Microbial Attachment to Meat Surfaces

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The attachment of microorganisms to various surfaces and the mechanisms of attachment have become of great interest to the food industry and to researchers during the past decade. Few things have a closer relationship to man than the many bacteria, yeasts and molds that we constantly contact in our food sources and environment. A large group of these organisms is considered as beneficial; they are used in food processing to produce fermented meat and dairy products, and breads, as well as alcohols and various pharmaceuticals. A second group contains organisms which infect certain plants or animals to produce disease and impact negatively on humans in reducing available food supplies. Many of these, however, are not transmitted through the food supply to produce infections in humans and, therefore, are regarded as relatively innocuous as a microbial food safety problem.

The third group, however, contains organisms which: (1) attach to food surfaces and produce *spoilage of the food products* following slaughter or harvesting and subsequent processing, or (2) are carried or transmitted through plant or animal food products to produce *illnesses in humans* following consumption of the contaminated product. Both of these sub-groups are important economically in that they result in loss of food products, loss of work time, and incurrment of medical expenses, as well as loss of nutritional substances. It is estimated that foodborne disease caused by *Salmonella* spp., *Campylobacter* spp., *Yersinia*, and pathogenic strains of *Escherichia coli* alone may account for 8 to 10 million cases of enteric diseases each year, and up to $2 billion from medical costs and lost productivity (Kvenberg and Archer, 1987).

In addition, the extensive media coverage of outbreaks of contaminated foods and resultant recalls, (recently with *Listeria monocytogenes* in dairy products and with *Salmonella spp* in meats), markedly affects the buying habits of the consumer and results in a subsequent decreased market for those products. Consequently, the attachment of pathogenic and spoilage organisms to meat and meat products is a prime concern of the U.S. Department of Agriculture and is being examined by our laboratory and by others with the eventual goal of developing methods to reduce such binding, thereby reducing foodborne disease, and improving food production and quality.

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Meatborne Organisms

Meats and meat products have a wide variety of organisms which may be isolated through various techniques, and which can vary with processing and storage conditions. The occurrence and importance of the microbial contamination also varies with the location and the types of processing conditions. In the United States, for example, work at the University of Kentucky (Newsome et al., 1984; Newsome et al., 1987) showed that the populations of organisms in six groups varied with processing of hot or cold boning, restructuring, vacuum packaging, and time of storage (Table 1). These data were combined from different experiments to show variation in organisms in the different isolation classes, and are reported as log values of the colony forming units/g meat product. Three of the groups (aerobic, anaerobic, and psychrotrophic) measure the effect of storage atmospheres and temperatures on the total microbial count, including both harmful and innocuous organisms. The other three groups (Enteric, *Staphylococcus*, and *Lactobacillus*) measure the content and growth of organisms that can produce sickness or spoilage.

The enteric group contains those organisms normally found within the gut of the animal, such as *E. coli, Enterobacter* spp, and *Salmonella* spp, and indicates fecal contamination during some stage of processing.

Staphylococcus aurerus is an organism that produces severe gastrointestinal food poisoning through production of several toxins, one of which survives boiling and cooking, although the organism itself may be completely destroyed by such treatments. This organism is also able to grow in high salt environments and is frequently associated with food poisonings from ingestion of high salt foods, such as hams. It is responsible for about 1/3 of the classified food poisoning cases in the United States during the past 20 years.

The genus *Lactobacillus* contains organisms that produce lactic acid as a metabolic byproduct and reduce the pH of the food. Several species are used for production of fermented meat (*Lactobacillus plantarum*) and dairy yogurt (*Lactobacillus bulgaricus*) products. Naturally occurring *Lactobacilli* can tolerate anaerobic conditions, and four strains (*L. divergens, L. carnis, L. sake,* and *L. curvatus*) are usually isolated from vacuum-packaged fresh meat products after long-term refrigerated storage (Schillinger and Lucke, 1987).

The collected data in Table 1 indicate that the restructuring process appears to increase the populations of all six groups when compared to the control samples (unrestructured). Hot boning samples compared vs cold boned samples showed small but significant increases in four of the groups. Refrigerated storage samples under anaerobic conditions (barrier bags) for 1 week showed little differences from the non-restructured control (HB or CB, 0 time). The

Table 1. Microbiological Quality of Raw Steaks from Various Treatments.[a]

	Aerobic Atmospheres				Barrier Bags		
Organisms Measured	*Control Meat*	*Restruct Meat*	*HB-0 Time*[b]	*CB-0- Time*	*HB-1 wk Storage*	*CB-1 wk Storage*	*CB-6 wk- Storage*
Staphylococcus	1.36[c]	3.30	2.92	2.22	2.52	2.19	2.07
Lactobacillus	1.98	3.79	1.57	0.90	1.51	1.25	5.68
Enteric	0.52	2.45	0.79	0.87	1.46	1.13	2.04
Psychrotrophs	3.90	5.31[d]	3.33	3.15	4.24	3.10	6.53
Aerobes	3.30	5.01	3.32	2.75	3.08	2.89	6.39
Anaerobes	3.27	4.68	3.12	2.60	3.12	2.66	6.44

a. Source: Newsome et al., J. Food Sci. 1987.
b. CB = Cold boned, HB = Hot boned.
c. Values are $\log_{10}$ CFU/g of meat product.
d. Underlined values indicate incipient spoilage.

vacuum-packaged cold boned product after 6 weeks had large increases in the psychrotrophs, anaerobes, and Lactobacilli, as might be expected, and also in the aerobes. An aerobic plate count with a log value of 5 or above indicates incipient spoilage of the meat. The authors have also reported that *Enterobacteriaceae* also occur in greater numbers in vacuum-packaged fresh meats than in conventionally aged meats.

Although meats can contain large populations of spoilage and non-spoilage microorganisms before showing visible changes in color or odor (Benedict et al., 1975), the pathogenic bacteria are important to human health, even if present in very low numbers. Bentley et al. (1987) found that whole pork sausage during refrigerated storage for four and eight weeks developed total bacterial counts of 10^9/g, with *Pseudomonas* species being the most prevalent. Although the psychrotropic pseudomonads have been responsible for decreased shelf life in meat products, certain species such as *Pseudomonas aeruginosa* can produce illnesses in humans (Krieg & Holt, 1984). Surveys in Canada (Lammerding et al., 1988) on the prevalence of *Salmonella* and thermophilic *Campylobacter* in red meats and poultry in that country during the years 1983 to 1986 indicated that *Salmonella* spp were isolated from 17.5% of the pork, 4.1% of the veal, 2.6% of the beef, 69.1% of the turkey, and 60.9% of the poultry carcasses, with *Salmonella typhimurium* being the most isolated serotype. With thermophilic *Campylobacter*, the incidence was 43.1% in veal, 22.6% in beef, 16.9% in pork, 73.7% in turkeys, and 38.2% in poultry, with *Campylobacter jejuni* being the most frequently isolated.

In Sweden, however, market surveys on the incidence of potential pathogens on raw pork, beef and chicken were conducted by Ternstrom and Molin (1987), and no *Salmonella* spp., *Listeria monocytogenes*, or *P. aeruginosa* were detected on the meat sampled. The lack for *Salmonella* apparently results from the extensive program on eradication of *Salmonella* from the meat supply that Sweden has been conducting since the 1960's. However, *Aeromonas hydrophila, E. coli, S. aureus*, hemolytic streptococci, *Yersinia enterocolitica, Bacillus cereus, Clostridium perfringens*, and *Erysipelothrix rhusiopathiae* were all detected. This last organism is the causative agent of swine erysipelas and can infect meat handlers. Of particular interest to the meat industry is the observation that many of these organisms remain viable and virulent under refrigerated storage (Palumbo, 1986).

Transfer of Pathogens to Meat

Except for extremely rare instances of slaughter and consumption of infected animals, the pathogenic and spoilage organisms are transferred to the meat during post-slaughter processing, storage and handling. Many pathogens that may be present in the intestinal contents of the animals during slaughter can contaminate the carcass and subsequently the processing tables and equipment. *S. typhimurium* was shown to be transferred from raw poultry skin to other surfaces (Carson et al., 1987). Attempts to reduce this contamination by the use of acetic acid in the scald water were ineffective (Lillard et al., 1987). *S. aureus*, a component of human nasopharyngeal secretions and abcesses, can be transferred by human contact with the meat during processing or by other fomites. *S. aureus* also adheres strongly to cotton and polyester fibers of work clothes (Hsieh and Merry, 1986), and to poultry defeathering machinery, where it becomes unusually resistant to chlorine sterilization (Bolton et al., 1988). *S. typhimurium* may also survive in the water or water/glycol mixtures used for cooling and refrigeration (Zottola and Smith, 1986). The procedures for equipment cleanup can also alter the mechanism of attachment of the organism. *Pseudomonas fragi* on stainless steel, after suboptimum cleaning and sanitizing, produced attachment fibrils within 24 hr at 21°C. (Stone and Zottola, 1985), whereas various cations affected the adherence of *Pseudomonas fluorescens* to glass (Fletcher, 1988). Furthermore, the circulating air and wash waters within the meat processing plant can carry in various organisms from outside the facility, including those not indigenous to the animal.

Microbial Adhesion

Bacteria can be separated through staining techniques into two classes: Gram Positive and Gram Negative organisms. This distinction is based on distinct cell surface differences, and allows a broad base for classification. With the meatborne organisms of interest, the Gram Positive organ-

isms are various species of *Staphylococcus, Clostridium, Listeria, Streptococcus, Lactobacillus* and *Bacillus*. Many of these organisms cause illness through the production of toxins that produce cramps, vomiting, diarrhea and other adverse effects. The remainder are included in the Gram Negative group, with many belonging to *Enterobacteriaceae* family. The latter includes various species of *Salmonella, Yersinia, Shigella, Proteus, Serratia*, and *E. coli*, which are responsible for food poisoning outbreaks and nosocomial infections. Other meatborne Gram Negative organisms of note are *Aeromonas, Pseudomonas, Campylobacter,* and *Erysipelothrix*. The Gram Negative organisms in human food poisonings often produce their effect by adhering to the intestinal mucosa followed by colonization, *in situ* production of toxins, and/or cellular or tissue invasion.

Both Gram Positive and Gram Negative organisms contain the normal cellular contents bounded by a lipid bilayer cytoplasmic membrane with protein and lipoprotein inserts and a surrounding covering of peptidoglycan (also known as murein). The Gram Positive organisms additionally contain teichoic acid which links together the peptidoglycans to form an interwoven network. This is covered by an additional surface layer. The Gram Negative cell envelope is more extensively developed. The peptidoglycan layer is surrounded by a periplasmic space and an extremely asymmetric lipid bilayer, containing lipopolysaccharides (LPS) and (in the family *Enterobacteriaceae*) the enterobacterial common antigen (ECA). LPS consists of 3 parts: lipid A, the core, and the 0 antigens, which are responsible for many of the bacteria's virulent effects. An outer membrane (also continuous in places with the asymmetric lipid bilayer) contains pores to allow transport of selected nutrients, and has openings for flagella and pili (attachment molecules). Protective capsular material and the adherent glycocalyx arise from the LPS layers. A more extensive treatment is not warranted here; for additional information, the reader may consult Savage and Fletcher (1985) or most recent microbiology textbooks.

In recent years, the importance of the glycocalyx in microbial adherence to tissue or inanimate objects has become recognized (Costerton et al., 1978, 1981). The glycocalyx is a highly hydrated polysaccharide or lipopolysaccharide structure external to the outer membrane of gram negative cells and the peptidoglycan of gram positive cells, which acts to protect the organism from being swept away by fluids in streams or animal digestive tracts. Animal tissues may also be considered to have glycocalyxes, also of polysaccharides or glycoproteins. Because this structure is so highly hydrated, it collapses onto the organism during the dehydration step for preparation of scanning electron microscopy and its normal structure is not seen. With transmission electron microscopy, special techniques of fixation using antibodies or polyanionic dyes, e.g., ruthenium red or alcian blue, can be employed during the stages of preparation to indicate the structure of the surrounding glycocalyx biofilm. The organisms and tissues, because of these films, actually exist in a protected environment away from harmful factors such as phagocytosis or antibiotics. Much of the research on the adherence of microorganisms to various surfaces has used isolated defined cultures which do not produce the glycocalyx under normal laboratory conditions (Smith, 1977; Costerton, 1978, Cheung and Fischetti, 1988). Consequently, the work with in vitro pure cultures may not be applicable to in vivo conditions of microbial adherence, but can be used both to illustrate the nature of the specific binding sites and indicate the mechanisms for adhesion.

Duguid and Gillies (1957) noted that the *E. coli* agglutination of red blood cells could be inhibited by addition of mannose or mannosides. Ofek et al. (1977) found that the attachment of *E. coli* to epithelial cells was mediated by mannose or mannose-like receptors on the surface of the epithelial cells. This observation allowed the division of microbes that adhere to cells into two groups: those containing pili that are sensitive to mannose inhibition (MS) (or Type 1 pili), and those that are resistant to mannose or mannoside inhibition (MR). Mannose is a terminal component of many glycoproteins and is a presumptive terminal signal carbohydrate for cellular recognition and clearance from the plasma by macrophages or cells of the reticuloepithelial system (Stahl and Schlesinger, 1980). Although up to 90% of the attachment of *E. coli* cells to epithelial cells was prevented by the addition of 0.25M mannoside to the suspension medium, it should be noted that this is only a one log value decrease, an insignificant decrease in many bacterial contaminations.

Following that observation, the mechanisms for attachment and its relevance to disease have been studied from many aspects. Many excellent reviews are available (Ofek & Beachey, 1980; Costerton et al., 1981; Rauvala, 1983; Lewin, 1984; Savage & Fletcher, 1985; Sharon, 1985, 1987; Gristina, 1987, Ofek and Sharon, 1988) but the mechanism is far from being known. Adhesion depends upon characteristics of the bacterium, the substrate surface to be colonized, and the surrounding fluid. The initial approach of the organism to the binding site may be by flagella in motile organisms or through fluid flow or external propulsion in motile and nonmotile organisms. Although both surfaces possess a net negative surface charge, favoring repulsion, variations in the isoelectric points through localized changes in the microenvironmental pH may allow the two surfaces to approach within the minimum van der Waals forces to permit attraction.

One role of salt in meat curing may be to interfere with this initial ionic bonding by swamping out the charges. Additionally, salts may interfere with the subsequent hydrophobic bonding between the surfaces. The close proximity of the two surfaces allows contact and adhesion, either through the extracellular polymeric films that may be present, or through the production of specific adhesins (microbial glues). Cell to cell attachment is involved in the development of the animal during morphogenesis and in subsequent communication between the cells in hormone and antibody actions. These reactions, as well as attachments to the cells by viruses, enzymes, toxins, lectins, bacteria, and desialyated glycoproteins, all appear to involve cell surface carbohydrates.

The mechanisms of binding and adhesion have been examined from the thermodynamic view (Absalom, 1983); from hydrophobicity and electrostatic attraction (Hermannsson et al., 1982; Miorner et al., 1982; Gristina, 1987); from the microbial surface components and microbial lectins and carbohydrates (Smith, 1977; Sharon, 1987); from the tissue surface carbohydrates (Rauvala, 1983; Fiezi and Childs, 1985); and from special binding substances. Staphylococci have a specific binding site on the fibronectin molecule (a connective tissue component and a plasma protein)

that also binds to collagen molecules (Vartio and Vaheri, 1983). Group B Streptococci, the cause of neonatal meningitis, appear to use the lipid portion of their lipoteichoic acid for adherence to epithelial cells (Teti et al., 1987). *C. jejuni*, a meatborne pathogen, appears to bind specifically to intestinal mucus components before it colonizes the mucus (Lee et al., 1986). Use of HeLa cells for studies of invasiveness by *S. typhirmurium* (Gianella et al., 1973) has shown that a mannose resistant adhesion is necessary for internalization of the organism by the HeLa cells (Jones and Richardson, 1981). Use of these techniques to develop blocking methods of microbial adherence has been attempted.

In addition to the antibodies against pili, various receptor analogues, lipoteichoic acid antibodies and sublethal levels of certain antibiotics have been used (Ofek and Beachey, 1980). More recent studies have examined the use of cholera antitoxin for neutralizing *Salmonella* enterotoxin (Jiwa and Mansson, 1983); oligomannoside-type glycopeptides from plant glycoproteins to inhibit type 1 pili adhesion (Neeser et al., 1986); and the application of taurolidine, a non-antibiotic agent derived from taurine, that reduces the adherence of certain bacteria to epithelial cells by changing the microbial surfaces (Gorman et al., 1987).

The adhesiveness of biopolymers can be quite impressive, such as the protein produced by the marine mussel, *Mytilus edulis*, which consists of a repeating decapeptide (Waite and Tanzer, 1981; Benedict and Waite, 1986). This segment, Ala-Lys-Pro-Ser-Tyr-Hyp-Hyp-Thr-DOPA-Lys, because of the large content of – OH and amino groups and the uncoiling effect of the proline rings on the secondary structure, effectively has both a hydrophobic and a hydrophilic side. This protein can even attach to the very hydrophobic substance Teflon. With bacterial adhesins, however, this adhesion initially results from carbohydrates in the form of glycoproteins or glycolipids. A report by Darfeuille-Michaud et al. (1986), however, has identified a nonfimbrial adhesive factor in an enterotoxigenic *E. coli* strain as a protein.

Binding Studies with Meat Surfaces

When compared to the studies of microbial binding to oral and mucosal tissues or to prosthetic devices (Savage and Fletcher, 1985), research conducted on the attachment of bacteria to meat surfaces is slight. The attachment to the meat surfaces has been examined in model systems in which the meat sample is placed in a bath containing the organisms of interest for a given time, following which the degree of attachment was measured by different methods. These studies have not been without controversy. Early studies showed that the higher the initial population in the bath medium, the greater was the attachment. The relationship of the extent of attachment with the length of time in the bath, with the pH or temperature of the bath, and with the motility of the test organism is less clear, various researchers having reported differing values. Farber and Idziak (1984) reported on the attachment of psychrotrophic meat spoilage organisms to *Longissimus dorsi* muscle by the bath method, using a method devised by Ruth Firstenberg-Eden (Firstenberg-Eden et al., 1978) to express the strength of attachment. In that paper, it was noted that chicken breast with fascia attached was the best surface for bacterial attachments. In this method, the meat samples were cut into pieces to selected sizes and were irradiated to produce sterile samples. After being placed in the bath medium containing the test organisms at a concentration of about 1 million/ml for 20 minutes at room temperature, the samples were removed and tested in duplicate by one of three methods.

Method (A) to determine loosely attached bacteria consisted of shaking the meat sample for 1 minute in 100 mls of peptone water for 200 shakes. Method (B) to determine the total count of loosely and strongly attached cells involved blending the cells in peptone water for 1 min. Since these two methods also carried over bacteria contained in the surface attached fluid, method (C) used 2 pre-rinses in peptone water each for 30 seconds before blending the rinsed sample. Aerobic plate counts were used to determine the number of organisms absorbed to a measured area of meat surface under the above conditions. The value for "S" (for attachment strength) was equal to "log C – log [A-(B-C)]".

The authors used seven isolated meat spoilage organisms – *P. fluorescens, Pseudomonas putida, Brochothrix thermospacta, Enterobacter agglomerans, Moraxella osloensis*, a non-fluorescent pseudomonad, and an *Acinetobacter* species. Their results indicated that *P. fluorescens*, which also is a plant pathogen, and *B, thermosphacta* were the most firmly attached, with the non-fluorescent pseudomonad the least attached. The authors commented that the strength of attachment is important in possible airborne contamination in the slaughterhouse during spraying and handling. The mechanisms for attachment of the psychrotrophic spoilage organisms in the meat studies were not examined, but the presence of mucoid material produced by some of the organisms on the agar plates was noted as possibly being of importance in attachment.

An alternative method was used by Butler and coworkers (Butler et al., 1979) in that pork skin or beef and lamb carcasses were immersed in the test solution for given times, after which the surfaces were removed and embedded in solidified wax that was subsequently used in colony determinations. The Gram Negative motile organisms showed a greater attachment to the surfaces than the Gram Positive non-mobile species, and the extent of attachment appeared not to be affected by temperature or pH.

Recent studies have concentrated on adhesion to poultry skin, because of the incidence of *Salmonella* noted on such products. Lillard (1985, 1986a; 1986b) reported that the organisms increased binding with time; this binding appeared independent of the presence of flagella, fimbriae or water uptake. Water, however, did appear to be involved in that an initial surface layer of water on the poultry skin during immersion acts to bring the organisms to the tissue.

Alternative studies have focused on preventing the adherence of *Salmonellae* to the chick gut (Soerjadi et al., 1982) by techniques of competitive exclusion, such as the "Nurmi" principle (Stavric, 1987, Savage, 1987). A recent method (Valentin-Weigand et al., 1987) may allow the in vivo quantitative determination of bacterial binding to human or animal epithelial cells by fluorescein labeling of the bacteria and the density gradient centrifugation separation of non-adhering cells from the cellular bound bacteria.

In recent studies in our laboratory, cultures of *S. typhirmurium* in contact with fresh meat surfaces showed by

use of scanning electron microscopy a selective binding to collagen fibers, particularly the "reticulin" type, and essentially no binding to the myofibrils. *Salmonella* allegedly have type 1 pili or mannose binding sites; the principal carbohydrate residues of collagen are: Glucose-galactose and Galactose (Tang & Williams, 1984). These micrographs may be artifactual, Campbell and coworkers (Campbell et al., 1987) and Lillard (1988) have reported that binding of laboratory cultures of selected Gram Negative pathogens to chicken muscle connective tissue is quite sensitive to ionic strength and that organisms which apparently bound in distilled water could be removed by rinses with saline media.

Future Studies

The study of microbial adherence to meat surfaces has only begun. Many questions still need to be answered: (1) Do pathogens bind more strongly than non-pathogens? (2) What are the various stages of adhesion in the various pathogens and spoilage organisms? (3) Are there key stages in the initial attachment that may be blocked? (4) Can organisms be removed from the tissues by specific enzymes or chemical treatments to dissolve the glue? (5) Do the same binding sites for these intestinal pathogens exist on animal tissues, such as meat, as in the human intestine? (6) What are the relative contributions of the glycocalyx and the adhesins in the in-vivo meat system? (7) Which of these mechanisms are chromosomally regulated, and which are regulated by plasmid or other extrachromosomal methods?

Some relevant comments: *Listeria* and *Salmonella* are both intracellular pathogens and appear to bind to the Peyer's patches of the intestine. The eventual effects, however, differ. Among the Enterobacteriaceae, *Yersinia, Salmonella, Shigella*, and enteroinvasive *E. coli* have all been shown to invade human epithelial cells. Although these organisms cause similar diseases, they appear to interact with host cells in different ways. *Salmonella* and *Yersinia* are thought to invade intestinal epithelial cells to gain access to the reticuloepithelial system, where they multiply and from which they may disseminate throughout the host. In contrast, *Shigella* and enteroinvasive *E. coli* usually remain in the colonic epithelial layer where they invade and multiply locally. *Yersinia* carries a high MW plasmid (40-48 MD) that is required for virulence in animal models.

Certain animal pathogens may have an advantage in being carried through the food processing system by being bound more tightly to the animal tissues than the non-pathogens. If meat binding sites can be identified, methods may be developed to block their attachment during processing and prevent food poisonings. Interestingly, Madden et al. (1986) have hypothesized the opposite, i.e., that pathogenic microbes attached tightly to meat membranous tissues should pass through the human gastrointestinal tract and not adhere to host receptors.

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Discussion

B. Killday: On the adding of salt – the fact that the salt might inhibit the binding due to electrostatic interaction – how would one separate the inhibitory factors between the electrostatic interactions or is it just the matter of lowering the water activity?

R. Benedict: I think that some of the work can be done with the equipment that's used for measuring soil particles. Anybody who wants to can do this work. The equipment is available. The soil precipitates in a profile sedimentation. It's a whole lot of work to measure the effect of various pH and ionic strengths on the binding of particles. This of course becomes important with clay type or mixed soil types where you have particles that repel particles that attract. Essentially, what you have is a microscope that focuses on the things, and going back to the old beta potential, you change the beta potential and get some of the bindings. That would be one method of measuring under different ionic strengths, different pH's and trying to extrapolate which factors are involved there. That's one possibility.

Killday: Can you separate the factors?

Benedict: As you point out, no factor is separate. Every factor that changes in pH as you increase in ionic strength might change some of the pH effects or cation effects, and of course, even kill the organism.