Detaching and de-clumping of Enterohemorrhagic Escherichia coli (EHEC) adhered to cattle hide and the surface of beef processing equipment

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**Background**

EHEC is a group of bacterial pathogens that are capable of causing severe manifestations such as hemolytic uremic syndrome (HUS). Unlike other foodborne illnesses, a high percentage of the patients contracting EHEC infections require a hospital stay and many require kidney dialysis. On average, 2-7% of the patients with HUS die. Among the high-risk population, the mortality rate can be as high as 50%. The major transmission vehicle of EHEC infection is contaminated food, especially raw and undercooked ground beef. During cattle production and beef processing, pathogens such as EHEC may attach and subsequently form biofilm on cattle hide as well as a variety of surfaces including stainless steel, aluminium, glass, buna N rubber, teflon, and nylon materials that are widely used in beef processing environments (Kumar and Anand, 1998).

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous, extracellular matrix (Donlan, 2002). For EHEC, the formation of the matrix involves at least two of the cell surface components; namely, curli and cellulose (Romling et al. 2000; Zogaj et al. 2001). The curli are expressed in response to nutrient limitation under conditions of low osmolarity, low growth temperature, and in the stationary phase of growth (Romling et al. 1998). Although the isolates of EHEC that express the curli are often capable of causing diseases in humans, a definite role of the fimbriae in EHEC pathogenesis has not yet been established (Collinson et al. 1991, 1996; Grund and Weber, 1998). The function of these fibers in adhesion to solid surfaces has however, been documented (Vidal et al. 1998), and cells with curli have been shown to have a better ability to interact with their contact surfaces. Cellulose, nevertheless, is produced by EHEC as an extracellular component for mechanical and chemical protection (Solano et al. 2002). It plays a structural role by conferring mechanical strength to biofilm (Solano et al. 2002). When curli and cellulose are co-expressed by EHEC, a matrix of tightly packed cells is covered in a well-structured hydrophobic network (Zogaj et al. 2001; Gerstel and Romling, 2003). This network is extremely important in biofilm formation and the persistence of EHEC on various surfaces.

Bacterial attachment and biofilm formation on animal hide and processing surfaces is of major significance for the overall quality of beef because over time, cells attached to a surface will detach and migrate to a new growth site (Bar-Or, 1990). Bacterial cells sloughing from a biofilm could be a source of sporadic contamination of beef products coming in direct contact with the surface on which a biofilm has formed. The contamination may be an important cause of beef spoilage or beef-borne diseases (Joseph et al. 2001).

Attachment and biofilm formation by bacterial cells on plastic, metal, glass, or rubber surfaces has been reported in earlier studies (Helke et al. 1993; Austin et al. 1998; Sinde and Carballo, 2000). However, the types of bacterial cells used in these studies were not in the most persistent physiological state. Effective measures to remove the bacterial cells firmly associated with their contact surface and enclosed in the amorphous, hydrophobic network has not yet been thoroughly investigated.

The stated objectives for this work were:

The goal of the project was to identify chemical and enzymatic agents that can effectively degrade EHEC cell surface components that are critical to biofilm formation. With these effective measures we can detach and de-clump EHEC cells adhered to the surface of beef.
processing equipment in order to increase the efficiency and warrant the success of cleaning and/or sanitizing treatments in the beef processing environment.

**Methodology**

**Phase I: Chemical and enzymatic degradation of curli and cellulose under laboratory conditions**

*Bacterial strains and growth conditions*

EHEC strains 5-1 1C+, 5-1 1C-, 7-52C+, 7-52C-, 7-57C+, 7-57C-, 6-8, 6-35, 17, 49, 50, and 51, all from our laboratory collection were used in this study. The cultures were grown on Luria Bertani (LB) no salt agar supplemented with Calcofluor (200 g ml-1) for qualitative assessment of cellulose produced by the STEC cells. Cellulose-producing STEC were then grown on LB no salt agar at 28ºC for 72 h. The resulting cultures were collected with phosphate buffered saline (PBS) (pH 7.4) and the harvested STEC cell suspensions were used in the cellulose degradation experiments described below.

*Degradation of cellulose and curli with enzyme, organic acids, and commercial detergents*

The efficiency of both enzymatic and chemical agents was evaluated in this study in the degradation of cellulose produced by the cells of the selected EHEC strains. Cellulase of Aspergillus niger was dissolved in 0.05M sodium acetate buffer (pH 5.0), followed by an overnight dialysis at a refrigerated temperature. Appropriate volumes of the prepared cellulase solution were added to the Petri plates containing each of the EHEC suspension to a final concentration of 0.5 and 3.75%, respectively. The treatments were conducted at 37°C for 1 h with gentle shaking.

*Quantification of cellulose*

The quantification of cellulose was carried out using a colorimetric method developed by Updegraff with some modifications. The EHEC cultures treated by the chemical and enzymatic agents were appropriately washed with PBS. The cultures were then centrifuged at 3,400 g for 25 min. The centrifuge tubes were inverted gently and then placed in a boiling water bath for 16 min after which they were placed into an ice bath. The absorbance of each sample at 620 nm (A620) was recorded using the Novaspec II Spectrophotometer. A standard curve of absorbance as a function of cellulose concentration was prepared. The quantities of cellulose remaining on the surface of EHEC cells were calculated by comparing the absorbance values of the standard with the values of the tested samples. All reagents used in the quantification of cellulose were purchased from Fisher Scientific unless otherwise specified.

*Quantification of curli*

Following treatment with the chemical and enzymatic treatments described above, the cells of EHEC were centrifuged at 4,000 g for 10 min. The quantities of unbound Congo red were determined by measuring the absorbance of the supernatant at 500 nm (A500) using a spectrophotometer (Novaspec II, Pharmacia Biotech, Cambridge, England).

*Statistical analysis*

All experiments were set up with appropriate controls, duplications and replications. Data obtained were analyzed using the general linear model of the Statistical Analysis Software (SAS 1999) at a 95% confidence interval. The effectiveness of the degradations achieved by the chemical and enzymatic treatments was calculated by comparing the overall mean absorbance of the cellulose and curli solutions.
Phase II: Detaching and de-clumping EHEC cells attached to cattle hide and the surface of beef processing materials

Control of biofilm formation using the chemical and enzymatic agents identified in Phase I of the study

Two ml of the broth cultures of EHEC cells were added to 24 well polystyrene tissue culture plates. The cells were allowed to form biofilm on the polystyrene surfaces for 7 d at 28°C. At the end of the 7-d incubation period, the biofilm formed by the EHEC cultures was treated with the chemical and enzymatic agents evaluated in phase I of the project. The biofilm left in the wells of the tissue culture plates after the treatments were quantified using the crystal violet binding assay. The biofilm that did not receive any treatment was included as controls.

Control of EHEC cells on cattle hide using the chemical and enzymatic agents identified in Phase I of the study

Cattle skin with hide was obtained from a beef slaughterhouse in south GA. The cattle skin was transported to our laboratory under refrigerated temperatures. The skin was cut into 5 cm X 5 cm blocks and stored at -20ºC until use. On the day of experiment, the cattle skin blocks were withdrawn from the storage and inoculated with four different strains of EHEC, respectively at an appropriate level. The inoculated skin blocks were kept at room temperature for approximately 30 min before being sprayed with appropriate concentrations of acetic acid, lactic acid, Quorum Yellow, and Zep formula 7961TM, respectively. The treatment solutions were allowed to be in contact with the cattle skin samples for 30 sec. After this period of time, the populations of total aerobic bacteria and Enterobacteriaceae on the skin blocks were determined. Cattle skin samples that were not inoculated with the EHEC cells or were not treated with the chemical and enzymatic agents were used as controls.

Findings
Phase I: Chemical and enzymatic degradation of curli and cellulose under laboratory conditions

Degradation of cellulose

The treatments with the chemical and enzymatic agents evaluated in the present study effectively degraded the cellulose on the surface of EHEC cells. The residual amounts of cellulose on the surface of EHEC cells after the treatments with cellulase, acetic acid, and lactic acid are shown in Fig 1 and 2, respectively. Lactic acid at a concentration of 4% was more effective \( (P < 0.05) \) than the lower concentrations tested in the study (Fig. 3). Fig. 4 shows the efficacies of the two commercial detergents in degrading the cellulose produced by the EHEC cells. Longer treatments with the commercial detergents significantly improved the efficiencies of the treatments \( (P < 0.05) \) (Fig. 5).

In summary, treatments with 3.75% cellulase reduced the overall mean cellulose produced by the cells of six STEC by 85% (Fig. 6). Acetic and lactic acid at 2% concentration reduced the overall mean cellulose by 36% and 52%, respectively (Fig. 6). Treatments with Quorum Yellow decreased the overall mean cellulose produced by the cells of six STEC by 80%, while the treatments with Zep formula 7961TM reduced the overall mean cellulose by 92% (Fig. 6).

Degradation of Curli

The treatment with the chemical and enzymatic agents effectively degraded curli fibers on the surface of EHEC cells. The efficacies of protease, acetic acid, and lactic acid in the degradation of curli are shown in Fig. 7, 8, and 9, respectively. Fig. 10 shows the effectiveness of the two commercial detergents in degrading the curli fibers on the surface of EHEC cells.
Phase II: Detaching and de-clumping EHEC cells attached to cattle hide and the surface of beef processing materials

Control of biofilm formation

The efficacies of cellulase and protease in the control of biofilm are shown in Fig. 11 and 12, respectively. While the control of bioilm formation using the two commercial detergents are shown in Fig. 13.

Control of EHEC cells attached to cattle skin and hide

This part of the project is currently on-going. The P.I. is currently attempting to identify new funding source to continue this part of the project, and has not been successful. According to the results that we have thus far, the treatments with the chemical and enzymatic agents evaluated in this study reduced the numbers of total aerobic bacteria and Enterobacteriaceae associated with cattle skin and hide. The numbers of total aerobic bacteria and Enterobacteriaceae killed by the chemical and enzymatic treatment are shown in Table 1.

Implications

All evaluated agents reduced the amounts of cellulose and curli produced by EHEC cells, even though some of the treatments were more effective than others. The evaluated agents also effectively controlled biofilm formation on a beef processing material in a laboratory setting.
Fig. 1. Degradation of cellulose produced by EHEC cells using different concentrations of cellulase.

Fig. 2. Degradation of cellulose produced by EHEC cells using organic acids.

Fig. 3. Degradation of cellulose produced by EHEC cells using different concentrations of lactic acid.
Fig. 4. Degradation of cellulose produced by EHEC cells using two commercial detergents.

Fig. 5. Effect of treatment time on the degradation of cellulose produced by EHEC cells.

Fig. 6. Percentage of cellulose reduction achieved by the chemical and enzymatic treatments.
Fig. 7. Degradation of curli produced by EHEC cells using protease.

Fig. 8. Degradation of curli produced by EHEC cells using organic acids.
Fig. 9. Degradation of curli produced by EHEC cells using different concentrations of lactic acid.

Fig. 10. Degradation of curli produced by EHEC cells using two commercial detergents.
Fig. 11. Control of biofilm formation using 3.75% cellulase solution

Fig. 12. Control of biofilm formation using different concentrations of protease.

Fig. 13. Control of biofilm formation using the diluted commercial detergents
<table>
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<tr>
<th>Sampled Microorganisms</th>
<th>Total aerobic bacteria</th>
<th>Enterobacteriaceae</th>
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<tbody>
<tr>
<td></td>
<td>Avetic acid</td>
<td>Lactic acid</td>
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<td>57C+</td>
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