COLONIZATION OF THE MUSCLE EXTRACELLULAR MATRIX COMPONENTS BY ENTEROHEMORRHAGIC *ESCHERICHIA COLI*

C. Chagnot[1,2], N. Caccia[1], T. Astruc[2] and M. Desvaux[1]

[1] INRA, UR 454 Microbiologie, 63122 Saint Genès Champanelle, France

[2] INRA, UR 370 Qualité des produits Animaux, 63122 Saint Genès Champanelle, France

Abstract – Enterohemorrhagic *Escherichia coli* (EHEC) are anthropozoonotic agents responsible for repeated food-poisoning cases often caused by contaminated burgers. EHEC are Shiga-toxin producing E. coli (STEC) responsible for foodborne poisoning mainly incriminated to the consumption of contaminated beef meat. Eight serogroups are especially of high-risk for human health, i.e. O157, O26, O45, O103, O111, O121, O145 and O104. Ruminants are the main natural reservoir for EHEC and primary bacterial contamination occurs at the dehiding stage of slaughtering. The extracellular matrix (ECM) is the most exposed part of the skeletal muscles in beef carcasses. Investigating bacterial colonization to the skeletal-muscle extracellular matrix (ECM) proteins, it appeared that environmental factors influenced specific and non-specific bacterial adhesion of O157 and non-O157 EHEC as well as biofilm formation. From this first comprehensive investigation of EHEC adhesion to ECM proteins with respect to muscle biology and meat processing, new research directions for the development of innovative practices to minimize the risk of meat contamination are further discussed.

Key Words – Meat contamination, Bacterial adhesion, Biofilm formation.

I. INTRODUCTION

Worldwide, the occurrence of food poisoning following the consumption of products contaminated with enterohemorrhagic *Escherichia coli* (EHEC) is recurrent [1]. EHEC outbreaks and sporadic cases incriminate some meat, milk or vegetable products as well as water-based drinks. Human infections, though, often follow the consumption of beef meat, especially minced meat, and often involve strains of the serogroup O157. While the EHEC reference strain O157 EDL933 was originally isolated from contaminated burgers (beef patty) responsible for an outbreak of hemorrhagic colitis in 1982 [2], it is only one of the over 300 distinct serotypes for Shiga toxin-producing *Escherichia coli* (STEC) isolated so far. However, only a very limited number of serogroups is associated with the majority of human diseases. By far, the most commonly isolated non-O157 EHEC belongs to O26, O45, O103, O111, O121 and O145 serogroups, the so-called "big six". From the last major European outbreaks in 2011, the serogroup O104 is watched closely. EHEC infection manifests clinically with diarrhea and abdominal cramps before proceeding to hemorrhagic colitis characterized by bloody diarrhea. Although it can occur at any age, this is predominantly a pediatric illness [3].

Ruminants such as bovines are the main natural reservoir for EHEC. Hygienic slaughtering practices reduce faecal contamination of carcasses (prevention of evisceration accidents, cross contamination and poor hygiene) but cannot guarantee the absence of *E. coli* O157 from meat. Primary bacterial contamination occurs often inevitably at the dehiding stage where bacteria can be transferred from hides to beef carcasses. According to the EC n°853/2004 regulation, animal slaughter and cutting of carcases into quarters can be carried out at room temperature. However, during further cutting, storage and/or transport, meat must reach and be maintained at 7°C. From then on the temperature of meat preparations and minced meat must not exceed 4°C and 2ºC respectively. While EHEC pathogenicity have been subjected to intense research, the molecular aspects of food contamination clearly lags behind.

ECM is composed of two main classes of macromolecules, the fibrous proteins and the proteoglycans [4]. In skeletal muscle tissue, fibrous proteins are the predominant components of the ECM, essentially comprised of collagens I, III and IV, insoluble fibronectin (i-fibronectin), laminin-α2 and elastin. The role of pili in mediating the attachment of EHEC cells to meat was suggested but not ascertained and other cell surface determinants related to the virulence may also be involved [5]. Importantly, the expression of virulence factors in EHEC is inducible and depends on growth conditions. Altogether, this prompted us to reinvestigate the adhesion of EHEC to the main ECM fibrous proteins present in beef meat.

II. MATERIALS AND METHODS

Bacterial strains and culture conditions. EHEC strains investigated in this study are listed in Table 1. Bacteria were cultured in different nutrient media either chemically defined, i.e. DMEM, M9 and MinCa, or complex undefined, i.e. LB, TSB, BHI), or *ex vivo*, i.e. LH, BCC (bovine caecum content) and

BJIC (bovine jejunum-ileum content) sterile media. Strains were plated on the relevant agar medium and incubated overnight at 39°C (bovine temperature) [6]. A preculture was set up from one bacterial colony grown in the respective nutrient broth medium at 39°C in an orbital shaker till stationary phase as determined using the logarithmic growth curve of the OD_{600nm}.

Coating of microtiter plates with ECM proteins. The ECM proteins consisted of collagen I, III and IV, laminin-α2, elastin and i-fibronectin. BSA (bovine serum albumin) was used as a control for specific adhesion to ECM proteins [7]. Basically, ECM proteins were solubilised in 0.1 M carbonate coating buffer (pH 9.6) and 250 µl was dispensed at a saturating concentration (50 µg ml-1) to the well surface of the microtiter plate and incubated overnight at 4°C. The wells were washed with PBS containing 0.05 % (v/v) Tween 20 (PBST, pH 7.3) at room temperature (rt) prior to blotting with 250 µl of 1% (w/v) BSA in PBST. After 2 h at 37°C, the wells were washed 3 times with PBST and used for bacterial adhesion or biofilm formation assays.

Table 1: List of O157 and non-O157 E. coli strains studied.

Strain	Serogroup	Origin	Virulence profile
EDL933	O157	Clinical	*stx1, stx2, eae, ehxA*
CM454	O157	Laboratory	*eae, ehxa*
ED180	O26	Clinical	*stx2, eae, ehxA*
12047	O45	Clinical	*stx1, eae, ehxA*
CH087	O103	Clinical	*stx1, eae, ehxA*
ED191	O111	Clinical	*stx1, stx2, eae, ehxA*
32316	O121	Clinical	*stx2, eae, ehxA*
PH27	O145	Clinical	*stx2, eae*
CB13348	O104	Clinical	*stx2*

Bacterial adhesion and biofilm formation assay

Precultures were diluted 1:100 and grown as described above. Relevant media were adjusted with NaOH (0.1 M) to reach a pH of 7 at the time of sampling. Sampling was performed during the exponential growth phase at an OD_{600nm} of 0.5. Chloramphenicol was added (170 µg ml^{-1}) to prevent *de novo* protein synthesis and growth. A MMT (minimal mechanical treatment) was applied to avoid vigorous shaking, vortexing and centrifugation and thus preserve cell surface supramolecular structures [8]. Bacterial cell suspension (200 µl) was deposited in relevant protein-coated wells of the microtitre plate using wide-bore tips and incubated statically at relevant temperature for 2 h. After incubation, bacterial suspension was removed by pipetting. Wells were further first washed with TS (tryptone salt) to remove loosely attached cells. Adherent bacteria were fixed with 200 µl absolute ethanol for 20 min. Wells were then emptied by pipetting and dried for 30 min prior to 20 min staining with 200 µl of an aqueous-solution of crystal violet (0.1 % w/v). Wells were emptied, washed a second time with TS to remove the excess of unbound crystal violet dye, and dried for 30 min. The bound dye was solubilized from stained cells using 200 µl of an aqueous solution of acetic acid (33 % v/v) for 1 min under orbital shaking. Contents of each well (150 µl) were transferred to a clean microtiter plate and absorbance was measured at 595 nm using a microtiter plate reader. The readings were corrected by substracting the average absorbance from control wells. For the biofilm formation assays, precultures in LB were diluted 1:100 and 200 µl of bacterial cell suspension were dispatched in protein-coated wells of the microtitre plate. To follow the bacterial sessile development at various incubation times, wells were subjected to the same sequential procedure described for the bacterial adhesion assay, i.e. washing with TS, fixation with absolute ethanol, air drying, staining with crystal violet solution, washing with TS, air drying, dye recovering in acetic acid solution and reading of the absorbance at 595 nm. To back up the results of the crystal violet assay, fluorescence microscopic observations were performed with the fluorescent strain *E. coli* CM454 pSARE-Red1 [7].

Image acquisition and analysis from microscopic observations. Sample preparations were inserted on the stage plate to take one image for each well. This operation was repeated 12 times in order to acquire satisfactory statistical information. Field of view was chosen in the center of the well in order to avoid artifacts such as edge optical aberrations or biased bacterial spatial distribution. Observations were performed in phase contrast transmitted light or fluorescence reflected light. The corresponding images were acquired using an inverted phase-contrast microscope coupled to a cooled CCD camera (Olympus IMT-2, DP30BW) optimized for high sensitivity fluorescence work. The fluorescence light source was a mercury short arc lamp and images were processed with ImageJ v1.43.

Statistical analysis. Statistical analysis was performed from Excel using XLSTAT v2009.3.02. Data of assays result from at least five independent experiments, i.e. five biological replicates. On the figures, error bars thus represent the standard deviation from five independent experiments. For each experiment, a value was calculated from the average of repetitions performed in triplicate at fewest. Data were statistically analyzed following Student's t-test with differences considered significant ($p<0.05$, *), very significant ($p<0.01$, **),

highly significant ($p<0.001$, ***) or very highly significant ($p<0.0001$, ****).

III. RESULTS AND DISCUSSION

Mechanical treatment greatly impacts bacterial adhesion assay of *EHEC* to immobilized ECM proteins. A SCVT (shaking-centrifugation-vortexing treatment) was compared with a MMT (minimal mechanical treatment) protocol using the reference strain *E. coli* O157 EDL933. Two conditions were tested, *i.e.* using bacterial cells grown (i) in LB, which induces bacterial specific adhesion to collagen I and III, and (ii) in DMEM inducing non-specific adhesion to ECM proteins (Fig. 1). In both LB and DMEM, bacterial adhesion was statistically very much lower upon SCVT than with the MMT protocol.

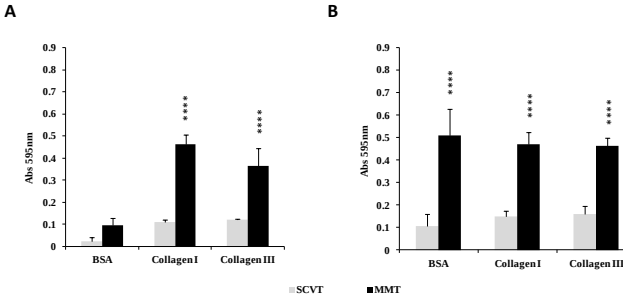

Figure 1: Effect of mechanical treatment on the adhesion of EHEC O157 EDL933 to collagens I and III. Bacterial adhesion in LB (A) and in DMEM (B) compare to the effect of SCVT (shaking-vortexing-centrifugation treatment) and MMT (minimal mechanical treatment) protocols.

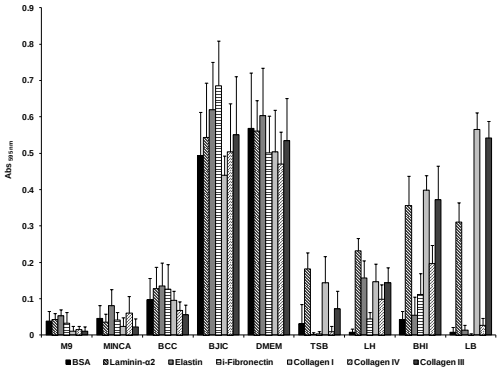

Figure 2: Adhesion of *E. coli* O157 CM454 grown in different media to immobilized ECM proteins.

Growth media influence bacterial adhesion to the main muscle ECM components. *E. coli* O157 CM454 grown in the chemically defined media M9 or MinCa could not adhere to the main muscle ECM components (Fig. 2). In contrast, bacterial cells grown in BCC, BJIC or DMEM adhered similarly to the different ECM proteins tested as well as to BSA, indicating bacterial adhesion was non-specific. The levels of non-specific bacterial adhesion were especially high with BJIC and DMEM. Autoaggregation occurred in DMEM and BJIC but not in LB. For the remaining media tested, specific adhesion of *E. coli* O157 CM454 to ECM proteins could be observed (Fig.2). The similar trend of bacterial adhesion to immobilized collagen I or III versus BSA were further backed up by fluorescent microscopy observations (Fig. 2).

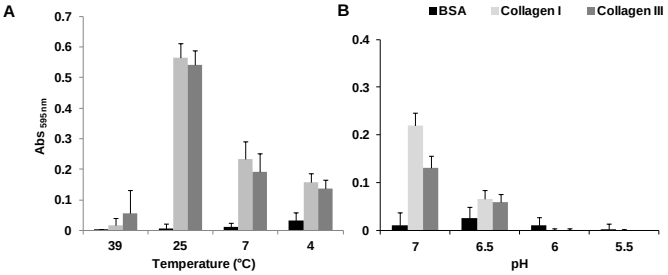

Figure 3: Effect of temperature (A) and pH (B) on adhesion to immobilized collagen I or III of *E. coli* O157 CM454.

Temperature and pH influence bacterial adhesion to collagens I and III. Besides decrease of the meat temperature along the beef production chain, the decrease to pH_u is one of the most prominent post-mortem physicochemical modifications occurring in skeletal muscles. No significant adhesion could be observed at 39°C but maximum specific bacterial adhesion occurred at 25°C (Fig. 3). It appeared further that maximal bacterial adhesion occurred at pH 7 and chiefly decreased at lower pH where no significant specific bacterial adhesion could be observed.

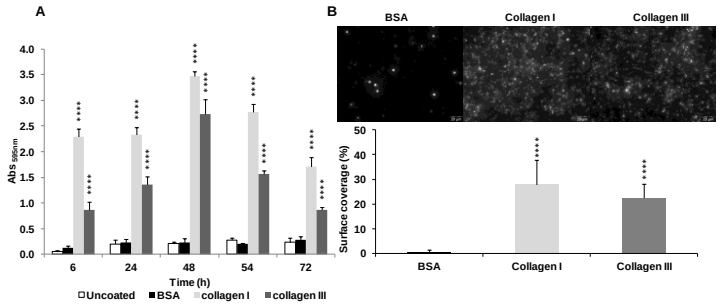

Figure 4: Colonization of immobilized collagen I and III by *E. coli* O157 CM454. Kinetics of biofilm formation (A) and percentage of surface coverage (B) from epifluorescence microscopy.

Collagens I and III promote biofilm formation. The presence of coated ECM proteins chiefly increased the amount of sessile biomass over time when compared to uncoated surface or coated with BSA, especially immobilized collagen I (Figure 4). There were very highly significant differences in biofilm formation on biotic surfaces made of

immobilized BSA versus collagen I or III, which clearly induced biofilm formation.

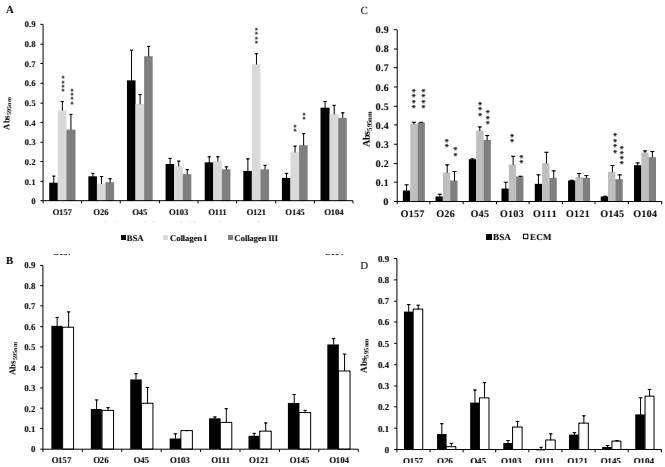

Figure 5: Adhesion (A, B) and colonization (C,D) by EHEC O157 EDL933 and the major non-O157 EHEC to immobilized ECM proteins. Specific and non-specific bacterial adhesion to collagens I and III or a reconstituted ECM were tested in LB (A, C) and in DMEM (B, D).

Differential adhesion and biofilm formation of non-O157 EHEC to immobilized ECM proteins. While *E. coli* O157 EDL933 exhibited specific adhesion ability towards collagen I or III in LB, EHEC O26 ED180 could not adhere to those ECM proteins in tested conditions (Figure 5). In contrast, the adhesion of EHEC O45 12047, O103 CH087, O111 ED191 and O104 CB13348 was non-specific. On the contrary, EHEC O157 EDL933 and O145 PH27 adhered specifically to both collagens I and III. In DMEM, except for EHEC O103 CH087 and O121 32316, non-specific bacterial adhesion occurred for all the remaining non-O157 EHEC tested. The presence of immobilized collagen I or III chiefly increased the amount of EHEC O157 EDL933 and O145 PH27 sessile biomass in LB compared to a surface coated with BSA (Figure 5). In DMEM, biofilm formation EHEC O45 12047 and O104 CB13348 formed a biofilm as well as EHEC O157 EDL933 but with significantly lower level of biomass adhered.

IV. CONCLUSION

This study clearly evidenced that mechanical treatment can influence and bias the results of bacterial adhesion assay when investigating the interaction of EHEC with ECM proteins. It clearly appeared that adhesion ability greatly depends on the growth media, pH and temperature. While collagens I and III promote biofilm formation, this also depends on growth conditions. A better understanding of the molecular and cellular mechanisms involved in adhesion of major EHEC to meat is necessary to limit the risk of food outbreak. The discrepancies in the colonization abilities of EHEC to ECM proteins could ultimately be taken into consideration to evaluate the risk of contamination and eventually lead to the development of more efficient preventing strategy of carcass contamination by bacterial pathogens in the meat industry

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