

Sausage Casings as a Model for Attachment of *Salmonella* to Meat

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ABSTRACT

Artificial sausage casings were used as a model for studying bacterial attachment to meat connective tissue. Sausage casings of known mass were exposed to suspensions of *Salmonella typhimurium* in 0.15 M NaCl under various time, temperature, and inoculum level regimes, then washed to remove unattached bacteria. Attached bacterial cells were enumerated using both plate counts and scanning electron microscopy. Bacterial cells attached to sausage casing surfaces within 1 min of incubation. Numbers of attached cells increased with increasing temperature and inoculum levels and with time. Rates of attachment of *S. typhimurium* to sausage casings were comparable with those reported for attachment to meat surfaces. Sausage casings appear to be a convenient model for examining mechanisms of bacterial attachment to meats.

Conventional microbiological techniques and electron microscopy (EM) have been used to study attachment of microorganisms to meat (13,18,19). The advantage of using EM is that cells are enumerated "in situ", unlike with plate counts where attached cells must be removed before being quantified. A major difficulty with the use of EM for study of bacterial attachment to meat is the inability to visualize cells which are entrapped within the connective tissue matrix or between muscle fibers. Another concern is the generation and interpretation of artifacts.

Bacteria have been shown to attach to collagen fibers (1,14). Type I collagen is the major collagen found in meat tissues. Located in the muscle epimysial and perimysial connective tissue sheaths, type I collagen represents approximately 1% of the wet weight of typical post rigor mortis adult mammalian muscle (9). Artificial sausage casings are an edible packaging material formed from bovine hide corium, which is rich in type I collagen. Although sausage casings contain components not found in meat, (i.e., cellulose, carboxymethylcellulose, and mineral oil), the predominant component (65%) is collagen (Devro, Inc., personal communication).

The aim of this research was to evaluate the use of sausage casings as a model for attachment of microorganisms to meat.

MATERIALS AND METHODS

Salmonella suspensions

Salmonella typhimurium strain 14028 cells were grown in tryptose phosphate broth (Difco, Detroit, MI) at 37°C for 18 h, washed, and resuspended in sterile isotonic saline. Standard plate

counts (SPC) were used to determine inoculum levels, using a Spiral Plater (Spiral Systems Instruments, Bethesda, MD) with tryptose phosphate agar (TPA) (Difco) as the solid medium. Plates were incubated at 37°C for 24 h, and colonies were counted using a laser bacteria colony counter with the computer assisted spiral bio-assay data processor (Model 500A Spiral Systems Instruments).

Source of sausage casings

Sausage casings (diameter 40 mm) were provided by Devro (Somerville, NJ).

Analysis of fine structure of sausage casings using transmission electron microscopy

Sausage casings were cut aseptically into disks (diameter 12 mm) using a sterile cork borer. They were immersed in sterile isotonic saline for 30 min at 37°C. Disks were fixed by immersion in 1.0 ml of 1% glutaraldehyde per 0.1 M sodium cacodylate buffer solution (pH 7.4) for at least 24 h. Disks were washed in 0.1 M cacodylate buffer, postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer, washed again in buffer, and dehydrated in a graded series of ethanol solutions. Samples were embedded in an epoxy resin mixture, cured, sectioned, and stained with 2% uranyl acetate solution and lead citrate solution. Images of thin sections of sausage casings were photographically recorded on film using a Zeiss 10B transmission electron microscope.

Effect of immersion in aqueous solutions on the microtopography of sausage casings

Sausage casings were cut aseptically into disks (diameter 12 mm) using a sterile cork borer. Disks were immersed in either isotonic saline or deionized water and incubated at 37°C for 30 min. Control disks (not hydrated) for the two conditions were either incubated at 37°C for 30 min or placed directly into fixative solution. For viewing under scanning electron microscopy (SEM) disks were processed as for transmission electron microscopy (TEM) (see above) through dehydration, followed by critical point drying with carbon dioxide. Dried disks were mounted on aluminum stubs with colloidal silver adhesive, coated with a thin layer of gold by DC sputtering, and viewed in the secondary electron imaging mode using a JEOL Model 840A scanning electron microscope.

Estimation of bacterial attachment using standard plate counts

Sausage casings (0.5 ± 0.01 g) were placed in sterile petri dishes with 45 ml sterile isotonic saline. *Salmonella* suspension in 5.0 ml of saline (approximately 10⁹ CFU/ml) was added to the saline suspension in the dishes containing the sausage casings to give a final inoculum level of approximately 10⁸ CFU/ml. Rate of attachment was determined by exposing sausage casings to *Salmo-*

nella typhimurium for 1, 15, 30, 45, or 60 min at either 4, 19, or 37°C. Bacterial counts were made as follows: Sausage casings were removed aseptically from the petri dishes and washed twice by agitation for 30 s in approximately 50 ml of sterile isotonic saline. Sausage casings were placed in stomacher bags with 9.9 ml of peptone water (0.1% wt/vol) and stomached for 2 min. Suspensions were diluted in 0.1% peptone water, then plated on to TPA using a Spiral Plater. Plates were incubated at 37°C for 24 h and counted. Counts were expressed as CFU/g.

Estimation of bacterial attachment using scanning electron microscopy

Sausage casings were cut aseptically into disks (diameter 12 mm) using a sterile cork borer. Casing disks were dipped into approximately 20 ml of sterile isotonic saline to allow them to rehydrate. From the *Salmonella* suspension (approximately 10^9 CFU/ml), 0.1 ml was placed onto the inner surface of the casing. Rate of attachment was determined by exposing sausage casings to the bacterial suspension for 1, 15, 30, 45, or 60 min at either 4, 19, or 37°C. Bacterial counts were made as follows: Sausage casing disks were washed twice by agitation for 30 s in approximately 20 ml of sterile isotonic saline to remove unattached cells. Disks were prepared for viewing using SEM as described above.

Sausage casings were viewed under X2,000 magnification. Forty fields were chosen at random and all organisms in each field of view were counted. A mean count per field was obtained. The field area was measured and estimated to be 0.00168 mm² and counts were expressed in terms of CFU/mm².

Counts made using the SEM were compared with those made using the SPC. Using SEM, only the inner side of the sausage casings were inoculated, while with the SPC sausage casings were immersed in the *Salmonella* suspension. To allow comparisons between the SPC and SEM to be made, a study was made, showing that cells attached equally well to either side of the sausage casings (unpublished data). SEM counts could then be multiplied by 2. Counts were converted into CFU/g, based on the observation that a disk of area 113 mm² weighed 0.006 g.

Effect of inoculum level

The effect of inoculum level was studied using a direct technique based on plate counts. Sausage casings were cut aseptically into squares (40 mm x 40 mm) and immersed in sterile isotonic saline. *S. typhimurium* suspension was added at levels of 10^1 , 10^3 , 10^5 , and 10^7 CFU/ml. Sausage casings were incubated with the *Salmonella* suspension for 30 min at 37°C, then washed twice by agitation for 30 s in approximately 50 ml of sterile isotonic saline to remove unattached cells. Casing squares were lifted carefully and placed on either bismuth sulphite agar (Difco) or xylose-lysine-deoxycholate (XLD) agar (Difco). On these selective agars, salmonellae appeared black, contrasting clearly with the sausage casings (Fig. 1). Sausage casings were incubated at 37°C for 24 h. Colonies growing on sausage casings were considered to be from attached cells, and they were counted with the aid of a magnifying lens.

Statistics

All experiments were replicated three times with each sample being assayed in duplicate. Analysis of variance and regression analysis were performed using SAS computer software (17).

RESULTS

Analysis of sausage casings using electron microscopy

The physical features of the inner surface of sausage casings were resolved in thin cross sections by TEM. In general, collagen fibrils showing typical banding patterns

were located at or near the surface (Fig. 2). Fibrils were embedded in a fine filamentous or granular matrix throughout the casing. Clear areas of varying sizes, possibly corresponding to cellulose fibrils, were observed occasionally at the surface without a continuous coating of collagen fibrils, but in general the surface was considered to be mainly collagen. Viewed under SEM, the inner surface of the casing appeared as very long fine fibrils, about 100 nm wide, and smooth

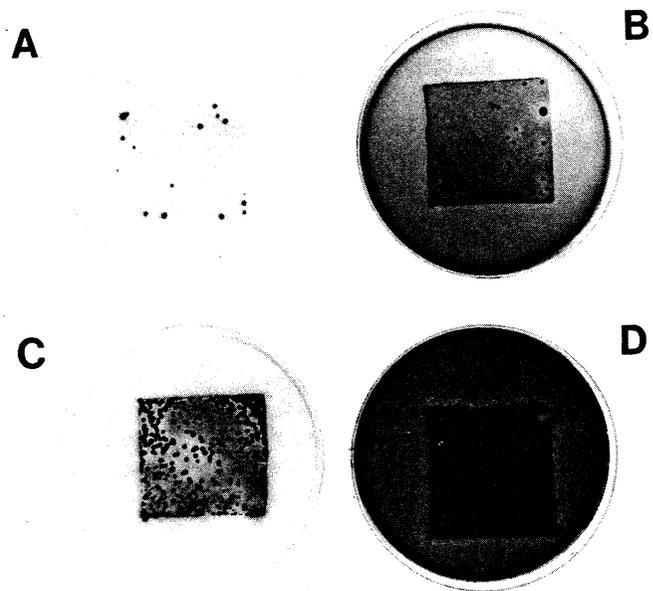


Figure 1. *Salmonella typhimurium* attached to sausage casings. Inoculum level is 10^3 CFU/ml (A) on XLD agar, 10^3 CFU/ml (B) on bismuth sulphite agar, 10^5 CFU/ml (C) on XLD agar, and 10^5 CFU/ml (D) on bismuth sulphite agar.

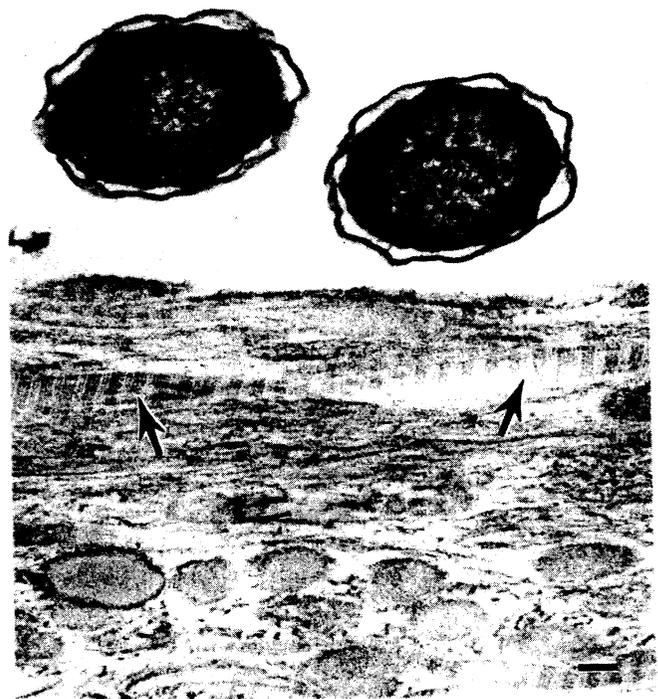


Figure 2. This section of sausage casing using TEM, with *S. typhimurium* cells attached, X50,000. Arrow indicates collagen fiber showing typical banding pattern. Bar = 100nm.

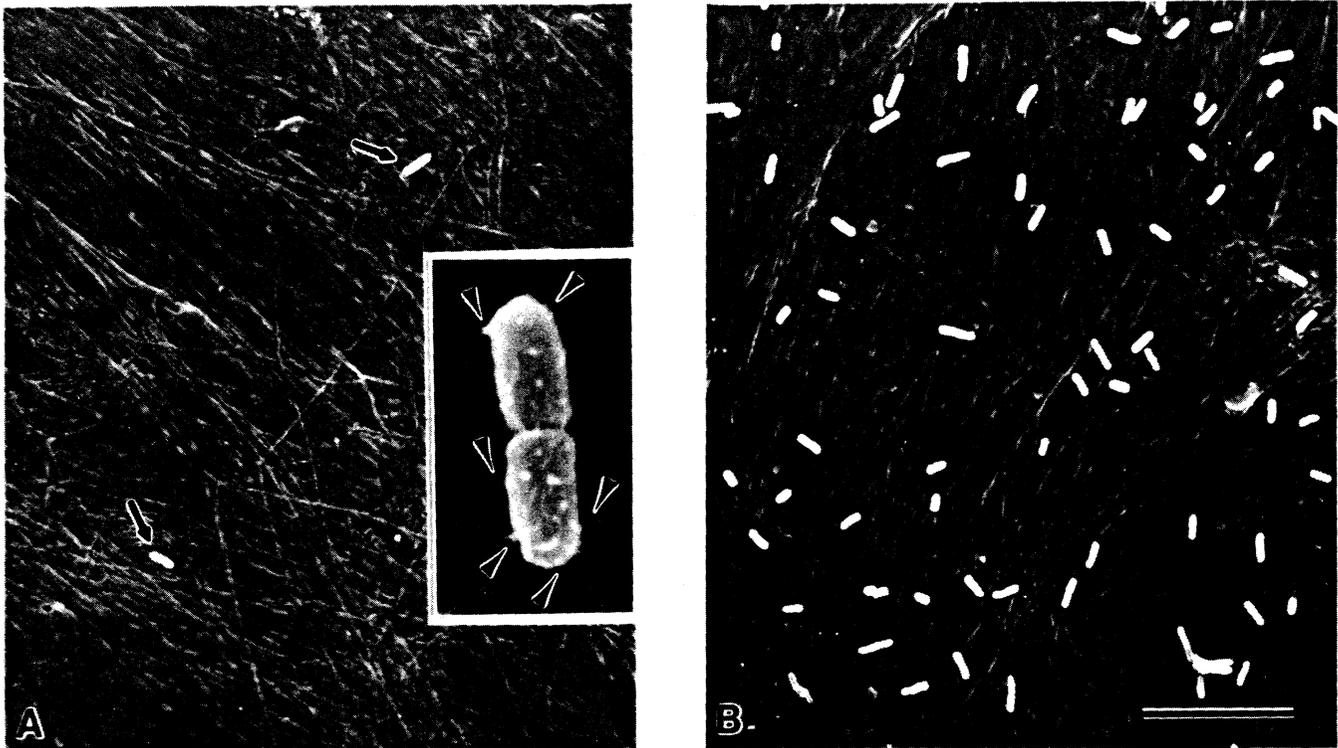


Figure 3. Scanning electron micrographs illustrating attachment of *S. typhimurium* to sausage casings after 1 min (A) after 60 min (B) X2,000. Inset shows cell with fibrils on its surface, X20,000. Bar = 10µm.

irregular areas. Outlines of large cellulose fibrils subtended the fine fibrillar and smooth regions (Fig. 3A). Immersion in isotonic saline or deionized water had no effect on the microtopography of sausage casings; all disks appeared similar when viewed using SEM.

Attachment of *S. typhimurium* to sausage casings

Attachment of *S. typhimurium* to sausage casings was measured using both SPC and SEM methods. Cells could be distinguished clearly from the background using SEM (Fig. 3A and B). On some cells fibrils were visible (Fig. 3A inset).

Bacteria were not evenly distributed over the surface of sausage casings (Fig. 4). Numbers of cells per field varied widely; at the most extreme, the distribution on one piece of casing ranged from 0 - 314 cells per field. Cells appeared singly or in groups (3 or more cells together, mean = 8.39 cells, maximum = 48 cells). However, the cells were not in direct contact with each other, indicating that the groups of

cells were not microcolonies. Of 120 pieces of casings observed with SEM, 37 (31%) had one or more fields containing groups of cells. The mean number of groups per sample (out of 40 fields of view) was 4.

Attachment of *S. typhimurium* to sausage casings increased with increasing time and incubation temperature (Fig. 5A and B). Analysis of variance shows a significant effect ($P < 0.05$) of temperature and time on counts made using both SPC and SEM. Good correlation was obtained between the SPC and the SEM counts ($r^2 = 0.89$) (Fig. 5C).

Effect of inoculum level on attachment of *S. typhimurium* to sausage casings

Bacterial attachment could be measured directly on the sausage casings by placing the inoculated squares on to both XLD and bismuth sulphite agars. Similar bacterial counts were obtained regardless of the agar used. The technique allowed low numbers of attached bacteria to be enumerated, i.e., 1 CFU/1600 mm². Where high numbers of attached cells were present, distinguishing between individual colonies became more difficult. When the inoculum level was greater than 10⁷ CFU/ml, it was not possible to make bacterial counts using this method. Attachment of *S. typhimurium* to sausage casings increased linearly with increasing inoculum level (Fig. 6).

DISCUSSION

Previous studies (1,14) have shown that salmonellae attach to collagen in poultry and meat tissues. It was hypothesized that sausage casings, composed mainly of type I collagen, could serve as a model for studying bacterial attachment. Our TEM studies have confirmed the presence of collagen fibers at the surface of sausage casings. Unlike meat

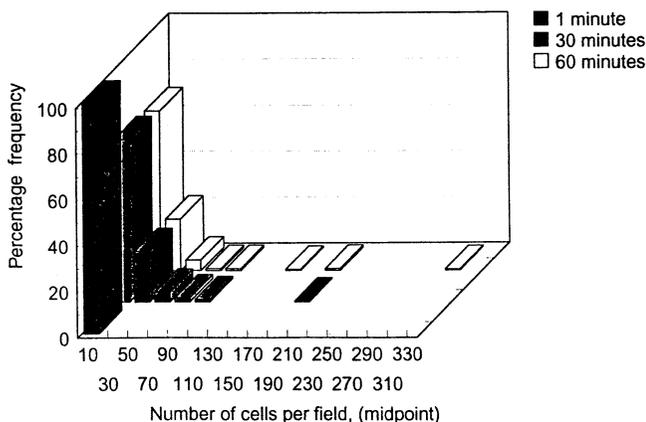


Figure 4. Comparison of distribution of *S. typhimurium* cells attached to sausage casings after incubation for 1, 30, and 60 min.

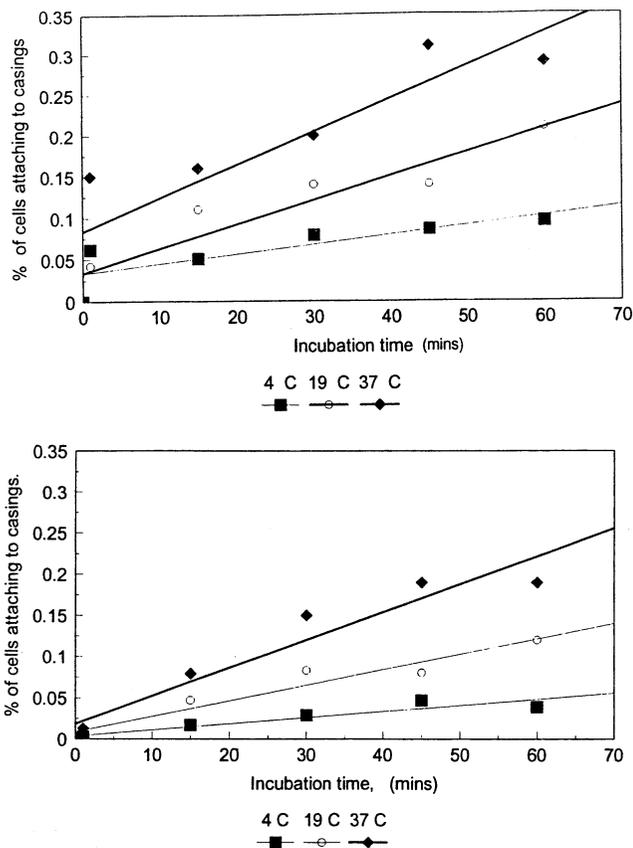


Figure 5. Rate of attachment of *S. typhimurium* to sausage casings, at 4, 19, and 37°C, measured using plate counts (A), and scanning electron microscopy (B).

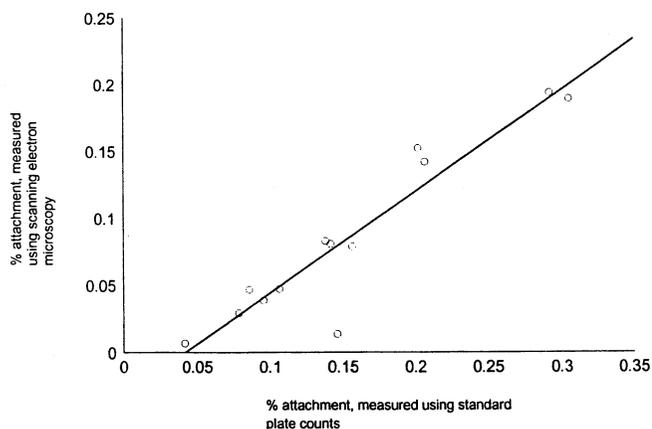


Figure 5C. Relationship between standard plate counts and counts made using scanning electron microscopy. ($SEM = 0.053 + 1.18 SPC$, $r^2 = 0.89$).

tissues, casings are sterile and were generally free from particulate material which could be mistakenly identified as bacteria in high resolution SEM images. Therefore, attachment of a pure culture could be studied without interference from indigenous flora or need to sterilize meat tissues.

No changes in the microtopography of sausage casings were observed after immersion in liquid media. Thomas and McMeekin (21), reported that with chicken muscle fascia, the microtopography was significantly altered by immersion in aqueous solutions, increasing the amount of water held at the surface. It was concluded that since bacteria are retained

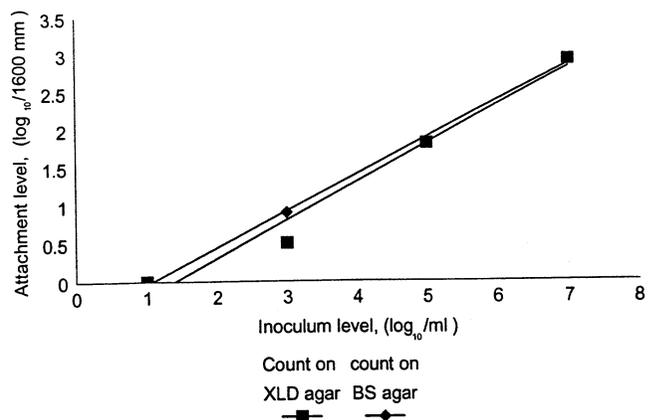


Figure 6. Effect of inoculum level on attachment of *S. typhimurium* to sausage casings.

within the liquid film, attachment levels are related to the amount of water held by the meat tissue. A film of water is associated with the sausage casings after their immersion in aqueous solutions which probably retains some bacteria, while other cells are localized on the collagen fibers.

The surfaces of sausage casings are more uniform than those of poultry and meat tissues, which reduces nonspecific entrapment of cells within fibers. Benedict et al. and Lillard observed that mechanical entrapment of cells occurs between muscle fibers or within the connective tissue matrix (1,11). As a result, trapped cells often could not be visualized easily using SEM. Entrapment of cells, therefore, reduces the accuracy of enumeration of attached bacteria using SEM, so sausage casings are more suitable for SEM studies than muscle tissue or chicken skin.

Cells attached to the sausage casings in a nonuniform pattern. Similar results were reported by Firstenberg-Eden et al. who observed that bacteria were not spread uniformly on the surface of cows' teats (4). Nonuniformity implies that bacterial attachment is not a random process, but rather, that cells are attaching to specific areas of the casings. This supports the theory that binding sites specific for microbial attachment are present on collagen fibers. As cells appeared in groups, it may be hypothesized that a number of cells are localizing close to a specific binding site or attractant. These findings, however, contrast with those of Piette and Idziak who observed that bacteria were distributed evenly on the surfaces of tendon slices and fat (16).

Using SEM, fibrils were observed on some bacterial cells. Fibrils were also observed by Firstenberg-Eden et al. on bacteria attached to cows' teats (4). These authors hypothesized that fibrils enhanced bacterial attachment to surfaces. Similar observations were noted by Schwach and Zottola and Zottola on bacteria attached to beef surfaces and stainless steel (18,22).

Numbers of *S. typhimurium* cells attached to sausage casings increased with time and incubation temperature, indicating that the attachment mechanisms were dependent on time and temperature. Thomas and McMeekin hypothesized that bacterial retention may be a function of the amount of water loosely bound by the connective tissue with increasing amounts of water being bound with time (21). Previous studies have shown that bacterial attachment increases with time (3,10,15,20), but the effect of temperature has not been

so well-defined. Fletcher observed that fewer cells of a marine pseudomonad attached to polystyrene at 3°C than at 20°C (5). Notermans and Kampelmacher observed that while attachment rate of *Escherichia coli* k12 to chicken skin depended on temperature, the optimum temperature was 20°C (15). Similar observations were made with *Yersinia enterocolitica* and *Listeria monocytogenes* attaching to stainless steel, where cells attached in greater numbers at 21°C than at 10 or 35°C (6,7). Conversely, Butler et al. observed little or no effect of temperature on attachment (2).

Attachment of *S. typhimurium* to sausage casings increased linearly with inoculum level. This is similar to findings by previous researchers (2,12,21). Our method to study the effect of inoculum level allowed very low numbers of cells to be detected. At higher inoculum levels, enumeration of bacteria proved difficult due to overgrowth of bacterial colonies. Although the piece of sausage casing was covered with colonies when a high inoculum was used, this does not necessarily mean that all the binding sites for individual bacteria were occupied.

Sausage casings are a restructured product; during processing, cattle hides undergo extensive chemical treatment to remove noncollagenous proteins and extraneous fatty tissue (8). Hair and grain layers are removed by treatment with strong alkalis causing disruption of collagen fiber bundles and denaturation of the collagen molecule. Further changes include hydrolysis of peptide bonds, hydrolysis of amides, hydrolysis of arginine, amino acid racemization, formation of double bonds, and formation of new amino acids. Since a significant amount of native fibrillar structure is retained and despite the above limitations, sausage casings appear to be a convenient model for studying bacterial attachment to meats. Studies on the specific mechanisms responsible for attachment of bacteria to sausage casings are continuing.

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