Attachment of *Escherichia coli* O157:H7 and Other Bacterial Cells Grown in Two Media to Beef Adipose and Muscle Tissues

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**ABSTRACT**

Three strains of *Escherichia coli* O157:H7 were grown in tryptic soy broth (TSB) or in a sterile cattle manure extract at 35°C for 18 ± 2 h. Aliquots from both inocula containing 10^6 CFU/ml were used to inoculate 1-cm³ cubes of beef muscle or adipose tissue by immersion for 20 min at 21°C. After removal from the inoculum, one-half of the samples were analyzed for bacterial cell numbers and pH, and the other half were stored at 4°C for 2 or 3 h before analysis. Samples were analyzed by enumerating bacteria present in liquid droplets deposited on the tissue and bacteria loosely or strongly attached to the tissue in order to determine attachment strength. Total numbers of cells on beef muscle tissue (bacteria in liquid droplets, as well as those loosely and strongly attached) were 5.65 ± 0.14 and 5.76 ± 0.26 log CFU/cm² for *E. coli* O157:H7 inocula grown in TSB and manure extract, respectively. The differences in attachment strength between inocula from the two media were not significant (*P* > 0.05). A 2-h storage period after exposure of muscle tissue to an *E. coli* O157:H7 inoculum did not influence attachment strength. Numbers of bacteria attached to adipose tissue and muscle (5.31 ± 0.08 and 5.48 ± 0.09 log CFU/cm², respectively) were not significantly different (*P* > 0.05). After 3 h at 4°C, the attachment strength of *E. coli* O157:H7 cells on muscle or adipose tissue had not changed. Overall, the culture medium and type of beef tissue did not affect the numbers of *E. coli* O157:H7 cells attached, nor the strength of their attachment, to muscle or adipose tissue.

Key words: *E. coli* O157:H7, attachment, beef tissue, culture media

Bacterial attachment is the first step in meat contamination. Without contact and attachment to meat surfaces, microorganisms would not be able to create an appropriate niche for multiplication, and their removal, control, or destruction would be easier to accomplish, which could result in a reduced incidence of foodborne illness. Although several steps in the food-processing chain can influence the potentiality of foodborne illness, an important preventive measure may be avoidance of initial contamination, or at least removal of bacteria, by treatments such as spray washing before attachment (13, 26). The attachment of bacteria to meat surfaces depends on factors such as bacterial species (7, 17, 23, 24), inoculum concentration (3, 12), temperature of the attachment medium (3), and type of meat or tissue (19).

Among other factors, bacterial morphology and structure are affected by the properties of the medium used in inoculum preparation (3, 6, 17, 21). In natural environments, bacteria are covered by a glycocalyx composed of a network of polysaccharide fibers (4). The glycocalyx is involved in the attachment of bacteria to surfaces, as well as in the formation of biofilms (20). When the environment has a low nutrient concentration, adhesion of bacteria to surfaces helps them avoid starvation (10). For this reason, even though there is an energy expense involved in formation of the glycocalyx, it is a worthwhile survival mechanism. However, in highly nutritious environments, synthesis of the polysaccharides may be a waste of energy which could otherwise be used to support bacterial multiplication (8). Thus, when bacteria are grown in highly nutritious media, they do not need to form a glycocalyx and may lose part of their ability for attachment (9). For this reason, results from laboratory work on attachment may not be applicable to bacterial attachment in wild environments (1).

Although bacterial species have been reported to attach at different rates to different surfaces, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Salmonella arizonae*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Listeria monocytogenes* have been reported to attach similarly to lean and fat beef tissues (2, 7, 16). Another report has indicated that bacteria seem to attach more readily to connective tissue fibers than to myofibrils, although some bacteria could easily be entrapped between muscle bundles (2). In addition, there are results showing attachment of more bacterial cells to chicken muscle fascia than to cut chicken muscle, but attachment of fewer cells to beef with intact fascia than to cut beef muscle (18, 19).

This study was designed to determine whether *Escherichia coli* O157:H7 inoculum grown in a sterile cattle-manure extract, compared to growth in a laboratory broth, had an effect in improving the ability of this bacterium to attach to beef muscle, and to determine whether bacterial cells attached similarly to beef muscle and adipose tissues.

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The results of such a study may be useful for developing methodologies to examine decontamination processes for carcasses during slaughter.

**MATERIALS AND METHODS**

**Test of effect of inoculum preparation medium**

**Meat preparation.** Biceps femoris beef muscle, vacuum packaged and frozen at −20°C for 4 weeks, was thawed overnight at 4°C and then cut into cubes 1 cm on a side with knives which had been dipped in boiling water to minimize contamination. Sets of forty cubes were put into sterile bags (Nasco Whirl Pak®, Nasco, Ft. Atkinson, WI) and frozen at −20°C until used (within 1 week). Before use, the beef cubes were thawed inside the bags at refrigeration temperature (4°C) for 2 h.

**Manure extract preparation.** Fresh cattle manure was collected from a Colorado State University farm (Fort Collins, CO) and squeezed with a pestle through cheesecloth placed on a sieve. The resulting manure paste was diluted in 3 parts of distilled water to yield a stock manure paste, which was stored (4°C) in air-tight containers and used within a week. The stock manure paste was diluted (10−2) with distilled water and 20-ml volumes were autoclaved for 4 min at 121°C. The 4 min of autoclaving time was found to be the minimum necessary to sterilize the manure extract.

**Preparation of inocula.** Escherichia coli O157:H7 ATCC 35150, ATCC 43895, and a wild isolate were grown separately in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) or in the sterile cattle manure extract at 35°C for 18 ± 2 h. All three strains grown in TSB were combined and diluted with 0.1% sterile peptone water (Difco) to yield a cell concentration of approximately 106 CFU/ml. Strains grown individually in sterile manure extract reached concentrations of approximately 106 CFU/ml, and the single-strain inocula were combined and used as the inoculum without further dilution.

**Inoculation and bacterial recovery.** Twelve cubes of beef muscle were each dipped into either 200 ml of the TSB inoculum, the manure extract inoculum, or 0.1% sterile peptone water for 20 min at 21°C. Immediately after inoculation (time 0) half of the beef cubes were drained by pressing them with alcohol-flamed tweezers against the beaker internal wall and processed for bacterial recovery. The other half of the cubes were removed from the inoculum suspension and held in sterile beakers for 2 h at 4°C before being processed for bacterial recovery.

**Bacteria were recovered by methods similar to those described by Farber and Idziak (17).** Bacterial cells were recovered from the tissue samples by three procedures; each recovery procedure was applied to one-third of the samples. Under procedure A, the beef cubes in 100 ml of 0.1% sterile peptone water were shaken in a water bath shaker (Shaking Waterbath 50, Precision Scientific, Chicago, IL) at 21°C and 200 oscillations per min for 1 min. Appropriate dilutions of the peptone water were surface plated on tryptic soy agar (TSA) (Difco) plates. Under procedure B, the beef cubes were blended (Stomacher Lab Blender 400, Tekmar® Company, Cincinnati, OH) with 100 ml of sterile peptone water. Appropriate dilutions of the blended samples were plated on TSA plates. Under procedure C, the beef cubes were immersed in 0.1% sterile peptone water for 30 s. After being drained against the flask wall with alcohol-flamed tweezers, the samples were blended in the stomacher for 2 min with 100 ml of sterile peptone water. Appropriate dilutions of the blended samples were surface plated on TSA plates. These procedures were applied to groups of beef muscle cubes immediately after exposure to the inocula and after 2 h of storage at 4°C. All plates were incubated at 35°C for 24 h. Measurements of pH were performed in samples immediately after blending for microbiological analysis. The experiment was replicated four times.

**Calculation of the attachment strength.** The bacteria recovered in procedure A were designated as cells loosely attached to meat and cells found in water droplets hanging onto the meat surface. These could be classified as unattached cells. The bacteria recovered by procedure B were designated as cells loosely (or reversibly) attached to the meat, and cells strongly (or irreversibly) attached to the meat, and cells in water droplets (unattached). Bacteria recovered by procedure C were designated as cells loosely (reversibly) and strongly (irreversibly) attached to meat (17).

The attachment strength (17) or index of irreversible attachment (IIA) was calculated as

\[
IIA = \log C - \log (A - (B - C)),
\]

where \( A \) is the number of bacteria recovered with procedure A, \( B \), the number of bacteria recovered with procedure B, and \( C \), the number of bacteria recovered with procedure C.

**Test of effect of beef muscle and adipose tissue**

**Meat preparation.** In addition to beef muscle cubes obtained as described above, beef brisket adipose tissue samples were obtained from a commercial slaughtering plant, frozen (−20°C) overnight, and cut in the frozen state into cubes 1 cm on a side with knives which had been dipped in boiling water. Sets of 40 cubes were placed in sterile bags and then frozen at −20°C for no longer than one week. Muscle and adipose tissue samples were thawed at 4°C for 2 h before inoculation and testing.

**Preparation of inoculum.** Escherichia coli O157:H7 ATCC 35150, ATCC 43895 and the wild isolate were grown, separately in TSB at 35°C for 18 h. Culture preparations of all three strains were composited and diluted with sterile peptone water (0.1%) to yield a cell concentration of approximately 106 CFU/ml.

**Inoculation and bacterial recovery.** Twelve muscle and 12 adipose tissue sample cubes were inoculated by immersion into 200 ml of the cell suspension or 0.1% sterile peptone water for 20 min at 21°C. After inoculation, half of the muscle and adipose tissue samples were drained by pressing them against the beaker internal wall with alcohol-flamed tweezers and were processed for bacterial recovery. The remaining samples were drained by pressing them against the internal beaker wall with alcohol-flamed tweezers, placed in sterile beakers, and held at 4°C for 3 h before being processed for bacterial recovery. Bacterial recovery from muscle and adipose tissue samples including pH measurements of the blended samples and determination of attachment strength followed the procedures described above. The study was replicated three times.

**Statistical analysis**

Least square means of the log CFU per gram of bacterial counts, attachment strength values, and pH values were calculated from the results of duplicate samples in the three or four replicates, for each growth medium and type of tissue, at each sampling time, separately. An analysis of variance was performed on the bacterial counts, attachment strength values, and pH values, utilizing the general linear models procedure of SAS (25). When differences were significant (\( P < 0.05 \)), means were separated with the least significant difference procedure.
RESULTS

Effect of inoculum preparation medium

At time 0 (immediately following exposure to the inoculum), the total number of bacterial cells (in droplets, loosely attached and strongly attached; or unattached, reversibly attached and irreversibly attached), respectively, from the TSB and manure inocula, that were recovered by procedure B from beef muscle samples were $5.65 \pm 0.14$ and $5.76 \pm 0.26$ log CFU/cm$^2$, respectively, and the attachment strengths or IIA on beef muscle of cells grown in TSB and cells grown in cattle manure extracts were statistically similar ($P > 0.05$) ($0.73 \pm 0.59$ and $0.88 \pm 0.45$, respectively) (Table 1). After 2 h of storage of inoculated samples at $4^\circ$C, the total numbers of bacterial cells (unattached, reversibly and irreversibly attached) did not change: $5.44 \pm 0.21$ and $5.62 \pm 0.33$ log CFU/cm$^2$, for bacteria grown in TSB and manure, respectively (Table 1). After the 2-h storage period, IIA values for cells grown in TSB and manure extract were $0.82 \pm 0.41$ and $0.45 \pm 0.36$, respectively. Therefore, E. coli O157:H7 and other natural contaminants did not change with respect to their attachment strength or IIA on beef muscle after 2 h of storage at $4^\circ$C, regardless of the culture medium used to grow the inoculum. The pH values (Table 1) of the uninoculated samples were similar at time 0 and after 2 h at $4^\circ$C ($6.08 \pm 0.29$ and $6.02 \pm 0.30$, respectively).

Overall, numbers of cells attached to beef muscle after immersion in the inoculum for 20 min as well as the IIA were similar for cells grown in TSB and cells grown in autoclaved manure extracts. After a 2-h period at refrigeration temperature, numbers of bacterial cells recovered from inoculated samples and attachment strengths or IIA did not differ significantly ($P > 0.05$) from numbers of cells recovered at time 0 or the time 0 IIA for bacteria grown in TSB or bacteria grown in manure extract.

Effect of beef muscle and adipose tissue

The levels of natural bacterial contamination of muscle and adipose samples were similar at time 0 as well as after 3 h of sample storage at $4^\circ$C (Table 2). At time 0, aerobic mesophilic counts in uninoculated beef muscle and adipose tissue samples were $2.98 \pm 0.49$ and $2.83 \pm 0.34$ log CFU/cm$^2$, respectively, while after 3 h at $4^\circ$C they were $3.14 \pm 0.35$ and $2.98 \pm 0.81$ log CFU/cm$^2$, respectively (Table 2).

After 20 min of immersion of the samples in the TSB inoculum, the total numbers of bacteria including E. coli O157:H7 present on beef muscle and adipose tissues recovered by treatment B were not significantly different ($P > 0.05$): $5.48 \pm 0.09$ and $5.31 \pm 0.08$ log CFU/cm$^2$, respectively (Table 2). Attachment strength or IIA immediately following exposure to the inoculum was $0.47 \pm 0.13$ and $0.99 \pm 0.88$ for muscle and adipose tissue samples, respectively. After 3 h at $4^\circ$C, total numbers of bacteria including the inoculum of E. coli O157:H7 present on the muscle and adipose tissues were $5.31 \pm 0.08$ and $5.38 \pm 0.38$ log CFU/cm$^2$, respectively (Table 2). Differences in bacterial attachment strength (IIA) between muscle and adipose tissue samples were not significant ($P > 0.05$); IIA values were $0.49 \pm 0.40$ and $0.32 \pm 0.17$, respectively. At time 0, immediately following inoculation, muscle samples had a pH (Table 2) of $6.35 \pm 0.53$, which was slightly lower than the pH of adipose tissue samples ($6.76 \pm 0.51$). After 3 h at refrigeration temperature ($4^\circ$C), the pH of adipose tissue samples remained constant ($6.78 \pm 0.03$), but there was a decrease in the pH of muscle samples ($5.82 \pm 0.13$).

Overall, the results indicated that numbers of cells attached to beef muscle and adipose tissue samples after immersion in the inoculum for 20 min, as well as the IIA, were similar in both types of tissue. As expected, since E. coli O157:H7 is not a psychrotrophic microorganism, cells did not multiply when samples were held at $4^\circ$C for 3 h, and their attachment strength did not change with 3-h storage of muscle and adipose tissues at $4^\circ$C. It is unknown, however, how a higher storage temperature or longer storage time would have affected numbers of bacteria and irreversible attachment.

DISCUSSION

It has been shown that the cell wall structures of bacteria grown in liquid laboratory media or on a solid support are different (5, 21), suggesting the importance of the environment on the expression of the cell wall proteins.
and the ability to attach. Costerton et al. (9) have suggested that bacteria grown in culture media are exposed to enough nutrients to lose part of their ability to attach, compared to wild bacteria. Results from this study showed no difference in bacterial attachment when beef muscle tissue samples were exposed to E. coli O157:H7 cultures prepared in a highly nutritious laboratory broth or a cattle manure extract. Other researchers have also found that growing bacteria in not ideally nutritive media or on solid surfaces had no effect on the attachment of these bacteria to muscle surfaces (14, 22). Notermans et al. (22) reported that culturing bacteria, including some strains of E. coli, in a laboratory medium or on the surface of chicken breast meat had little influence on the attachment to chicken breast meat. However, cells obtained from 48-h orally infected chicks had a higher attachment ability. Dickson and Frank (14) reported no effect of growing E. coli O157:H7 in a starvation medium on the ability of the cells to attach to beef adipose tissue or muscle. The similarity in attachment ability of E. coli O157:H7 grown in TSB or in sterile bovine fecal extract could indicate that autoclaved feces contained adequate nutrients for E. coli O157:H7 development. It could also result from growing the bacterial inoculum monoxenically; competition among bacterial species in natural environments may confer a greater attachment ability on wild bacteria. It should be kept in mind that the results might have been different if the manure had not been autoclaved before inoculation with E. coli O157:H7, which is the reason that the autoclaving time was kept to the minimum of 4 min. If no autoclaving was performed, then the E. coli O157:H7 inoculum might have been outgrown by other bacteria during manure extract incubation.

In this study, E. coli O157:H7 attached similarly to beef muscle and adipose tissues, which is in agreement with findings by Dickson (12) and Dickson and Frank (14). Working with L. monocytogenes and S. typhimurium, they found that numbers of these bacteria attached to beef muscle were only 0.5 log units higher than numbers attached to adipose tissue. Dickson and Koohmaraie (15), working with S. typhimurium, also found attachment to beef adipose and muscle tissues to be similar, and in the more recent study of Dickson and Frank (14), it was shown that there was similar attachment of L. monocytogenes and E. coli O157:H7 to beef adipose and muscle tissues. Although there was an unexplained high variability in irreversible attachment of cells attached to adipose tissue, there were no significant differences in attachment of E. coli O157:H7 cells to muscle and adipose tissue. However, results from another study involving L. monocytogenes, S. typhimurium, S. aureus and S. marcescens showed a greater effect of NaOH and KOH in washing these microorganisms from beef adipose tissue than from beef muscle, suggesting a stronger attachment to muscle than to adipose tissue (11).

Three hours at 4°C had no effect on total numbers of bacteria attached or on attachment strength. Since E. coli O157:H7 is not a psychrotrophic microorganism, cell metabolism may be inhibited sufficiently to not allow formation of the exopolysaccharide structures that lead to stronger attachment. Although the mean pH of muscle samples decreased from 6.35 to 5.82 during storage, it did not have any effect on total numbers of bacteria attached or on the attachment strength. The results of this study should be beneficial in developing experimental designs and methodologies to study intervention treatments to decontaminate carcasses during the slaughtering process.

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