

## ATTACHMENT AND REMOVAL OF *SALMONELLA* SPP. ON MEAT AND POULTRY TISSUES

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### ABSTRACT

*Muscle pieces from beef, pork, and chicken were exposed to Salmonella strains in various aqueous solutions to determine mechanisms of microbial attachment and release. Binding was measured by scanning electron microscopy and by bacteriological methods. Bacteria appear to attach preferentially to connective tissue fibers, rather than to myofibrils. Muscle fiber swelling and shrinkage during processing permits some microbial entrapment between muscle bundles. Mannose and salt solutions were examined as potential inhibitors of attachment or as removal agents. Mannose inhibited attachment slightly and isotonic saline rinses removed some attached cells, but either method effected only about a one log reduction (90%). Application of 41 rinsings only effected a 4 log reduction. The apparent variety of attachment mechanisms by Salmonella hinders complete removal from meat tissues by simple rinsing procedures.*

### INTRODUCTION

*Salmonella* serovars are responsible for substantial illness in North America, being the bacterial foodborne disease estimated as having the greatest cost in the United States and the second greatest in Canada (Bean *et al.* 1990; Todd 1989a, 1989b). The cost associated with an estimated 2–4 million *Salmonella* infections in the United States each year has been calculated from \$1 to \$4 billion for lost worktime, medical care, and fatalities in humans alone (Todd 1989b; Roberts 1989), with costs from unsaleable food products, product recall, and lack of consumer confidence in food safety being additional.

Most foodborne salmonellosis outbreaks are associated with consumption of foods of animal origin, including meats (Archer and Young 1988; Bryan 1988). *Salmonella* spp. have been isolated from retail meat products (Lammerding *et al.* 1988; Dubbert 1988), and in some instances, *Salmonella* strains responsible for human outbreaks have been traced back to a particular infected herd or animal (Holmberg *et al.* 1984; Spika *et al.* 1987). In most instances, however, contamination of meat occurs during postmortem processing or handling, with transfer of pathogens from skin, feathers, hair, or intestinal contents to equipment and carcasses (Stolle 1987).

*Salmonella* spp. are difficult to remove from contaminated hides, feathers, muscle tissue, or post-slaughter equipment surfaces during processing. Even with use of detergents, high pressure water sprays with or without chlorine germicides, or pH alterations in the washes and rinse solutions, viable organisms remained on the meat or carcass surface (Butler *et al.* 1980; Chung *et al.* 1989; Dickson 1988; McHan *et al.* 1988; Okrend *et al.* 1986; Schackelford *et al.* 1988).

Various mechanisms have been proposed to explain these results, including postulating the specific binding of the organism to the meat surface thereby inhibiting easy removal (Beachey 1981; Butler *et al.* 1979; Firstenberg-Eden 1981). Although nonspecific attachment of bacteria may occur during postmortem processing from animal protein denaturation products, e.g., dried or clotted blood, most researchers believe that a specific binding involving carbohydrate-containing surface components is responsible for the adhesion process. These components (glycoproteins, glycolipids, collagen, lipoproteins, and polysaccharides) bind by a variety of physico-chemical and biochemical mechanisms. Use of the simple carbohydrate mannose has been shown to competitively inhibit binding by organisms that contain type 1 pili (Ofek *et al.* 1978). The isolation of other specific binding carbohydrate-containing components has not yet resulted in effective binding inhibitors or removal agents for use with foods.

In this report we examine by electron microscopy and bacteriological methods the binding and subsequent removal of *Salmonella* strains to determine the relative importance of various binding mechanisms. These data were used to assess the likelihood that potential blocking agents could impact significantly the reduction of the risk of food contamination.

## MATERIALS AND METHODS

### Meat Substrates

Beef, pork, and chicken meats were purchased from local retail markets and were boned manually. Skin, excess adipose tissue, and epimysial tissues were separated from the lean tissues and each tissue type was vacuum-packaged in-

dividually in plastic barrier bags in 1, 10, 50, and 100 g samples. Samples were frozen at  $-20^{\circ}\text{C}$ , irradiated (2 Mrad with  $\text{Cs}^{137}$  irradiation source), and stored at  $-20^{\circ}\text{C}$  until used.

### Salmonella Strains

*Salmonella typhimurium* TML R66 was the principal organism used, although other strains (*S. enteritidis* 9186 and *S. newport* 6902) were examined in these studies. These three are the principal species associated with meat- and poultry-borne salmonella outbreaks (Todd 1989a). Organisms were grown in tryptose phosphate broth ( $37^{\circ}\text{C}$  for 18 h) to a density of approximately  $10^8$  colony forming units/mL (cfu/mL), centrifuged and washed with sterile isotonic (0.15 M) saline solution to remove medium, and resuspended to desired levels in the various test media used to infect the meat surfaces.

### Binding Studies

The thawed, irradiated meat samples were edge-trimmed and cut aseptically with a Stady-Riggs tissue slicer to an approximate thickness of 1–2 mm. Samples were then cut aseptically to a size approximately  $1\text{ cm}^2$ , and weighed. These meat samples were incubated for a given time (usually 10 or 30 min) in 10 mL of the microbial suspension, removed, shaken to remove excess fluid, and rinsed with 10 mL of the test solution. The rinsed meat was either homogenized with a Virtis blender and microbial counts determined on the homogenate supernatant or removed to sterilized Buchner filters (11 cm diameter) containing sterilized Whatman #1 filter discs and rinsed repeatedly with 10 mL rinses on filters. For rinses, 10 mL were pipetted on the sample, swirled for 30–60 s, filtered under negative pressure, and collected aseptically. In some runs meat slices were removed and processed for electron microscopy. Following the last rinse the meat was homogenized for plating to determine final microbial counts. Rinse solutions were surface plated with a Spiral plater, Model D (Spiral Systems Instruments, Inc., Bethesda, MD) on tryptose phosphate agar. Plates were counted after incubation ( $32^{\circ}\text{C}$  for 18 h), and counts reported for area or weight. Suspension and rinse media were varied by alteration of the ionic strengths, salt concentrations, or presence of potential attachment inhibitors.

To measure the continued effect of extensive rinsing on muscle tissue, chunks ( $4 \times 4\text{ cm}$ ) of irradiated poultry breast muscle were inoculated with *Salmonella typhimurium* TMLR66 suspension. With the use of sterile cork borers, disks of surface area  $2.01\text{ cm}^2$  (in triplicate) were removed, sliced with a Stady-Riggs tissue slicer, the surface slice weighed and homogenized in 20 mL sterile saline, and plated. This procedure was performed at intervals immediately after inoculation, a brief saline rinse, and after 10, 30, and 60 min. During this period,

the tissues were being shaken (1000 rpm) in 300 mL of sterile saline, with changes of rinse solution every 10 min.

### **Electron Microscopy**

Scanning electron microscopy (SEM) and negative staining transmission microscopy were conducted on untreated and treated meat samples according to procedures developed in this laboratory (Jones *et al.* 1976) and elsewhere (Rowe 1984; Schwach and Zottola 1982). Samples were rinsed by dipping briefly in sterile saline, drained, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2. Samples for SEM were processed through critical point drying. Meat tissue surfaces were examined after staining with either sodium phosphotungstate or methylamine tungstate (1% aqueous solutions). Negative staining to demonstrate flagella or pili was done on samples applied to carbon coated copper grids using a Zeiss EM-10B TEM at 100 kV.

### **Chemicals**

Mannose was obtained from Sigma Chemicals, and bacteriological media were purchased from Difco. All other chemicals were reagent grade.

## **RESULTS AND DISCUSSION**

### **Attachment of Salmonella**

Chicken breast muscle was prepared by immersion of sliced irradiated muscle samples in *Salmonella* cultures in tryptose phosphate broth for 30 min. Poultry contain the highest incidence of *Salmonella* infection (up to 70% of market ready birds), but are a lesser vector than red meats in producing the disease in humans, since they are generally cooked to a higher final temperature (Benedict 1988). Analysis by scanning electron microscopy of surface-attached microorganisms required a minimum cfu/cm<sup>2</sup> of about  $5 \times 10^6$ , so inoculation solutions contained levels higher than would normally be present under commercial conditions. Scanning micrographs (Fig. 1) indicated that bacteria attached to the endomysial "reticulin" fibrils rather than to the muscle fibers. Although sample preparation may have selectively removed bacteria attached to muscle fibers, this process was not more severe than might be encountered during commercial processing of poultry carcasses. Endomysial reticulin fibrils are composed of type I collagen and stain with silver-containing reagents in light micrographs from reaction with their content of reducing carbohydrates (Swatland 1978). Although some spherical objects attached to the muscle fibers were noted in the micrograph, these for the most part are muscle mitochondria rather than microorganisms. Research by McMeekin and Thomas (McMeekin and Thomas 1978; McMeekin *et al.*

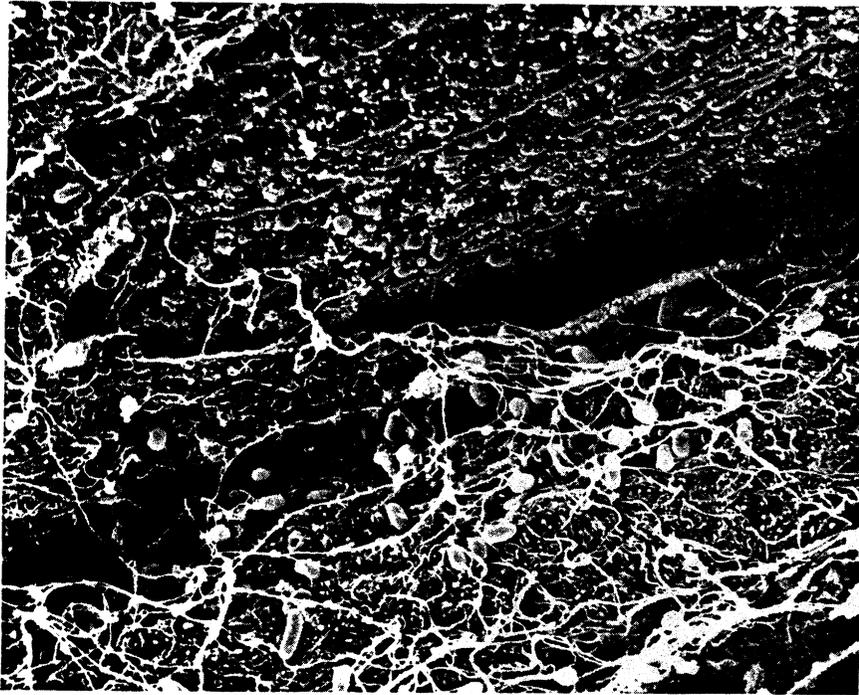


FIG. 1. SCANNING ELECTRON MICROSCOPY OF CHICKEN BREAST MUSCLE AFTER IMMERSION IN CULTURE OF *SALMONELLA TYPHIMURIUM* TML R66 FOR 30 MIN  
Magnification 7500 X.

1979, 1986; Thomas and McMeekin 1980, 1981a, 1981b, 1984) and by Lillard (1986b) using electron microscopy indicated that microbes approach the surface of chicken skin and muscle in a thin water layer following immersion in aqueous suspensions or from various post-slaughter cleaning regimens. With muscle surfaces hydrophilic electrostatic attraction or van der Waal's forces between the microbe and tissue surface after initial approaches may be involved (Gristina 1987), although both surfaces have net negative charges favoring repulsion. Microbial flagella and fimbriae (pili) are of limited effect in initial approach (Lillard 1986a), but may be involved in subsequent attachment (Finlay and Falkow 1988). For skin, hide, and adipose tissues, the surfaces allow hydrophobic attachment sites as well as clefts and crevices that would protect absorbed bacteria from removal by subsequent rinsings or washes.

In addition to this specific attachment to fibrils, some *Salmonella* appeared to be entrapped within clefts between muscle bundles and the latticework of collagen fibrils (Fig. 2). Such a mechanical entrapment of microbes can occur by flagellar

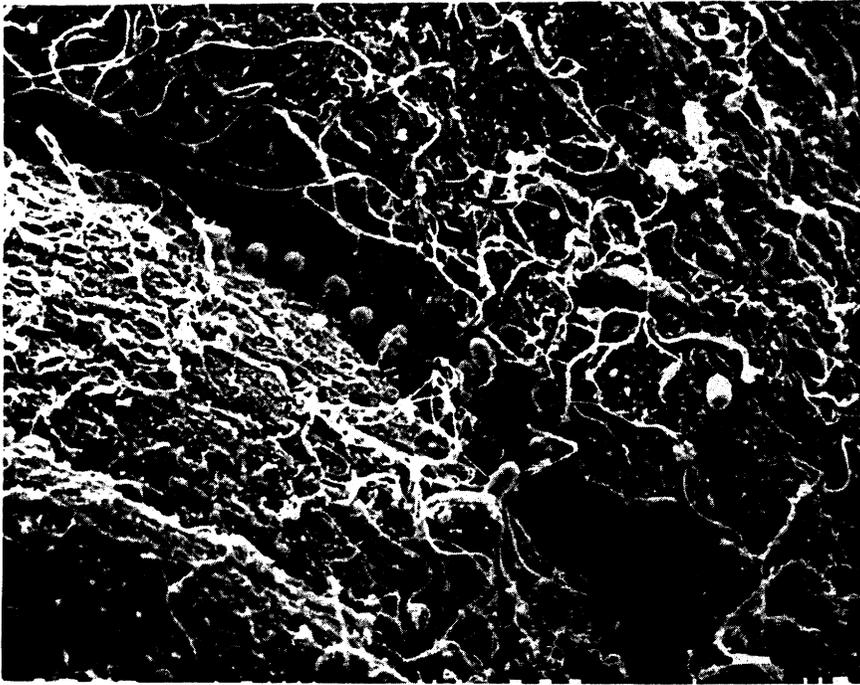


FIG. 2. SCANNING ELECTRON MICROSCOPY OF *SALMONELLA TYPHIMURIUM* TML R66 ENTRAPPED WITHIN CLEFT BETWEEN MUSCLE FIBRILS AND ATTACHED TO ENDOMYSIAL FIBRILS  
Magnification 10000 X.

movement of the organism and subsequent entanglement within clefts, but may also result during postmortem processing of carcasses. In defeathering and washing of poultry carcasses, birds are exposed to solutions of differing tonicity that can produce swelling and shrinkage of the tissues (Lillard 1988, 1989). In such baths any bacteria present can approach the hydrophobic membranes of the carcasses in the surface water film and be subsequently entrapped when the fibers change volume. Similar effects may occur with red meat carcasses exposed to high pressure water sprays that force bacteria between fibers. Gill *et al.* (1984) noted that changes in muscle structure occurring during rigor could allow invasion of bacteria into the tissues.

Carbohydrate components of glycoproteins and glycolipids have been implicated in binding mechanisms of bacteria to tissue surfaces. The addition of mannose to suspension solutions has been used to inhibit attachment of *Escherichia coli* and other organisms containing type 1 pili to various animal tissue

surfaces (Ofek *et al.* 1977; Sharon and Lis 1989; Finlay and Falkow 1988; Ernst *et al.* 1990). To determine if addition of mannose to suspension solutions would alter the attachment site of the bacteria, muscle samples were incubated for 30 min in the same immersion medium supplemented with 25 mg mannose/mL. The site of attachment of the organism to the endomysial fibrils in the mannose-containing medium appears similar to that in solutions without added mannose (Fig. 3), although an estimate of total attached organisms in micrographs from the two media indicated about 50–80% fewer cells on muscle surfaces in the mannose media. Lillard (1988) has discussed the pitfalls of estimating actual bacterial populations from electron micrographs, and these observations may not be significant. The additional 138 milliosmols contributed by mannose may also have had an osmotic effect. Employment of a negative staining technique to demonstrate flagella and pili of the organisms (micrographs not shown) showed peritricheous flagella in cells incubated in both mannose (+) and mannose (–)

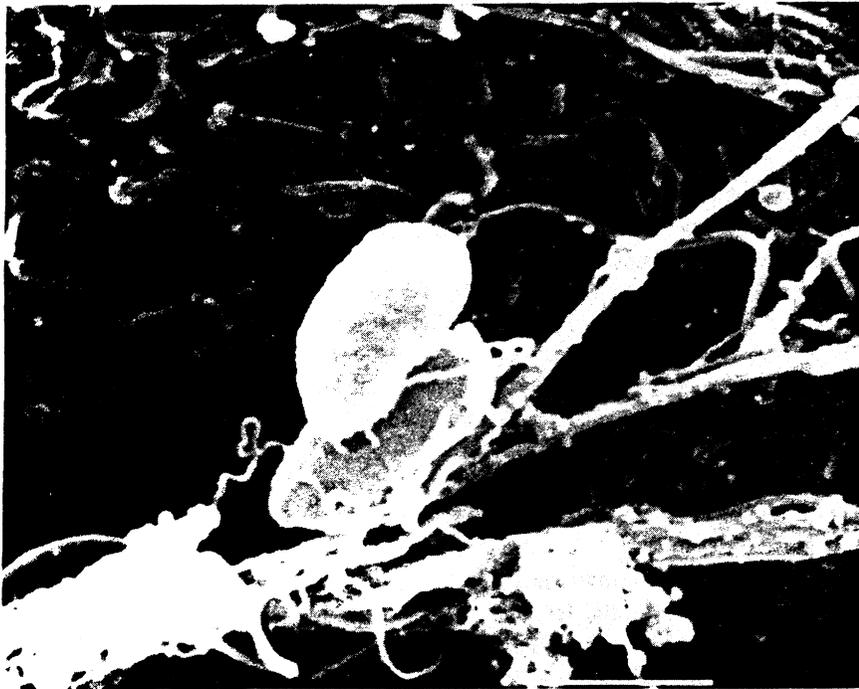


FIG. 3. SCANNING ELECTRON MICROSCOPY OF *SALMONELLA TYPHIMURIUM* TML R66 ATTACHED TO ENDOMYSIAL FIBRILS OF CHICKEN BREAST MUSCLE AFTER IMMERSION IN CULTURE CONTAINING 1% ADDED MANNOSE  
Magnification 25000 X.

suspension solutions. Although pili were present in the mannose (–) micrographs, their presence in the mannose (+) medium was occluded by an absorption of stain in the pili region for reasons unknown. Such an effect may possibly result from a reaction of absorbed mannose with EM fixatives, etc., during preparation and may not be relevant to attachment mechanisms.

### Removal of *Salmonella*

The mechanism of attachment of the bacteria to the surface becomes of importance when attempting removal (Benedict 1988). Once the microorganisms become firmly attached to the muscle or carcass surface through entrapment or specific binding mechanisms, they resist removal by normal processing methods. Vanderzant *et al.* (1986) studied the role of lean and fatty surfaces on the attachment of individual meat bacteria on red meat surfaces, and Dickson and co-workers (Chung *et al.* 1989; Dickson and Koomaraie 1989) have examined the role of cell surface change in initial attachment to meat surfaces. Although bacteria can attach by electrostatic bonds to the meat surfaces, such binding should be affected by pH or ionic strength alteration of the wash solutions. Studies in which the pH or content of sanitizer in scald or rinse tanks for poultry have indicated that such treatments are of very limited effect in reducing the level of attachment of *Salmonella* to poultry tissues (Humphrey *et al.* 1987; Morrison and Fleet 1985; Obafemi and Davies 1986). Disruption of hydrophobic attachment by use of detergent solutions have also proved ineffective (Dickson 1988; Lillard 1988). The effectiveness of the ionic strength of the rinsing solution in removal of adhering organisms from skin and muscle samples of chicken is shown in Fig. 4. The muscle tissue samples after a 30 min immersion in the microbial suspension were rinsed once with the various test solutions, and microbial counts determined on both rinse solutions and rinsed samples. Saline solutions in general removed about 0.6 log cycles more than distilled water. Although the graph indicates that isotonic saline (0.15 M) was the most effective concentration for removal, this only achieved a reduction in the total count on the muscle of approximately 1 log value (90%). Removal was slightly better with muscle tissue than with skin tissues.

With chicken breast muscle examined by the punch method with lower initial counts in the suspension medium, results showed that with an initial level of  $2.9 \pm 0.2 \times 10^4$  cfu/cm<sup>2</sup> of surface tissue (average of triplicate determinations), there were  $3.6 \pm 1.4 \times 10^4$  present after an initial saline rinse, and  $2.2 \pm 0.0 \times 10^4$  after 10 min rinsing,  $0.6 \pm 0.4 \times 10^4$  after 30 min, and  $0.4 \pm 0.2 \times 10^4$  after 60 min rinsing with shaking. These data indicate only about an 85–90% removal under these conditions. Similar experiments using saline with addition of 0.1% Tween 80 (a nonionic detergent) gave results almost identical, indicating that

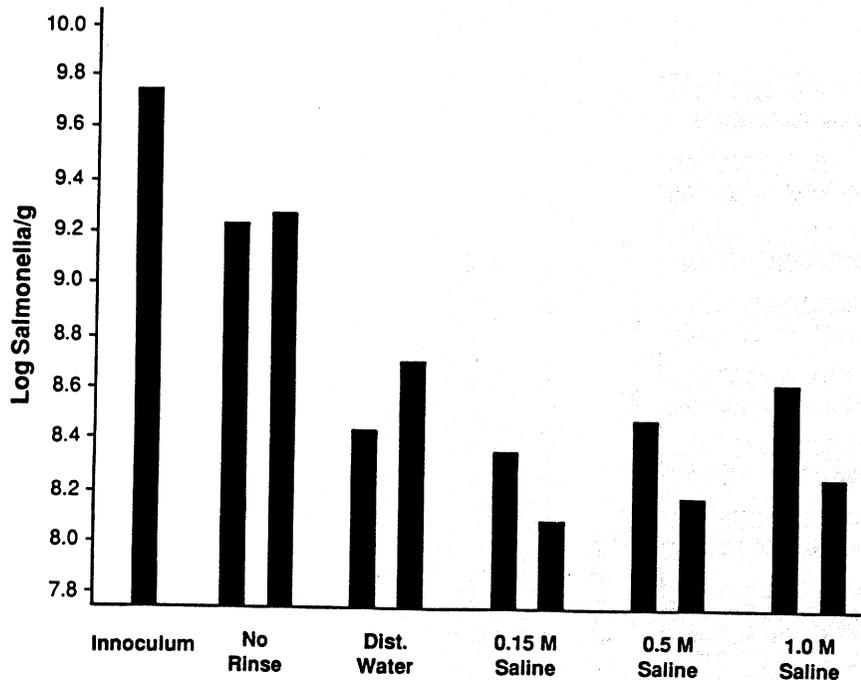


FIG. 4. PLOT OF EFFECT OF SALINE CONCENTRATION ON REMOVAL OF SALMONELLAE FROM CHICKEN SKIN AND MUSCLE  
The left bar in each rinse figure is for chicken skin; the right bar is for muscle free of skin.

hydrophobic and hydrophilic mechanisms of attachment may not be significant after initial binding.

Attachment and removal of *Salmonella* spp. to specific meat surfaces were examined using both beef muscle and chicken muscle samples. After suspension of the meat sample in the inoculum, samples were removed and rinsed as described. Total numbers of organisms removed in the rinses were calculated and shown for selected rinse numbers for beef and for chicken (Fig. 5). Summation of the counts from the various rinses indicated that most of the organisms (95–99% of total removed) were removed during the first 5 rinses with isotonic saline. Even after 41 rinses, however, a low level of bacteria were still present on the meat or skin surface, although at a  $10^5$  to  $10^6$  log cycle reduction. This asymptotic decline would indicate that complete removal of high levels of attached bacteria on the tissue surfaces would be doubtful with the use of present processing methods. The 41 rinses were performed within 180 min at  $15^\circ\text{C}$ , decreasing the probability of extensive growth during rinsing.

Because of restrictions both from microscopy and microbiological methods, relatively high salmonellae inoculum levels were employed in these experiments

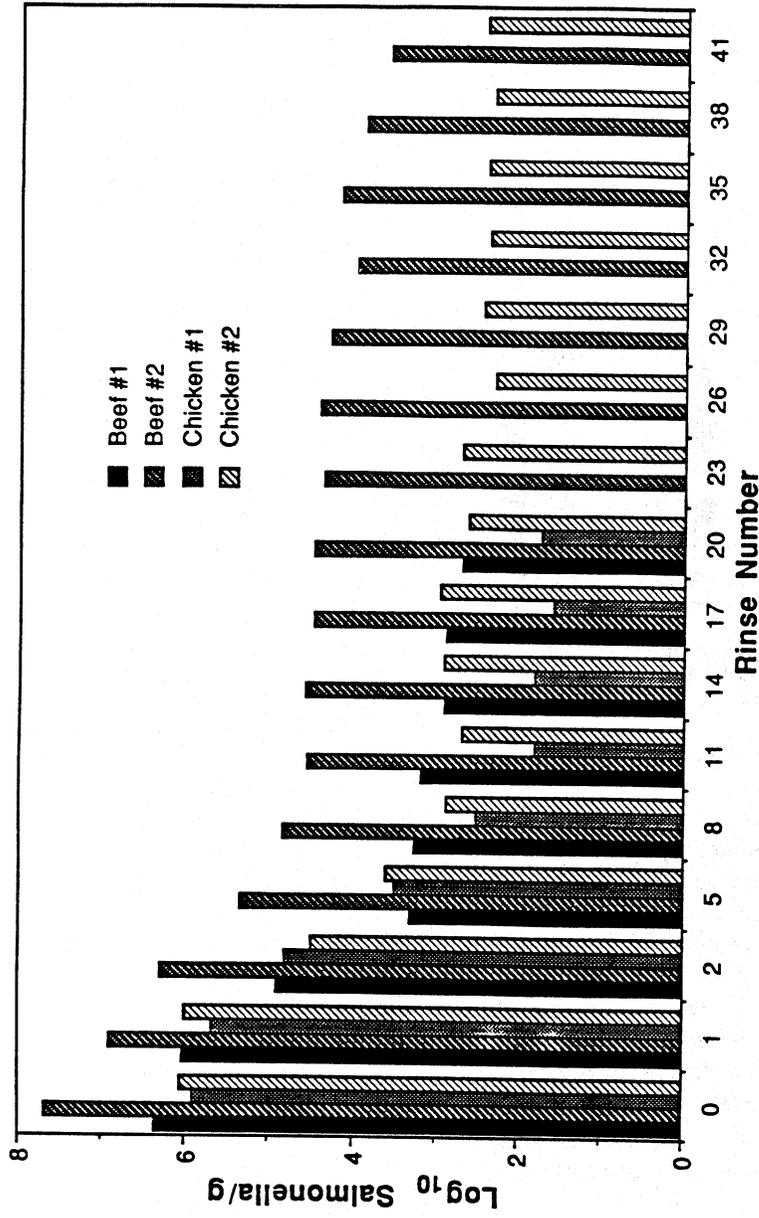


FIG. 5. PLOT OF REMOVAL OF BOUND SALMONELLAE WITH MULTIPLE RINSE ON BEEF AND POULTRY MUSCLE TISSUE SAMPLES  
 Ordinate values are log<sub>10</sub> of salmonellae present in the inoculum and selected rinses of the meat samples with isotonic saline. Values are means of duplicate determinations. The four samples are: beef rinsed 20 times, beef rinsed 41 times; poultry rinsed 20 times, and poultry rinsed 41 times.

for satisfactory micrographs or colony counts, levels that normally would not be present in commercial meat processing. When much lower initial inoculum levels were used, statistically invalid attachment levels were noted that suggested a greater degree of attachment with lower inoculum levels. However, this is probably an artifact of the procedural requirement. Lillard (1988) has indicated that accurate estimates of attached cells cannot be obtained from electron micrographs.

The difficulty of removal or inhibition of attachment of the bacteria by a single method implies that attachment is multifactorial, probably involving hydrophilic and hydrophobic mechanisms, entrapment, and specific binding sites in various combinations. The attachment of the microbes to collagen fibrils might result from a specific lectin linkage between the carbohydrates of the endomysial collagen glycoprotein and the microbial surface. Although the consultant carbohydrates in collagen may be involved, recent research has indicated the possible involvement of fibronectin as an intermediate attachment molecule between the microbe and collagen surfaces (Baloda 1988; Baloda *et al.* 1988; Vercelloti *et al.* 1985). We are presently examining the relative contributions of both fibronectin and the collagen carbohydrates in attachment of *Salmonella* spp. to endomysial collagen fibers.

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