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Attachment and Proliferation of Bacteria on Meat

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ABSTRACT

The attachment of bacteria (*Serratia marcescens, Staphylococcus aureus, Streptococcus faecalis, Salmonella arizonae, Pseudomonas aeruginosa,* and *Listeria monocytogenes*). to lean muscle tissue and fat tissue was investigated. The number of cells attached to the meat was directly proportional to the initial cell concentrations present. There was no significant difference in the number of cells attached between the lean muscle tissue and fat tissues among the organisms tested. All bacteria tested except *P. aeruginosa* proliferated better on the lean muscle tissues than on the fat tissue at ambient temperature for 72 h. No significant attachment competition to tissue samples was seen between *L. monocytogenes and P. aeruginosa,* however, the numbers of *P. aeruginosa* were greater than *L. monocytogenes* (after 24 h). Similarly, no competitive attachments between *S. aureus* and *S. marcescens, S. faecalis* and *S. arizonae* were observed; but the numbers of *S. marcescens* were greater than *S. aureus,* and *S. arizonae* were greater than *S. faecalis,* when the inoculated meat was incubated at room temperature for 24 h.

Microbial contamination of raw meat has always been an important issue for food safety. One measure to ensure good meat quality is to rely on effective washing of carcasses in order of decrease the microbial population on the surface of the meat (*5,11*). Although methods and devices have been developed to clean carcass surfaces (*1,2,3*), complete sterilization of raw meat is difficult to achieve. To ascertain if washing succeeds, basic information on microbial attachment to meats is essential. Although rates of bacterial attachment to meat, especially chicken, have been studied, (*12,18*), there is limited information on the bacterial attachment to fat tissue in comparison to lean muscle tissue of red meat. Fat tissue may provide a better surface than lean muscle tissue for bacterial attachment since their surface structures are entirely different. Microbial spoilage of meat is influenced not only by their initial attachment to the surface, but also by subsequent proliferation after attachment. It is, therefore, important to learn how bacteria proliferate on meats.

The objective of this study was to investigate whether fat tissue would be more susceptible than lean tissue to bacterial attachment, and to study the proliferation of bacteria after attachment to meat. Emphasis was also placed on microbial interactions regarding both attachment and proliferation on meat. The ultimate goal is to provide information for the study of decontamination of bacteria on meat surfaces.

MATERIALS AND METHODS

Meat

Fresh beef was obtained from the abattoir at the U.S. Meat Animal Research Center. Lean muscle and fat tissue were stored at -15°C. Before experiments, samples were thawed at room temperature and cut with a sterile scalpel into 1.0 cm x 1.0 cm x 0.5 cm pieces. These samples contained fewer than 100 colony forming units per sample.

Bacterial Strains

Serratia marcescens ATCC 8100, *Staphylococcus aureus* ATCC 25923, *Streptococcus faecalis* ATCC 19433, *Salmonella arizonae,* ATCC 13314, and *Pseudomonas aeruginosa* ATCC 27853 were obtained from Difco Laboratories (Detroit, MI). *Listeria monocytogenes* (strain Scott A) was obtained from FDA, Division of Microbiology, Bacterial Physiology Branch, Cincinnati, Ohio. The bacteria were maintained on Tryptic Soy Agar (TSA, Difco).

Attachment experiment

Organisms were grown in Tryptic Soy Broth (TSB, Difco) at 37°C except for *L. monocytogenes,* which was grown at room temperature, and *S. aureus,* which was grown at 32°C. They were incubated for 18 to 24 h. The cultures were centrifuged at 3,000 g at 4°C for 10 min. The supernatants were decanted and cell pellets suspended in 20 ml of attachment medium (*18*). Suspended cultures had approximately 10^8 cells per ml, with some variation from strain to strain. Cell suspensions were diluted in the same medium to appropriate concentrations for use.

Meat pieces were aseptically transferred to a sterile beaker containing 20 ml of cell suspension at appropriate concentrations, and were held at room temperature for 10 min (or as otherwise specified). At the end of holding, samples were aseptically picked and gently rinsed in 10 ml of sterile 0.87% NaCl (normal saline) solution and then drained at the edge of the beaker for 20 seconds. Organisms remaining on the meat were considered to be attached. Rinsed samples were immediately transferred to a sterile bag

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containing 99 ml of sterile Butterfield's phosphate buffer (*8*) and stomached for 2 min in a Stomacher 400 (Tekmar, Inc., Cincinnati, OH). Suspensions were then serially diluted in 9 ml of sterile buffer to appropriate concentrations and surface plated on TSA plates.

Inoculated plates were incubated at 37°C (room temperature for *L. monocytogenes* and 32°C for *S. aureus*) for 24 to 48 h. Culture counts of the inoculated bacteria [colony forming units (CFU)] were the average of duplicate plates that showed 25 to 300 colonies.

Bacterial proliferation in meat

Bacteria were attached to the meat samples as outlined above. Inoculated meats were put into sterile petri dishes, sealed with Parafilm to prevent dehydration, and incubated at room temperature. Culture counts were conducted a 0, 24, 48, and 72 h. Two pieces of meat were tested per culture per incubation time in each experiment.

Competitive Attachment and Proliferation:

Three sets of experiments for competitive attachment to and proliferation of bacteria on meat were conducted. They were 1. *L. monocytogenes* vs. *P. aeruginosa*, 2. *S. aureus* vs. *S. marcescens*, 3. *S. arizonae* vs. *S. faecalis*.

The organisms were grown as described above, and were diluted to approximately 10^7 per ml for test. In each set, attachments were carried out in three ways: pure culture, sequential, and simultaneous. For sequential attachment, the first organism was allowed to attach; then, after being rinsed gently with normal saline solution and drained, the sample was immersed immediately into the cell suspension of the 2nd organism. Attachments were carried out at room temperature for 30 min for each organism. At the end of holding, meat samples were aseptically picked, rinsed and diluted for plating as described before. Samples of meat inoculated with bacteria were incubated for 24 h at ambient temperature and for one week at 5°C. At the end of incubation, culture counts were conducted.

Culture counts were conducted on selective media. For *P. aeruginosa*, TSA with crystal violet (2 µg/ml) was used (*6*). The culture count of *L. monocytogenes* was obtained on modified McBride Agar, in which cycloheximide was omitted (*15*). Baird-Parker Agar Base (Difco) with EY-tellurite enrichment was used for *S. aureus*, and Violet Red Bile Agar (VRBA, Difco) was used for *S. marcescens*. SS agar (Difco) was used for *S. arizonae*, and TSA (with 0.04% Na-azide) was used for *S. faecalis*.

Statistical analysis

Statistical analysis was performed according to SAS (*19*). Unless otherwise noted, significance is expressed at the 5% level. Each experiment was performed in duplicate. The data presented were at least average of 2 experiments, with most being performed in 4 experiments.

RESULTS

Bacterial attachment

Attachments to meat by *L. monocytogenes* and *S. marcescens* were measured by using two different initial cell concentrations. Results for *L. monocytogenes* are shown in Fig 1. There was no significant difference in the numbers of attached cells between fat and lean tissues at either inoculum level. There was no significant (P>0.05) increase in the number of cells attached to the lean tissue between 0 and 10 min with both inoculum levels. There was a significant increase in the number of attached cells between 0 and 20 min with both tissues at the high inoculum level. For *S. marcescens*, there was no significant difference between the number of cells attached to lean and fat tissues for either inoculum level. (data not shown).

The effect of initial cell concentration on bacterial attachment was studied by varying cell concentration (Table

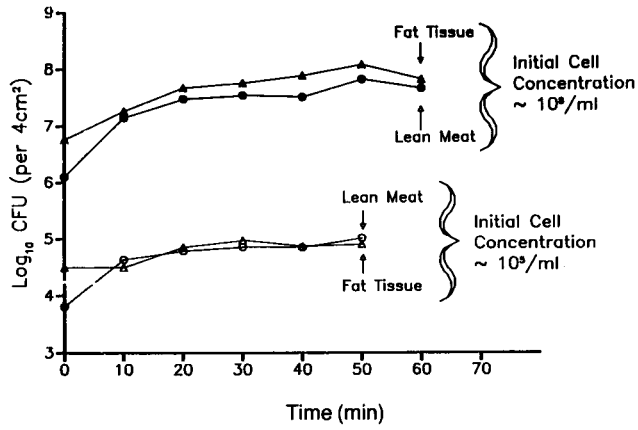

Figure 1. *Effect of holding time on the attachment of* Listeria monocytogenes *to meat.*

TABLE 1. *Effect of initial bacterial concentrations on the attachment to meat.*

Tissue	Initial Cell Concentration	10^5/ml	10^6/ml	10^7/ml	10^8/ml
		Number of Cells Attached Log_{10} CFU (per 4 cm^2)			
Serratia marcescens					
Lean Muscle		4.02[a1,2]	5.05[b]	5.95[c]	7.05[d]
Fat		4.45[e]	5.16[b]	6.06[c]	7.16[d]
Staphylococcus aureus					
Lean Muscle		3.56[a]	4.67[b]	5.96[c]	6.71[d]
Fat		3.49[a]	4.86[b]	5.69[c]	6.72[d]
Salmonella arizonae					
Lean Muscle		4.41[a]	5.47[b]	6.41[c]	7.39[d]
Fat		4.30[a]	5.29[b]	6.34[c]	7.36[d]
Streptococcus faecalis					
Lean Muscle		4.35[a]	5.29[b]	6.43[c]	7.23[d]
Fat		4.34[a]	5.23[b]	6.28[c]	7.30[d]
Pseudomonas aeruginosa					
Lean Muscle		4.15[a]	5.31[b]	6.38[c]	7.28[d]
Fat		4.44[a]	5.18[b]	6.30[c]	7.46[d]
Listeria monocytogenes					
Lean Muscle		4.71[a]	5.67[b]	6.49[c]	7.33[d]
Fat		4.89[a]	5.79[b]	6.69[c]	7.79[d]

[1]Means with different superscripts are significantly different (P<0.05) for lean and fat tissue.
[2]Each bacterial species analyzed separately.

1). In almost every case, there was no significant (P>0.05) difference between the counts on fat and lean tissue for a given bacterium at a given initial cell concentration. The 2 exceptions were *S. marcescens* at 10^5 and *S. aureus* at 10^7. There was a significant (P<0.05) increase in numbers as the initial cell concentration increased for each bacterium tested.

Bacterial proliferation

When meat was inoculated with bacteria, bacterial growth took place. Lean muscle supported bacterial proliferation better than fat tissues for all bacteria tested except *P. aeruginosa*. *P. aeruginosa* grew much better in fat tissue than in the lean meat. (Fig. 2).

Competitive attachment and proliferation

When *L. monocytogenes* and *P. aeruginosa* were inoculated on to lean muscle, no competitive interactions were observed (Table 2). The attachment of one bacterium was neither inhibited nor enhanced by the presence of the other. After 24 h at room temperature, *P. aeruginosa* reached higher cell densities than *L. monocytogenes*. However, after 7 d at 5°C, the numbers of *L. monocytogenes* were higher than those of *P. aeruginosa*, although there was no significant difference between the pure culture and mixed culture populations for a specific bacterial species. The same basic pattern of results was seen when the bacteria were inoculated on fat tissue (data not shown).

S. aureus and *S. marcescens* produced slightly different results. There were no significant differences between the pure culture and mixed culture experiments on lean tissue after initial attachment (0 h) or after 24 h at room temperature (Table 3). However, after 7 d at 5°C, the population of *S. aureus* was significantly (P<0.05) higher when inoculated simultaneously with *S. marcescens* than when *S. marcescens* was inoculated first. The population of *S. marcescens* did not differ significantly between the initial inoculum and after 7 d at 5°C. Although neither bacterium reached as high a population in fat tissue, the pattern of growth was similar.

When *S. faecalis* and *S. arizonae* were inoculated on to lean tissue, *S. faecalis* grew to a lower population after 24 h when *S. arizonae* was inoculated first (Table 4). The population of *S. arizonae* did not differ between pure and mixed culture experiments after 24 h at room temperature. When the same experiment was conducted on fat tissue, *S. faecalis* did not grow as well after 24 h for either of the two sequential attachment experiments when compared to the pure culture (data not shown). The population of *S. arizonae* was slightly less after 24 hours when it was inoculated first on to the tissue. No growth of *S. arizonae* took place after 1 week at 5°C on meat (Table 4).

TABLE 2. *Competitive attachment and proliferation of* Listeria monocytogenes *and* Pseudomonas aeruginosa *on lean meat.*

Organism for attachment	Log_{10} (CFU) (per 4 cm^2) 0-H	24-H (Rm Temp)	1 wk (5°C)
Listeria monocytogenes alone	$6.43^{a,1}$	8.36^{b}	8.63^{b}
Pseudomonas aeruginosa alone	$6.67^{c,d}$	9.31^{f}	$7.50^{c,d,e}$
Listeria monocytogenes first followed by *Pseudomonas aeruginosa*	*Listeria monocytogenes* 6.30^{a}	8.44^{b}	8.37^{b}
	Pseudomonas aeruginosa $6.44^{c,d}$	9.12^{f}	7.97^{e}
Pseudomonas aeruginosa first followed by *Listeria monocytogenes*	*Listeria monocytogenes* 6.50^{a}	8.34^{b}	8.24^{b}
	Pseudomonas aeruginosa $6.55^{c,d}$	9.03^{f}	$7.39^{c,d,e}$
Listeria monocytogenes and *Pseudomonas aeruginosa* simultaneously	*Listeria monocytogenes* 6.46^{a}	8.62^{b}	8.47^{b}
	Pseudomonas aeruginosa 6.34^{c}	9.19^{f}	$7.06^{c,d,e}$

[1]Means with different superscripts within columns are significantly different (P<0.05).

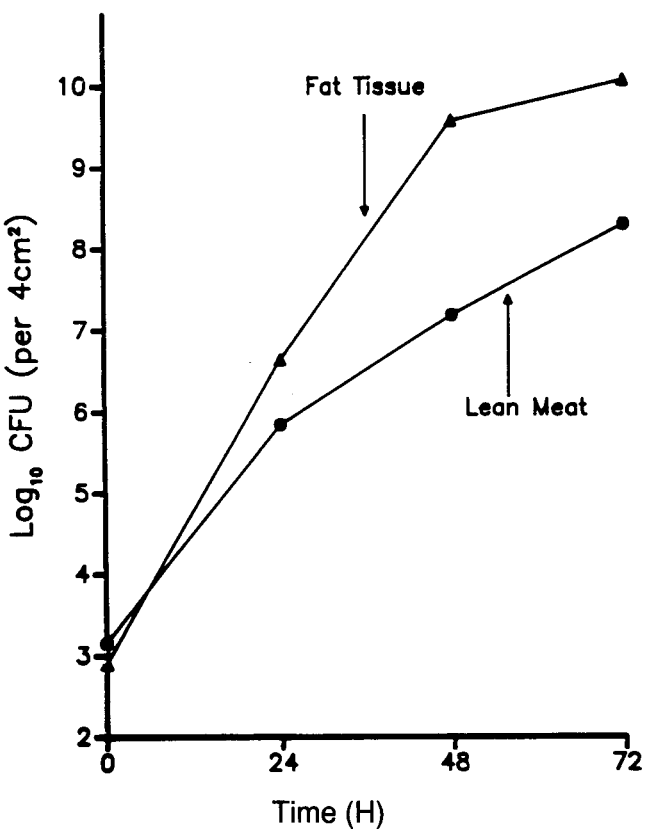

Figure 2. *Proliferation of* Pseudomonas aeruginosa *on meat.*

DISCUSSION

The measurement of the rate of attachment of organisms is difficult. The method employed in this study might reflect merely the organism entrapped in the beef fibers. However, the behaviors of these pathogens entrapped in the beef fiber is valid information in regard to beef safety.

Bacteria tend to attach to the meat surfaces in a manner that they are not easily removed (*18*). Many factors affect the attachment. Some bacteria are able to attach to meat surfaces

TABLE 3. *Competitive attachment and proliferation of* Staphylococcus aureus *and* Serratia marcescens *on lean meat.*

Organism for attachment	Log_{10} (CFU) (per 4 cm^2) 0-H	24-H (Rm Temp)	1 wk (5°C)
Staphylococcus aureus alone	$6.41^{a,b1}$	9.47^{g}	$6.51^{a,c,d,e}$
Serratia marcescens alone	6.09^{h}	10.11^{i}	6.74^{h}
Staphylococcus aureus first followed by *Serratia marcescens*	*Staphylococcus aureus* $6.44^{a,b}$	8.70^{g}	$6.13^{a,d,e,f}$
	Serratia marcescens 6.11^{h}	9.95^{i}	6.66^{h}
Serratia marcescens first followed by *Staphylococcus aureus*	*Staphylococcus aureus* $6.22^{a,b}$	8.55^{g}	$6.04^{e,f}$
	Serratia marcescens 6.02^{h}	9.43^{i}	6.23^{h}
Staphylococcus aureus and *Serratia marcescens* simultaneously	*Staphylococcus aureus* $6.57^{a,c}$	8.93^{g}	$6.56^{a,c,d}$
	Serratia marcescens 6.19^{h}	9.92^{i}	6.91^{h}

[1]Means with different superscripts within columns are significantly different (P<0.05).

TABLE 4. *Attachment and proliferation of* Streptococcus faecalis *and* Salmonella arizonae *on lean meat.*

Organism for attachment	Log_{10} (CFU) (per 4 cm^2) 0-H	24-H (Rm Temp)	1 wk (5°C)
Streptococcus faecalis alone	6.33^{a1}	8.96^{c}	7.90^{b}
Salmonella arizonae alone	6.15^{e}	9.55^{h}	$4.98^{f,g}$
Streptococcus faecalis first followed by *Salmonellae arizonae*	*Streptococcus faecalis* 6.35^{a}	8.71^{c}	7.63^{b}
	Salmonellae arizonae 6.27^{e}	9.53^{h}	$5.32^{f,g}$
Salmonella arizonae first followed by *Streptococcus faecalis*	*Streptococcus faecalis* 6.30^{a}	8.52^{d}	7.65^{b}
	Salmonellae arizonae 6.11^{e}	9.41^{h}	5.78^{g}
Streptococcus faecalis and *Salmonellae arizonae* simultaneously	*Streptococcus faecalis* 6.46^{a}	$8.73^{c,d}$	7.74^{b}
	Salmonella arizonae 6.24^{e}	9.30^{h}	6.35^{g}

[1]Means with different superscripts within columns are significantly different (P<0.05).

better than others (*9*). Kinetics of attachment depend on the individual bacterial species as well as the meat surfaces. Of the 6 species of bacteria tested, all of them attached to the meat surface instantly when meat pieces were immersed into the cell suspension (Table 1). If the time of incubation was prolonged, a slight increase in numbers of the cells attached to the meat was observed with *L. monocytogenes* (Fig. 1) but not *S. marcescens* (data not shown). Species difference might account for this variation. Although fat tissue is pliable and hydrophobic, there was basically no differences in attachment between fat and lean tissue. Bacteria attached to the fat tissue may be more difficult to wash off, because fat tissue is hydrophobic.

Other factors important to bacterial attachment have also been studied. Firstenberg-Eden et al., (*10*) demonstrated that extracellular polymers are important for bacterial attachment. Several reports indicated the flagellated bacteria attach more readily than nonflagellated bacteria to poultry and red meat surfaces (*4,7,18*). However, McMeekin and Thomas (*17*) were not able to confirm these results, and Lillard (*12*) indicated that nonflagellated bacteria attached as readily as flagellated bacteria to poultry skin (*12*). Recently, Lillard (*13*) also showed that a transfer of water and bacteria from surface films to skin is possible during prolonged water immersion (*13*). In her recent work, she concluded that bacterial attachment to poultry skin was a complex phenomenon that involves mechanisms other than fimbriae, flagellae, or water uptake, although a combination of these and other factors may be involved (*14*). By using Scanning Electron Microscopy techniques, Schwach and Zottola observed that attachment fibrils were involved with the bacterial attachment to the contacting surfaces (*20*). The recent work of Van Loosdrecht et al., (*21,22*) indicated that bacterial cell wall hydrophobicity is important in bacterial adhesion. They demonstrated that hydrophobic cells attached to various surfaces to a greater extent than hydrophilic cells.

In our investigation, a direct comparison was made between lean muscle and fat tissue under identical conditions. Although the surface qualities of fat tissue and lean meat are entirely different, bacterial attachment was not significantly different with the assay method employed. Evidence indicates that fat tissue is just as susceptible to bacterial attachment as the lean muscle tissue. This phenomenon should be considered when washing methods are developed to obtain the most hygienic meat possible.

Subsequent proliferation of bacteria, after attachment, also creates a microbial hazard in meat. Although the temperature used for the test is outside the normal storage temperature of refrigerated beef, the fact is that all of the species tested proliferated rapidly on meat stored at the abuse temperature. The finding that most bacteria tested with the exception of *P. aeruginosa* proliferated more rapidly on the lean tissue than on the fat tissue, might be due to the fact that either more nutrients are available to the bacteria from lean muscle or the moisture content is higher in the lean muscle portion.

Another consideration is that in the natural environment, meat may be exposed to mixed microbial populations rather than in the pure culture conditions of the laboratory. McEldowney and Fletcher (*16*) recently showed that the attachment of each bacterial species was increased, decreased, or not affected by simultaneous or by sequential attachment

of another species. We found that in the simultaneous presence of both *L. monocytogenes* and *P. aeruginosa*, no significant competitive attachment between these two species occurred (Table 2). After incubation of the inoculated meat at the abuse temperature, the numbers of *P. aeruginosa* were significantly greater than *L. monocytogenes* (Table 2). This indicates that meat at room temperature is a better habitat for the reproduction of *P. aeruginosa* than *L. monocytogenes*.

When *S. marcescens* and *S. aureus* were co-attached to the meat, no significant competition could be observed on lean muscle tissue after 24 h. However, *S. marcescens* grew better than *S. aureus* when the inoculated meat was stored at room temperature (Table 3). Similarly, *S. arizonae* grew better than *S. faecalis* after attachment when both were allowed to attach to meat at the same time (Table 4). Although only 3 kinds of combinations were studied in this investigation, there are probably innumerable interactions between different species that can occur in the natural environment.

Microbial attachment, subsequent proliferation and interactions, all play roles in the bacterial spoilage of food. Bacteria that attach more readily to meat may not necessarily proliferate more readily.

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