

## Research Article

# Shigatoxigenic and Enteropathogenic *Escherichia coli* Biofilm Formation: Effects of Different Stress Conditions on Polystyrene and Stainless Steel Surfaces

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**Abstract**

Biofilms constitutes one of the main problems for food industry and public health due to the survival and the ability of pathogenic microorganisms to form these communities on different surfaces and culture composition. Shigatoxigenic (STEC) and Enteropathogenic (EPEC) *Escherichia coli* are some of the microorganisms that could form biofilms and cause serious diseases in humans. The aim of this study was to determine the capability to form biofilms of native STEC and atypical EPEC (aEPEC) strains on polystyrene and stainless steel and to evaluate the effects produced by different stress conditions. The expression of the fimbria curli was also studied. All strains were strong biofilm formers at 37°C on polystyrene, except STEC O145:NM and aEPEC O130:H11 which were moderated biofilm formers. No one strain formed biofilm at heat stress (54°C) or refrigeration (5°C) temperatures. Stainless steel surface reduced significantly the capability to form biofilms regardless the culture composition. Most of the STEC and aEPEC strains were curli-negative at 37°C while they showed curli-positive phenotypes at 20°C. Both curli expression and biofilm formation were significantly influenced by temperature and incubation time. STEC and aEPEC strains demonstrated to be able to form biofilm and produce fimbria curli under different conditions of medium and temperature, which is important due the risk of survival and transmission of these pathogens from reservoirs and food to humans.

**Keywords:** Shigatoxigenic *Escherichia coli*; atypical enteropathogenic *E. coli*; biofilms; culture; surface; curli fimbriae

**Abbreviations**

A/E: Adherence and Effacement; aEPEC: Atypical Enteropathogenic *E. coli*; aw: water activity; CH: Hemorrhagic Colitis; EPEC: Enteropathogenic *E. coli*; HUS: Hemolytic Uremic Syndrome; LB: Luria Bertani; MBF: Moderate-Biofilm-Forming; NBF: Non-Biofilm-Forming; OD: Optical Density; ODc: cut-off OD; SBF: Strong-Biofilm-Forming; SF: Physiological Solution; STEC: Shigatoxigenic *E. coli*; WBF: Weak-Biofilm-Forming

**Introduction**

Biofilms are complex communities of microorganisms that grow embedded in an extracellular matrix, adhered to biotic or abiotic surfaces. These microorganisms have an altered phenotype compared to their planktonic counterparts respect to the rate of growth and gene transcription. Biofilms could be composed of a single or several different microbial species [1]. Biofilms constitutes one of the main problems for industry and public health due to the survival and the ability of pathogenic microorganisms to form these communities on different surfaces, utensils and equipment in contact with food or on medical devices such as catheters and prostheses, among others. In the food industry, biofilms could be formed on surfaces such as plastic, glass, wood, stainless steel and on food [2]. Insufficient or inadequate disinfection of surfaces or utensils causes an increase in product contamination and the development of food poisoning [3].

Shigatoxigenic (STEC) and Enteropathogenic (EPEC) *Escherichia coli* are some of the microorganisms that could form biofilms. STEC is an emerging pathogen related to public health that causes serious diseases in humans such as Hemorrhagic Colitis (CH) and Hemolytic Uremic Syndrome (HUS) by the production of Shiga toxins and other virulence factors. Cattle are the main reservoir, being able to transmit STEC to the human through cattle derived foods and direct contact with the animals or their environment [4-6]. EPEC strains do not produce Shiga toxins but just like STEC, produce histopathological lesions of “adherence and effacement” (A/E) in the intestinal tract cells. It is transmitted via the fecal-oral route through contaminated hands, water and food. Children with diarrhea and asymptomatic children or adult carriers as well as chicken and derived food have been documented as EPEC reservoir [7,8].

STEC and EPEC harbor virulence and adherence factors that allow adhesion and colonization to both living and inert surfaces and favor the biofilm formation, such as, the IrgA homolog adherence-conferring protein (*iha*) [9], the enterohemorrhagic *E. coli* factor of adherence (*efa1*) [10], the type 1 fimbria (*fim*) [11] and the long polar fimbria (*lpf*) [12]. They also can harbor several proteins belonging to the Autotransporter (AT) protein family such as, Antigen 43 (*agn43*), the Calcium-binding antigen 43 homolog (*cah*) [13,14] and the Enterohemorrhagic *E. coli* (EHEC) autotransporter (*ehaA*) [15], which favor the agglomeration and cell-to-cell interactions.

The extracellular matrix in *E. coli* biofilms is formed by curli as the main component and cellulose [16]. Curli expression is regulated by different genes that code for protein subunits, such as its transcriptional regulator (*csgD*) and the indirect regulator of this operon (*crl*) [17]. Crl was proposed as a thermal sensor, which protein is more stable at low temperatures; therefore, it is considered that the maximum expression of curli occurs at less than 30°C, although it can be variable depending on the isolation, its pathogenicity and the working conditions [18].

Biofilms have been studied for decades using several *in vitro* models in an effort to understand the mechanisms of bacteria to survive and persist in the environment or into a host. Such studies reported the capability of the microorganisms to form biofilms is influenced by many factors, such as culture medium (nutrient composition, temperature, pH), hydrodynamics (continuous flow vs. batch system), substratum (roughness, chemistry), incubation times, among others [19-21]. The results obtained will depend on the combination of these factors, even using the same microorganism. For example, different abilities to form biofilms have been observed in STEC isolates from different origins according to the composition of the culture medium and the incubation times [22,23]. The effects of temperature, oxidative and nitrosative stress, pH, salt content and water activity (aw) have been the most studied factors that affect the survival and growth of STEC [24-28]. Previous studies with STEC strains isolated from cattle and food were carried out using different organic and inorganic acids, fruit and alcohol juices, and at extreme temperatures (acidic, alcoholic and thermal stress) to observe the viability of this bacterium against different stress conditions. The results showed that STEC no-O157 serotypes were more resistant to high temperatures (54°C) than O157:H7, which is the serotype routinely used to standardize the thermal inactivation protocols of these pathogens [29]. In addition, no-O157 serotypes had a better behavior to acidic pH than O157:H7 being able to survive at least 8 h at pH=2.5; suggesting the great resistance that they would have during the digestion process in the host and their potential to cause disease. In addition, the ability of these bacteria to neutralize the surrounding acidic environment has been proven, which would contribute to greater host survival [26].

In EPEC strains, biofilm formation studies were performed using different methodologies, demonstrating the ability of these bacteria to adhere to cell lines with different patterns and to form biofilm on surfaces such as polystyrene and glass [30]. The role of the expression of the BFP and EspA in the biofilm formation on polystyrene under static and continuous flow conditions has been determined in typical EPEC strains, although the quorum sensing mechanism was also necessary to form biofilm [31]. However, little is known about the influence of different stress situations or changes in temperature or pH on the biofilm formation of this *E. coli* pathotype, particularly in atypical strains.

The aim of this study was to determine the capability to form biofilms of native STEC and atypical EPEC strains on polystyrene and stainless steel and to evaluate the effects produced by different stress conditions. In addition, the expression of the fimbria curli was studied.

## Materials and Methods

### Bacterial Strains

A total of fifteen STEC and atypical EPEC strains (aEPEC) isolated from different sources were randomly selected from a collection of different *E. coli* pathotypes belonging of the Laboratorio de Inmunología y Biotecnología (UNCPBA, Tandil, Buenos Aires, Argentina); the reference STEC strain O157:H7 EDL933 was also included. All the strains were previously serotyped and characterized in their classic virulence factors *-stx1, stx2, eae, ehxA-* [4,5,8,32,33], in other putative virulence genes present in the megaplasmid *-katP, espP, subA, stcE-* and in several adhesins and fimbriae involved in colonization and biofilm formation *-efa1, iha, fimCD, ehaA, agn43, agn43EDL933, csgA, crl-* [34,35]. Strains were grouped according the pathotype and the presence/absence of *eae*: STEC (*eae* positive/negative) and aEPEC (Table 1).

### Biofilm Formation Assays

The detection and quantification of biofilms were performed in 96-well polystyrene microtiter plates and stainless steel coupons under previously described laboratory conditions [23], with modifications. Briefly, the experiments were performed in two sections, according to different stress conditions and surfaces. In section 1 (polystyrene surface), biofilm formation assay was performed under different temperatures of incubation: standard (37°C), heat stress (54°C) and refrigeration temperature (5°C) and different culture conditions: standard, acidic or alcoholic at 37°C. Acidic and alcoholic conditions were achieved adding acetic acid (0,05 M, pH=5,5) or ethyl alcohol (6%) to Luria Bertani (LB) medium (with and without glucose 0,5%) respectively. In section 2 (stainless steel surface), biofilm formation was performed under different culture conditions: standard, acidic and alcoholic as described above. In this section, 24-well polystyrene plates were used as support for stainless steel coupons (1x1cm, finishing number 304#4) which were previously cleaned and sterilized according to Joseph, et al. [36].

In each section, the surfaces were washed, fixed and stained with crystal violet (0.1%) according to the protocol described previously [23]. The dye adhered to the biofilms was eluted with 200 µl of ethylic alcohol 96% and the OD<sub>570</sub> was read in a Labsystem Multiscan EX microplates reader (I.C.T, S.L. Instrumentación Científica Técnica, S.L.). In section 2, only the stainless steel coupons were staining to obtain the OD corresponding to the biofilm on this surface.

Three consecutive wells were used for each strain. The OD of each well was averaged and corrected by a cut-off OD (average OD of three wells with sterile medium and 3 times its standard deviation). According to the corrected OD, the strains were classified into four categories: Non-Biofilm-Forming (NBF), Weak-Forming (WBF), Moderate-Forming (MBF) and Strong Biofilm-Forming (SBF) [37]. Three independent experiments were made and data obtained were statistically analyzed by ANOVA InfoStat 2015E (National University of Cordoba, Argentina).

### Curli Expression Assays

The curli phenotype of STEC and aEPEC strains was studied by the Congo red assay at optimum growth temperature (37°C) and at room temperature (≈20°C) and then, the biofilm formation from colonies incubated at both temperatures was evaluated. Briefly, one

**Table 1:** STEC and aEPEC serotypes used in this study.

<i>E. coli</i> group	Serotype	Virulence profiles
eae-positive STEC	O103:H2	<i>vt1, vt2, ehxA, eae, espP, subA, efa1, fimCD, ehaA, agn4, csgA, crl</i>
	O111:NM	<i>vt2, ehxA, eae, katP, espP, efa1, iha, fimCD, ehaA, agn43<sub>EDL933'</sub>, agn43, csgA, crl</i>
	O145:NM	<i>vt2, ehxA, eae, katP, espP, efa1, iha, fimCD, ehaA, agn43<sub>EDL933'</sub>, agn43, csgA, crl</i>
	O26:H11	<i>vt1, vt2, ehxA, eae, katP, espP, efa1, iha, fimCD, ehaA, agn43<sub>EDL933'</sub>, agn43, csgA, crl</i>
	O157:H7	<i>vt2, ehxA, eae, katP, espP, stcE, efa1, iha, fimCD, ehaA, agn43<sub>EDL933'</sub>, agn43, csgA, crl</i>
eae-negative STEC	O91:H21	<i>vt2, ehxA, saa, espP, iha, fimCD, ehaA, agn43, csgA, crl</i>
	O113:H21	<i>vt2, ehxA, saa, espP, subA, iha, fimCD, ehaA, agn43, csgA, crl</i>
	O130:H11	<i>vt1, vt2, ehxA, saa, espP, subA, iha, fimCD, ehaA, agn43, csgA, crl</i>
	O178:H19	<i>vt1, vt2, ehxA, saa, espP, subA, iha, fimCD, ehaA, agn43, csgA, crl</i>
	O8:H19	<i>vt2, ehxA, espP, iha, fimCD, ehaA, agn43, csgA, crl</i>
aEPEC	O19:H11	<i>eae, fimCD, agn43, csgA, crl</i>
	O130:H11	<i>eae, fimCD, agn43, csgA, crl</i>
	O40:NM	<i>eae, fimCD, agn43, csgA, crl</i>
	O40:H10	<i>eae, fimCD, agn43, csgA, crl</i>
	O108:H9	<i>eae, fimCD, csgA, crl</i>
EDL933 <sup>†</sup>	O157:H7	<i>vt1, vt2, ehxA, eae, katP, espP, stcE, efa1, iha, fimCD, ehaA, agn43<sub>EDL933'</sub>, agn43, csgA, crl</i>

<sup>†</sup>Reference STEC strain O157:H7 EDL933 was incorporated to the eae-positive STEC group in order to a better analysis of the results.

aliquot of each strain stored at -70°C was reactivated in 500µL of LB for 2 h, at 37°C with moderate agitation. Then, they were seeded in Congo Red Agar plates (CRA) and incubated for 24 h at 37°C, after that they were incubated an additional 24 h at room temperature (≈20°C). The phenotypes expressed at each temperature were observed and colonies were classified as *rdar* ("red, dry and rough"; curli and cellulose production), *sar* ("red and smooth"; only curli production) or *saw* ("white and smooth"; not curli or cellulose production).

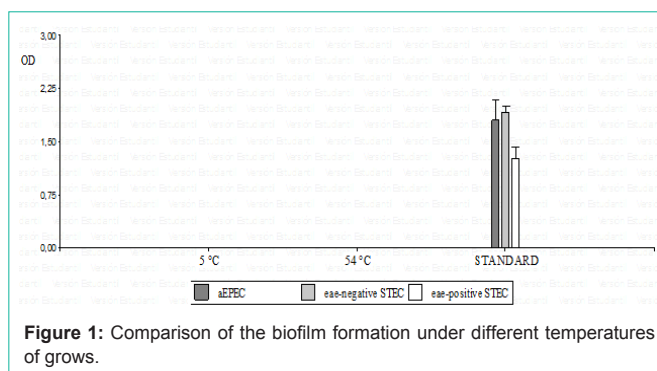
One individual colony from CRA incubated at 37°C and then at 20°C was taken for each strain. Colonies were separately homogenized in 1 ml of physiological solution (SF). From that homogenate, 50µl was seeded in 24-well polystyrene plates (three consecutive wells were used for each strain and six wells for culture medium controls). The plates were incubated at 37°C and 20°C statically for 48 h following the same protocol used above. Biofilm formation was estimated by the violet crystal technique as previously described and the OD obtained was related to the curli expression phenotypes.

## Results and Discussion

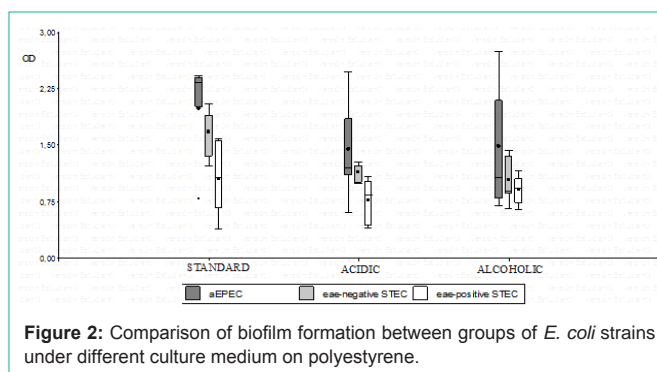
### Biofilm Formation

The biofilm formation for STEC and aEPEC strains was variable according the culture conditions on polystyrene. Indeed, under different temperatures of incubation all strains were SBF at 37°C, except STEC O145:NM and aEPEC O130:H11 that resulted MBF. No one strain formed biofilm at thermal stress (54°C) or refrigeration (5°C) temperatures (Figure 1). When the average of biofilm formation was compared by *E. coli* group (*eae*-positive STEC, *eae*-negative STEC, and aEPEC strains), it was found that *eae*-negative STEC were SBF at 37°C than aEPEC and *eae*-positive STEC strains (Figure 1).

Under different culture conditions -standard, acidic or alcoholic- all strains were MBF or SBF; aEPEC group resulted more biofilm-forming than the STEC groups in all conditions (Table 2, Figure 2).



**Figure 1:** Comparison of the biofilm formation under different temperatures of grows.



**Figure 2:** Comparison of biofilm formation between groups of *E. coli* strains under different culture medium on polystyrene.

In the standard condition the strongest biofilm formers were the strains belonging to the serotypes O40:H10, O91:H21 and O157:H7 from aEPEC, *eae*-negative STEC and *eae*-positive STEC, respectively. Under acidic conditions, the aEPEC serotypes O108:H9, O19:H11 and O40:NM were SBF; while O157:H7 and O113:H21 were the best biofilm formers from the *eae*-positive and *eae*-negative STEC, respectively. Under alcoholic conditions, the aEPEC strains O108:H9 and O19:H11, *eae*-negative STEC O130:H11 and *eae*-positive STEC

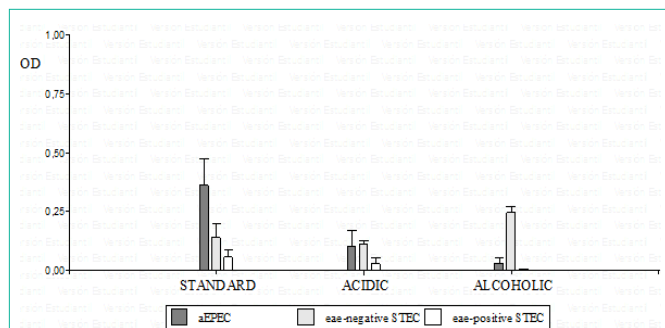
**Table 2:** The estimated biofilm formation ( $OD_{570}$ ) under different culture conditions according to the surfaces used.

<i>E. coli</i> Group	Serotype	Polystyrene <sup>*</sup>			Stainless Steel <sup>‡</sup>		
		Standard	Acidic	Alcoholic	Standard	Acidic	Alcoholic
<i>eae</i> -positive STEC	O157:H7	1,590	1,083	1,157	0	0	0
	O26:H11	1,567	0,425	0,645	0,0632	0	0
	O103:H2	0,986	1,021	1,054	0	0	0
	O111:NM	0,662	0,779	0,731	0,1257	0	0
	O145:NM	0,387	0,395	0,989	0,1527	0,1606	0,0153
	EDL933	1,175	0,896	0,874	0,0158	0	0
<i>eae</i> -negative STEC	O91:H21	2,053	1,227	1,357	0	0,0511	0,1609
	O113:H21	1,905	1,220	0,853	0,0898	0,1130	0,2890
	O130:H11	1,889	0,988	1,425	0,0920	0,1146	0,2549
	O178:H19	1,221	1,273	0,898	0,2102	0,1287	0,2426
	O8:H19	1,347	1,004	0,656	0,3170	0,1433	0,2848
aEPEC	O40:H10	2,425	1,101	0,789	0,4529	0	0
	O19:H11	2,399	1,853	2,101	0,6016	0,3563	0,0930
	O108:H9	2,336	2,479	2,744	0,1000	0,1129	0
	O40:NM	2,008	1,195	1,077	0,5673	0	0,0700
	O130:H11	0,789	0,611	0,702	0,0964	0,0531	0

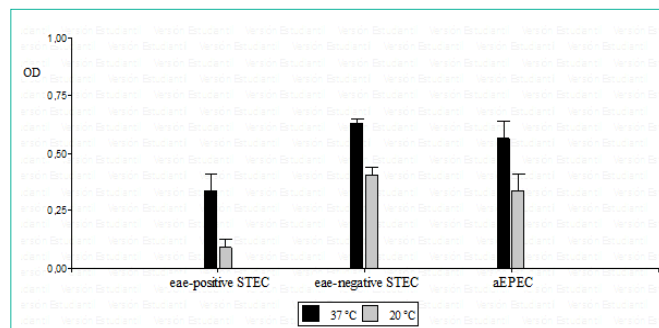
**References:**

<sup>\*</sup>Strains were considered as biofilm formers when their OD was greater than the established cut-off OD ( $OD_c=0,192$ ) on polystyrene. Strains whose values were included to  $2OD_c$  (0,385) were considered WBF; values between  $2OD_c$  and  $4OD_c$  (0,770) were considered MBF, and more than  $4OD_c$  were classified as SBF.

<sup>‡</sup>Strains were biofilm former when their OD were greater than  $OD_c=0,541$  on stainless steel. Strains whose values were included to  $2OD_c$  (1,082) were considered WBF. No one strain could be classified as MBF or SBF since no value was greater than  $2OD_c$  on this surface.



**Figure 3:** Biofilm formation under different culture conditions on stainless steel surface. It is notably that the OD's average obtained were never greater than 0.50 in the three groups.



**Figure 4:** Differences in the biofilm formation between *E. coli* groups according with the temperature of incubation.

O157:H7 were SBF.

The biofilm formation on stainless steel was significantly lower than that obtained on polystyrene ( $p<0.0001$ ). The highest OD values were observed in aEPEC strains which ranged from 0,4 to 0,6 in standard culture conditions (Table 2). The 81% of the strains were considered WBF under standard culture and the rest were NBF (19%). Under acidic and alcoholic conditions, the strains decreased their biofilm formation (WBF in a 56 and 50%, respectively) and increased the percentage of NBF (44 and 50%, respectively). The aEPEC group formed more biofilm under standard culture medium, while *eae*-negative STEC were better in acidic and alcoholic mediums. The *eae*-positive STEC group were the lowest biofilm former in all condition studied (Figure 3).

### Curli Expression

In this assay, data showed that temperature significantly influenced both biofilm formation and curli expression (Table 3). The biofilm formation was greater at  $37^{\circ}C$  than at  $20^{\circ}C$  ( $p=0.001$ ) and both *eae*-negative STEC and aEPEC strains were better biofilm-forming than *eae*-positive STEC at both temperatures ( $p=0.003$ ) (Figure 4). The curli phenotype was affected by temperature inversely to biofilm formation, so more positive curli colonies ("sar" and "rdar") were observed at  $20^{\circ}C$  than at  $37^{\circ}C$  ( $p=0.0007$ ). A direct association between the curli phenotype and the biofilm-forming capacity could not be established ( $p=0.059$ ).

The ability of bacteria to produce biofilms depends on many factors inherent to the microorganism, the environment and the surface where it can adhere. The studies of biofilm by STEC and EPEC have been based especially on the risk that these bacteria represent for



**Table 3:** The estimated biofilm formation ( $OD_{570}$ ) and fimbria curli expression under different temperatures of incubation.

<i>E. Coli</i> group	Serotype	Incubation temperatures					
		37°C			≈20°C		
		Curli expression	Biofilm formation	Classification	Curli expression	Biofilm formation	Classification
eae-positive STEC	O103:H2	saw-sar	0,610	MBF	sar	0,267	WBF
	O111:NM	saw	0,171	WBF	saw	0,050	WBF
	O145:NM	saw	0,331	WBF	saw	0,038	WBF
	O26:H11	saw-sar	0,480	MBF	sar	0,085	WBF
	O157:H7	saw	0,150	WBF	saw	0,046	WBF
	EDL933	saw	0,288	WBF	sar	0,062	WBF
eae-negative STEC	O91:H21	saw	0,663	MBF	sar	0,446	MBF
	O113:H21	saw	0,631	MBF	sar	0,419	MBF
	O130:H11	saw	0,562	MBF	saw	0,263	WBF
	O178:H19	saw	0,609	MBF	sar	0,479	MBF
	O8:H19	saw	0,676	SBF	sar	0,400	MBF
aEPEC	O19:H11	saw	0,415	MBF	sar	0,508	MBF
	O130:H11	saw-sar	0,779	SBF	sar	0,128	WBF
	O40:NM	saw-sar	0,677	SBF	sar	0,461	MBF
	O40:H10	sar	0,387	MBF	sar	0,395	MBF
	O108:H9	saw-sar	0,564	MBF	rdar	0,198	WBF

public health worldwide. Most STEC biofilm studies have focused on serotype O157:H7 [38-42] and few reports have been published on STEC no-O157 or EPEC [30,43,44]. In these studies, variables such as the culture medium, different surfaces, the expression of several adherence factors such as curli and cellulose, have been studied to find out how these pathogens form biofilm.

This study was aimed to investigate the ability to form biofilms of a set of native STEC and aEPEC strains under different conditions of culture media, surfaces and temperatures in order to know whether these conditions affect their biofilm formation. The temperature was the variable that more affected the biofilm formation, since at extreme temperatures (heat and cold) the strains did not form biofilms. Previous studies have evaluated the biofilm formation at refrigeration temperature (4 and 15°C) but they used longer incubation times (up to 16 days) finding a greater biofilm formation at 15°C more than at 4°C, regardless of the surface and substrate [20,42]. Other authors have used temperatures that favored the production of fimbria curli and cellulose, and greater adhesion and biofilm formation were obtained at 28°C, 30°C or 37°C on semi-solid and solid surfaces such as, stainless steel, teflon and glass [41, 44]. In agreement with our results, these findings suggest that temperature have a great influence on the ability to form biofilms, since STEC and aEPEC strains were favored by temperatures near those found in their natural environment (animal host or human) and not by temperatures that would constitute a stress condition.

Another variable that can influence the development of biofilms is the culture medium. Several studies evaluated STEC biofilms in mediums with different nutrient availability, from minimal medium to food products fluids such as milk, minced meat, meat juice etc., and demonstrated that there was a greater biofilm formation in nutrient medium than in minimal or diluted medium [38,42]. Other studies

have evaluated the bacteriostatic effects of organic acid solutions on pathogenic strains such as *Escherichia coli* and *Salmonella spp.* on cattle carcasses surfaces, and they have found that lactic acid was more effective than acetic acid [45,46]. The ability to survive in solutions with low concentrations of acid and alcohol allows the biofilm development on surfaces commonly used in industry and the domestic environment, constituting a risk to underestimate the presence of STEC or aEPEC in foods. Biofilm studies in EPEC strains are limited to use minimal medium such as DMEM or nutritive such as LB, since the study of biofilms in this pathotype, especially atypical EPEC, is quite recent [30,31,47-49].

In this study, STEC and aEPEC strains were subjected to different medium with pH and chemical composition (low pH, presence of acetic acid and alcohol). No significant differences were found in biofilm formation under standard culture medium condition and its acid and alcoholic variants, although STEC has been able to grow and survive for more than 24 h at the same acetic acid and ethyl alcohol concentrations [27]. The behavior of the strains was very variable within each *E. coli* group. It was observed that certain serotypes such as O108:H9 (aEPEC), O91:H21 and O157:H7 (eae-negative and eae-positive STEC, respectively) were the best biofilm formers on polystyrene surface. This is in agreement with results obtained by Wang, et al. [50], who showed that eae-negative O113 and O91 serogroups exhibited a greatest potential to form biofilms and by Molina, et al [27] who found that STEC O91:H21 was the most resistant serotype against acidity conditions. STEC O145:NM formed more biofilm under standard, acidic and alcoholic conditions on stainless steel than the other strains. Among the eae-negative STEC, O8:H19 was the serotype that formed the greater biofilm in the three conditions studied on stainless steel, however this serotype was one of the lowest biofilm-forming on polystyrene. STEC O157:H7 is the most frequently serotype used as a reference for the development of

control and thermal inactivation protocols of STEC in food [51,52]. In this study, this serotype showed a differently behavior according to its origin: the bovine strain was more biofilm former than the reference strain EDL933 O157:H7 isolated from human.

During the adhesion to the surface and maturation of the biofilm, the fimbriae plays an important role in favoring cell aggregation and interaction with cellulose to create hydrophobic networks in the extracellular matrix [53]. Curli has been studied in the biofilm formation of *E. coli*, especially in STEC O157:H7, on surfaces commonly used in the food industry such as stainless steel, polystyrene, glass and rubber [54], finding that the production of this fimbriae is more favored by temperatures below 30°C, varying not only according to the serotype, but within the same serotype [39,55]. Curli expression and biofilm formation at different temperatures from colonies incubated in CRA at 37°C and 20°C were compared. The results showed that most STEC and aEPEC strains were curli-negative at 37°C, but 12 of 16 strains (mostly *eae*-STEC and aEPEC) were moderate or strong biofilm. However, at 20°C, most of the strains were curli-positive (“sar” or “rdar”) but they were weak biofilm-forming. These results differ with those found by other authors where a relationship between curli expression and biofilm formation could be established [41,56,57]. However, other authors did not find a clear association between curli production and an increased biofilm formation [22,47]. In agreement with others studies, we suggest that curli could need more time of incubation to play an important role in the adhesion and maturation of biofilm under certain laboratory conditions [58]. The production of curli, cellulose, or both, may not be an indispensable factor to form biofilm, however they were suggested as important contributing factors [39,59].

## Conclusion

Native STEC and aEPEC strains demonstrated to be able to form biofilm and produce fimbriae curli under different conditions of culture medium and temperature. This is important because of the risk of transmission and survival of these pathogens from reservoirs and food to humans. The recreation of food industry environment into the laboratory result a useful tool to understand the behavior of these foodborne pathogens and to find new strategies for their prevention and control.

## Acknowledgment

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## References

- Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev.* 2002; 15: 167-193.
- Chia TW, Goulter RM, McMeekin T, Dykes GA, Fegan N. Attachment of different *Salmonella* serovars to materials commonly used in a poultry processing plant. *Food Microbiol.* 2009; 26: 853-859.
- Chmielewski RAN, Frank JF. Biofilm formation and control in food processing facilities. *Compr Rev Food Sci Food Saf.* 2003; 2: 22-32.
- Padola NL, Sanz ME, Blanco JE, Blanco M, Blanco J, Etcheverría AI, et al. Serotypes and virulence genes of shiga toxin-producing *Escherichia coli* (STEC) isolated from a feedlot in Argentina. *Vet Microbiol.* 2004; 100: 3-9.
- Fernández D, Rodríguez EM, Arroyo G, Padola NL, Parma AE. Seasonal variation of Shiga toxin-encoding genes (stx) and detection of *E. coli* O157 in dairy cattle from Argentina. *J App Microbiol.* 2009; 106: 1260-1267.
- Polifroni R, Etcheverría AI, Sanz ME, Cepeda RE, Krüger A, Lucchesi PMA, et al. Molecular characterization of Shiga toxin-producing *Escherichia coli* isolated from the environment of a dairy farm. *Curr Microbiol.* 2012; 65: 337-343.
- Ochoa TJ, Contreras CA. Enteropathogenic *Escherichia coli* infection in children. *Curr Opin Infect Dis.* 2011; 24: 478-483.
- Alonso MZ, Lucchesi PMA, Rodríguez EM, Parma AE, Padola NL. Enteropathogenic (EPEC) and Shiga toxin-producing *Escherichia coli* (STEC) in broiler chickens and derived products at different retail stores. *Food Control.* 2012; 23: 351-355.
- Tarr PI, Bilge SS, Vary JC, Jr, Jelacic S, Habeeb RL, Ward TR, et al. Iha: a novel *Escherichia coli* O157:H7 adherence conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect Immun.* 2000; 68: 1400-1407.
- Stevens MP, Van Diemen PM, Frankel G, Phillips AD, Wallis TS. Efa1 influences colonization of the bovine intestine by Shiga toxin-producing *Escherichia coli* serotypes O5 and O111. *Infect. Immun.* 2002; 70: 5158-5166.
- Cookson AL, Cooley WA, Woodward MJ. The role of type 1 and curli fimbriae of Shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. *Int J Med Microbiol.* 2002; 292: 195-205.
- Torres AG, Blanco M, Valenzuela P, Slater TM, Patel SD, Dahbi G, et al. Genes related to long polar fimbriae of pathogenic *Escherichia coli* strains as reliable markers to identify virulent isolates. *J Clin Microbiol.* 2009; 47: 2442-2451.
- Henderson IR, Meehan M, Owen P. Antigen 43, a phase-variable bipartite outer membrane protein, determines colony morphology and autoaggregation in *Escherichia coli* K-12. *FEMS Microbiol Lett.* 1997; 149: 115-120.
- Torres AG, Perna NT, Burland V, Ruknudin A, Blattner FR, Kaper JB. Characterization of Cah, a calcium-binding and heat-extractable autotransporter protein of enterohaemorrhagic *Escherichia coli*. *Mol Microbiol.* 2002; 45: 951-966.
- Wu Y, Hinenoya A, Taguchi T, Nagita A, Shima K. Distribution of virulence genes related to adhesins and toxins in Shiga-toxin producing *Escherichia coli* strains isolated from healthy cattle and diarrheal patients in Japan. *J Vet Med Sci.* 2010; 72: 589-597.
- Bokranz W, Wang X, Tschäpe H, Römling U. Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. *J Med Microbiol.* 2005; 54: 1171-1182.
- Hammar MR, Arnqvist A, Bian Z, Olsén A, Normark S. Expression of two *csg* operons is required for production of fibronectin and congo red-binding curli polymers in *Escherichia coli* K-12. *Mol Microbiol.* 1995; 18: 661-670.
- Dyer JG, Sriranganathan N, Nickerson SC, Elvinger F. Curli production and genetic relationships among *Escherichia coli* from cases of bovine mastitis. *J Dairy Sci.* 2007; 90: 193-201.
- Donlan RM. Biofilms: microbial life on surfaces. *Emerg Infect Dis.* 2002; 8: 881-890.
- Simpson Beauchamp C, Dourou D, Geornaras I, Yoon Y, Scanga JA, Belk KE, et al. Transfer, attachment, and formation of biofilms by *Escherichia coli* O157:H7 on meat contact surface materials. *J Food Sci.* 2012; 77: 343-347.
- Giaouris E, Chorianopoulos N, Nychas GJ. Impact of acid adaptation on attachment of *Listeria monocytogenes* to stainless steel during long-term incubation under low or moderate temperature conditions and on subsequent recalcitrance of attached cells to lethal acid treatments. *Int J Food Microbiol.* 2014; 171: 1-7.
- Polifroni R. Caracterización fenotípica de *Escherichia coli* verocitotoxigénica (VTEC) y su persistencia en el medio ambiente de tambo [dissertation]. Doctoral Theses. Facultad de Ciencias Veterinarias, UNCPBA, Argentina, 2012.

23. Cáceres ME, Etcheverría AI, Padola NL. Efectos del medio de cultivo y de la metodología aplicada sobre la formación de biopelículas de 2 cepas de *Escherichia coli* diarreagénicas. *Rev Argent Microbiol.* 2019; 51: 208-213.
24. Foster JW. Microbial responses to acid stress. Storz G and Hengge-Aronis R, editors. In: *Bacterial Stress Response*. American Society for Microbiology Press. 2000; 99-116.
25. Yura T, Kanemori M, Morita MT. The heat shock response: Regulation and function. Storz G and Hengge-Aronis R, editors. In: *Bacterial Stress Response*. American Society for Microbiology Press. 2000; 3-18.
26. Molina P, Parma AE, Sanz ME. Survival in acidic and alcoholic medium of Shiga Toxin-producing *Escherichia coli* O157:H7 and non-O157:H7 isolated in Argentina. *BMC Microbiol.* 2003; 3: 17.
27. Molina PM, Sanz ME, Lucchesi PMA, Padola NL, Parma AE. Effects of acidic broth and juices on the growth and survival of verotoxin-producing *Escherichia coli* (VTEC). *Food Microbiol.* 2005; 22: 469-473.
28. Villegas NA, Baronetti J, Albesa I, Polifroni R, Parma A, Etcheverría A, et al. Relevance of biofilms in the pathogenesis of Shiga-toxin-producing *Escherichia coli* infection. *Sci World J.* 2013.
29. Molina PM. Estudio de la sensibilidad al estrés ácido y térmico de cepas autóctonas de *Escherichia coli* verocitotoxigénica [dissertation]. Doctoral Theses. Facultad de Ciencias Veterinarias, UNCPBA, Argentina. 2005.
30. Culler HF, Mota CM, Abe CM, Elias WP, Sircili MP, Franzolin MR. Atypical enteropathogenic *Escherichia coli* strains form biofilm on abiotic surfaces regardless of their adherence pattern on cultured epithelial cells. *BioMed Res Int.* 2014.
31. Moreira CG, Palmer K, Whiteley M, Sircili MP, Trabulsi RL, Castro AFP, et al. Bundle-forming pili and EspA are involved in biofilm formation by enteropathogenic *Escherichia coli*. *J Bacteriol.* 2006; 188: 3952-3961.
32. Fernández D, Irino K, Sanz ME, Padola NL, Parma AE. Characterization of Shiga toxin-producing *Escherichia coli* isolated from dairy cows in Argentina. *Lett App Microbiol.* 2010; 51: 377-382.
33. Fernández D, Sanz ME, Parma AE, Padola NL. Short communication: characterization of Shiga toxin-producing *Escherichia coli* isolated from newborn, milk-fed, and growing calves in Argentina. *J Dairy Sci.* 2012; 95: 5340-5343.
34. Cáceres ME, Etcheverría AI, Fernández D, Rodríguez EM, Padola NL. Variation in the distribution of putative virulence and colonization factors in Shiga toxin-producing *Escherichia coli* isolated from different categories of cattle. *Front Cell Infect Microbiol.* 2017; 7: 147.
35. Cáceres ME. Formación de biofilms por cepas *Escherichia coli* verocitotoxigénica (VTEC) y *Escherichia coli* enteropatogénica (EPEC) sobre distintas superficies, sometidas a distintas condiciones de estrés. [dissertation]. Doctoral Theses. Facultad de Ciencias Veterinarias, UNCPBA, Argentina, 2019.
36. Joseph B, Otta SK, Karunasagar I, Karunasagar I. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Inter J Food Microbiol.* 2001; 64: 367-372.
37. Gómez J, Gómez-Lus ML, Bas P, Ramos C, Cafini F, Maestre JR, et al. ¿Es la cuantificación del biofilm un elemento diferenciador en la patogenia de bacilos gramnegativos? *Rev Esp Quimioter.* 2013; 26: 97-102.
38. Hood SK, Zottola EA. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. *Inter J Food Microbiol.* 1997; 37: 145-153.
39. Ryu JH, Kim H, Beuchat LR. Attachment and biofilm formation by *Escherichia coli* O157:H7 on stainless steel as influenced by exopolysaccharide production, nutrient availability, and temperature. *J Food Prot.* 2004; 67: 2123-2131.
40. Ryu JH and Beuchat LR. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: effects of exopolysaccharide and curli production on its resistance to chlorine. *Appl Environ Microbiol.* 2005; 71: 247-254.
41. Uhlich GA, Cooke PH, Solomon EB. Analyses of the red-dry-rough phenotype of an *Escherichia coli* O157:H7 strain and its role in biofilm formation and resistance to antibacterial agents. *Appl Environ Microbiol.* 2006; 72: 2564-2572.
42. Dourou D, Simpson Beauchamp C, Yoon Y, Geornaras I, Belk KE, Smith GC, et al. Attachment and biofilm formation by *Escherichia coli* O157:H7 at different temperatures, on various food-contact surfaces encountered in beef processing. *Int J Food Microbiol.* 2011; 149: 262-268.
43. Chen CY, Hofmann CS, Cottrell BJ, Strobaugh TP Jr, Paoli GC, Nguyen L-H, et al. Phenotypic and genotypic characterization of biofilm forming capabilities in non-O157 Shiga toxin-producing *Escherichia coli* strains. *PLoS ONE* 2013; 8: e84863.
44. Uhlich GA, Chen CY, Cottrell BJ, Nguyen LH. Growth media and temperature effects on biofilm formation by serotype O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli*. *FEMS Microbiol Lett.* 2014; 354: 133-141.
45. Cutter CN, Rivera-Betancourt M. Interventions for the reduction of *Salmonella Typhimurium* DT 104 and non-O157:H7 enterohemorrhagic *Escherichia coli* on beef surfaces. *J Food Prot.* 2000; 63: 1326-1332.
46. Signorini M, Costa M, Teitelbaum D, Restovich V, Brasesco H, García D, et al. Evaluation of decontamination efficacy of commonly used antimicrobial interventions for beef carcasses against Shiga toxin-producing *Escherichia coli*. *Meat Sci.* 2018; 142: 44-51.
47. Nascimento HH, Silva LE, Souza RT, Silva NP, Scaletsky IC. Phenotypic and genotypic characteristics associated with biofilm formation in clinical isolates of atypical enteropathogenic *Escherichia coli* (aEPEC) strains. *BMC Microbiol.* 2014; 14: 184.
48. Schiebel J, Böhm A, Nitschke J, Burdukiewicz M, Weinreich J, Ali A, et al. Genotypic and phenotypic characteristics associated with biofilm formation by human clinical *Escherichia coli* isolates of different pathotypes. *Appl Environ Microbiol.* 2017; 83: e01660-17.
49. Culler HF, Couto SC, Higa JS, Ruiz RM, Yang MJ, Bueris V, et al. Role of SdiA on biofilm formation by atypical enteropathogenic *Escherichia coli*. *Genes* 2018; 9: 253.
50. Wang J, Stanford K, McAllister TA, Johnson RP, Chen J, Hou H, et al. Biofilm formation, virulence gene profiles, and antimicrobial resistance of nine serogroups of non-O157 Shiga toxin-producing *Escherichia coli*. *Foodborne Pathog Dis.* 2016; 13: 316-324.
51. Vasan A, Leong WM, Ingham SC, Ingham BH. Thermal tolerance characteristics of non-O157 Shiga toxigenic strains of *Escherichia coli* (STEC) in a beef broth model system are similar to those of O157:H7 STEC. *J. Food Prot.* 2013; 76: 1120-1128.
52. Li K, McKeith AG, Shen C, McKeith R. A comparison study of quality attributes of ground beef and veal patties and thermal inactivation of *Escherichia coli* O157: H7 after double pan-broiling under dynamic conditions. *Foods.* 2018; 7: 1.
53. Gualdi L, Tagliabue L, Bertagnoli S, Ilerano T, De Castro C, Landini P. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. *Microbiol.* 2008; 154: 2017-2024.
54. Pawar DM, Rossman ML, Chen J. Role of curli fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. *J Appl Microbiol.* 2005; 99: 418-425.
55. Biscola FT, Abe CM, Guth BEC. Determination of adhesin gene sequences and biofilm formation in O157 and non-O157 Shiga toxin-producing *Escherichia coli* strains isolated from different sources. *Appl Environ Microbiol.* 2011; 77: 2201-2208.
56. Silagyi KS. Biofilm formation by *Escherichia coli* O157:H7 [dissertation]. University of Maryland, Master's Thesis, 2007.
57. Weiss-Muszkat M, Shakh D, Zhou Y, Pinto R, Belasov E, Chapman MR, et al. Biofilm formation by and multicellular behavior of *Escherichia coli* O55:H7, an atypical enteropathogenic strain. *Appl Environ Microbiol.* 2010; 76: 1545-1554.
58. Wang R, Bono JL, Kalchayanand N, Shackelford S, Harhay DM. Biofilm

- formation by Shiga toxin-producing *Escherichia coli* O157:H7 and non-O157 strains and their tolerance to sanitizers commonly used in the food processing environment. *J Food Prot.* 2012; 75: 1418-1428.
59. Macarisin D, Patel J, Bauchan G, Giron JA, Sharma VK. Role of curli and cellulose expression in adherence of *Escherichia coli* O157:H7 to spinach leaves. *Foodborne Pathog Dis.* 2012; 9: 160-167.