Attachment of Salmonella typhimurium and Listeria monocytogenes to beef tissue: effects of inoculum level, growth temperature and bacterial culture age

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Attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to beef tissue: effects of inoculum level, growth temperature and bacterial culture age

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The effects of inoculum level, growth temperature and culture age on the attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to beef tissue surfaces were evaluated. An increase in inoculum level resulted in an increase in the number of attached cells for both bacteria which was proportional to the increase in inoculum. Bacteria grown at 23°C attached in higher numbers (P<0.05) to fat tissue than bacteria grown at 37°C or at 37°C followed by 24 h at 5°C. Growth temperature did not affect attachment to lean tissue for either bacterium. Overnight cultures of both bacteria attached in greater numbers (P<0.05) to both tissue surfaces than cultures incubated for 66 h, with a difference of > 1 log10 cycle between 18- and 66-h L. monocytogenes cultures.

**Introduction**

Salmonellae historically have been associated with raw animal products, including meat and meat products (National Academy of Sciences, 1975) and their significance as food-borne pathogens is well documented (Wagner and McLaughlin 1986). More recently, other pathogens, including *Listeria monocytogenes* (Doyle 1985) have been associated with animal products. Although more commonly known for its significance in dairy products, *L. monocytogenes* is associated with animals (Brackett 1988) and has been isolated from samples of ground beef (Nicolas 1985). The Food Safety and Inspection Service recently initiated a survey of meat products to determine the extent of contamination at the retail level (Food Safety and Inspection Service 1987).

Initial contamination of meat occurs during slaughtering of the animal. Sources of contamination include the hair, hide, and hooves of the animal, as well as rumen fluid or intestinal material (Ayres 1955). Much of this initial contamination contacts fatty tissue, since after dehiding a large portion of the carcass is covered by fat.

Bacterial attachment is presumed to be the first step in the contamination of solid surfaces. Attachment is generally considered to be a two step process, consisting of an initial, reversible attachment to the surface followed by a more permanent, irreversible attachment (Marshal et al. 1971). The attachment and subsequent survival and growth of the contaminating bacteria is of importance for the overall safety of the meat supply. Much of the previous research on attachment to surfaces has focused on intrinsic properties of the bacterial surface.
cells. Fletcher and Floodgate (1973) described an extracellular acidic polysaccharide of marine bacteria which was involved in attachment. The presence of extracellular structures (flagella) has also been implicated in attachment (Notermans and Kampelmacher 1974, Schwach and Zottala 1982), although this may not be universally true for all bacteria. Several researchers (Meadows 1971, Lilliard 1985) have been unable to demonstrate a direct relationship between the presence of fimbriae or flagella and attachment, although Butler et al. (1979) reported that motile bacteria showed greater attachment to meat surfaces. The net negative charge on the bacterial cell surface has also been demonstrated to be positively correlated with bacterial attachment (Dickson and Koohmaraie 1989) and relative hydrophobicity of the bacterial cell (Fett 1985, Rosenberg and Kjelleberg 1986) has been shown to have significant effects on bacterial attachment to other diverse surfaces.

Environmental factors could influence bacterial attachment by altering the physiological or metabolic state of the bacterium. The objective of this study was to determine the effects of several physical factors, including cell numbers and culture growth conditions, on attachment of *Salmonella typhimurium* and *Listeria monocytogenes* to meat surfaces. It was intended that this information would provide a better understanding of the mechanism of bacterial attachment to meat surfaces.

### Materials and Methods

#### Tissue preparation

Post-rigor beef tissue was obtained as boneless trim from the abattoir at the Roman L. Hruska U.S. Meat Animal Research Center (RLHUSMARC). The tissue was separated into lean and fat tissue, sliced into 0.5-cm thick slices, frozen in sterile sealed bags, sterilized with γ-radiation at a minimum dose of 42 kGy, and stored at −20°C until use. Prior to use, the slices were cut into 1.0 × 1.0-cm squares (final tissue size 1.0 × 1.0 × 0.5 cm) and thawed at room temperature.

### Bacterial cultures

*Salmonella typhimurium* (ATCC 14028) was grown and maintained in tryptic soy broth (TSB, Difco). *Listeria monocytogenes* strain Scott A (FDA, Division of Microbiology, Cincinnati, OH) was grown and maintained in tryptic soy broth + 0.5% yeast extract (TSBYE). Cultures were transferred to TSB or TSBYE 18 h prior to use and incubated at 37°C (*S. typhimurium*) or 23°C (*L. monocytogenes*), unless otherwise specified in the methods. The attachment of these bacteria were determined in independent experiments.

#### Inoculation and enumeration

Cultures were diluted in Butterfield's phosphate buffer (Food and Drug Administration 1984) to achieve cell populations of approximately 10⁸ cfu ml⁻¹. Diluted cultures were prepared in 20-ml volumes in sterile beakers for inoculation purposes. Samples were inoculated by immersion in the diluted culture for 5, 10, and 20 min, transferred to dilution bottles containing 99 ml phosphate buffer, and the bottles gently inverted 25 times to remove planktonic (unattached) bacteria. The samples were removed from the dilution bottles, and bacteria remaining on the tissue surfaces were considered to be attached. Samples were stomached in 99 ml sterile buffer in a Stomacher 400 (Tekmar, Cincinnati, OH) for 2 min, and enumeration was carried out using the pour plate technique (Busta et al. 1984), with tryptic soy agar (TSA, Difco) as the growth medium. Plates were incubated at 23°C (*L. monocytogenes*) and 37°C (*S. typhimurium*) for 24–48 h. The bacterial population in the inoculum was also determined at 5 and 20 min, with the population of bacteria at 10 min being estimated as the average of the 5 and 20 min counts. Enumeration of the inoculum was determined using TSA and the pour plate method.

### Experimental design

**Inoculum level.** Attachment was evaluated using three levels of inoculum diluted in phosphate buffer. Samples were analysed at 5, 10 and 20 min intervals.
Bacterial attachment to beef tissue

Bacterial growth temperature. Cultures of both bacterial species were transferred to duplicate TSB or TSBYE tubes and incubated at 23 and 37°C for 18 h. A third broth culture of each species was incubated at 37°C for 18 h and then stored at 5°C for an additional 24 h. This incubation protocol was used to simulate conditions where a bacterium would be carried into an abattoir by a live animal, cooled in the chiller, and subsequently contaminate another carcass.

Culture age. Cultures were inoculated into TSB or TSBYE and incubated as described above for 66 h prior to use. Control experiments were conducted using cultures which had been incubated for 18 h.

Statistical analysis
The numbers of bacteria attached to the tissue surfaces were converted to log10 cfu cm⁻² values. Statistical analysis was conducted using the General Linear Models procedure of the Statistical Analysis System (1985), using models appropriate to the completely randomized design of the experiments. The numbers of bacteria in the inoculum at each time interval were used as a covariant in the analysis, which corrected the means for any differences in the initial inoculum levels between replications. Reported means are the average of three independent replications of each experiment. Unless stated otherwise, significance is expressed at the 0.05% level.

To further evaluate attachment with different inoculum levels, attachment was also expressed as a ratio of the initial inoculum at each time interval, which was calculated as (number of attached cells on the tissue per cm²)/(number of cells in the inoculum per ml) x 100. Attachment ratio was also analysed using the General Linear Models procedure.

Results and Discussion
Bacterial attachment to animal tissues is a complex phenomenon which, at present, is not fully understood, although properties of the individual bacterial cell (Dickson and Koohmaraie 1989) and substrate (Fletcher and Loeb 1979) affect the rate and degree of this process. Bacteria which become associated with tissue surfaces exist as either free cells in the water film on the tissue surface, or as cells which are physically attached to the surface. These cell types have been described as 'loosely' and 'strongly' attached cells (Firstenberg-Eden et al. 1978, Farber and Idziak 1984), or as planktonic and sessile (Costerton and Lappin-Scott 1989).

Most researchers consider 'attached' bacteria to refer to strongly-attached bacteria, and usually rinse tissue samples after treatment to remove any bacteria which are simply associated in the water film on the surface, i.e. the loosely-attached bacteria (Firstenberg-Eden et al. 1978, Butler et al. 1979, Farber and Idziak 1984, Lilliard 1986, Chung et al. 1989). In the research presented here, a washing step was also employed to remove the loosely-attached bacteria, and therefore 'attached bacteria' refers to bacterial cells which are physically attached to the tissue surface, i.e. strongly-attached cells.

The numbers of bacteria attached to the tissue increased significantly (P<0.05) in proportion to initial inoculum level for both S. typhimurium (Fig. 1) and L. monocytogenes (Fig. 2). There was an increase (P<0.05) over time with all levels of inocula for both bacterial species, typical of what was previously reported by Chung et al. (1989). However, in contrast to previous findings, more bacteria attached to lean tissue than to fat tissue. While these differences were statistically significant, the numerical differences were less than 0.5 log10 cycles and the actual biological significance of these differences is questionable.

When attachment was expressed as a ratio to the initial inoculum level, there was generally no difference between attachment at approximately 10⁴ and 10⁶ cfu ml⁻¹ inoculum levels for S. typhimurium within tissue type (Table 1). The attachment ratio with the 10⁸ cfu ml⁻¹ inoculum was lower than that of the 10⁶ level, although not statistically different from that of the 10⁴
Fig. 1. Attachment of *Salmonella typhimurium* to beef tissue as influenced by inoculum level. (a) Lean tissue; (b) fat tissue. (○—○) log 4; (□—□) log 6; (△—△) log 8.

Fig. 2. Attachment of *Listeria monocytogenes* to beef tissue as influenced by inoculum level. (a) Lean tissue; (b) fat tissue. (○—○) log 4; (□—□) log 6; (△—△) log 8.
Table 1. Ratio of attached Salmonella typhimurium and Listeria monocytogenes as affected by inoculum level. Average inoculum levels at $10^8$, $10^6$ and $10^4$ were, respectively log$_{10}$ 4-67, 6-66 and 8-69 for S. typhimurium and log$_{10}$ 4-53, 6-56, and 8-61 for L. monocytogenes.

<table>
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<tr>
<th>Bacterium Tissue</th>
<th>Inoculum level</th>
<th>Attachment ratio$^a$</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>SEM$^b$</th>
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<td>Fat $10^8$</td>
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<td>0.60</td>
<td>0.42</td>
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<td>$10^6$</td>
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<td>$10^4$</td>
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<td>5.66</td>
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$^a$ [(number of attached bacteria/cm$^2$)/(number of bacteria/ml of inoculum)] $\times$ 100.

$^b$ s.e. mean

inoculum level. As previously noted, there was a general increase in attachment over time ($P<0.10$), and a distinct difference between tissue type. With L. monocytogenes, the only difference noted was between tissue types. This indicates that the mechanism of attachment is generally mediated by individual cell properties, and not by the dynamics of the overall population. While it is true that a higher initial inoculum produces a higher level of attached cells, this increase in attachment is solely attributable to the increase in initial cell numbers.

Growth temperature also affected the attachment of both bacteria, although the differences were relatively small and primarily with fat tissue only. Higher numbers of S. typhimurium cells attached to lean tissue when the bacterium was grown at 37°C ($P<0.05$), although the range of values between the growth temperatures was a maximum of 0.2 log$_{10}$ cycles (Fig. 3). Higher numbers of cells grown at 23°C attached to fat tissue at all time intervals, with a difference of approximately 0.5 log$_{10}$ cycles at 20 min between the 23°C cells and the other two growth temperature cells. A similar pattern was noted with the results from L. monocytogenes (Fig. 4). There was no difference in attachment to lean tissue after 10 min, irrespective of growth temperature. As previously noted, bacteria grown at 23°C attached in greater numbers to fat tissue than bacteria grown at the other temperatures, with a difference of approximately 0.4 log$_{10}$ cycles at 20 min.

Growth temperature is known to affect the composition of the bacterial cell, with fatty acid composition of Escherichia coli reported to be influenced by temperature (Marr and Ingraham 1962). The motility of L. monocytogenes is also temperature dependent (Peel et al. 1988), with few if any flagella pro-
Fig. 3. Attachment of *Salmonella typhimurium* to beef tissue as influenced by growth temperature. (a) Lean tissue; (b) fat tissue. (○○) 37°C than 5°C; (□□) 23°C; (△△) 37°C.

Fig. 4. Attachment of *Listeria monocytogenes* to beef tissue as influenced by growth temperature. (a) Lean tissue; (b) fat tissue. (○○) 37°C then 5°C; (□□) 23°C; (△△) 37°C.
duced when the bacterium is grown at temperatures exceeding 30°C. However, these physiological factors apparently had only minor influence on the ability of bacteria to attach to beef tissue surfaces. Although statistically significant, the biological significance of a difference of 0.5 log₁₀ cycle is questionable. The growth temperatures used in this study produced comparable cell densities for a given bacterium, with no significant difference (P<0.05) in the populations in the inocula between growth temperatures.

The age of the bacterial culture affected attachment of *S. typhimurium* to both tissue surfaces, with more cells attaching from the 18-h cultures than from the 66-h cultures (Fig. 5). As previously noted, fewer bacteria of either age attached to the fat tissue than to the lean. After 20 min of inoculation, the numbers of attached 18-h cultures were approximately 0.5–0.6 log₁₀ cycles higher than those of the 66-h cultures. Culture age had a similar effect on attachment of *L. monocytogenes* to beef tissue (Fig. 6), with even larger differences in numbers of attached cells between the two growth temperatures. After 20 min of inoculation, the numbers of attached 18-h cultures were approximately 1.0–1.2 log₁₀ cycles higher than those of the 66-h cultures.

The bacterial populations in the 66-h cultures were higher than those in the 18-h cultures for both bacterial species, although the differences were only 0.2 log₁₀ cycles. This indicates that the 18-h cultures were in either late logarithmic growth or transitional phase between logarithmic growth and stationary phase. The 66-h cultures were assumed to be in stationary phase. The difference in attachment between the two different stages of growth may be attributable to basic physiological and metabolic differences between actively growing and nongrowing cells.
Physical factors can affect bacterial attachment to beef tissue surfaces, with culture age and subsequent phase of growth having a major influence on this attachment. The population in the initial inoculum has a direct affect on total numbers of attached cells, although the ratio of attachment between different inoculum levels is essentially the same. Bacterial growth temperature affected attachment only on fat tissue, and the reasons for this effect are not clear. These results emphasize the need for consistent growth conditions for bacteria when studying the phenomenon of bacterial attachment to meat surfaces, and also indicate that attachment to beef carcasses under actual processing conditions may vary from predicted laboratory values.

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References

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