh produces, including *ulam*, are increasing worldwide. The biofilm formation or bacterial attachment to plant surface is the initial step towards the contamination in fresh produce. The biofilm phenotype of bacteria grown on congo red agar is termed as red, dry and rough (rdar) morphotype. The binding of congo red dye with both biological proteins and inert surfaces is due to the presence of curli fimbriae and cellulose as the main extracellular components. The objective of this study was to determine the rdar morphotypes, biofilm ability and the role of *csg*A gene of *Escherichia coli* and *Salmonella* spp. isolated from *ulam* or Malaysian herbs. A total of 29 isolates, including 23 *E. coli* and 6 *Salmonella* spp. were analyzed for their ability to produce biofilm by colony morphology test, microtiter plate biofilm assay and qualitative biofilm test (pellicle formation). The presence of the *csg*A gene of *E. coli* was identified by PCR, which demonstrated the potential gene that able to produce curli fimbriae. Results revealed that 16 (69.6%) *E. coli* isolates were categorized as strong biofilm producers, 2 (8.7%) as moderate biofilm producers, 3 (13%) as weak biofilm producers, whereas 2 (8.7%) as negative biofilm producers (did not produce biofilm). While 4 (66.7%) *Salmonella* spp. isolates were identified as strong biofilm producers, 1 (16.7%) as moderate biofilm producers and 1 (16.7%) as negative biofilm producers. Majority of the *E. coli* strains (69.6%) were identified as strong biofilm producers and able to express rdar morphotypes. The ability of the of *E. coli* and *Salmonella* spp. isolates to form biofilm reveals the ability of these isolates to persist on the fresh vegetables and become hosts for the disease transmission to humans or/and animals.

1. Introduction

In Malaysia, a type of fresh vegetables that eaten raw or without cooking is called as *ulam*. Usually, people consume *ulam* as a side dish with rice and it acts as an appetizer. In the Southeast Asia countries, more than 120 plant species have been considered as *ulam* which may include leaves, shoots, seed, and fruits of vegetables (Reihani and Azhar, 2012). Studies have been proven that *ulam* has a lot of nutritional benefits and capabilities to prevent from diseases, resulting in an increased intake among people (Abas *et al*., 2006). However, foodborne outbreaks associated with the consumption of ready-to-eat fruits and vegetables have been increasing worldwide. In Brazil, thirty fruits and vegetables related outbreaks resulted in 2926 illnesses and 347 hospitalisations from 2008 to 2014 (Elias *et al*., 2018). Minimally processed fruits and vegetables are usually consumed without cooking and require no further treatment, making the presence of pathogens is a major concern. Raw vegetables can serve as vehicles for many different foodborne pathogenic microorganisms such as *E. coli* O157: H7, *Salmonella* spp., *Shigella* spp., *Listeria monocytogenes*, *Campylobacter* spp., *Staphylococcus aureus* and *Clostridium botulinum* (Mritunjay and Kumar, 2015). The vegetables can be contaminated from various sources such as soil, water (crop irrigation, application of pesticides and flood) and feces of animals (Ingham *et al.,* 2005).

Faecal contamination is specifically indicating the presence of *E. coli* (Mukherjee *et al.,* 2004). In 2006, *E. coli* O157: H7 contamination of lettuce and spinach resulted in 81 and 199 cases (3 deaths) in the United

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States of America (CDC 2006; FDA 2006; Sela *et al*., 2009; CDC, 2011). In 2008, there was an *E. coli* O157: H7 outbreak linked to lettuce resulted in 134 cases in Canada (Warriner and Namvar, 2010). Most of the cases were associated with the packaged product. Products had been washed and disinfected with hypochlorite before packaging, but the process was insufficient to eliminate *E. coli* O157: H7 and leading to microbial infections to humans (Rodriguez *et al.,* 2011). The reason behind this packaged product remained unclear, however, biofilm is believed to be the major culprit.

Biofilm is microbiologically derived sessile communities characterized by cells that are irreversibly attached to a substratum or interface to each other and embedded in a matrix of extracellular polymeric substances that they have produced. They are capable to exhibit an altered phenotype due to the effects of growth rate and gene transcription (Donlan and Costerton, 2002). Bacteria are capable to survive environmental stress, such as nutritional and oxidative stresses, desiccation, UV light exposure, and sanitizing agents with biofilms formed (Fatemi and Frank, 1999). Substrates' properties, the surface of the bacteria and genetic mechanism play an important role in biofilm formation (Shi and Zhu, 2009). According to Uhlich *et al.* (2006), *E. coli* O157: H7 can form biofilms on various types of surfaces because of its ability to attach and colonized on the surface. Biofilm can be formed by *E. coli* O157: H7 not only on abiotic surfaces, but also on biotic surfaces such as spinach, lettuce, Chinese cabbage, celery, leeks, basil, and parsley (Pawar *et al.,* 2005). *Salmonella* is also capable of adhering and forming biofilms on fresh produce leading to persistence and resistance to disinfection treatment, which subsequently can cause human infection (Yaron and Romling, 2014). Islam *et al.* (2004) reported the high persistence of nature of *Salmonella enterica* serovar Typhimurium in various types of vegetables such as lettuce, radish, carrot and parsley. The *S. enterica* serovar Typhimurium were persisted for more than 6 months in a compost-amended soil on which the vegetables were grown because the biofilm reached maturation stage.

The term 'rdar' (red, dry and rough) morphotype comes from distinctive colony appearance which is formed by the bacteria from the family *Enterobacteriaceae* on agar supplemented with Congo red dye. The occurrence of the rdar morphotype is vital for the survival of pathogenic bacteria outside of the host organism, and it is comparable with the process of spore formation in Gram-positive bacteria (Milanov, Prunic, Velhner, Pajic *et al.,* 2015). This morphotype could play a role in the transmission of bacteria between hosts, and it is also significant for the pathogenesis of specific infection. The rdar morphotype can express curli fimbriae and cellulose as the extracellular matrix components. The expression of curli fimbriae is performed by two divergently operons *csg*DEFG and *csg*ABC (Bokranz *et al*., 2005). Curli fimbriae are mostly made up of *csg*A, and it consists of a β helix of five repeat units. Curli fimbriae are essential for host cell adhesion, biofilm formation and important stimulants of the host inflammatory response (Dueholm *et al*., 2011).

This work examined the ability of *E. coli* and *Salmonella* spp. to form a biofilm, rdar morphotypes and the role of *csg*A gene of *E. coli* and *Salmonella* spp. in stress resistance.

2. Material and methods

2.1 Preparation of samples

Each *ulam* sample taken from sampling site was put separately to avoid cross-contamination, kept in the polystyrene box containing ice packs and transported to the laboratory immediately. No additional washing steps were applied to the samples after collection as this would represent the actual microflora present in the *ulam* samples. Samples were stored at 4-8ºC and were processed within 2 hrs of collection. A total of 25 g of each cut of *ulam* (Figure 1) was weighed into a sterile stomacher bag. Then, the 225 mL of sterile buffered peptone water (BPW) (Merck, Germany) was added and then stomached for 2 mins using a stomacher (BagMixer 400, Interscience, Singapore).

2.2 Isolation of Escherichia coli from fresh vegetables

Thirty samples of fresh vegetables were collected from different sampling sites in Kuala Terengganu during June-August 2016. The sampling sites included supermarkets, wet markets and mini markets/groceries. The isolation of *E. coli* was done by following the method described in the Bacteriological Analytical Manual (Jackson *et al*., 2001). *E. coli* ATCC 25922 was used as a positive control.

2.3 Isolation of Salmonella spp. from fresh vegetables

Twelve samples of fresh vegetables were collected from different sampling sites in Kuala Terengganu during May 2017-February 2018. The sampling sites included supermarkets and wet markets. The isolation of *Salmonella* spp was done by referring to the method based on BS EN 12824 (Roberts and Greenwood, 2003). *Salmonella* ATCC 14028 was used as a positive control.

2.4 Gram Staining

The standard protocol of Gram staining was done by

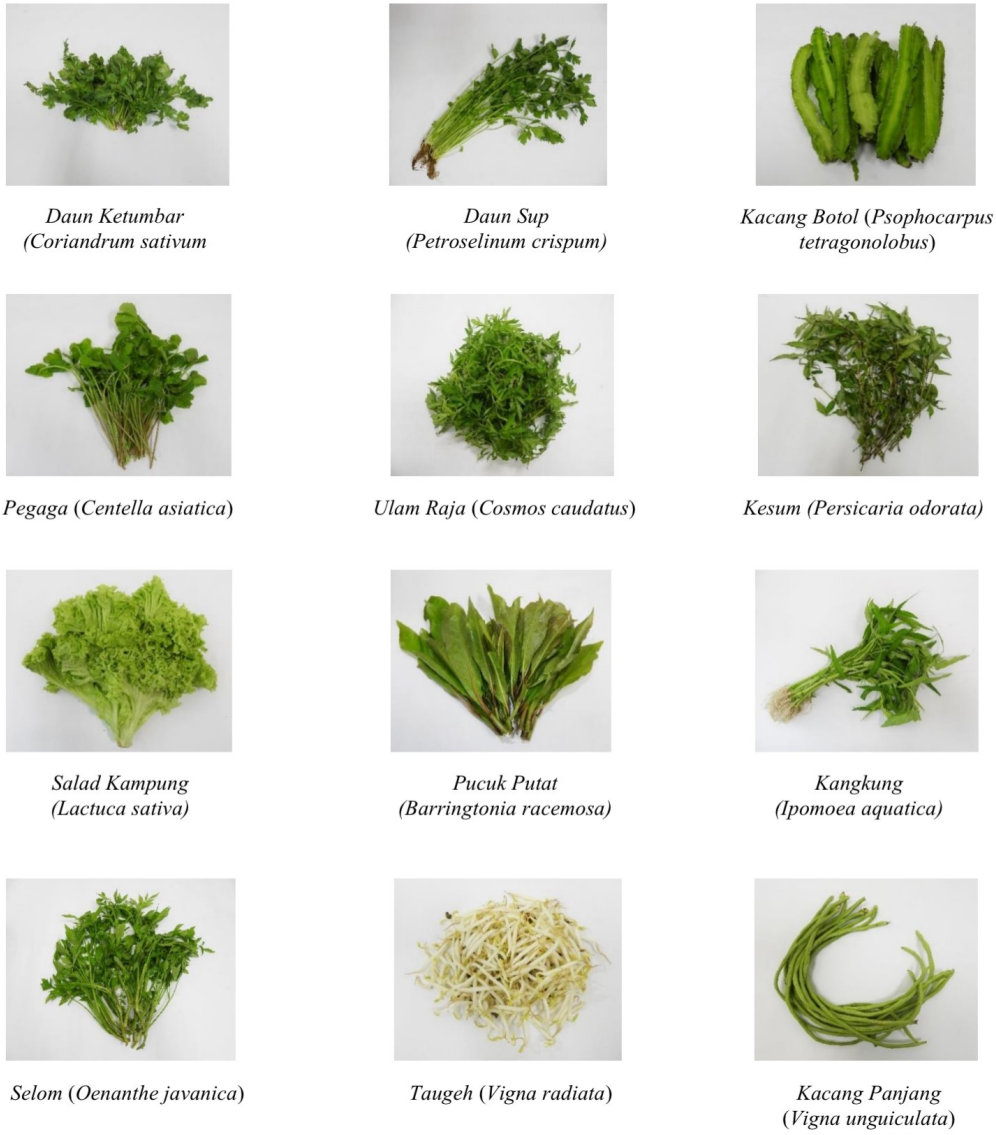


Figure 1. The Malaysian Traditional Vegetables (*Ulam*)

referring to the method described by James and Natalie (2014). The results of staining were observed under oil immersion using a light microscope with a total magnification of 1000x (Leica DME, Matrix Optics (M) Sdn Bhd, Malaysia).

2.5 Colony morphology

Colony morphology on Congo red-supplemented agar was used to detect the formation of fimbriae in colonies. LB agar without NaCl consists of 10 g/L tryptone (Merck, Germany), 6 g/L technical agar (Oxoid, UK), and 5 g/L of yeast extract (HiMedia Laboratories Pvt. Ltd, India) was supplemented with 0.04 g/L Congo red (Sigma Aldrich Inc., Germany). Then, 1 µL of the overnight culture was inoculated into congo red agar and incubated at 25ºC for 5 days (Abdullah *et al.,* 2017). Their colony morphologies were observed at day 3 and 5. The morphotypes observed were red, dry and rough (rdar) and smooth and white morphotype (saw). Rdar morphotypes able to form multicellular communities and their colonies were dark red colour, with a rough surface and undulated margins. While saw morphotypes showed white colonies and expression of matrix component was absent.

2.6 Biofilm formation protocol

2.6.1 Qualitative method

Biofilm formation on glass was tested as follows: 10 ml Luria Bertani (LB) broth without salt contained 10 g/ L tryptone (Merck, Germany) and 5 g/L yeast extract (HiMedia Laboratories Pvt. Ltd, India) was prepared in 25 mL Universal bottle. Then, one loopful of the overnight culture was inoculated into the broth and incubated at 25ºC for 4 days. Pellicle formation was recorded before and after staining with 0.1% (w/v) aqueous crystal violet solution (Merck, Germany).

2.6.2 Quantitative method

The following quantitative method was used to test biofilm formation on a microtiter plate (O'Toole and Kolter, 1998): a culture of strain was grown overnight in a rich medium of Luria Bertani (LB) broth consist of 10 g/L tryptone (Merck, Germany), 10 g/L sodium chloride (NaCl) (Merck, Germany) and 5 g/L yeast extract

(HiMedia Laboratories Pvt. Ltd, India). The overnight culture was diluted 1:100 (1%) into fresh medium for biofilm assays. Biofilm assays medium for the strain is LB broth without salt. Then, 200 µL of the dilution were added per well in 96 micro-well plates (flat bottom), typically used 6 replicate wells for each different strain. LB broth without salt (bacteria-free medium) was put together as a control. The 96 flat-bottom microplates were incubated at the 25ºC in the Humidity Chamber Model HCP (Memmert, Germany) and at static condition for 4 days.

After 4 days, the liquid cultures were discarded, and the plates that previously used for biofilm formation were washed twice with 225 µL of distilled water to discard the broth. Plates were inverted on a paper towel to remove excess moisture. Plates were then allowed to air dry in laminar flow (AVC-4DI, Esco, US) for 30 mins. Biofilm formation of different strains of *E. coli* and *Salmonella spp.* were stained using 0.1% aqueous crystal violet. Then, 200 µL of 0.1% aqueous crystal violet was added per well and left for 20 mins. Stains were discarded and plates were rewashed twice with 225 µL of distilled water. Plates were then submerged in a bath of distilled water twice, and water has been replaced between washes. Plates were tapped on a paper towel to remove excess moisture and were allowed to air dry under the laminar flow for 30 mins. The adherent stained biofilms were dissolved with 200 µL of 95% ethanol (R & M Chemicals, UK). The optical density was measured at 620 nm (OD 620 nm) in a microplate reader (Multiskan^{TM} GO Microplate Spectrophotometer, Thermo Fisher Scientific, US). The higher the absorbance, a stronger biofilm was formed.

*2.7 The sequence of csg*A *gene for detection of curli fimbria gene (biofilm)*

2.7.1 Polymerase chain reaction

The *csg*A gene (Forward primer 5'-ACTCTGACTTGACTATTACC -3' and reverse primer 5'- AGATGCAGTCTGGTCAAC -3') were used for the amplification of the DNA fragment. The *csg*A primers were synthesized by Integrated DNA Technologies (IDT). PCR reaction mixture, which comprised of 12.5 µL of 2x PCR Buffer, 5 µL of dNTP mix, 2 mM each, 1 µL of forward primer, 1 µL of reverse primer, 1 µL of DNA template, 0.5 µL Thermostable DNA polymerase (1U/µL) and 4 µL of Nucleus free water to make up the reaction volume of 25 µL. All PCR mixture was purchased from Promega (USA), and PCR amplifications were performed in an MJ Mini Personal Thermal Cycler (Bio-Rad Laboratories, US). The cycling conditions after gradient PCR were optimized with an initial denaturation at 94ºC for 2 mins, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 54.5ºC for 1 min, and extension at 68ºC for 30 s.

2.7.2 Gel electrophoresis

Aliquots of 1 µL PCR products were run on 1.7% TAE agarose gel (Hydragene, USA) at 100 V, 60 minutes. The size of the amplified product was confirmed by comparison with a standard DNA marker (100 bp ladder) (Promega, USA). The presence of amplicon at 198 bp were found positive for curli structural gene *csg*A. The sequencing process was sent to Apical Scientific Sdn. Bhd., Seri Kembangan, Selangor, Malaysia.

3. Results and discussion

In this study, we investigated in particular 23 isolates of presumptive *E. coli* and 6 isolates of *Salmonella* spp. for their ability to form biofilm and to produce the extracellular structures essential for adhesion to solid surfaces through colony morphology test. The graphical abstract is exhibited in Figure 2.

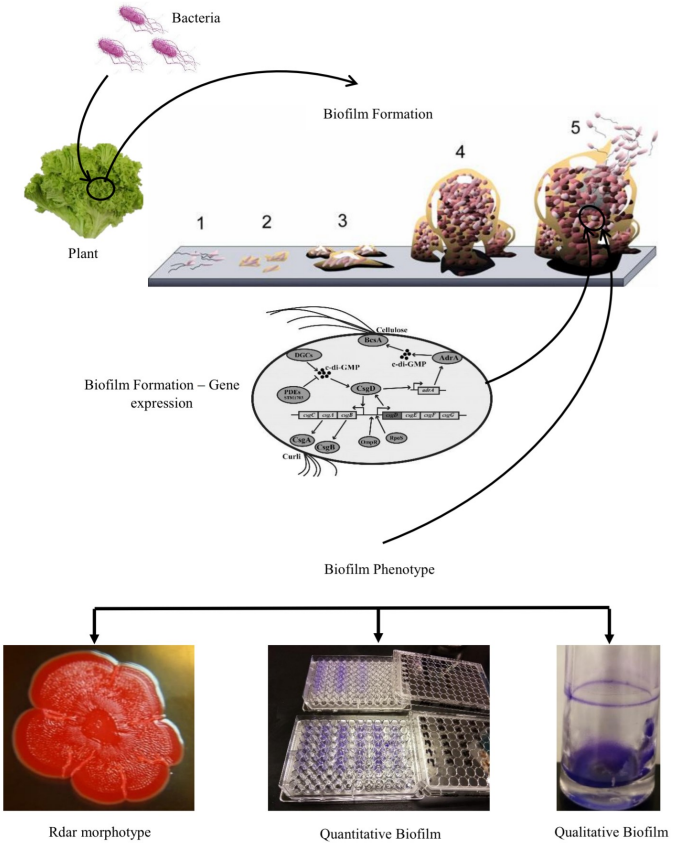


Figure 2. Graphical abstract of biofilm formation on plant surface (Gantcharova *et al.,* 2010; Unosson, 2015).

3.1 Colony morphology test

The colony morphology test was performed for the screening of biofilm formation. This test is fast and easy to implement among the phenotypic methods, its specificity, sensitivity, and accuracy are low compared to other quantitative and qualitative biofilm methods. Phenotypic production by all the isolates was analyzed

and interpreted according to standard colour variation from white to red. In this study, three distinctive colony morphologies were observed red, dry and rough (rdar), intermediate and smooth and white (saw) morphotypes. Red colonies represented biofilm formers, while white represented non-biofilm formers. The colonies formed by the intermediate morphotype was not as visually distinctive in morphotype as either rdar morphotypes or saw morphotypes. The observation was carried out after 120 hrs of incubation, as recommended by Abdullah *et al.* (2017). After 120 hrs incubation at 25ºC on Luria Bertani (LB) agar without NaCl supplemented with congo red, three distinctly different morphotypes were observed in the colonies formed by the *E. coli* strains isolated from vegetables; 78.3% (18/23) expressed red, dry and rough (rdar) morphotypes, 13% (3/23) intermediate morphotypes and 8.7% (2/23) smooth and white (saw) morphotypes. The assessment of colony morphology by *Salmonella* spp. showed that 100% (6/6) showed intermediate morphotypes after inoculated on congo red agar plates presented in Table 1.

Table 1. Prevalence of Congo Red morphotypes of *Escherichia coli* and *Salmonella* spp.

Isolate	Morphotypes Prevalence		
	rdar	intermediate	saw
E. coli	18/23 (78.3%)	3/23 (13%)	2/23 (8.7%)
Salmonella spp.		6/6 (100%)	

Congo red supplemented agar used to visualize curli and cellulose production in the colonies. Rdar colonies isolate producing both curli fimbriae and cellulose as an extracellular matrix component. Table 2 and 3 show rdar morphotype was expressed by 18 (78.3%) isolates of *E. coli* (SMDS2, SMDS4, WMDS, SMKG1, SMKG2, SMKG3, SMKG4, SMKB2, SMKB4, SMKB5, SMKB8, SMK2, SMK3, SMU2, SMT4, WMSK4, WMSK7 and WMPP2) and rdar morphotypes absent in all *Salmonella enterica*. Verma *et al.* (2018) reported that rdar morphotype characterized by both expressions of curli fimbriae and cellulose production was identified in 28 isolates (38.3%) of *E. coli* isolated from fresh vegetables and fruits samples.

The binding of the dye congo red to *E. coli* and *Salmonella* spp. have been reported to correlate with their production of curli and cellulose. Curli are thin aggregative surface fibers which are involved in adhesion of *E. coli* and *Salmonella* to its contact host (Gophna *et al.,* 2002). Curli fibers bind the congo red dye and specific host matrix proteins including fibronectin, laminin, plasminogen, and major histocompatibility complex class I molecules to initiate adherence and colonization in the host (Olsen *et al.,* 1998; Gophna *et al.,* 2001). Cellulose is an exopolysaccharide in which individual glucose monomers connected by a 1-4-β-glycosidic bond, which in bacteria is synthesized and excreted by a membrane-inserted cellulose synthase complex consisting of two subunits, BcsA and BcsB (Morgan *et al.,* 2013; Whitney and Howell, 2013). As a second matrix-component *Salmonella* and *E. coli* strains secrete cellulose as an extracellular component for mechanical and chemical protection (Zogaj *et al*., 2001). Previous studies have shown that curli and cellulose also play a role in *E. coli* attachment to plant surfaces as well. Jeter and Matthysse (2005) reported that *E. coli* K12 isolates carrying a plasmid encoding a gene for curli biosynthesis manifest a stronger attachment to tomato, *Arabidopsis* seedlings and alfalfa sprouts compared to those not producing curli.

While three isolates of *E. coli* (SMP1, SMKB3 and WMSK6) and six isolates of *Salmonella* spp. (WMS.HE.TT.2, SMKP.XLD.4, SMT.XLD.1, SMT.XLD.2, SMT.XLD.3 and SMT.XLD.RVS.1) expressed intermediate morphotype. The attempt had been done to prolong incubation to see whether intermediate morphotype can change to rdar morphotype; however, no changes were observed. Instead of clear rdar or saw morphotypes, these strains expressed some red binding but at levels substantially less than rdar morphotypes. The presence of red in these colonies often was higher in some regions of the colony growth, as observed by the ring of red on SMKB3/EC, WMSK6/EC and SMT1/S.

When no matrix components are expressed, the phenotypes are smooth and white (saw). It was consistent with the absence of curli on the cell surface. Saw morphotype was observed in two isolates (8.7%) of *E. coli* (SMDS1 and WMSK1). In another study, a higher percentage of saw morphotype was observed. It was found that 9 isolates (12.3%) of *E. coli* isolated from fresh vegetables and fruits sample did not bind with congo red dye and declared as negative (Verma *et al.*, 2018).

3.2 Quantitative biofilm

Several studies have shown that *E. coli* and *Salmonella* spp. are capable of adhering and forming biofilms on multiple surfaces including metal, rubber surfaces and glass (Hood and Zottola, 1997; Joseph *et al.,* 2001; Stepanovic *et al.,* 2004). The assessment of biofilm formation of *E. coli* and *Salmonella* spp. In this study showed that most of them were able to form biofilms. In this study, quantitative biofilm was assessed using 96-microtitre plate. The bacteria isolates were able to form biofilm on microtiter plate heighten its ability to form biofilm on different surfaces. The microtitre plate has the advantage of being simple and can be easily modified to growth conditions and examined multiple

Table 2. Gram stain, colony morphology and biofilm formation on the air-liquid interface of *Escherichia coli*

Source	Laboratory Designation	Gram Stain	Colony Morphology[a]	Thin Aggregative Fimbriae Biosynthesis	Pellicle on air-liquid interface[b]
Pegaga	SMP1	Negative	intermediate	-	+
Daun Sup	SMDS1	Negative	Saw	-	-
	SMDS2	Negative	rdar, convoluted	++	+++
	SMDS4	Negative	rdar, convoluted	+	++
	WMDS	Negative	rdar, convoluted	++	+++
Kangkung	SMKG1	Negative	rdar, convoluted	++	+++
	SMKG2	Negative	rdar, convoluted	++	+++
	SMKG3	Negative	rdar, convoluted	++	+++
	SMKG4	Negative	rdar, convoluted	++	+++
Ketumbar	SMKB2	Negative	rdar, convoluted	++	+++
	SMKB3	Negative	intermediate	-	+
	SMKB4	Negative	rdar, convoluted	++	+++
	SMKB5	Negative	rdar, convoluted	++	+++
	SMKB8	Negative	rdar, convoluted	++	+++
Kesum	SMK2	Negative	rdar, convoluted	++	+++
	SMK3	Negative	rdar, convoluted	++	+++
Ulam Raja	SMU2	Negative	rdar, convoluted	++	+++
Taugeh	SMT4	Negative	rdar, convoluted	++	+++
Salad Kampung	WMSK1	Negative	Saw	-	-
	WMSK4	Negative	rdar, convoluted	++	+++
	WMSK6	Negative	intermediate	-	+
	WMSK7	Negative	rdar, convoluted	+	++
Pucuk Putat	WMPP2	Negative	rdar, convoluted	++	+++

Table 3. Gram stain, colony morphology and biofilm formation on the air-liquid interface of *Salmonella* spp.

Source	Laboratory Designation	Gram Stain	Colony Morphology[a]	Thin Aggregative fimbriae biosynthesis	Pellicle on air-liquid interface[b]
Selom	WMS.HE.TT.2	Negative	Intermediate	-	+++
Kacang Panjang	SMKP.XLD.4	Negative	Intermediate	-	+++
Taugeh	SMT.XLD.1	Negative	Intermediate	-	+
	SMT.XLD.2	Negative	Intermediate	-	+++
	SMT.XLD.3	Negative	Intermediate	-	++
	SMT.XLD.RVS.1	Negative	Intermediate	-	+++

[a]Morphotypes on Congo Red agar (rdar) red, dry and rough, intermediate and (saw) smooth and white; [b]Phenotype on air-liquid interface: (-) the complete absence of pellicle – lack of ability to produce biofilm; (+) formation of very thin pellicle – weak biofilm producer; (++) formation of thin pellicle – moderate biofilm producer, (+++) formation of strong and thick pellicle – strong biofilm producer.

strains in each experiment. The microtiter plate method for detection of biofilm production demonstrated that a lower percentage of isolates as strong biofilm producer compared to colony morphology test results, indicating the specificity of the method.

The isolates of *E. coli* and *Salmonella* spp. varied in their ability to produce biofilm on the surface of a microtiter plate with optical density measured at 620 nm ranging from 0.051-0.955; 0.123-0.418 respectively. The values for optical density (OD) of *E. coli* and *Salmonella* spp. isolates are represented in Figures 3 and 4. After incubation in LB broth without salt at 25°C, 16 (69.6%) *E. coli* isolates were categorized as strong biofilm producers, 2 (8.7%) as moderate biofilm producers, 3 (13%) as weak biofilm producers, whereas 2 (8.7%) as negative biofilm producers (did not produce biofilm). While 4 (66.7%) *Salmonella* spp. isolates were identified as strong biofilm producers, 1 (16.7%) as moderate biofilm producers and 1 (16.7%) as negative biofilm producers (Tables 4 and 5).

Table 6 shows the significant moderate correlation in biofilm formation of *E. coli* strains with the same morphotypes ($r=0.718$, $p<0.001$). Results of the correlation indicate that rdar morphotypes are associated with higher biofilm formation. All rdar and intermediate *E. coli* and *Salmonella* spp. isolates were identified as

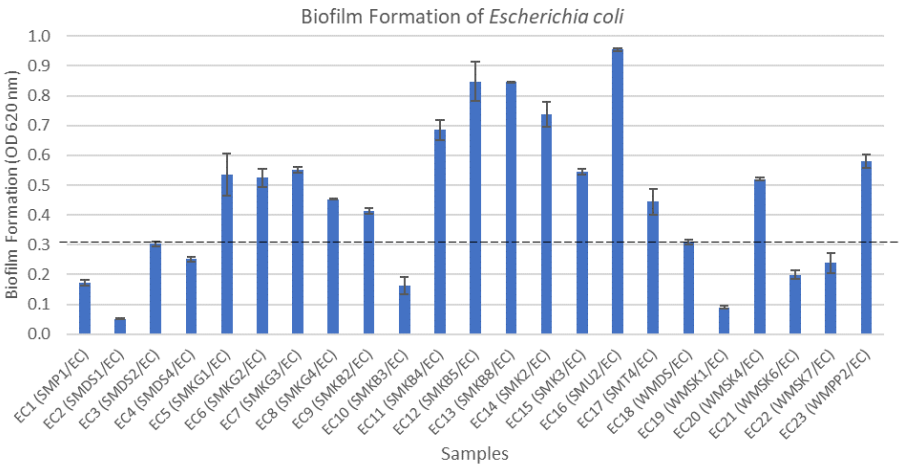


Figure 3. The Biofilm Formation of *E. coli* strains. The Biofilm formation was calculated by using formula; Biofilm Formation (BF) BF = AB – CW; AB, stained attached bacteria; CW, stained control wells.
*Value more than 0.30 indicate strong biofilm.

Table 4. Screening of biofilm formers of *Escherichia coli*

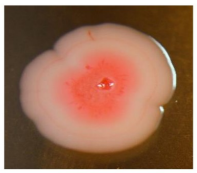
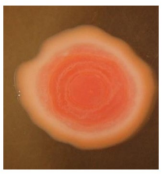
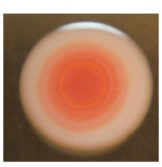
Morphotypes	Colony Morphology	*E. coli*	Biofilm Production	Universal Bottle	*E. coli*
Smooth and white (saw)		2 (8.7%)	Negative		2 (8.7%)
Intermediate		3 (13%)	Weak		3 (13%)
Red, dry and rough (rdar)		18 (78.3%)	Moderate		2 (8.7%)
			Strong		16 (69.6%)

Table 5. Screening of biofilm formers of *Salmonella* spp.

Morphotypes	Colony Morphology	*Salmonella* spp.	Biofilm Production	Universal Bottle	*Salmonella* spp.
			Weak		1 (16.7%)
Intermediate		6 (100%)	Moderate		1 (16.7%)
			Strong		4 (66.6%)

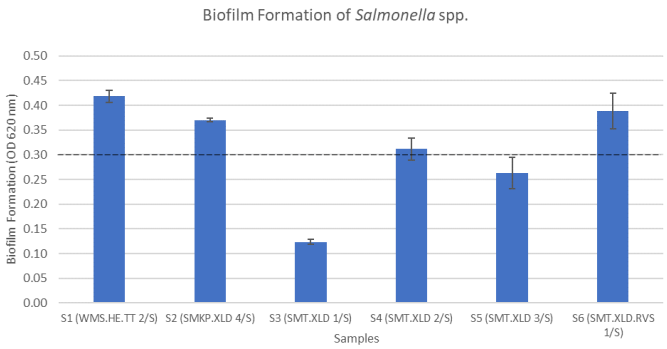


Figure 4. The Biofilm Formation of *Salmonella* spp. strains. The Biofilm formation was calculated by using formula; Biofilm Formation (BF) BF = AB – CW; AB, stained attached bacteria; CW, stained control wells.
*Value more than 0.30 indicate strong biofilm.

biofilm producers. Majority of the *E. coli* strains (69.6%) were identified as strong biofilm producer and also able to express rdar morphotypes. While 2 (8.7%) *E. coli* isolates were recognized as saw morphotypes and did not produce biofilm. The current results were in accordance to previous authors that described the correlation between a phenotypic appearance on congo red agar and biofilm formation (Bokranz *et al*., 2005; Milanov, Prunic, Velhner, Todorovic *et al.,* 2015; Amrutha *et al*.,2017). Bokranz *et al.* (2005) demonstrated that *E. coli* strains expressing rdar morphotype showed a higher capacity to form biofilms than saw strains.

3.3 Qualitative biofilm

Table 2 and 3 present the result for biofilm phenotype at the air-liquid interface i.e., ability to form a pellicle at the air-liquid interface at 25°C after 120 hours incubation in LB broth without salt. A visible purple film lining the walls and bottom of the universal tubes indicated the positive result for biofilm formation. The amount of biofilm formed was categorized as weak, moderate and strong, depending on the intensity of the

Table 6. Correlation between colony morphotypes and biofilm production of *E. coli*

			Colony Morphotypes	Biofilm Formation
Spearman's rho	Colony Morphotypes	Correlation Coefficient	1	.718**
		Sig. (2-tailed)	.	0
		N	69	69
	Biofilm Formation	Correlation Coefficient	.718**	1
		Sig. (2-tailed)	0	.
		N	69	69

visible film. The formation of a strong and thick pellicle (+++) was observed in 16 (69.6%) *E. coli* isolates (SMDS2, WMDS, SMKG1, SMKG2, SMKG3, SMKG4, SMKB2, SMKB4, SMKB5, SMKB8, SMK2, SMK3, SMU2, SMT4, WMSK4, WMPP2) and 4 (66.7%) *Salmonella* spp. isolates (WMS.HE.TT.2, SMKP.XLD.4, SMT.XLD.2, SMT.XLD.RVS.1). Amrutha *et al*. (2017) reported 22.8% *E. coli* and 22.2 % *Salmonella* isolated from fresh fruits and vegetables were strong biofilm former. Those isolates also formed a strong and thick pellicle on the test tube.

In 2 of *E. coli* isolates (SMDS4, WMSK7) and 1 of *Salmonella* spp. isolate (SMT.XLD.3) the formation of a moderate or thin pellicle (++) was observed. 3 of *E. coli* isolates (SMP1, SMKB3, WMSK6) and 1 of *Salmonella* spp. (SMT.XLD.1) formed a weak or very thin pellicle (+). While, 2 isolates of *E. coli* (SMDS1, WMSK1) did not form a pellicle at the air-liquid interface (Table 4 and 5). These findings were consistent with those observed in the microtiter plate.

*3.4 csg*A *gene for detection of curli fimbria gene (biofilm)*

The *csg*A gene is essential for the expression of the major subunit protein of the fibre (*csg*A subunit protein) and encoding curli fimbriae. Previous studies have shown that curli produced by *E. coli* O157: H7 and *S. enterica* play a significant role in the attachment to plant surfaces (Jeter and Matthysse, 2005). Fink *et al.* (2012) reported that the *csg*A mutant in *E. coli* K12 and O157: H7 was weakened in their ability to attach and colonize on lettuce leaves. A series of primer was designed by Maurer *et al.* (1998) to recognize the curli structural gene *csg*A. The expected size of the PCR product for *csg*A is 200 bp (Figure 5).

One isolate from *E. coli* (SMK2/EC) and *Salmonella* (WMS.HE.TT.2/S) was selected to detect the presence of the curli selected gene. These isolates are among the highest reading of optical density (OD) when tested with the microtiter plate. *Csg*A gene was present in *E. coli* isolate (SMK2/EC) but not detected in *Salmonella* isolate (Figure 5). This result is consistent with those results from colony morphology test as *E. coli* isolate showed the obvious formation of curli fimbriae. *Salmonella* isolate (WMS.HE.TT.2/S) was categorized

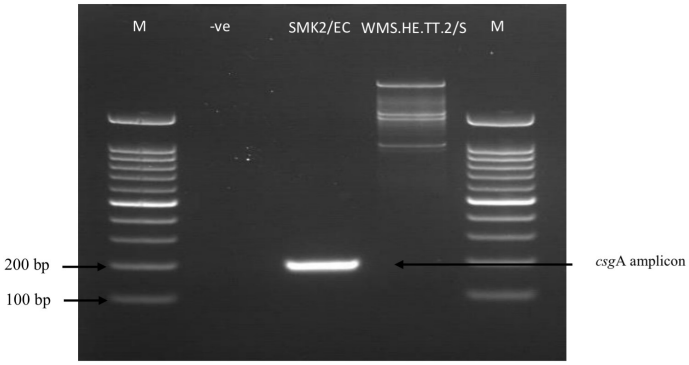


Figure 5. Representative agarose gel analysis of PCR assay targeting *csg*A gene in *Escherichia coli* (EC) and *Salmonella* spp. (S) isolates. Lane 1 and 5: 100 bp DNA ladder, Lane 2: Negative control, Lane 3: Sample 14/EC, Lane 4: Sample 1/S.

as strong biofilm producer, but during the colony morphology test, the formation of curli fimbriae was absent. This result was confirmed with the lack of the *csg*A gene (WMS.HE.TT.2/S).

Even though this isolate (WMS.HE.TT.2/S) lack of the *csg*A gene, but its biofilm-forming potential is not because of this particular gene. This happens due to the other genes may be involved in the biofilm formation, which is not taken into account in this study. As biofilm is highly organized and complex structure, several genes related to biofilm formation such as *csg*D (master regulator for biofilm formation), *fli*C (Flagella), *fim*H (Type 1 fimbriae), *bcs*A (cellulose) and many more (Niba *et al.,* 2007; Schiebel *et al*., 2017). For example, the *csg*D is a transcriptional protein that activates the biosynthesis and expression of both curli and cellulose. *Csg*D activates transcription of the *csg*BAC operon, which encodes curli structural subunits, and transcription of *adr*A gene, involved in cellulose biosynthesis (Brombacher *et al*., 2006; MacKenzie *et al.,* 2017).

4. Conclusion

The present study has investigated biofilm forming potential of *E. coli* and *Salmonella* spp. isolated from fresh vegetables. The findings demonstrated that the microorganism that associated with fresh vegetables are capable of biofilm formation. The *E. coli* (SMK2/EC) isolates from *Kesum* sample was one of the highest reading of optical density (OD). Besides, the presence of the *csg*A gene in the *E. coli* isolate (SMK2/EC) was consistent with the formation of curli fimbriae. This

isolate represents a public health concern because it can survive and distribute through the food chain to reach the consumer causing the food contamination and disease outbreak. The use of sanitizers (sodium hypochlorite, aqueous chlorine dioxide, hydrogen peroxide) is widely used in the food industry and effective for eradicating these biofilms on plant surfaces. However, the overuse of sanitizers may be one of the driving force concerning the emergence of antibiotic-resistant in bacteria. Therefore, a better understanding of bacterial attachment to fresh produce is necessary for the development of alternative washing treatments to control *E. coli* and *Salmonella* spp. contamination during handling and processing of food products. Besides, a regular and hygienic handling practice of fresh vegetables along the supply chain is important for public health concern and maintaining the standard of food safety.

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