SANITIZER EFFICACY AGAINST BACTERIA ATTACHED TO SYNTHETIC MEAT PROCESSING SURFACES

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A dissertation submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Food Science and Technology

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June 7, 1993

Blacksburg, Virginia

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ABSTRACT

The effectiveness of selected sanitizers against bacteria attached to synthetic meat processing surfaces was determined. In initial experiments, *in vitro* suspensions of Shw. putrefaciens, P. fragi, S. typhimurium, and L. monocytogenes were challenged with sanitizers according to the AOAC Germicidal and Detergent Sanitizer Test (GDST). Germicides employed were chlorine (200 ppm), iodophor (25 ppm), quaternary ammonium compound and phosphoric acid (200 ppm), and peracetic acid (185 ppm). In subsequent studies, the same sanitizers were tested against bacteria attached to polyvinyl chloride, polyurethane, and high density polyethylene surfaces. Test surfaces in sterile poultry slurries were inoculated with bacterial cultures, agitated (100 rpm) for 2 hr at 18°C, and then incubated for 22-28 hr at 26°C (Shw. putrefaciens and P. fragi) or 37°C (S. typhimurium and L. monocytogenes). Scanning electron microscopy (SEM) was employed to determine microbial-surface interactions. Attached organisms remaining on surfaces after vortexing were challenged with germicides. In some cases, surfaces were treated with detergents before applying sanitizers. Impedance microbiology was used to estimate surviving bacterial populations remaining after chemical treatments. All test sanitizers reduced levels of suspended bacterial cultures >5 logs after 30 sec, and thus, were deemed acceptable according to GDST guidelines. From SEM

micrographs, the 22-28 hr surface biofilms of Shw. putrefaciens, S. typhimurium, and L. monocytogenes could be characterized as single adherent cells or cell monolayers. Conversely, the P. fragi biofilm occurred as cell aggregates or microcolonies. Although sanitizers were effective according to GDST results, in many instances, attachment to surfaces increased bacterial resistance to germicidal agents (a 5 log reduction was not observed even after 1 min of chemical exposure). Peracetic acid, overall, was the most effective sanitizer in reducing levels of attached bacteria. Where resistance to other germicides could be observed for up to 20 min, peracetic acid typically eliminated biofilm populations after 1 min. Treatment of surfaces with detergents and then sanitizers led to more effective reductions of attached bacteria. The extensive fibril production by attached S. typhimurium (versus other attached organisms in this research) may explain the greater overall sanitizer resistance of this organism as compared to other test bacteria. However, higher initial numbers of surface bacteria (time 0) may have been the reason for greater sanitizer resistance (higher survivor levels) in some experiments. It is hoped that results of this study can aid processors in developing sanitizer schemes to minimize processing surface contamination with organisms of quality and safety concern.

ACKNOWLEDGEMENTS

I sincerely appreciate the time, advice, and encouragement given by Committee Chairman, Dr. John A. Marcy, during the course of my research and dissertation preparation. Gratitude is also extended to Dr. J. Russell Bishop, Dr. James R. Claus, Dr. R. Michael Hulet, Dr. Janet M. Johnson, and Dr. Merle D. Pierson for their attention and helpful suggestions.

I would like to acknowledge Dr. Rob Byrne, Ms. Tracy Mosteller, and Ms. Pat Rule for their help with impedance assays. Special thanks is given to Kyle Thornham for sharing his expertise in scanning electron microscopy. I also appreciate the valuable technical assistance of Mr. Walter Hartman and Mr. John Chandler. Thanks is due Dr. Paul P. Graham for reading portions of this manuscript and offering helpful advice.

Much appreciation is owed to the Virginia Poultry Processing/Products Research Group for funding the research described in this manuscript, and to bioMerieux Vitek, for providing supplies used in impedance assays.

Finally, I want to thank my wife and "best friend," Angela, for her love, support, and understanding.

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JUSTIFICATION

The impact of microbial adhesion has been acknowledged in marine environments (Marshall et al., 1971a; Marshall et al., 1971b; Fletcher and Floodgate, 1973), wastewater/sewage treatment (Tomlinson and Snaddon, 1966), fermentation (Atkinson and Fowler, 1974), tissue infection (Woods et. al., 1980; Freter and Jones, 1983), oral microbiology (Gibbons and Van Houte, 1975; Costerton et al., 1978), heat transfer unit technology (Characklis, 1981a), and food processing (Notermans and Kampelmacher, 1974; Thomas and McMeekin 1981; Zoltai et al., 1981; Stone and Zottola, 1985; Mafu et al., 1990a). Consequently, several scientific disciplines, including microbiology, physical chemistry, engineering, cell biology, and medical technology have investigated the properties of attached microorganisms (sometimes referred to as biofilms).

Early studies such as those conducted by Henrici and Johnson (1935) and Zobell (1943) described the attachment of aquatic microorganisms to solid surfaces and laid the foundation for microbial biofilm research. Following this pioneering work, much of the initial experimentation on microbial adhesion flourished in the fields of marine, medical, and oral microbiology. In the mid 1970's, food microbiologists began to examine the phenomenon. Researchers investigated the development of biofilms on animal tissues

(e.g., poultry skin and beef) and food-contact surfaces (e.g., stainless steel).

The formation of biofilms on food-contact surfaces has become a considerable concern to the food processing industry. Microorganisms attached to these substrata are a potential source of contamination to food products (Zottola, 1991; Dunsmore et al., 1981).

Proper sanitation plays a major role in maintaining the safety and quality of processed foods. Appropriate use of sanitizing agents can reduce the incidence of spoilage and pathogenic microorganisms on equipment surfaces, and therefore, improve the shelf-life and safety of processed products. Studies, however, have indicated that conventional cleaning and sanitizing procedures may not adequately control microbial biofilms (LeChavallier et al., 1988; Mustapha and Liewen 1989; Frank and Koffi, 1990; Krysinski et al., 1992; Mosteller and Bishop, 1993). Bacteria attached to synthetic polymer surfaces used in poultry and meat processing may have an increased resistance to germicides. Experimentation is required to determine the nature of biofilm development on such surfaces and the degree of sanitizer resistance afforded any attached microorganisms.

LITERATURE REVIEW

BIOFILMS

If microbial adhesion to a solid substrate is extensive enough, a slime or biofilm can develop. In nature, biofilms can be characterized as complex systems in which the number of organisms, the type of microbial species, and the extent of microbial interaction will depend upon where the biofilm developed (Kent, 1988). Any changes in the surrounding environment are typically reflected in the composition of the biofilm.

Biofilms may be quite diverse, containing a variety of organisms such as bacteria, aquatic fungi, protozoa, and algae (Kent and Duddridge, 1981) or they may simply contain one organism type, such as bacteria. Bacterial biofilms are the ones of primary interest to the food processing industry. Zottola (1991) described these as microcolonies of bacteria closely associated with an inert surface and attached to it by a matrix of complex polysaccharide material. The matrix may also contain entrapped debris or nutrients.

Jones (1977) proposed that bacterial adhesion is an initial event in the colonization of a habitat, and the mechanism by which organisms attach to surfaces is of ecological importance. Consequently, it appears that microorganisms can use attachment to gain a survival or proliferation advantage.

Costerton et al. (1978) found that some bacteria attach to surfaces by producing a mass of tangled polysaccharide fibers known as a "glycocalyx". It was postulated that the glycocalyx could channel nutrients to organisms while allowing release of toxins and enzymes. Flexibacter, a gliding bacterium, produces an extracellular slime that permits lateral migration of this organism across surfaces (Humphrey et al., 1979).

Streptococcus mutans can synthesize an insoluble glucan on dental enamel that allows this bacterium to firmly adhere to teeth (Costerton et al., 1978). S. mutans utilizes the fructose monomers from sucrose disaccharides for energy requirements and the glucose monomers as building units for the glucan polymer. Iron-oxidizing bacteria such as those of the genera Gallionella and Sphaerotilus can create biofilms on metal surfaces and, if oxygen is depleted, cause corrosion by creating differential oxygen cells at the surface (Kent, 1988).

Microbial Attachment to Inert Surfaces

Microbial attachment to surfaces (or biofilm development) can occur both in still fluids and in dynamic flow systems (Stone and Zottola, 1985; Kent, 1988). It is a very complex phenomenon, and typically, it is influenced by a number of factors. Characklis (1981a) suggested that

microbial biofilms in a turbulent flow system evolve in the following manner: 1) adsorption of organic material to a wetted surface, 2) transport of microorganisms to the wetted surface, 3) attachment of microorganisms to the surface, 4) metabolism and growth of attached microorganisms, 5) detachment or reentrainment of the biofilm by fluid shear stress. Except for the final step, one can assume that biofilm development occurs much in the same way in less dynamic or stationary systems.

The above sequence of biofilm formation was proposed by Characklis (1981a) for substrata exposed to microorganisms that reside in aquatic environments (e.g., heat exchangers). However, a similar type of biofilm synthesis probably occurs in some food processing systems (e.g., milk pipelines). Creation of biofilms on non-liquid or non-flowing food processing surfaces (e.g., conveyer belting) probably features some of the same characteristics of dynamic systems, except that solid product contact dictates transfer of microorganisms to and from a surface. Some of the factors affecting microbial adhesion to surfaces are discussed below.

<u>Adsorption of Organic Substances to Surfaces</u> Generally, within a few minutes of exposure to natural waters containing low concentrations of nutrients, a surface will adsorb a monolayer of organic molecules (Kent, 1988). Characklis

(1983) indicated that this film is no more than 0.1 μm thick. Nevertheless, the absorbed layer of organics influences the substratum enough to alter many of its properties such as wettability and surface charge (Baier, 1970). As a result, the altered or "conditioned" surface determines to a greater degree the type and extent of microbial interaction that will occur versus the original or clean surface.

Transport of Microorganisms to Surfaces In order for microbial adhesion to occur, organisms must be transported from a suspension to a surface. Once in close proximity of a surface, repulsive forces must be overcome to allow more intimate contact between an organism and a substratum (Marshall et al., 1971b; Harbron and Kent, 1988).

For particles of bacterial size (0.2 to several μm in length), mechanisms of transport to surfaces include fluid flow forces, Brownian motion, chemotaxis, sedimentation, and cell surface hydrophobicity (Marshall, 1985; Harbron and Kent, 1988). Under turbulent conditions, fluid dynamic forces are chiefly responsible for particle transport to surfaces, whereas in still or low-shear systems the other aforementioned mechanisms (e.g., such as chemotaxis) play a more significant role.

1. Fluid Flow Forces Fluid flow forces can be characterized in terms of drag, inertia, lift, drainage, or downsweeps (Kent, 1988). In turbulent flow, eddy diffusion (a minor counter-flow within a major current) disperses cells within the turbulent flow core and propels them into the *viscous sublayer*, a zone of relatively still fluid existing near the solid surface (Marshall, 1985). Organisms must penetrate this sublayer in order to be deposited onto solid surfaces.

Frictional drag forces impede bacteria as they approach the surface; however, since these organisms are small and their density is similar to that of water, inertial and lift forces have little effect (Kent, 1988). Nevertheless, if bacteria are traveling faster than the fluid in the region of the surface, lift forces may direct bacteria toward the surface (Characklis, 1981a). Drainage forces generally tend to repel bacteria from a surface (Kent, 1988).

Once in the viscous sublayer, the most important mechanism directing organisms to the surface are the downsweeps of fluid from the turbulent core (Kent, 1988; Marshall 1985).

2. Brownian Motion Brownian motion is a peculiar dancing motion exhibited by a suspension of finely divided particles or bacteria as they are being bombarded by fluid

molecules (Pelczar et al., 1986). Under turbulent flow conditions, Brownian motion probably exerts little role in the transport of bacteria; nevertheless, it may be a significant form of transport within the viscous sublayer (Characklis, 1981a).

3. Sedimentation This process is only significant in low-shear systems with relatively large particles (i.e. very large microbes or aggregates of normal size bacteria). Sedimentation is unlikely to influence bacterial attachment in turbulent flow conditions (Characklis, 1981a).

4. Chemotaxis Chemotaxis is the movement of an organism toward or away from a chemical (Hazelbauer and Parkinson, 1977; Brock and Madigan, 1988). Many bacteria are motile by the propulsive action of flagella. As a consequence, flagellated organisms display a positive chemotactic response to certain nutrient sources and a negative chemotactic response to harmful chemicals (Brock and Madigan, 1988).

Zobell (1943) indicated that nutrients could accumulate at surfaces. Therefore, it would appear that motile cells could respond to nutrient gradients at surfaces faster than non-motile cells and possibly adhere more rapidly and in greater numbers. Chemotaxis probably does not play a major

role in turbulent flow conditions (Characklis, 1981a), but it may be significant in transporting bacteria through the viscous sublayer (Characklis, 1981b).

5. Cell Surface Hydrophobicity Marshall and Cruickshank (1973) demonstrated that bacteria could orient themselves perpendicularly to the non-aqueous interface of air-water, oil-water, and solid-water systems. Moreover, cells formed rosettes in aqueous phases; a process similar to micelle production in surfactant solutions. These observations suggested that bacterial cells are hydrophobic and may be rejected from suspensions and attracted towards nonaqueous phases, such as a solid surfaces. Therefore, hydrophobicity may be a mechanism influencing transport of bacteria to surfaces.

Topography of Attachment Surface Surface texture appears to be a chief factor influencing the degree of microbial/surface interaction. Dunsmore et al. (1981) indicated that the physical nature (e.g., porosity, surface finish, and hardness) of a substrate is an important factor affecting soil accumulation. These researchers reported ten times more soil on rubber than on smoother glass or stainless steel surfaces. Thus, it can be inferred that crevices on

rubber that accumulate soil can also provide refuge for bacteria.

Gaspar-Rolle (1991) observed greater levels of P. fragi attached to buna-N rubber than to teflon. This worker postulated that the irregular topography of rubber provided more harborages for microbial entrapment versus the smoother teflon surface. Lewis and Gilmour (1987) also found higher numbers of attached mesophilic and psychrotrophic bacteria on rubber than on stainless steel, but the difference was not statistically significant.

Although Mafu et al. (1990a) found no correlation between surface conditions (irregularities) and the ability of Listeria monocytogenes to attach to a surface, scanning electron micrographs generally revealed that this bacterium adhered in higher numbers to rougher rubber and stainless steel surfaces than to smoother glass and polypropylene surfaces. However, it is not known if the micrographs were truly representitive of the bacterial surface population.

Physicochemical Aspects Bacteria, as well as most natural surfaces, are negatively charged (Harbron and Kent, 1988). A counterbalance of such charges occurs in aquatic environments due to the presence of oppositely charged ions. These ions are loosely attracted to surfaces to create a diffuse double layer of ions (Rutter and Vincent, 1984; Marshall, 1985).

When two negatively charged bodies are in close proximity, they may be either attracted to or repelled by each other. The thickness of the double layer of ions determines if attraction or repulsion between the charged bodies will occur. Valency and concentration of counter-ions dictate how thick the double layer will be (Marshall, 1985).

Long-range forces are evident in the initial stages of microbial adhesion. In the absence of steric forces, long-range interaction between two surfaces charged alike include London-van der Waals and electrostatic forces (Rutter and Vincent, 1984; Harbron and Kent, 1988).

1. Long-Range Forces The Derjagouin-Landau-Verwey-Overbeek (DLVO) or colloidal stability theory considers the interaction of London-van der Waals and electrostatic forces that occurs between two particles of similar charge (Jones, 1977). It relates the stability of colloidal dispersions to the total potential energy of interaction. Total interaction energy is the sum of two terms: one term is due to London-van der Waals forces (dispersion forces) and the other is attributable to the overlap of double layers (electrostatic repulsion) associated with the charged surfaces (Harbron and Kent, 1988).

Since bacteria may be characterized as "living colloidal particles," bacterial association with surfaces may be

explained somewhat by the DLVO theory. However, the presence of steric forces as a result of organic materials adsorbing to inert surfaces complicates attempts to understand attachment solely by London-van der Waals and electrostatic forces. As a result, it would seem that in most natural environments that the DLVO theory fails to fully explain long-range interactions between cells and a "conditioned" (organically soiled) surface.

2. Short-Range Forces If long-range forces allow a microorganism to come close enough to a surface, short-range forces can take effect in the attachment process. Short-range forces may be divided into three categories: 1) chemical bonds, such as electrostatic, covalent, and hydrogen bonds; 2) dipole interactions, such as dipole-dipole, dipole-induced dipole, and ion-dipole interactions; 3) and solvation forces, including hydration forces and hydrophobic bonding (Tadros, 1980).

The above forces become evident in microbial adhesion when external structures such as pili, fimbriae, flagella, and extracellular polymers are present. Such polymeric components, in some instances, are thought to allow firm anchorage of cells to surfaces (Zobell, 1943; Marshall et al., 1971b; Piette and Idziak, 1991). Extracellular structures will be further addressed as a separate topic.

3. Surface Free Energy Surface free energy (surface tension) has been used to describe microbial adhesion (Fletcher and Loeb, 1979; Absolom et al., 1983; Busscher et al., 1984; Mafu et al., 1991a). According to a thermodynamic model (Absolom et al. 1983), microbial adhesion will be favored if the process itself causes the free energy to decrease. For systems where the effect of electrical charges and specific biochemical interactions (e.g., receptor-ligand) are ignored, the change in the free energy function (F_{adh}) may be described by:

$$F_{adh} = \gamma bs - \gamma bl - \gamma sl$$

where F_{adh} is the free energy of adhesion, γbs is the bacterium-surface interfacial free energy, γbl is the bacterium-liquid interfacial free energy, and γsl is the surface-liquid interfacial free energy. The above equation however is nothing more than a free energy balance and is not useful in itself for obtaining research data.

Experimentally, surface free energy can be calculated by measuring the contact angle of a drop of water or other liquid on a test surface. Contact angles are the function of three surface tensions as specified by Young's equation (Neumann et al., 1974):

$$\gamma sv - \gamma sl = \gamma lv \cos \theta$$

where γsv, γsl, and γlv, are the solid-vapor, solid-liquid, and liquid-vapor interfacial tensions, respectively. θ is the contact angle of the liquid on the solid. However, only θ and γlv are determined readily by experimentation (Absolom et al., 1983).

Fowkes (1964) proposed a non-thermodynamic relationship that allows estimation of solid surface free energy (e.g., γsv and γsl). Incorporation of this relationship has led to the generation of some useful data for predicting microbial adhesion (Absolom et al., 1983; Busscher et al., 1984)

Surface hydrophobicity in most cases is inversely related to surface free energy. Typically, a more hydrophobic surface will have a lower surface tension (van Loosdrecht et al., 1987). Consequently, contact angle measurements themselves can be used to estimate surface hydrophobicity of bacteria or contact substrata.

Bacterial hydrophobicity may also be ascertained by partitioning organisms between two aqueous phases (Gerson, 1980) or by determining the bacterial concentration adhering to a droplet of organic solvent (Rosenberg, 1984). Knowing the hydrophobicity level of bacteria or attachment substrata of interest can provide information for predicting the degree of microbial adhesion.

van Loosdrecht et al. (1987) compared contact angle values to chemical methods and found agreement among the techniques relative to bacterial hydrophobicity. Also, contact angles correlated well ($r^2 = 0.80$) with the degree of bacterial adhesion. Employing contact angle measurements, Fletcher and Loeb (1979) indicated that hydrophobic surfaces overall were more susceptible to microbial attachment than were hydrophilic ones; nevertheless, exceptions are known to exist (Fletcher, personal communication).

Microbial Appendages 1. Flagella These organelles are responsible for the motility of microorganisms. Bacterial flagella are composed of subunits synthesized from the protein flagellin. The shape and wavelength of a flagellum are controlled by the flagellin protein structure (Brock and Madigan, 1988).

The role of flagella in bacterial attachment to surfaces has received some attention (Notermans and Kampelmacher; 1974; McMeekin and Thomas, 1978; Farber and Idziak, 1984; Lillard, 1986b). Notermans and Kamplemacher (1974) reported that motile bacteria attached to poultry skin much more avidly than did non-motile bacteria. This observation was further supported by the research of Butler et al. (1979) and Farber and Idziak (1984) who demonstrated greater attachment to meat surfaces of flagellated bacteria versus non-

flagellated bacteria. Piette and Idziak (1991) found that flagellated cells adhered in greater numbers to beef tendon than did non-flagellated cells. This increased adhesion was attributable to the ability of motile bacteria to reach the tendon surface in greater numbers versus non-flagellated organisms.

In some instances, it has even been surmised that flagella can form adhesive bonds with surfaces (Kent and Duddridge, 1981; Piette and Idziak, 1991). This suggests that increased attachment of flagellated organisms to surfaces may involve more than just motility.

Lillard (1986b) hypothesized that microbial attachment may be influenced by surface structures such as flagella and fimbriae. However, this worker found that some non-flagellated Salmonella typhimurium cells attached to poultry surfaces at similar rates to those organisms possessing flagella. Other studies by McMeekin and Thomas (1978) and Lillard (1985) also suggested that motile cells had no adhesion advantage over non-motile types.

2. Fimbriae and Pili Fimbriae are non-flagellar filaments extending from the surface of bacteria (Duguid and Anderson, 1967; Brock and Madigan, 1988). These proteinaceous structures are considerably shorter than flagella, but more numerous. Besides their apparent role as

attachment appendages, fimbriae are thought to enable microorganisms to form scums or pellicles on the surfaces of liquids (Brock and Madigan, 1988).

Pili are structurally similar to fimbriae, and the two terms are often used interchangeably. However, pili are usually longer than fimbriae and are present in lower numbers on the bacterial surface (Brock and Madigan, 1988). These structures are significant in the attachment of some pathogenic bacteria to human tissues (Jones, 1977).

3. Extracellular Polymers Extracellular polymers have been implicated in the firm adhesion of microorganisms to surfaces (Marshall et al. 1971b; Fletcher and Floodgate, 1973; Costerton et al., 1978; Zoltai et al., 1981). Biopolymers can be simple polysaccharides synthesized from identical monomeric units or more complex structures such as muco-lipo-proteinaceous polymers and protein-nucleic acid polymers (Harris and Mitchell, 1973). Many different monomers have been found in microbial extracellular polymers (Harbron and Kent, 1988). These include neutral sugars (e.g., D-glucose, D-galactose, D-mannose, L-fucose, and L-rhamnose), amino sugars (e.g., N-acetyl-glucosamine and N-acetyl-galactosamine), and uronic acids (e.g., D-glucuronic and D-galacturonic acids). Combined phosphate, acetate,

formate, or pyruvate have also been isolated from microbial extracellular polysaccharides (Harbron and Kent, 1988).

Synthesized only from glucose units, the water-insoluble glucan produced by S. mutans (Costerton, 1978) is an example of a simple bacterial exopolysaccharide. On the other hand, the extracellular slime of Flexibacter is an intricate glycoprotein containing fucose, glucose, galactose, and uronic acid (Humphrey et al., 1979).

Marshall et al. (1971) reported that an attached marine pseudomonad produced polymeric attachment fibrils over time. They found that mechanical shearing of bacteria from test surfaces left a "footprint" or area where extracellular polymer material remained, suggesting that the strength between the polymer and the surface was greater than that between the polymer and bacterium.

Fletcher and Floodgate (1973) also observed an adhesive fibril on the exterior of marine bacteria, but went further by characterizing the nature of the polymer. Ruthenium Red and Alcian Blue stains revealed that the polymer was an acidic polysaccharide. Furthermore, these workers described two types of attachment polymers, primary and secondary fibrils. They postulated that compact primary fibrils evolved into fibrous secondary fibrils that were instrumental in a time-dependent firm attachment.

Costerton et al. (1978) reported that some bacteria produced a feltlike mass of tangled polysaccharide fibers known as a "glycocalyx". The glycocalyx allows bacteria to adhere to surfaces existing in natural environments, potentially giving such organisms a competitive advantage. In addition to its attachment role, it was proposed that the glycocalyx could channel nutrients to organisms, release toxins and enzymes, and protect attached cells from outside stresses (Costerton et al., 1978).

Phases of Microbial Attachment Information from the above discussions on physicochemical forces and cellular appendages aids in the interpretion of how microbial adhesion occurs. Bacterial attachment has been shown to occur in two phases (Zobell, 1943; Marshall et al., 1971; Fletcher and Floodgate, 1973; Zoltai et al., 1981). The first phase involves a reversible attachment to surfaces. Generally, this type of attachment is effected immediately and involves a balance between London-van der Waals and electrostatic long-range forces. Microorganisms reversibly adhering to a substrate are easily removed by washing (Marshall et al., 1971).

In a time-dependent second phase, microorganisms are able to completely overcome repulsive forces and firmly attach to a surface by producing an extracellular polymeric bridge. Such organisms are not easily removed by washing;

therefore, they are characterized as irreversibly attached cells (Marshall et al., 1971).

Although the aforementioned is a description of the microbial attachment process typically agreed upon by several researchers, one must assume that steric long-range forces probably influence adhesion in some capacity since most surfaces are "conditioned" in nature. Also, organisms that possess fimbriae, pili, and/or flagella may use these structures to aid in short-range interactions (Harbron and Kent, 1988).

Microbial Attachment to Skin and Muscle Tissue Surfaces

To this point, most of the discussion has focused on the attachment of microorganisms to inert or inaminate surfaces. Nevertheless, several researchers have evaluated bacterial attachment to animal tissue surfaces (Notermans and Kamplemacher, 1974; McMeekin and Thomas, 1978; Firstenburg-Eden, 1978; Lillard, 1985; Lillard 1986a and 1986b; Dickson, 1991; Piette and Idziak 1991). Since firmly attached microorganisms resist removal by typical washing procedures (Marshall et al., 1971b), they can remain on processed tissue surfaces to potentially engage in foodborne illnesses or spoilage. Therefore, the focus of this research area has been to gain better insight into microbial/tissue interactions, as well as determine methods to reduce attached

microbial levels. In addition to the previously discussed role of flagella in attachment, some other factors that have been investigated include: bacterial concentration in tissue adhesion suspensions, bacterial physiology, attachment temperature and pH, and tissue type.

McMeekin and Thomas (1978) and Notermans and Kampelmacher (1974) reported that higher initial bacterial levels in an attachment suspension correlated to higher levels of attached cells on poultry skin. Also, these authors observed a linear increase in bacterial attachment with increasing time. Red meat studies by Butler et al. (1979), Chung et al. (1989), and Dickson (1991) further substantiate the relationship between initial bacterial suspension concentrations and tissue attachment levels.

Microorganisms grown at 23°C (before exposure to meat surfaces) attached in higher numbers to beef fat tissue than those grown at 37°C (Dickson, 1991). Growth temperature, conversely, had little effect on the attachment of bacteria to beef lean tissue. Bacteria incubated for 18 hr (before exposure to meat surfaces) attached to tissues better than those incubated for 66 hr. Dickson (1991) suggested that this difference could be due to the physiological and metabolical states of actively growing versus non-growing cells.

Notermans and Kampelmacher (1974) indicated that changing the pH and temperature of the attachment suspension affected the rate and degree of bacterial attachment to meat surfaces. Butler et al. (1979) reported conflicting data suggesting that pH had little effect on the extent of bacterial adhesion.

Firstenberg-Eden (1978) observed that chicken breast with fascia was a better surface for microbial attachment as compared to chicken meat without fascia and beef with or without fascia. Nevertheless, multiplication of attached bacteria was greatest on chicken without fascia, possibly as a result of nutrient and pH differences among tissues.

Postulating that fat may support more bacterial attachment than lean muscle, Chung et al. (1989) determined the extent of microbial association to these tissues. There was no difference ($p>0.05$) in the number of attached cells between fat and lean tissues. After 72 hr at room temperature, all bacteria grew better on lean tissue than on fat tissue, except for *Pseudomonas aeruginosa*. The authors attributed this result to the greater nutrient availability and moisture content of lean tissue.

On the contrary, Dickson (1991) found that more bacteria attached to lean tissue than to fat tissue. In this study, no attempts were made to determine the growth activities of

bacteria following their attachment to tissues.

CLEANING AND SANITATION

Cleaning and sanitation of a food processing surface involves a four-step process: (1) a prerinse with high-pressure water to remove gross soil, (2) physical removal of soil by detergents and mechanical aids, (3) another rinse to remove detergent and loose soil, and (4) application of sanitizers to prevent recontamination of surfaces before processing (Giese, 1991).

No one detergent or sanitizer is effective in every situation. Selection of detergents and sanitizers for a sanitation scheme depends on many factors such as water hardness, type of processing surface, type of soil, and organism(s) of greatest concern relative to spoilage and/or safety.

Detergents

To be effective, detergents should be able to: (1) wet and penetrate soil, (2) emulsify fat, (3) disperse and suspend soil, and (4) counteract water hardness (Giese, 1991). Many types of detergents exist and their application depends mainly on the type of soil and surface. The following are some of the more commonly used detergents.

Neutral Detergents These compounds have a pH near 7.0 and are not widely applied in the food industry, except on glass and black metal surfaces. They can be used in conjunction with abrasive materials for light soil removal when scouring with brushes or metal sponges (Marriott, 1989a). Neutral detergents generally have good fat emulsification properties for effective cleaning.

Alkaline Detergents Alkaline detergents may be classified as strong, moderate, or mild according to their degree of alkalinity. They remove organic soils such as proteins, carbohydrates, grease, and oils from processing surfaces and equipment.

Strongly alkaline compounds (pH > 13) are employed for removal of heavy burnt-on soils found in commercial ovens and smokehouses (Marriott, 1989a). The active agents of these cleaners are sodium hydroxide and silicates having large $N_2O:SIO_2$ ratios (Anonymous, 1976). Strongly alkaline detergents have strong dissolving powers and are very corrosive (Marriott, 1989a).

Moderately (heavy-duty) alkaline detergents (pH 10-12) including sodium metasilicate, sodium hexametaphosphate, and trisodium phosphate are slightly corrosive or non-corrosive cleaners (Marriott, 1989a). These detergents are frequently

utilized in cleaning-in-place systems or other mechanized systems found in poultry plants (Marriott, 1989a).

Compounds with a pH of 7-10, mild alkaline detergents, are used for manual cleaning of lightly soiled areas (Giese, 1991). Active ingredients include sodium carbonate, tetrasodium pyrophosphate, alkyl aryl sulphonates, and sodium sesquicarbonate (Anonymous, 1976).

Acid Detergents Strong acid cleaners (i.e., hydrochloric and hydrofluoric acids) remove encrusted surface matter and mineral scale (Anonymous, 1976). Because of their corrosiveness to stainless steel, the application of strongly acid detergents are limited to heavy scale deposits found on steam-producing equipment, boilers, and some processing equipment (Giese, 1991). Organic acids such as citric and hydroxyacetic are less corrosive and are incorporated in manual cleaning (Giese, 1991).

Sanitizers

A broad array of chemicals have been employed as sanitizing agents in the food processing industry. Chlorine compounds, quaternary ammonium compounds, iodophors, peracetic acid, fatty acids, aldehydes, mixed halogen solutions, and hydrogen peroxide are some of these.

Chlorine Elemental chlorine is a greenish-yellow gas with a characteristic irritating and penetrating odor. The disinfecting properties of chlorine (in the form of chloride of lime) were not realized until the nineteenth century (Dychdala, 1991). In 1846, Semmelweis used chloride of lime to battle puerperal fever in Vienna and chlorinated lime was applied in the treatment of sewage in London as early as 1854 (Dychdala, 1991). Koch demonstrated that pure cultures of bacteria could be destroyed by hypochlorites.

The most widely utilized sanitizers are the chlorine containing compounds. Chlorine sanitizing agents include chlorine dioxide, organic and inorganic chloramines, and hypochlorites. Hypochlorites are the most commonly used sanitizers in the food processing industry (Banwart, 1989; Marriott, 1989b).

The hypochlorites are powerful germicides controlling a broad spectrum of microorganisms. They are effective against bacterial spores, molds, yeasts, and some viruses. Gram-negative bacteria are more sensitive to hypochlorites than are Gram-positive bacteria (Banwart, 1989).

When sodium hypochlorite is added to water the following reaction occurs:

$$1)\quad NaOCl + H_2O \rightarrow HOCl + NaOH$$

$$2)\quad HOCl \rightleftarrows H^+ + OCl^-$$

The destructive mechanisms of chlorine against microorganisms have been postulated by several investigators. Baker (1926) reported that chlorine could combine with proteins of cell membranes and form N-chloro compounds, which interfere with cell metabolism and cause cell death. Furthermore, chlorine has been found to cause membrane damage, inducing leakage of cellular components (Banwart, 1989) and impairing uptake of extracellular nutrients (Camper and McFeters, 1979). Dychdala (1991) cited an early study which speculated that hypochlorites functioned in two subsequent stages: 1) penetration of an active germicidal ingredient into the cell followed by 2) reaction of the germicide with the protoplasm of the cell to form toxic complexes. Knox et al. (1948) suggested that chlorine inhibited essential enzyme systems by oxidizing enzymatic SH groups, while Wlodkoski and Rosenkranz (1975) proposed that chlorine oxidized purine and pyrimidine molecules in DNA.

Generally, a 200 ppm solution of sodium hypochlorite is used to sanitize food-contact surfaces. However, higher concentrations may be applied to floors and walls. The maximum temperature at which chlorine may be used is 115°F, and its optimum activity is at a pH of 6-7.5 (Bradley, 1992)

Although chlorine is an effective sanitizer and is relatively inexpensive, disadvantages do exist concerning its application. Chlorine is corrosive, irritating to the skin

and other tissues, has reduced activity in the presence of organics, and chloramines may be produced. In addition, chlorine requires a tight pH and concentration control in order to assure its effectiveness. It is also adversely affected in the presence of very hard water (>500 ppm calcium carbonate)(Bradley, 1992).

<u>Iodophors</u> Iodine is a nonmetallic element that, in nature, occurs sparingly in the form of iodides in seawater, in Chilean saltpeter and nitrate-bearing earth, in brines from old sea deposits, and in brackish waters from oil and salt wells (Gottardi, 1991). The first use of iodine in medicine was as a remedy for bronchocele. Later, Koch experimented with iodine as a disinfectant against anthrax spores (Gottardi, 1991).

Iodine undergoes the following reactions in solution:

1) $I_2 + H_2O \rightleftarrows HOI + H^+ + I^-$ (iodine hydrolysis)
2) $3I_2 + 3H_2O \rightleftarrows IO_3^- + 5I^- + 6H^+$ (iodate formation)
3) $I_2 + I^- \rightleftarrows I_3^-$ (triiodide formation)

The main bactericidal species are I_2 and HOI (Black et al., 1968), whereas OI^- and I_3^- possess less antimicrobial activity (Gottardi, 1991).

Relative to antimicrobial action, iodine is assumed to react with N-H groups of some amino acids (e.g., lysine, histidine, arginine) and nucleotide bases to form N-iododerivatives (Gottardi, 1991). As a result, important positions for hydrogen bonding are blocked, and a lethal disruption of protein structure probably occurs. SH groups in the amino acid cysteine may be oxidized by iodine, causing loss of protein chain interaction by disulfide bridges (Gottardi, 1991 citing Krusé et al., 1970).

Iodine may react with the phenolic group of tyrosine to form mono- or diiododerivatives. Consequently, the bulkiness of iodine atom(s) in the ortho position causes steric hindrance in the hydrogen bonding of the phenolic OH group (Gottardi, 1991). Apostolov (1980) indicated that iodine could react with carbon-carbon double bonds of unsaturated fats and change the physical properties of lipids and membrane immobilization.

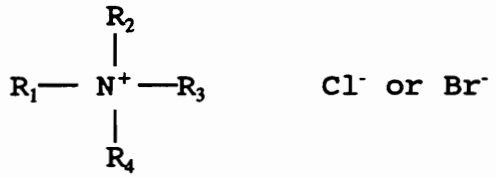
In the food industry, iodine is normally applied in the form of iodophor compounds. An iodophor is a loose complex of elemental iodine or triiodide with a carrier molecule. The carrier increases the solubility of iodine and provides a sustained-release reservoir of the halogen (Gottardi, 1991). Carriers are neutral polymers, such as polyvinyl pyrrolidone, polyether glycols, polyvinyl alcohols, and polyamides (Gottardi, 1991). Iodophors exhibit surface-active

properties that improve the wetting characteristics of solutions. Thus, they may be classified as detergent-sanitizers (Marriott, 1989b)

Similar to chlorine sanitizers, iodophors have a broad spectrum of activity against microorganisms, but are less effective against bacterial spores and bacteriophages than are hypochlorites (Banwart, 1989). Iodophors are generally applied at concentrations of 12.5 to 25 ppm and show greatest bacteriocidal activity under acidic conditions (Marriott, 1989b). They are less sensitive to organic matter than hypochlorites and are not affected by hard water salts.

Iodophors may discolor surfaces as well as impart off-flavors even at low concentrations (Bradley, 1992, Banwart, 1989). Foaming can be a problem and sublimation of iodine can occur at solution temperatures of 50°C and above.

Quaternary Ammonium Compounds Quaternary ammonium compounds are products of a nucleophilic substitution reaction of alkyl halides with tertiary amines (Merianos, 1991). Four carbon atoms are linked chemically to the nitrogen atom by covalent bonds, while the anion in the original alkylating agent becomes linked to the nitrogen by electrovalent bonding. The general formula for these compounds is:



The nitrogen atom with its attached alkyl groups creates the positively charged cation that acts as the functional portion of the molecule (Merianos, 1991)

Quaternary ammonium compounds are good penetrants and natural wetting agents with built-in detergency characteristics; the cationic detergents are the most common types of these sanitizers (Marriott, 1989b). Benzalkonium chloride, N-alkyl dimethylbenzyl ammonium chloride, and N-alkyl ethyl benzyl ammonium chloride are examples of quaternary ammonium compounds.

Quaternary ammonium compounds are effective against Gram-positive organisms, but do not kill spores and are of limited effect against viruses and some Gram-negative bacteria (Anonymous, 1986). Mold growth is more effectively reduced by these sanitizers than by halogen agents (Marriott, 1989b).

The mechanism by which quaternary ammonium compounds destroy microorganisms is related to their physical and chemical properties. They lower the surface tension of solutions and the cationic portion adsorbs to the cell altering its permeability, causing death by release of

cellular contents (Anonymous, 1986; Scharff and Maupin, 1960). Also, it is thought that these agents may denature proteins and inactivate enzymes.

Quaternary ammonium compounds have a broad pH range of activity, going from weakly acidic to fairly alkaline. In addition, they are not corrosive to metals, will not attack plastics (Bradley, 1992), and are more stable in the presence of organic material than are chlorine and iodine sanitizers (Marriott, 1989b). They have good residual activity but are inactivated by hard water.

Peracetic Acid This product is a stabilized combination of hydrogen peroxide and acetic acid (Bradley, 1992). It is a colorless liquid with a characteristic pungent odor. Peracetic acid is a strong oxidizing agent and is highly explosive in the undiluted state.

Just as the halogen sanitizers, it is effective against all microbial groups including hard-to-destroy bacillus and clostridium types. Its destructive mechanism involves wall penetration and oxidation of proteinaceous components (Anonymous, 1986).

Peracetic acid can be effectively used at cold temperatures (i.e, 2-10°C). It is active over a broad pH range and is generally non-corrosive to food-contact metal surfaces at use concentrations (Anonymous, 1986).

SANITIZER EFFECTIVENESS AGAINST MICROBIAL BIOFILMS ON FOOD PROCESSING SURFACES

In many cases, surface association may protect microorganisms from cleaning and sanitizing procedures. Bacteria and soil can accumulate in cracks and crevices on processing surfaces, making microbial contact with cleaning and sanitizing agents difficult. Furthermore, cells on the surface of a biofilm may shelter those within the slime from germicidal action (Sakagami et al., 1989).

Mosely et al. (1976) reported that bacteria attached to stainless steel were more resistant to sanitizers than were cells in suspension. In these experiments, halogen sanitizers were more effective than quaternary ammonium compounds, especially against Gram-negative bacteria.

Mustapha and Liewen (1989) noted a 4 log decrease in the levels of *in vitro* (suspended) L. monocytogenes after cultures were exposed to sodium hypochlorite (200 ppm) for 1 min. However, attachment to stainless steel chips increased the resistance of L. monocytogenes to sodium hypochlorite. In some experiments, not even a 1 log reduction in attached listeriae numbers was observed following treatment of stainless steel chips with 200 ppm sodium hypochlorite for 1 min. In contrast to the activity of sodium hypochlorite against suspended and attached listeriae, a quaternary

ammonium compound (50 ppm) was found to be equally effective against *in vitro* and attached L. monocytogenes.

L. monocytogenes cells adhering to glass were more resistant to benzalkonium chloride and anionic acid sanitizers than were organisms in suspension (Frank and Koffi, 1990). *In vitro* listeriae cultures were completely destroyed ($\geq$6 log reduction) in 30 seconds following treatment with the lowest test concentration of each sanitizer (100 ppm for benzalkonium chloride; 200 ppm for anionic acid). Following a 3 to 5 log decrease in 30 sec, adherent single cells required a total of 12 to 16 min exposure to germicides before they were completely inactivated. Microcolony cells survived for at least 20 min after an initial reduction of 2 to 3 logs during the first 30 sec of exposure.

A recent study by Mosteller and Bishop (1993) further substantiates the germicidal resistance of attached microorganisms. These authors reported that Pseudomonas fluorescens, Yersinia enterocolitica, and L. monocytogenes attached to dairy pipeline teflon and rubber gaskets were less sensitive to sanitizer treatment versus bacteria in suspension. Relative to the destruction of surface biofilms, the reduction goal in this research was 3 logs (99.9%). In many instances, the reduction goal was not met after exposing soiled gaskets to germicides for 30 sec.

Stedman et al. (1954) indicated that longer sanitizer exposure times were required to inactivate bacteria attached to porous substrates versus those attached to nonporous types. This agrees with Mafu et al. (1990b) who observed that bacteria on nonporous glass and stainless steel surfaces were less resistant to sanitizer treatment than were organisms on porous polypropylene and rubber surfaces.

Krysinski et al. (1992) studied the ability of cleaners and sanitizers to destroy L. monocytogenes biofilms existing on stainless steel, polyester, and polyester/polyurethane surfaces. These researchers observed that listeriae attached to the polyester/polyurethane belting were more resistant to cleaners and sanitizers than those adhering to stainless steel and polyester materials. Polyester/polyurethane contained many harborages for cells to embed, probably explaining the greater survival of L. monocytogenes on this surface. The least effective sanitizers were those most commonly employed in food processing (chlorine, iodophor, and neutral quaternary ammonium compound). Complete biofilm destruction was observed in many situations where a cleaning step was followed by sanitizer treatment.

The above studies appear to support Gilbert et al. (1987) who suggested that antimicrobial susceptibility testing should better simulate the environment or conditions in which microorganisms are expected to be destroyed.

Challenging suspensions of microorganisms with germicides, which is normally done in sanitizer efficacy tests, may not be a representative analysis.

MICROORGANISMS ASSOCIATED WITH POULTRY AND MEAT PRODUCTS

Poultry can harbor a variety of microorganisms. Before processing, mesophilic and psychrotrophic bacteria are found on the feet, feathers, and skin of birds. Bacteria initially isolated from processed carcases belong to the genera Acinetobacter, Moraxella, Pseudomonas, Corynebacterium, Micrococcus, Staphylococcus, and Flavobacterium (Barnes, 1960 and 1975). As refrigerated storage proceeds, psychrotrophic bacteria increase in concentration and become the predominate organisms on poultry products. Representative psychrotrophic genera include Pseudomonas, Flavobacterium, Aeromonas, Acinetobacter, Moraxella, and Alcaligenes (Banwart, 1989). Pseudomonads, nevertheless, are the bacteria chiefly responsible for the spoilage of refrigerated poultry products (Nagel et al., 1960; Barnes and Impey, 1968; Barnes, 1976; McMeekin, 1977). The spoilage flora of fresh red meat products is similar to that of poultry products, with pseudomonads being the main spoilage types (Banwart, 1989).

Pathogenic bacteria that may be associated with poultry include Salmonella, Campylobacter jejuni, Staphylococcus aureus, Clostridium perfringens, and Yersinia enterocolitica

(Cox and Bailey, 1987). In addition, Listeria monocytogenes has recently been isolated from poultry products (Bailey et al., 1989; Genigeorgis et al., 1990). Red meat items may also harbor the aforementioned pathogenic bacteria (Fung, 1986).

Of the above pathogenic bacteria, the presence of Salmonella has created the greatest concern in the poultry and meat processing industries; however, Campylobacter jejuni (especially regarding poultry products) and Listeria monocytogenes are eliciting greater attention. The spoilage and pathogenic organisms that will be analyzed for their sanitizer resistance in the attached state are further discussed below.

Pseudomonas fragi

Pseudomonas spp. are aerobic, Gram-negative, nonsporulating, non-fermentative rods; many of which are psychrotrophic. These bacteria are catalase positive and usually oxidase positive. Their growth range is normally between 4 to 43°C, and they have a DNA G + C mol% of 58-71 (Palleroni, 1984). Pseudomonas fragi is a representative organism of the non-pigmented pseudomonads and is therefore classified in Schewan group II (Schewan et al., 1960). Spoilage of refrigerated poultry and meat products is

primarily caused by the growth of both non-pigmented and pigmented pseudomonads (Schewan group I).

P. fragi has been isolated from the spoilage flora of poultry and meat products (Ayres et al., 1950; Ayres, 1960; Barnes, 1976). Barnes (1976) indicated that P. fragi could produce sweetish, urine-like, stale, rotting, fungal, and potato sack off-odors in minced chicken leg.

Shewanella putrefaciens

Members of the genus Shewanella are Gram-negative, straight or curved rods, motile by a single polar flagellum (Jay, 1992a). Metabolism is strictly respiratory. Shewanella putrefaciens (formerly in the genus Pseudomonas and then later reclassified in the genus Alteromonas) has been recognized as an important poultry and meat spoilage organism (Banwart, 1989).

Barnes and Impey (1968) found that Shw. putrefaciens grew better in poultry leg muscle than in poultry breast muscle. These workers attributed the slower proliferation rate of this bacterium in breast muscle to the lower pH of this tissue (5.7-5.9). Barnes (1976) characterized Shw. putrefaciens growth in chicken leg tissue as strongly proteolytic; putrid, fecal, and strong sulfurous off-odors were noted.

McMeekin (1977) reported that although Shw. putrefaciens remained a small fraction of the refrigerated chicken microbial flora, this organism must be considered an important part of the spoilage association because of its psychrotrophic ability and production of strong off-odors. However, Barnes and Melton (1971) found that Shw. putrefaciens was the predominate spoilage organism on chicken carcasses stored at 1°C and wrapped in oxygen impermeable film. It has been suggested that higher levels of carbon dioxide encourage poultry spoilage by Shw. putrefaciens (Barnes and Impey, 1968).

Salmonella spp.

Members of the genus Salmonella are short, Gram-negative, facultatively anaerobic, asporogenous rods which are usually motile (S. pullorum and S. gallinarium are nonmotile) by peritrichous flagella. Gas and acid are produced from glucose, but generally not from lactose (Minor, 1984). Nearly 2000 serotypes of Salmonella can be distinguished according to their biochemical and serological characteristics.

Salmonellae may be divided into three groups according to host preference: 1) those primarily adapted to humans (i.e., typhoid and paratyphoid agents), 2) those primarily adapted to a particular animal host (i.e., S. dublin), and 3)

those unadapted to a host (Committee on Salmonella, 1969). Foodborne gastroenteritis is most often caused by members of the unadapted group.

The primary habitat of salmonellae are the intestinal tracts of animals and humans. Although their main reservoir is the intestinal tract, they may be found in other parts of the body (Jay, 1992b). Because salmonellae are intestinal bacteria, these organisms are excreted in feces and may be disseminated by insects, rodents, and other living creatures. Furthermore, salmonellae may be transferred to sewage and water through fecal material. A cycle develops when water and foods contaminated with Salmonella are ingested by humans and animals. This leads to illness or asymptomatic conditions, and the organism is excreted and re-enters the environment. Augmentation of this cycle through the international shipment of animal products and feeds is greatly responsible for the worldwide distribution of this pathogen and its consequent problems (Jay, 1992b).

Raw foods, particularly those of animal origin, have long been known to be primary vehicles of foodborne salmonellosis. Foods of animal origin often incriminated as Salmonella sources are poultry, beef, and pork (Cox and Bailey, 1987). Consumption of raw milk and eggs contaminated with salmonellae have led to several outbreaks of gastroenteritis (Flowers, 1988).

A primary source of Salmonella for poultry is contaminated feed or feed ingredients (Cox and Bailey, 1987). Although commercial feed is frequently contaminated, percentages of turkeys and chickens entering the processing plant with salmonellae in their intestinal tracts are low (Sadler et al., 1961; Sadler and Corstvet, 1965). However, once birds are inside the plant, there is a widespread dissemination of Salmonella due to product to product cross contamination during processing (Dougherty, 1974; Cox and Bailey, 1987). Poultry carcasses once free of salmonellae are now contaminated with this organism resulting in a much higher percentage of Salmonella-positive carcasses than before the inception of processing.

Listeria monocytogenes

Listeria sp. are Gram-positive, facultatively anaerobic, asporogenous rods capable of proliferation at psychrotrophic temperatures. Their growth range is from 1 to 45°C and their optimum growth temperature is between 30 and 37°C (Seeliger and Jones, 1986). Listeria sp. are ubiquitous in the environment and have been isolated from silage, vegetation, soil, sewage, water, and slaughter-house waste (Seeliger and Jones, 1986).

In recent years, L. monocytogenes has emerged as a pathogen of significant public health interest. Once thought

to be primarily a infectious agent of livestock, L. monocytogenes has been implicated in a number of human foodborne outbreaks (Schlech, et al., 1983; CDC, 1985; Fleming et al., 1985; Ho et al., 1986; Barnes et al., 1989). Gray and Killinger (1966) reported that L. monocytogenes was isolated from more than 40 mammalian species and at least 17 different avian species including domesticated chickens and turkeys.

L. monocytogenes is a unique foodborne pathogen, causing diseases in some infected individuals that, in many cases, have advanced beyond classical gastroenteritis. Primary manifestations of listeriosis include meningitis, spontaneous abortion, encephalitis and septicemia, while conditions such as cutaneous lesions, conjunctivitis, endocarditis, and peritonitis have been known to occur (Gray and Killinger, 1966; Nieman and Lorber, 1980). Subpopulations most susceptible to listeriosis are pregnant women, newborns and infants, and immunocompromised persons (Seeliger and Finger, 1976).

Recent work has reported the presence of L. monocytogenes in poultry products (Bailey et al., 1989; Genigeorgis et al., 1990), suggesting that L. monocytogenes may be a potential etiologic agent to humans when it is associated with poultry. Indeed, a case of listeriosis has been linked to the consumption of turkey frankfurters (Bailey

et al., 1989); however, no large scale outbreaks have been associated with L. monocytogenes-contaminated poultry.

L. monocytogenes has shown inconsistent behavior in muscle tissue products suggesting that muscle tissue pH, nutrient availability, or microbial competition could be influencing factors. Kahn et al.(1973), Buchanan et al. (1987), Harrison and Carpenter (1989), and Carpenter and Harrison (1989) noted growth of L. monocytogenes in meat products that included sterile lamb and poultry. Gouet et al. (1978), Johnson et al. (1988), and Shelef (1989) observed little or no multiplication in products that included ground beef and liver. Survival, slight growth, or exceptional growth were reported for L. monocytogenes in a variety of meat products analyzed by Glass and Doyle (1989).

SCANNING ELECTRON MICROSCOPY

Scanning electron microscopy (SEM) is particularly useful for examining the surfaces of specimens in that it provides a three dimensional image of the surface topography (Mohanty, 1982). A small focused electron beam is generated by the microscope to scan the surface of a specimen in a predetermined raster pattern. Signals at or near the specimen surface are created and include secondary and back-scattered electrons. These two electron types as well as those absorbed by the specimen can be used for SEM imaging.

However, low energy secondary electrons are more efficiently collected by the SEM detector, and thus, are more commonly employed. Imaging is similar to that of a television; collected electrons are amplified and converted into an electron signal that is displayed on a cathode ray tube (Mohanty, 1982).

Several researchers have used SEM to better understand microbial attachment and biofilm development (Fletcher and Floodgate, 1973; Firstenberg-Eden et al., 1979; Zoltai et al., 1981; Schwach and Zottola, 1982; Stone and Zottola, 1985; Herald and Zottola, 1987; Herald and Zottola, 1988; Mustapha and Liewen, 1989; Gaspar-Rolle, 1991). The photomicrographs from such work have revealed how cells interact with surfaces, soil, or with one another; the texture of attachment surfaces; and the nature of extracellular microbial attachment structures.

IMPEDANCE MICROBIOLOGY

Several methods have been used to remove and/or estimate attached microbes from inanimate surfaces. These methods include shaking (Mosely et al., 1976), vortexing (Mustapha and Liewen, 1989), aggitation with glass beads (Krysinski, 1991), scraping (Frank and Koffi, 1990), and the direct epifluorescent filter technique (Mostellar and Bishop, 1993). Recent studies have indicated that impedance microbiology is

a reliable method for determining microbial surface concentrations (Mostellar and Bishop, 1993).

The use of impedance technology to measure microbial activity is a relatively modern procedure when considering implementation of instrumental methods. As early as 1898, Stewart demonstrated that defibrinated blood undergoing putrefaction had a ten-fold increase in conductivity after 10 days (Firstenberg-Eden and Eden, 1984). Many years later, Ur and Brown (1974) introduced continuous impedance monitoring to the field of clinical microbiology.

Impedance is the resistance to flow of an electrical current through a conducting medium; it is the vectorial combination of a conductive element and a capacitive element (Firstenberg-Eden, 1984). Impedance microbiology determines electrical changes in a growth medium to allow detection of initial microbial concentrations in a sample. As microorganisms degrade nutrients in a medium, smaller metabolic molecules are produced and impedance is decreased, or there is a corresponding increase in the conductance and capacitance elements (Firstenberg-Eden and Eden, 1984). In an impedance monitoring unit, conductance signals electrical changes occurring in the bulk solution, whereas capacitance detects changes occurring near the electrodes (Firstenberg-Eden and Eden, 1984).

Threshold bacterial levels of 6-7 log CFU/mL are required to produce an impedance detection time (IDT) on a computer generated curve (Wood et al., 1977). At these levels, an IDT generally corresponds to the moment of upward acceleration from a base line (inflection point). IDTs can then be correlated to initial cell concentrations (as determined by plate counts); the lower the IDT, the higher the initial microbial number and vice versa.

RESEARCH JUSTIFICATION

Laboratory studies of sanitizer efficacy against attached bacteria must be conducted to produce a sanitation operation effective in reducing microbial biofilms. Microorganisms associated with surfaces have shown increased resistance to sanitation procedures. As a result, they can remain on processing surfaces after cleaning and sanitation; proliferate, especially if a nutrient source is available (e.g., residual soil from inadequate sanitation); and be released from the biofilm to contaminate food products.

Most research examining microbial adherence to food-contact surfaces has been limited to the study of stainless steel, rubber, or glass. It can be assumed that bacterial attachment to all food-contact surfaces does occur, and control of this phenomenon would be advantageous to the meat

and poultry industries with respect to product quality and safety.

The objectives of this research were to:

1) use SEM to determine the nature of bacterial adhesion to synthetic meat processing surfaces.
2) employ impedance microbiology to evaluate the effectiveness of sanitizers against spoilage and pathogenic bacteria attached to synthetic meat processing surfaces.

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SECTION 1.0: Sanitizer Efficacy Against Bacteria in Suspension

INTRODUCTION

From a microbiological standpoint, reliable sanitation programs are necessary to ensure the production of high quality and safe muscle food products. Procurement of adequate sanitizers requires stringent testing of chemicals against representative microorganisms.

Sanitizer efficacy is typically ascertained by challenging a suspension of microorganisms with a germicide solution of known concentration. One such analysis is the AOAC Germicidal and Detergent Sanitizer Test (GDST). The GDST indicates the minimum concentration of a chemical required to sanitize a precleaned, non-porous surface. Additionally, this test can be used to determine if a chemical agent at an established concentration is effective against microbial genera or groups of specific concern.

Most, if not all food processing surfaces contain imperfections (pores or crevices) that may serve as harborages for soil and bacteria. Microorganisms may also irreversibly attach to surfaces (Marshall et al., 1971; Fletcher and Floodgate, 1973; Zoltai et al., 1981). Attached bacteria have been shown to be more resistant to antimicrobial agents than bacteria in suspension (Stedman et

al., 1954; Mosely et al., 1976; Frank and Koffi, 1990; Mosteller and Bishop, 1993).

Gilbert et al. (1987) suggested that antimicrobial susceptibility tests should reflect the environmental growth conditions of a microorganism (e.g., the situation that is expected to exist when a microorganism is challenged with a germicide). Thus, the GDST may be of limited value when evaluating sanitizer efficacy against microorganisms located in harborages and/or firmly attached to surfaces. Nevertheless, the GDST is an approved and commonly employed procedure for evaluating sanitizers, and many food processors rely on its results when selecting germicides.

Using the GDST, the objective of this study was to evaluate sanitizer effectiveness against suspensions of four microorganisms of quality or safety concern to the meat and poultry industries: *Shewanella* {*Alteromonas*} *putrefaciens*, *Pseudomonas fragi*, *Salmonella typhimurium*, and *Listeria monocytogenes*.

MATERIALS AND METHODS

Shewanella putrefaciens ATCC 8071 and Pseudomonas fragi ATCC 4973 were selected as representative spoilage organisms. Salmonella typhimurium ATCC 19585 and Listeria monocytogenes Scott A (R.E. Brackett, University of Georgia, Athens, Ga.) were the pathogens tested. Sanitizer solutions were prepared by diluting stock liquids with sterile distilled water in chemically cleaned glass bottles. Final concentrations were 200 ppm for sodium hypochlorite (Gil Super Chlor, Gilmer Co., Harrisonburg Va.), 25 ppm for iodophor (Gil-O-Fact, Gilmer Co.), 200 ppm for quaternary ammonium compound with phosphoric acid, (Gil Acid, Gilmer Co.), and 185 ppm for peracetic acid (P3 Oxonia, Klenzaid Inc., St. Paul, Mn.).

The GDST (AOAC, 1984), with some modifications, was used to determine the effectiveness of sanitizers against bacterial suspensions. Briefly, bacterial cultures were grown on nutrient agar {trypticase soy agar (BBL, Cookeysville, Md.) supplemented with 0.6% yeast extract (BBL; TSAY) was used for L. monocytogenes} flats in French square bottles for 48 hr. After incubation, organisms were removed from each slant using sterile glass beads and 2 mL of phosphate buffer (PB, pH 7.2). PB bacterial suspensions were filtered and 1-ml aliquots were challenged with 99 mL of a swirling sanitizer solution. After 30 and 60 sec exposure intervals, 1 mL of the sanitizer solution was transferred to

9 mL of neutralizer.

Neutralized cultures were pour-plated with nutrient agar (TSAY was used for L. monocytogenes) to determine the number of survivors. A "numbers control" tested in 99-mL of PB for 30 sec was pour-plated to determine the initial challenge concentration. A sanitizer was deemed effective if a 5 log (99.999%) reduction in counts was observed after 30 sec. Two replications of each study were conducted.

RESULTS AND DISCUSSION

All sanitizers reduced bacterial levels >5 logs (Tables 1-4). In many cases, no colonies were observed on pour plates. This was indicated by a tabulated value of <1.00 log CFU/ml.

Although sanitizers effectively decreased bacterial numbers (>5 logs), the suspension test employed is not truly representative of the conditions occurring in food processing. Additional experiments were conducted to determine the efficacy of sanitizers against bacteria attached to synthetic meat processing surfaces.

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Table 1. Sanitizer efficacy against a suspension of *Shw. putrefaciens* ATCC 8071[a].

Sanitizer	Log CFU/ml	
	30 sec[b]	60 sec
Sodium hypochlorite (200 ppm)	0.40	<1.00
Quaternary ammonium/ phosphoric acid (200 ppm)	<1.00	<1.00
Iodophor (25 ppm)	<1.00	<1.00
Peracetic Acid (200 ppm)	<1.00	<1.00

[a]Challenge concentrations - 7.23 to 7.51 log CFU/mL
[b]Exposure time to sanitizer solutions.

Table 2. Sanitizer efficacy against a suspension of *P. fragi* ATCC 4973[a].

Sanitizer	Log CFU/ml	
	30 sec[b]	60 sec
Sodium hypochlorite (200 ppm)	<1.00	0.40
Quaternary ammonium/ phosphoric acid (200 ppm)	1.30	<1.00
Iodophor (25 ppm)	0.40	0.70
Peracetic Acid (200 ppm)	<1.00	<1.00

[a]Challenge concentrations - 8.15 to 8.62 log CFU/mL
[b]Exposure time to sanitizer solutions.

Table 3. Sanitizer efficacy against a suspension of *S. typhimurium* ATCC 19585[a].

Sanitizer	Log CFU/ml	
	30 sec[b]	60 sec
Sodium hypochlorite (200 ppm)	<1.00	<1.00
Quaternary ammonium/ phosphoric acid (200 ppm)	1.48	<1.00
Iodophor (25 ppm)	0.88	<1.00
Peracetic Acid (200 ppm)	<1.00	<1.00

[a]Challenge concentrations - 7.83 to 8.08 log CFU/mL
[b]Exposure time to sanitizer solutions.

Table 4. Sanitizer efficacy against a suspension of *L. monocytogenes* Scott A[a].

Sanitizer	Log CFU/ml	
	30 sec[b]	60 sec
Sodium hypochlorite (200 ppm)	<1.00	<1.00
Quaternary ammonium/ phosphoric acid (200 ppm)	0.70	<1.00
Iodophor (25 ppm)	0.88	<1.00
Peracetic Acid (200 ppm)	<1.00	<1.00

[a]Challenge concentrations - 7.58 to 7.79 log CFU/mL
[b]Exposure time to sanitizer solutions.

SECTION II: Sanitizer Efficacy Against *Shewanella putrefaciens* and *Pseudomonas fragi* Attached to Synthetic Meat Processing Surfaces

ABSTRACT

Experiments were conducted to determine the effectiveness of selected sanitizers against biofilms of *Shewanella putrefaciens* and *Pseudomonas fragi* on synthetic meat processing surfaces. Test surfaces (polyvinyl chloride, polyurethane, and high density polyethylene) in sterile poultry slurries were inoculated with bacterial cultures, agitated (100 rpm) for 2 hr at 18°C, and then incubated for 22-28 hr at 26°C. Scanning electron microscopy was employed to determine bacterial-surface interactions. Attached organisms remaining on surfaces after vortexing were challenged with chlorine (200 ppm), iodophor (25 ppm), quaternary ammonium compound and acid (200 ppm), and peracetic acid (185 ppm) sanitizers. In some cases, surfaces were treated with detergents before sanitizers. Impedance microbiology was used to estimate surviving bacterial populations remaining after chemical treatments. Overall, peracetic acid was the most effective sanitizer against surface biofilms, eliminating attached bacteria after 1 min in most instances. When detergents were used in conjunction with sanitizers, more effective reductions in biofilm numbers occurred. These results should aid processors in developing sanitizer schemes to minimize processing surface contamination with problem meat spoilage bacteria.

SECTION II: Sanitizer Efficacy Against *Shewanella putrefaciens* and *Pseudomonas fragi* Attached to Synthetic Meat Processing Surfaces

INTRODUCTION

Bacterial attachment to food-contact surfaces has elicited major concern in the food processing industry. Attached bacteria (sometimes referred to as biofilms) may be a substantial source of contaminating organisms during processing and, as a consequence, could influence the quality and safety of food products.

Microbial adherance to food-contact surfaces has been investigated by several workers (Zoltai et al., 1981; Speers et al., 1984; Stone and Zottola, 1985; Gaspar-Rolle, 1991). Such research has revealed mechanisms by which bacteria attach to surfaces and has improved our understanding of biofilm development.

A number of researchers have conducted sanitizer efficacy tests using attached microorganisms (Mosely et al., 1976; LeChavallier et al., 1988; Mustapha and Liewen, 1989; Frank and Koffi, 1990; Krysinski et al., 1992; Mostellar and Bishop, 1993). Contaminated surfaces researched in these studies included such materials as stainless steel, glass, rubber, and teflon. In general, attached microorganisms were reported to have increased resistance to test germicides. Limited work has been performed to determine the efficacy of sanitizers against bacteria adhering to synthetic meat

processing surfaces. The objectives of this research were to employ: 1) scanning electron microscopy (SEM) to observe the attachment nature of two meat and poultry spoilage bacteria, Shewanella (Alteromonas) putrefaciens and Pseudomonas fragi, to synthetic processing surfaces and 2) impedance microbiology to quantitatively determine the effectiveness of selected sanitizers against surface biofilms of these organisms. It was hoped that SEM images of microbial biofilms would help explain any potential differences in bacterial sanitizer resistance.

MATERIALS AND METHODS

Processing Surfaces

New (unused) polyvinylchloride (PVC; Burrell Belting, Marietta, Ga.) and polyurethane (PU; Eagle Belting Co., Des Plains, IL.) belting materials were acquired directly from a commercial poultry processing plant. High density polyethylene (HDPE; McMaster Inc., New Brunswick, N.J.) represented a meat cutting board surface. An arch die was used to cut discs from flat sections of PVC or HDPE and a utility knife was employed to slice strands of PU into small cylinders. The dimensions of each material were 2.17 mm (height) x 6.50 mm (diameter) for PVC, 8.70 mm (height) x 3.44 mm (diameter) for PU, and 1.61 mm (height) x 6.50 mm (diameter) for HDPE. These dimensions correlated to surface areas in cm^2 of 1.11 for PVC, 1.13 for PU, and 0.99 for HDPE.

Poultry Slurry Preparation

The poultry slurry was prepared by macerating (Stomacher Lab Blender 400, Tekmar Co., Cincinnati, Oh.) 25 g of ground dark turkey meat in 275 mL of distilled water for 3 min. This solution was then diluted further with 700 mL of distilled water and dispensed into milk dilution bottles in 100-mL portions. The slurry was sterilized by steaming at 100°C for 30 min on two consecutive days; intermittent incubation was 18-24 hr at 30°C (Carpenter, 1977) between

steaming treatments. Fractional sterilization was conducted instead of autoclaving to minimize coagulation of proteins caused by higher sterilization temperatures. The final pH range of the slurry was 6.0-6.4.

Bacterial Culture Preparation for Scanning Electron Microscopy and Surface Sanitizer Tests

Shewanella (Alteromonas) putrefaciens ATCC 8071 and Pseudomonas fragi ATCC 4973 were maintained at 4°C and subcultured monthly on trypticase soy agar (BBL, Cookeysville, Md.) slants supplemented with 0.6% yeast extract (BBL). Refrigerated cultures were activated in trypticase soy broth supplemented with 0.6% yeast extract (TSBY) by three consecutive transfers (18-24 hr, 26°C at each transfer).

Soiling of Surfaces

Surface pieces were vigorously rinsed with distilled water and autoclaved at 121°C for 15 min. PU and PVC surfaces were placed in sterile 250-mL flasks and immersed in 50 mL of sterile poultry slurry. HDPE surfaces were transferred to sterile 250-mL beakers, covered with polyester mesh (McMaster Inc.) baskets fitted to the interior of the beakers, and immersed in 50 mL of sterile poultry slurry. The mesh kept floatable HDPE pieces submerged during soiling.

Each vessel contained 36-52 pieces.

A 1 mL portion of an 18-24 hr TSYB culture (either Shw. putrefaciens or P. fragi) incubated at 26°C was added to each slurry. This gave a bacterial concentration of 6.0-6.5 log CFU/mL. The inoculated slurries were agitated at 100 rpm for 2 hr at 18°C in an environmental shaker (Model G-26, New Brunswick Scientific Co., Edison, N.J.) to soil surfaces as well as deposit bacteria onto pieces. After the 2-hr soiling, the poultry slurry was incubated an additional 22-28 hr at 26°C without agitation. This was done to encourage proliferation of attached bacteria into surface biofilms.

Scanning Electron Microscopy

Surfaces were soiled as described above and rinsed by moderate vortexing in 0.01M phosphate buffered saline (PBS, pH 7.6) for 30 sec and/or by gentle agitation (twice) in 0.1 M sodium phosphate buffer (SPB, pH 7.2). Rinsed pieces were fixed for 30 min in a 2.5% glutaraldehyde solution prepared with SPB. The glutaraldehyde solution was used within 24 hr of preparation.

Following glutaraldehyde fixation, samples were rinsed again in SPB and dehydrated in a 25, 50, 70, 95, and 100% graded ethanol series for 10 min in each alcohol concentration. The surfaces were further dehydrated in a critical point dryer (Ladd Research Industries, Burlington,

Vt.), mounted on aluminum stubs, coated with conductive silver paint, and dried at 70°C for 15 min. Resulting specimens were sputter-coated (Hummer X, Anatech LTD, Alexandria, Va.) with 20 nm of gold and viewed with a Phillips model 505 (Holland) scanning electron microscope at 15-20 kV.

Sanitizers and Detergents

Sanitizer solutions were prepared by diluting stock liquids with sterile distilled water in chemically cleaned glass bottles. Final concentrations were 200 ppm for sodium hypochlorite (Gil Super Chlor, Gilmer Co., Harrisonburg Va.), 25 ppm for iodophor (Gil-O-Fact, Gilmer Co.), 200 ppm for quaternary ammonium compound with phosphoric acid, (Gil Acid, Gilmer Co.), and 185 ppm for peracetic acid (P3 Oxonia, Klenzade Inc., St. Paul, Mn.). A neutral detergent (P3-topax 41, Klenzade Inc.) and an alkaline detergent (P3-topax 47, Klenzade Inc.) were formulated according to the instructions of the manufacturer. Prepared solutions were refrigerated and used within 24 hr.

To ensure that sanitizers were at active concentrations, solutions were checked approximately every two weeks using titration kits (Ecolab, St. Paul, Mn.). If required, solution concentrations were adjusted according to titration results.

Suspension Tests

The effectiveness of sanitizers against bacteria in vitro was determined by the Germicidal and Detergent Sanitizer Test (GDST; AOAC, 1984) briefly described in Section I. For a germicide to be considered effective by the GDST, a $\geq$5 log reduction (99.999%) in microbial numbers must be observed within 30 sec. The GDST is considered a standard analysis relative to the evaluation of germicidal efficacy.

Sanitizer Efficacy Against Contaminated Surfaces

Soiled surfaces (8-15 pieces) were transferred to 125 x 20 mm glass culture tubes containing 10 mL of PBS and moderately vortexed for 30 sec. Organisms remaining after this treatment were considered irreversibly or firmly attached to pieces. A portion of the soiled pieces were drained of excess moisture and retained as unwashed surfaces (not vortexed). These samples represented the total bacteria concentration (reversibly and irreversibly attached cells) on surfaces.

Rinsed surfaces were transferred to sterile plastic petri dishes (100 X 10 mm) and exposed to 30 mL of sanitizer for 0, 1, 2, 6, 10, 14, and 20 min. When testing HDPE, perforated, sterile, plexiglass plates with handles were fitted to 10 x 100 mm plastic petri dish bottoms to keep free-floating pieces submerged in sanitizer solutions. Tests

were conducted at 23-27°C.

Using sterile forceps, duplicate pieces (including unwashed and time 0 surfaces) from each exposure interval were transferred to vessels containing 2 mL of neutralizing buffer (AOAC, 1984) to inactivate sanitizers. After at least 3 min in neutralizing buffer, surfaces were placed in the wells of a Bactometer module (bioMerieux Vitek, Inc., Hazelwood, Mo.). Each well contained 1 mL of modified plate count broth (MCPB; Bishop et al., 1984). Modules were incubated at 26°C in a Bactometer Model 128 (bioMerieux Vitek) to generate impedance detection time (IDT) curves for samples tested. IDTs were correlated to log CFU/mL using calibration curves created for each bacterium (Fig. 1 and 2). The curves were obtained by conducting pour-plate counts for a dilution range of 10^{-1}-10^{-8}. All experiments were repeated three to four times.

Since HDPE floated in neutralizing buffer and MPCB, these media were modified with 0.2% agar. This made their consistency semi-solid and prevented HDPE from floating. For media preparation and reporting convenience, these two media were employed for all surface materials after initial experiments. Semi-solid MPCB calibration curves were also generated for each organism.

Cleaning and Sanitizing Tests

Soiled materials (P. fragi attached to PVC and PU) were moderately vortexed in PBS for 30 sec, placed in sterile 250-mL beakers, and submerged for 10 min in 50-ml of either a neutral detergent or an alkaline detergent. Detergent solutions were at 50°C. The pieces were agitated at 200 rpm for the first min of detergent exposure, and allowed to rest during the remainder of the 10 min period. This treatment was done to measure the physical attributes of detergency against biofilms.

Following the detergent step, surfaces were gently rinsed with PBS and then treated with the above sanitizers at the previously discussed time intervals (up to 10 min). Distilled water controls were run in conjunction with detergent tests to factor out temperature effects. Samples were neutralized as mentioned previously.

Statistical Analysis

Statistical analyses were conducted using the General Linear Models procedure of the Number Cruncher Statistical System (NCSS, 1990). IDTs were converted to log CFU/mL using calibration equations (Fig. 1 and 2). A split-plot analysis of variance (ANOVA) was used to evaluate log CFU/cm^2 numbers resulting from division of log CFU/mL data by appropriate surface area measurements. When significance ($p<0.05$) was

detected by the ANOVA procedure, least significant difference (LSD) was employed as the mean comparison test.

Time intervals with no detection of bacterial activity were assigned an IDT that correlated to 0 log CFU/mL. Those values exceeding the maximum IDT, and thus the calibration range (indicating that there was at least one surviving bacterium, but quantification was not possible), were designated an IDT that correlated to an arbitrary log value of 0.03. To rule out module well contamination, survivors detected out of the calibration range were confirmed by typical colony appearance and Rapid NFT biochemical strips (bioMerieux Vitek, Inc.).

RESULTS AND DISCUSSION

Scanning Electron Microscopy

Fig. 3A-D represent scanning electron micrographs of test surfaces before soiling. All materials were very smooth to the physical touch, and appeared that way to the unaided eye. Even at high magnifications, PVC still appeared to be quite smooth (Fig. 3A). Conversely, rough areas on PU (Fig. 3B) and HDPE (Fig. 3C) were observed at higher magnifications. Deep crevices were sometimes seen on HDPE (Fig. 3D).

Following a 22-28 hr incubation at 26°C, the Shw. putrefaciens biofilm on all surface types could be characterized as a layer of single adherent cells (Fig. 4-6). Occasionally, aggregates or microcolonies of this bacterium were found. Attachment fibrils were sometimes seen extending from Shw. putrefaciens cells to test surfaces (Fig. 5).

Unlike Shw. putrefaciens, P. fragi formed mostly cell aggregates or microcolonies on surfaces (Fig. 7-10). Furthermore, the overall fibril production displayed by P. fragi (e.g., Fig. 10) was more extensive than that exhibited by Shw. putrefaciens. Vortexing surfaces for 30 sec (versus gentle rinsing) did not seem to alter the general pattern of aggregate formation by P. fragi cells (e.g., Fig. 10).

Microbial attachment is thought to occur in two phases (Zobell, 1943; Marshall et al., 1971; Fletcher and Floodgate,

1973; Zoltai et al., 1981). The first phase involves an instantaneous reversible attachment to surfaces. In a time-dependent second phase, microorganisms irreversibly adhere to surfaces by producing a polymeric bridging material. The attachment of Shw. putrefaciens and P. fragi to synthetic meat processing surfaces could have occurred in a similar manner.

Marshall et al. (1971) indicated that irreversibly (firmly) attached bacteria are not removed by rinsing. In the present study, a number of organisms that remained on surfaces following vortexing did not exhibit extracellular fibrils. However, the bridging substance involved in anchoring these cells to surfaces could have been less mature (no obvious fibril formation).

Sanitizer Efficacy Against Bacterial Suspensions

All sanitizers were deemed effective according to GSDT guidelines. Test germicides reduced cell suspension levels of both Shw. putrefaciens and P. fragi more than 5 logs (99.999%) within 30 sec (results in Section I).

Sanitizer Efficacy Against Attached Bacteria

Shw. putrefaciens bacterial levels before and after vortexing (rinsing) are illustrated in Fig. 11. The rinsing step in this study could be considered analogous to the pre-

rinsing procedure in a sanitation scheme. Bacteria remaining after rinsing were deemed firmly (irreversibly) attached.

Initial Shw. putrefaciens concentrations on unwashed surfaces ranged from 5.3 to 6.5 log CFU/cm^2, and were highest on unwashed HDPE (Fig. 11). Rinsing surfaces reduced counts 1.5 to 2.0 log CFU/cm^2.

Shw. putrefaciens may have attached to HDPE in higher numbers because of the hydrophobic nature of HDPE and/or the deep crevices on this surface (Fig 3D). Fletcher and Loeb (1983) indicated that hydrophobic surfaces, such as polyethylene, are typically more susceptible to bacterial attachment. Crevices on HDPE could potentially serve as harborages for bacteria, making the removal of some organisms during rinsing more difficult.

When treated with sanitizers, attached Shw. putrefaciens displayed some degree of survival on all surfaces. The initial attachment level (time 0 or concentration following rinsing) of Shw. putrefaciens on PVC was only 3.5 logs. Nevertheless, approximately 1 log of attached bacteria remained after exposing contaminated PVC to chlorine and iodophor sanitizers for 1 min (Fig. 12). Very low residual Shw. putrefaciens activity was noted for up to 14 min after application of quaternary ammonium compound and acid (quat-acid) (Fig. 12).

Peracetic acid and quat-acid sanitizers were more

effective ($p<0.05$) against Shw. putrefaciens attached to HDPE than were chlorine and iodophor germicides (Fig. 13). All sanitizers reduced Shw. putrefaciens levels on PU to 0.5 logs or less after 1 min, with little bacterial viability being observed thereafter (results not shown).

The interaction of sanitizer level and surface level was significant ($p<0.05$) for Shw. putrefaciens (Fig. 14). Although initial numbers of attached Shw. putrefaciens were higher (Fig. 11) than those on other surfaces, it is evident from the interaction plot (Fig. 14) that the iodophor sanitizer was much less efficient against Shw. putrefaciens on HDPE than it was against this bacterium on PVC and PU surfaces.

P. fragi bacterial counts on unwashed and rinsed surfaces are illustrated in Fig. 15. Unwashed and rinsed HDPE possessed the highest P. fragi concentrations. As indicated for attached Shw. putrefaciens (Fig. 11), this could be attributable to the deep crevices on HDPE and/or the hydrophobic nature of this material.

Just as noted for attached Shw. putrefaciens, there were surviving P. fragi populations on each material following germicide treatment. After 1 min exposure, chlorine, iodophor, and quat-acid treatments only reduced initial PVC levels of P. fragi by 3 logs (Fig. 16). Residual concentrations of this bacterium were observed for up to 6

min with chlorine, and after 6 min with iodophor and quat-acid. Peracetic acid only required 2 min to completely destroy the biofilm of P. fragi on PVC. However, peracetic acid overall was not significantly more effective ($p>0.05$) than the other sanitizers against P. fragi on PVC.

After 1 min of exposure (except for quat-acid), all sanitizers were effective in reducing P. fragi levels on PU below 1 log (Fig. 17). All sanitizers reduced P. fragi concentrations on HDPE to 0.5 logs or less after 1 min, with little bacterial viability being observed thereafter (results not shown). Furthermore, the interaction of sanitizer level and surface level was not significant ($p>0.05$) for P. fragi (results not shown).

Distilled water (DW) effected a 1.5 log reduction in the P. fragi biofilm of rinsed PVC, and neutral detergent (ND) caused a 2.5 log decrease (Fig. 18). Alkaline detergent (AD) virtually eliminated attached P. fragi on PVC. The same treatments were even more effective against P. fragi on PU, with AD completely destroying the biofilm (Fig. 19). DW and ND treatments in conjunction with sanitizer application generally destroyed biofilms on either surface after 2 min (results not shown). After 1 min, sanitizers completely eliminated P. fragi attached to PVC and PU surfaces previously treated with AD.

Sanitizers in the GDST were deemed successful in

reducing suspensions of test organisms. In many instances, however, adherence of Shw. putrefaciens and P. fragi to synthetic meat processing surfaces enhanced the tolerance of these bacteria to germicides. This finding seems logical since bacteria in the attached state have less surface area that can be exposed to a sanitizing agent. Conversely, all sides of a microbial cell in a mixed suspension will be exposed to a germicide. Moreover, the concentration of organic materials (in this study, poultry soil and bacteria themselves) on a solid substrate could further buffer cells from the lethal effect of sanitizers.

The formation of aggregates (microcolonies) of cells would seem to shelter cells within from sanitizer lethality. Furthermore, extracellular polymeric fibrils surrounding bacteria (sometimes referred to as a glycocalyx) may protect cells from antimicrobial compounds (Costerton et al., 1978; Hoyle et al., 1990). Although attached P. fragi normally formed cell aggregates and produced more fibril material, it did not appear to possess any more resistance to sanitizers versus attached Shw. putrefaciens. In addition, the fact that HDPE possessed deep crevices, which could potentially serve as harborages for bacterial cells, did not seem to be a major factor affecting bacterial sanitizer resistance in this study.

The results of this research further support previous

work demonstrating the increased germicidal resistance of bacteria attached to food processing surfaces (Mosely et al., 1976; LeChavallier et al., 1988; Mustapha and Liewen, 1989; Frank and Koffi, 1990; Krysinski et al., 1992; Mosteller and Bishop, 1993). Kryzinski et al. (1992) found that several sanitizers were not effective in reducing L. monocytogenes counts on stainless steel and synthetic surfaces. For some sanitizers, these authors observed <1 log reduction of listeriae after a 10 min exposure. Less active germicides included chlorine, iodophor, and quaternary ammonium compound. However, peracetic acid was found to be one of the more effective germicides against L. monocytogenes attached to these surfaces.

Mosteller and Bishop (1993) reported that bacteria adhering to buna-rubber and teflon gasket surfaces were more resistant to a variety of sanitizers than those in suspension. In many cases, the goal reduction (≥3 logs) of attached organisms was not achieved after 30 seconds of sanitizer exposure.

ND displayed some ability to remove attached bacteria. This indicates that the action of a detergent alone (with no germicidal activity) could contribute to the removal of biofilm material. In the present study, detergent treatment (especially AD application) used in conjunction with sanitizing led to effective decreases in surface biofilms.

This agrees with Kryzinski et al. (1992) who found that using a detergent step prior to germicide application led to greater reductions in bacterial biofilm numbers as compared to sanitizer treatment alone.

Impedance microbiology has been acknowledged as a reliable method for estimating attached microbial numbers (Mosteller and Bishop, 1993). Since this procedure measures microbial metabolic activity, it accounts for all viable organisms associated with a sample. Therefore, physical techniques such as vortexing with glass beads and scraping are not required to obtain estimates of attached bacteria.

Nevertheless, biofilm cells injured by detergent or sanitizer treatments could have an altered (slower) metabolism (even in a non-selective media), and thus, a delayed IDT may result. This could lead to potential underestimations of initial surface counts.

Using impedance procedures, Mosteller and Bishop (1993) performed injury recovery experiments with biofilm bacteria. They employed a 2 hr preincubation with nutrient broth. However, the results of these studies were inconclusive in that the preincubation period did not yield significantly different (higher) numbers. Additional research is needed to ascertain if a preincubation step is required in the impedance monitoring of microorganisms surviving germicidal treatments.

CONCLUSIONS

Although not always statistically significant, peracetic acid, in general, was the most effective (fastest acting) sanitizer against the biofilms of Shw. putrefaciens and P. fragi. Other sanitizers varied in effectiveness according to the attached bacterium/surface. Peracetic acid usually eliminated biofilms on test surfaces after 1 min. Sanitizer treatment following detergent application effectively reduced biofilms of both organisms. In practical terms, peracetic acid may be quite effective against bacteria on synthetic processing surfaces when only a pre-rinse (no detergent step) is carried out prior to sanitizing (e.g., mid-shift break). Nevertheless, application of alkaline detergent and then treatment of surfaces with any of the sanitizers analyzed in this study should prove effective in decreasing biofilms of key spoilage organisms. Therefore, results from this research should aid meat and poultry processors in enhancing the efficacy of their sanitation programs.

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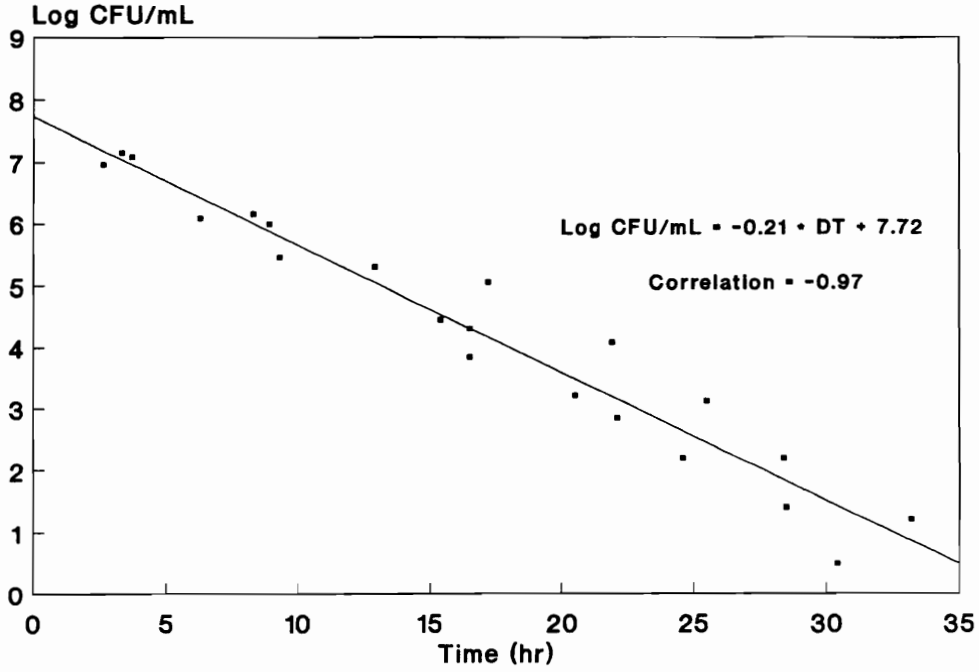


Figure 1. Calibration curve of impedance detection time (IDT) versus Log CFU/mL for Shw. putrefaciens. Equation is Log CFU/mL = -0.21 * IDT + 7.72; Correlation = -0.97.

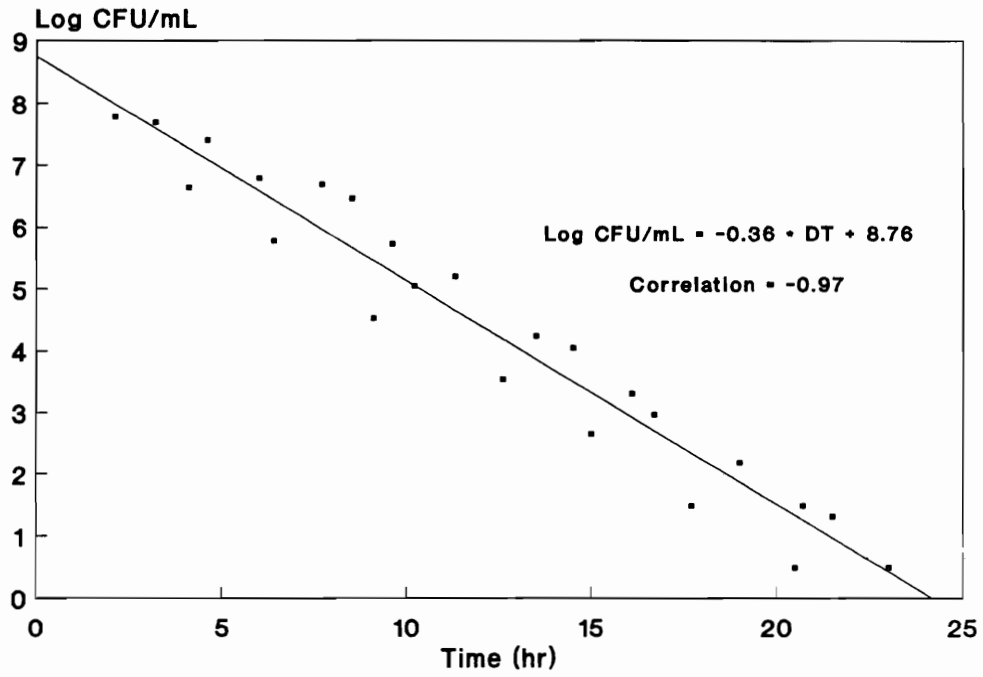
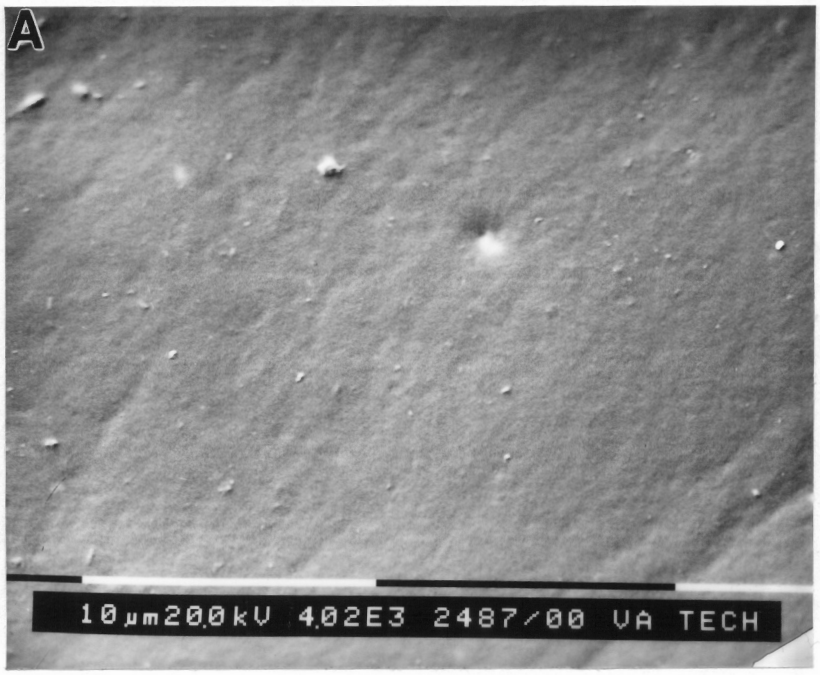


Figure 2. Calibration curve of impedance detection time (IDT) versus Log CFU/mL for P. fragi. Equation is Log CFU/mL = -0.36 * IDT + 8.76; Correlation = -0.97.



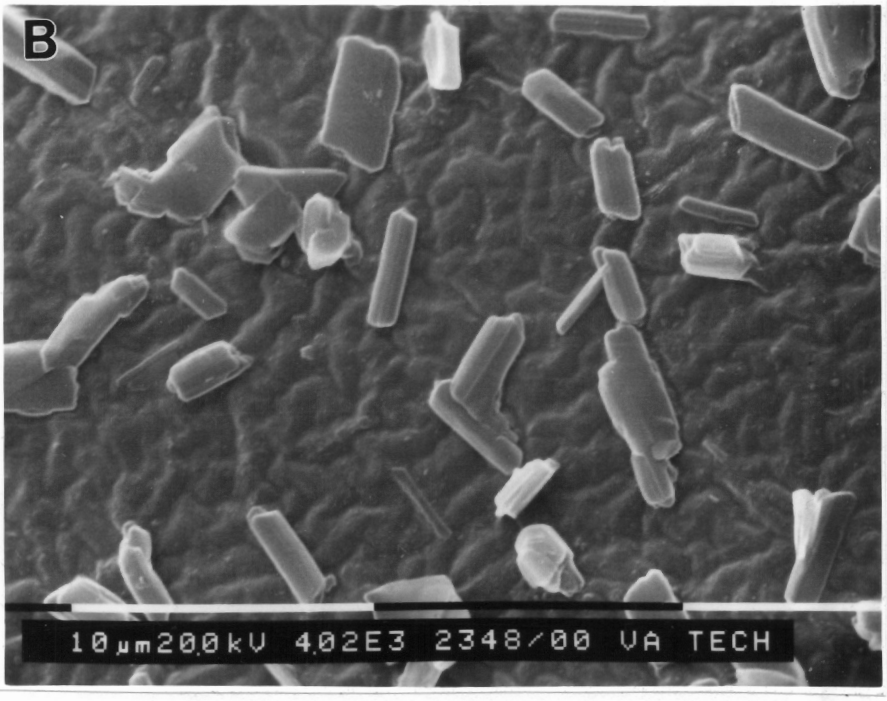
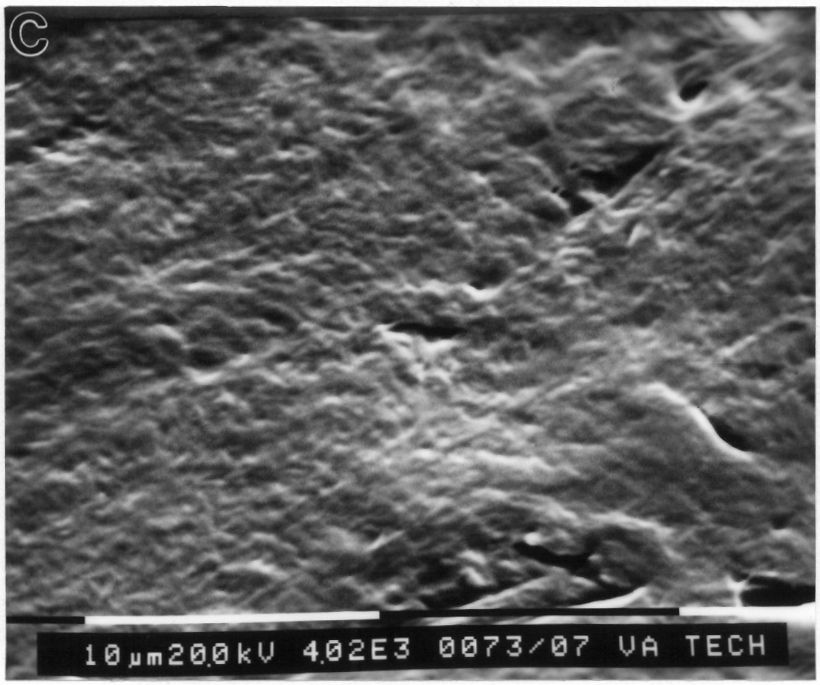
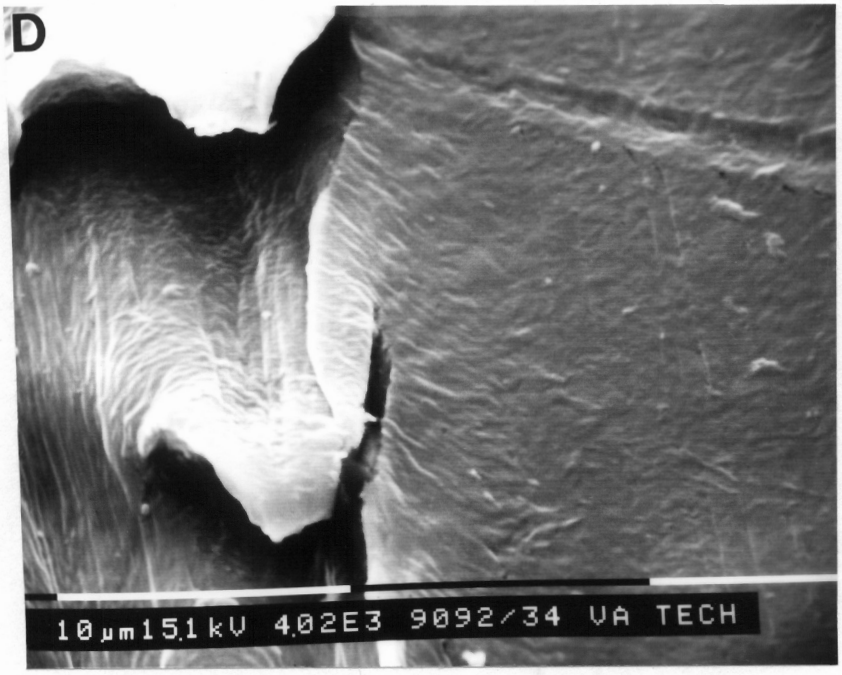


Figure 3. Scanning electron micrographs of sterile A) polyvinyl chloride and B) polyurethane. Magnification is 4020X.





Figures 3C and 3D. Scanning electron micrographs of sterile high density polyethylene. Magnification is 4020X.

Figure 4. Scanning electron micrograph of Shw. putrefaciens on polyvinyl chloride. Magnification is 4020X.

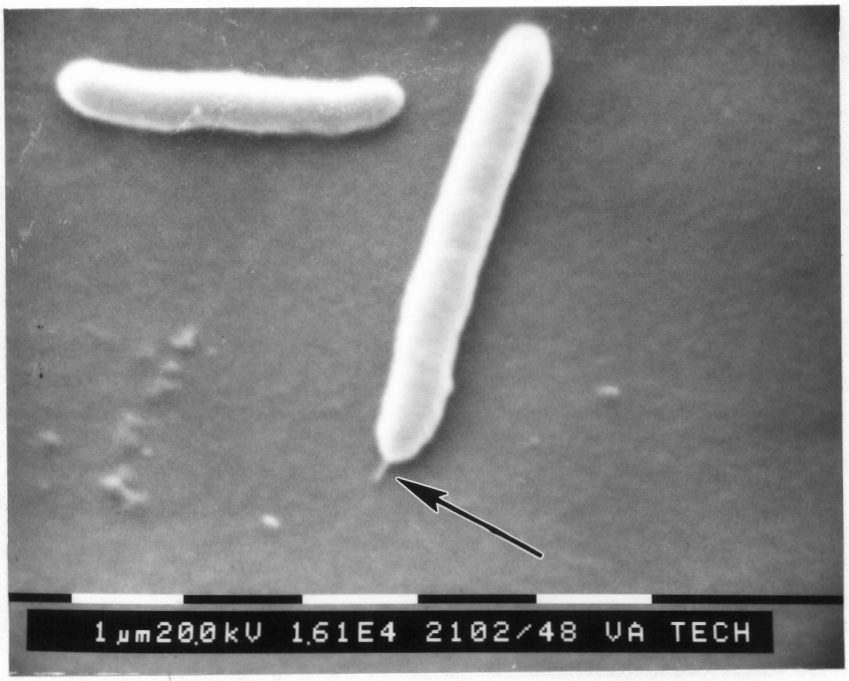


Figure 5. High magnification scanning electron micrograph of Shw. putrefaciens on polyvinyl chloride. A single fibril extends from the longer cell. Magnification is 16,100X.

Figure 6. Scanning electron micrograph of Shw. putrefaciens on high density polyethylene. Magnification is 4020X.

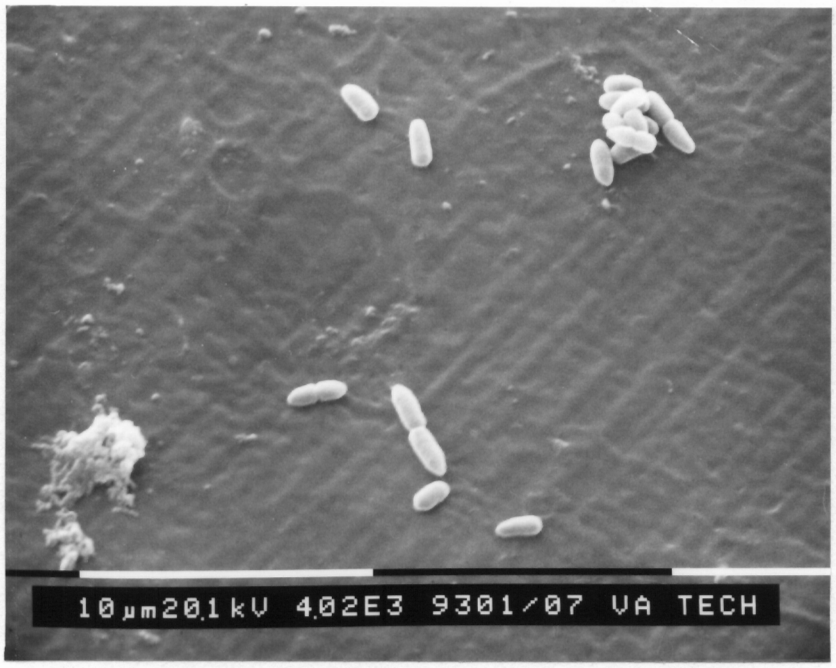


Figure 7. Scanning electron micrograph of P. fragi on polyvinyl chloride. Magnification is 4020X.

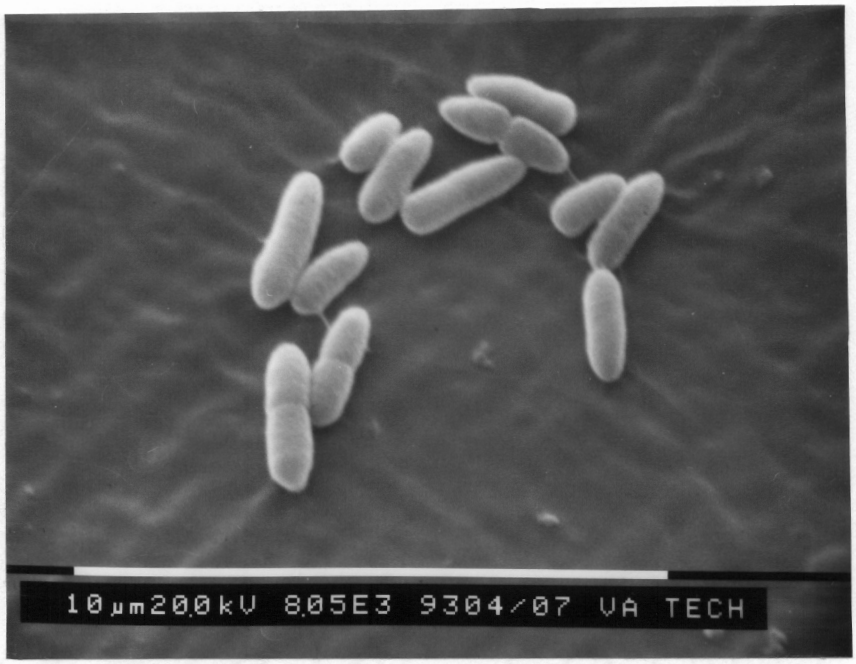


Figure 8. High magnification scanning electron micrograph of P. fragi on polyvinyl chloride. Fibrils are evident on some cells. Magnification is 8050X.

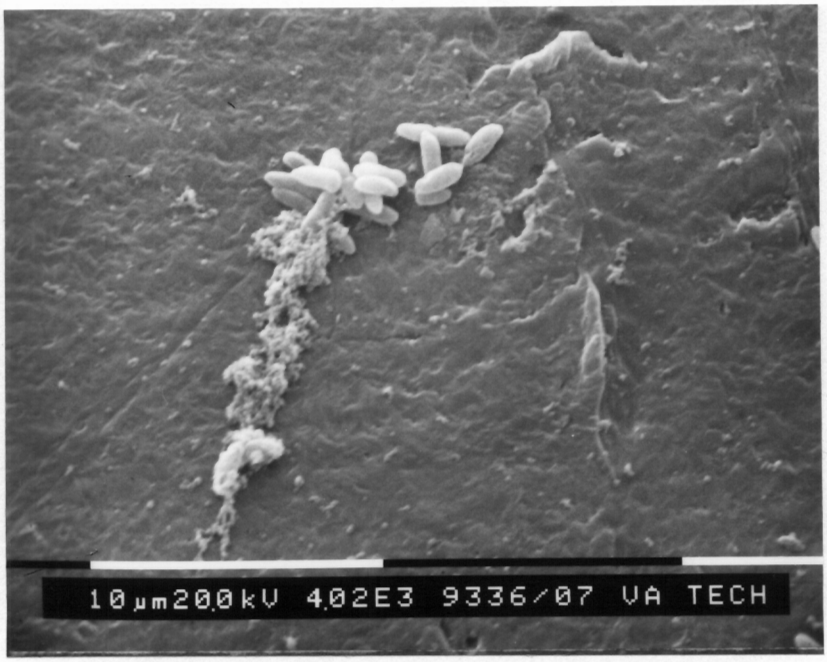


Figure 9. Scanning electron micrograph of P. fragi on high density polyethylene. Poultry soil can be seen trailing aggregate of cells. Magnification is 4020X.

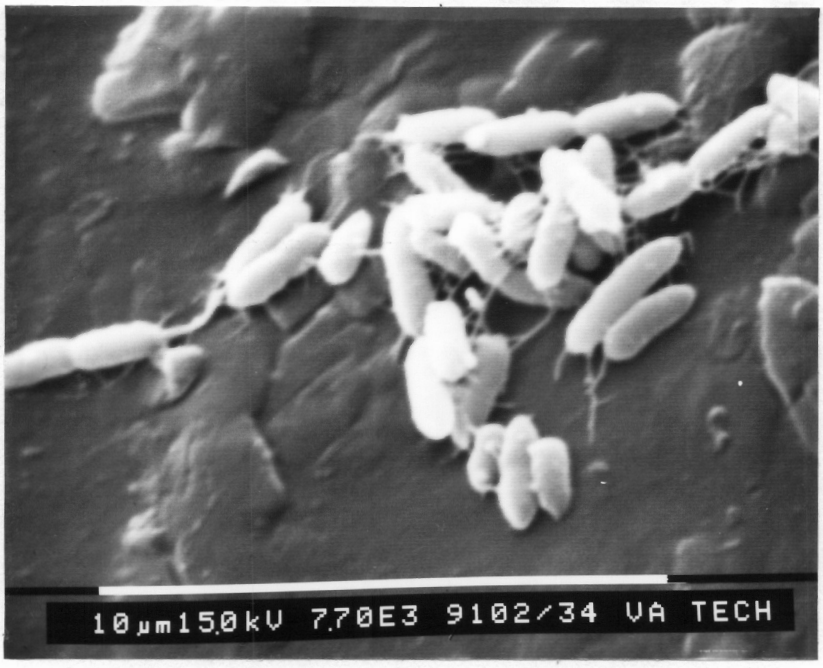


Figure 10. Scanning electron micrograph of P. fragi on polyurethane. Extensive fibril production by cells in aggregate. Magnification is 7700X.

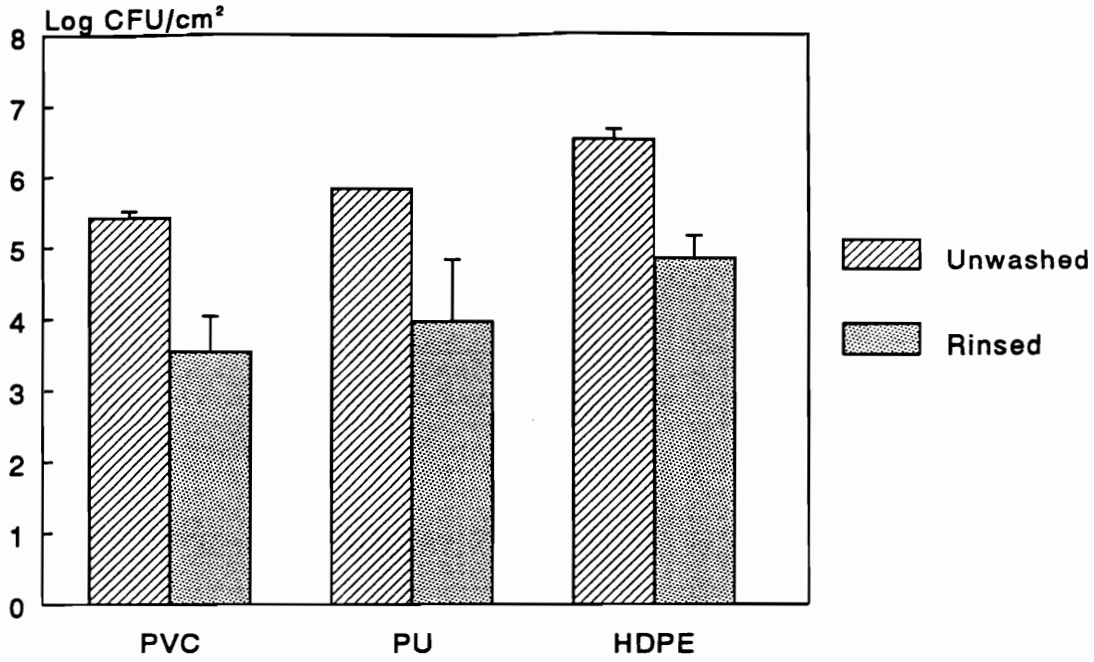


Figure 11. Levels of Shw. putrefaciens on unwashed and rinsed surfaces: polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE). Rinsed surfaces were vortexed for 30 sec.

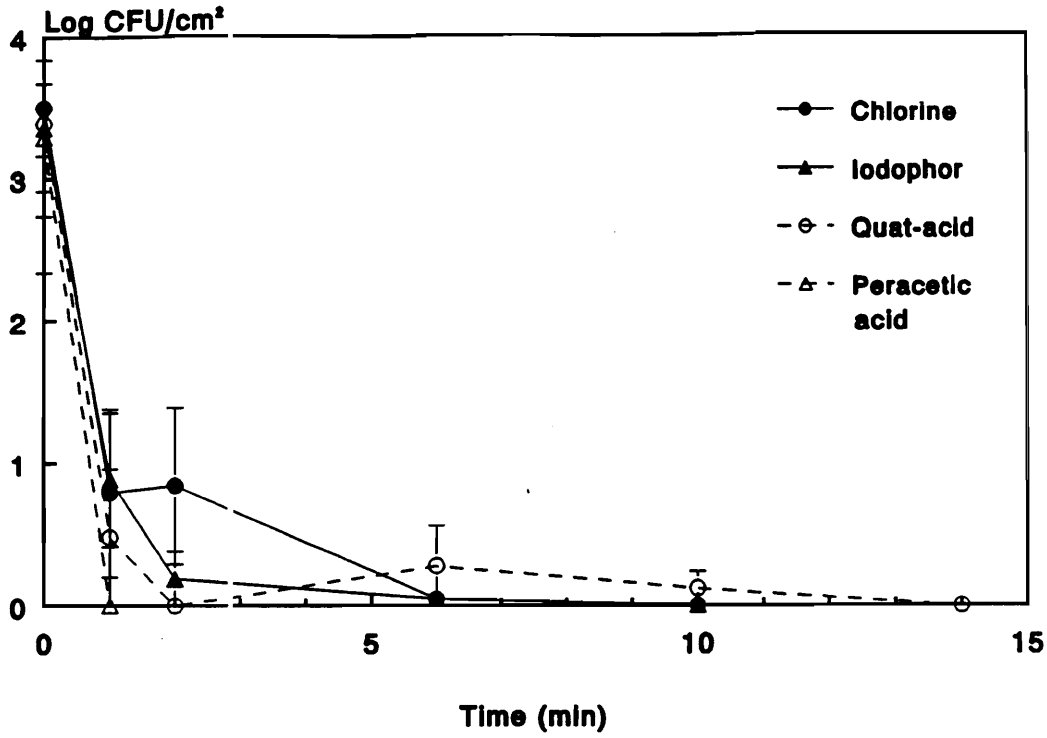


Figure 12. Sanitizer efficacy against Shw. putrefaciens attached to polyvinyl chloride.

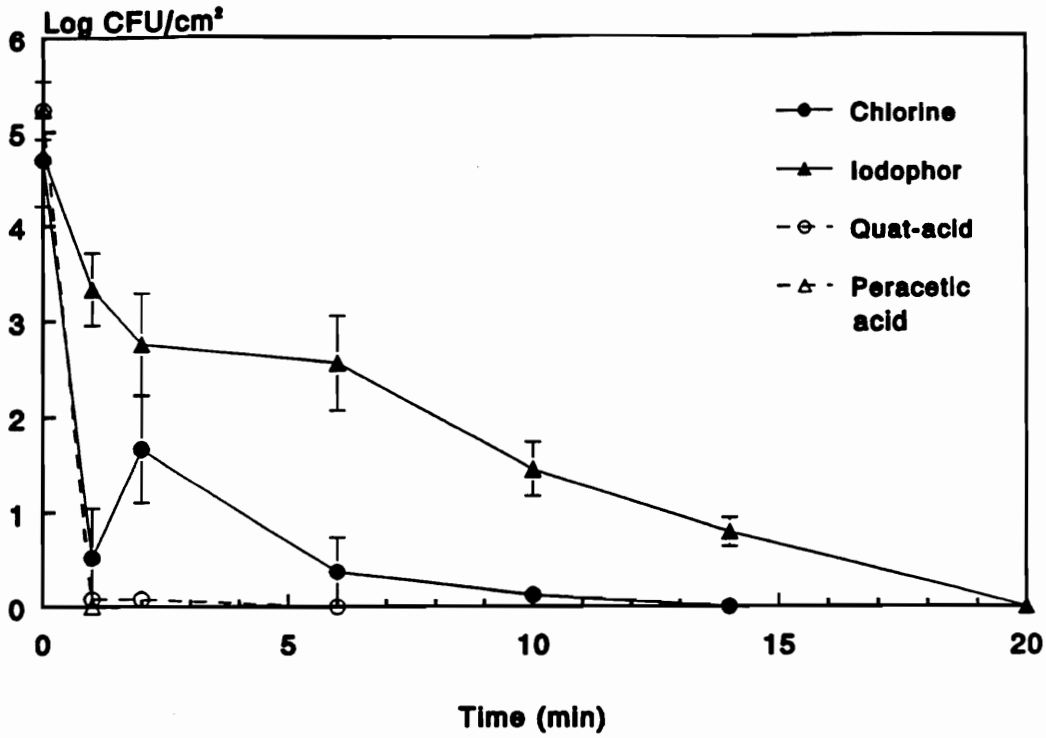


Figure 13. Sanitizer efficacy against Shw. putrefaciens attached to high density polyethylene.

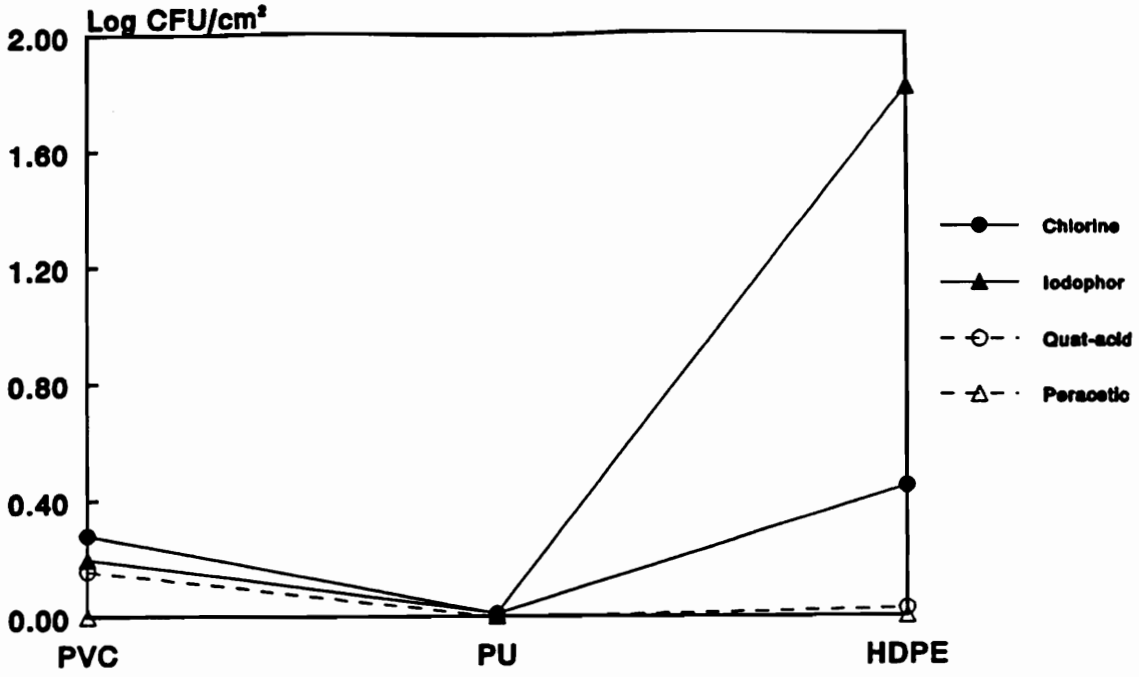


Figure 14. Interaction of sanitizer and surface levels for Shw. putrefaciens. Data points were obtained by averaging across all time intervals and replications. Surfaces are polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE).

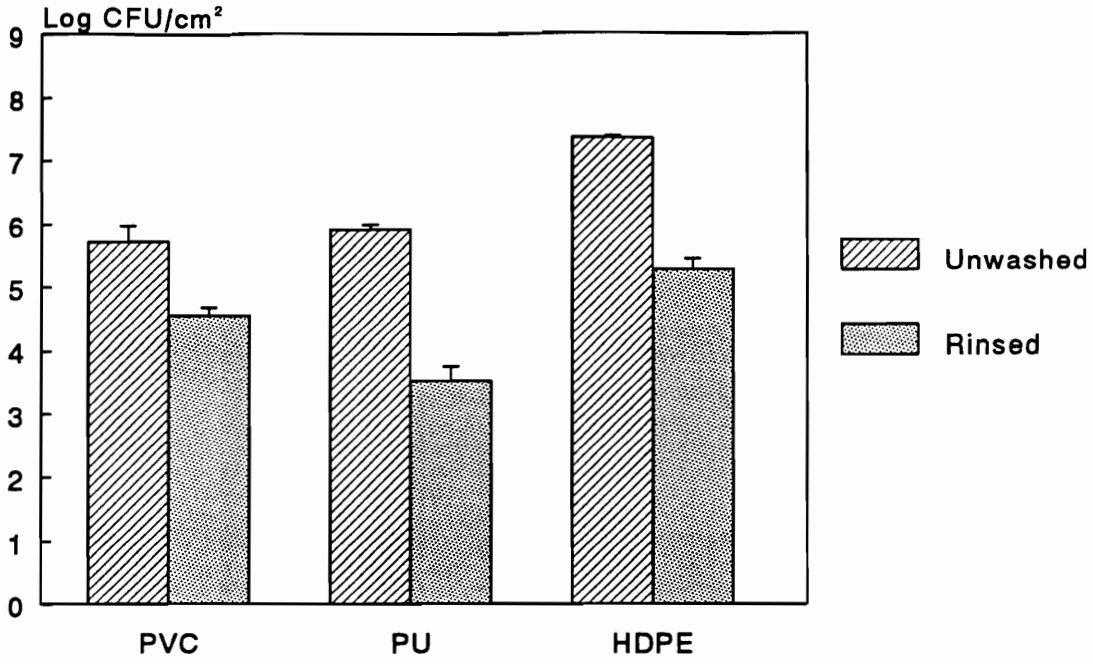


Figure 15. Levels of P. fragi on unwashed and rinsed surfaces: polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE). Rinsed surfaces were vortexed for 30 sec.

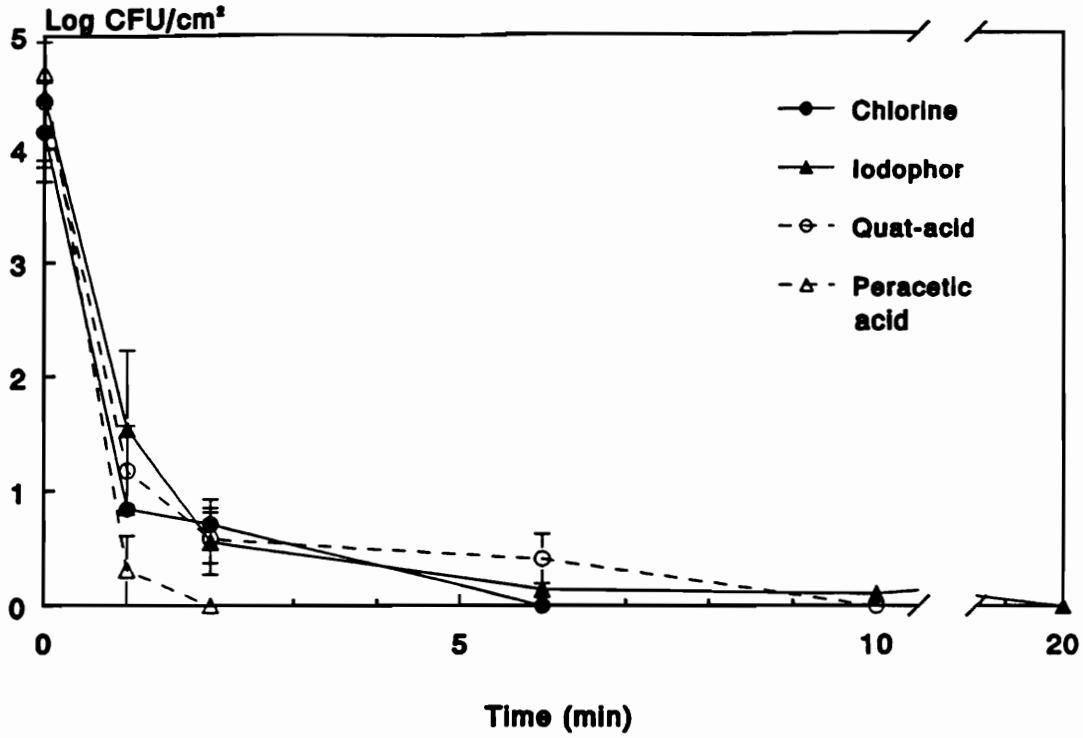


Figure 16. Sanitizer efficacy against P. fragi attached to polyvinyl chloride.

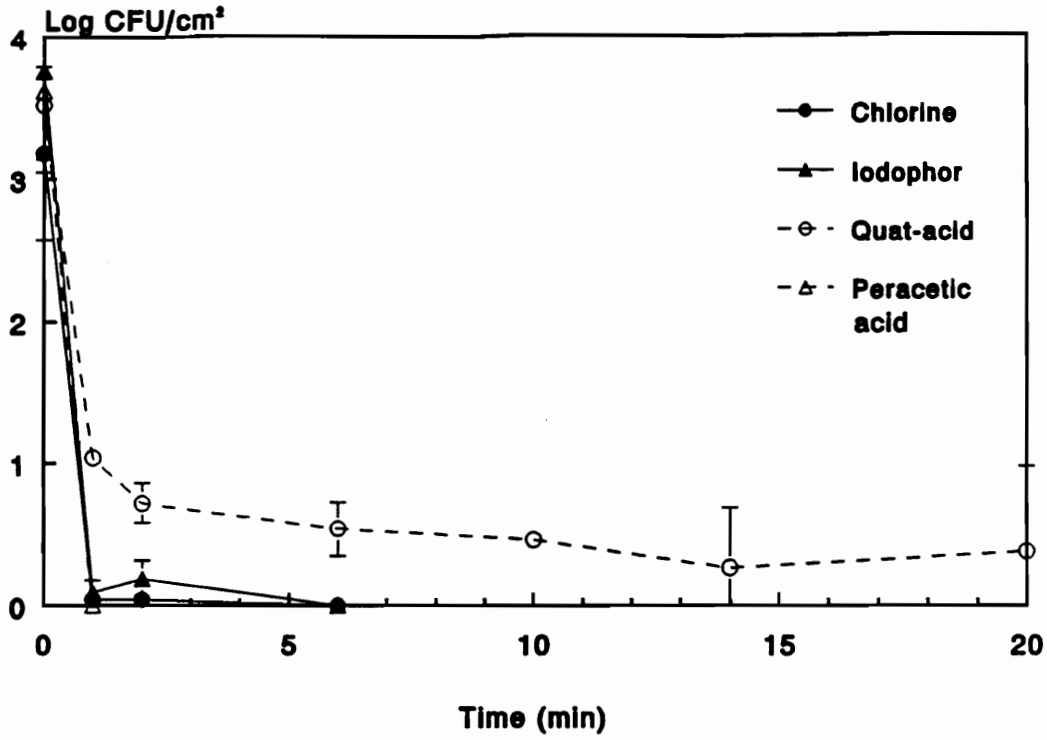


Figure 17. Sanitizer efficacy against P. fragi attached to polyurethane.

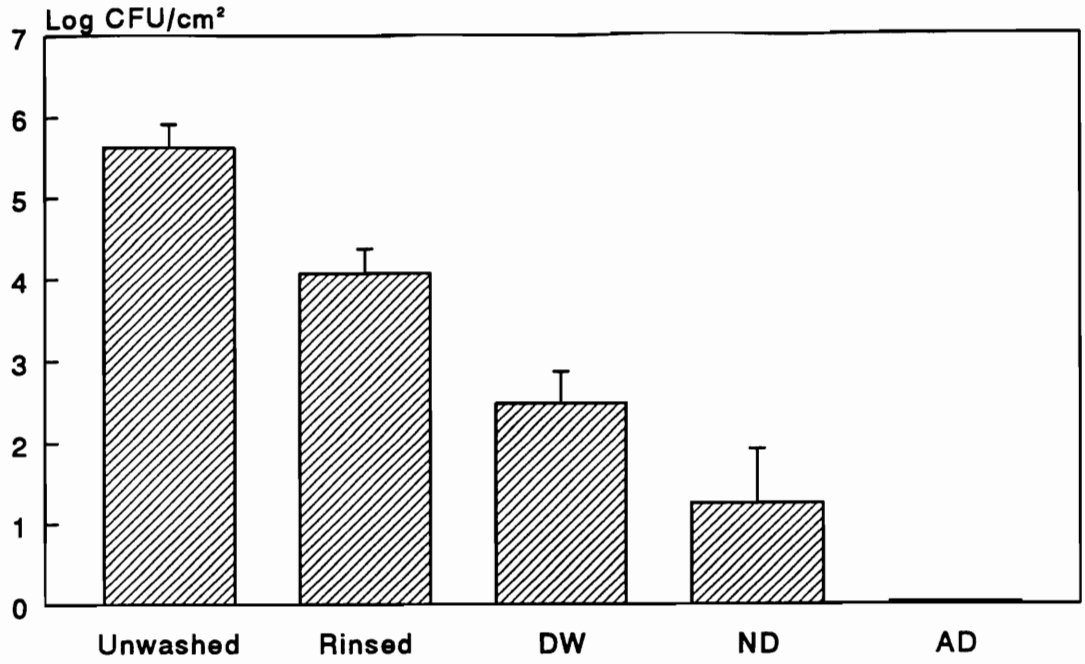


Figure 18. Detergent efficacy against P. fragi attached to polyvinyl chloride. Distilled water (DW), neutral detergent (ND), and alkaline detergent (AD) treatments were 10 min at 50°C.

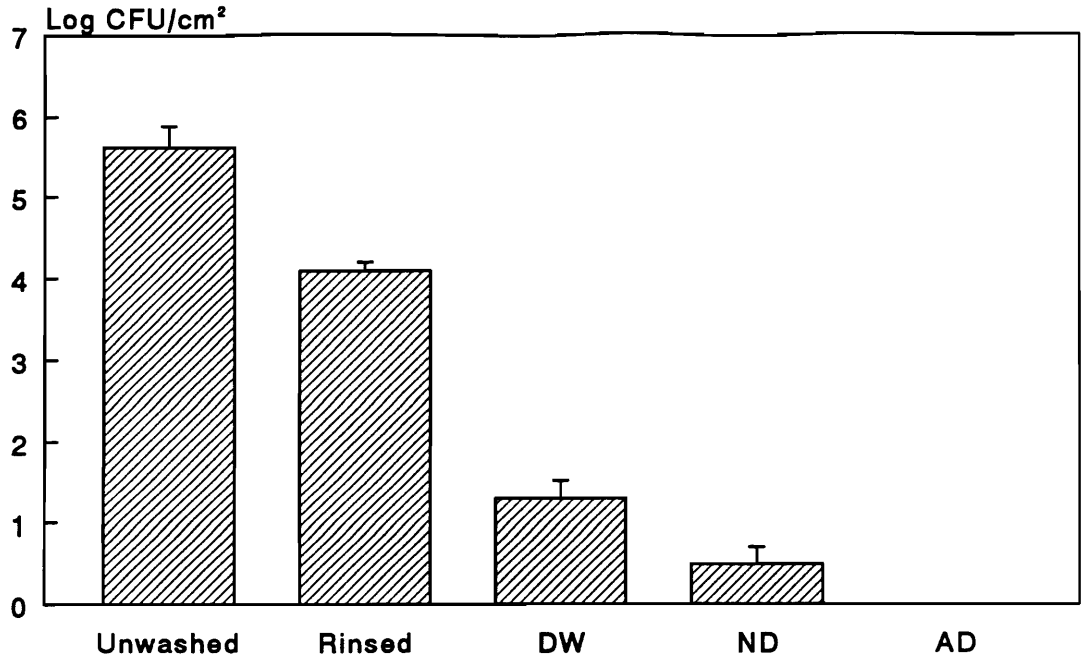


Figure 19. Detergent efficacy against P. fragi attached to polyurethane. Distilled water (DW), neutral detergent (ND), and alkaline detergent (AD) treatments were 10 min at 50°C.

SECTION III: Sanitizer Efficacy Against *Salmonella typhimurium* and *Listeria monocytogenes* Attached to Synthetic Meat Processing Surfaces

ABSTRACT

Experiments were conducted to determine the effectiveness of selected sanitizers against biofilms of *Salmonella typhimurium* and *Listeria monocytogenes* on synthetic meat processing surfaces. Test surfaces (polyvinyl chloride, polyurethane, and high density polyethylene) in sterile poultry slurries were inoculated with bacterial cultures, agitated (100 rpm) for 2 hr at 18°C, and then incubated for 22-28 hr at 37°C. Scanning electron microscopy was employed to determine bacterial-surface interactions. Attached organisms remaining on surfaces after vortexing were challenged with chlorine (200 ppm), iodophor (25 ppm), quaternary ammonium compound and acid (200 ppm), and peracetic acid (185 ppm) sanitizers. In some cases, surfaces were treated with detergents before sanitizers. Impedance microbiology was used to estimate surviving bacterial populations remaining after chemical treatments. Overall, peracetic acid was the most effective sanitizer against biofilms, eliminating attached bacteria after 1 min in most instances. When detergents were used in conjunction with sanitizers, more effective reductions in biofilm numbers occurred. *S. typhimurium* was generally more resistant to sanitizers than *L. monocytogenes*. This may be related to the

much greater fibril production exhibited by *S. typhimurium*. Results of this study should aid processors in developing sanitizer schemes to minimize processing surface populations of *S. typhimurium* and *L. monocytogenes*.

SECTION III: Sanitizer Efficacy Against *Salmonella typhimurium* and *Listeria monocytogenes* Attached to Synthetic Meat Processing Surfaces

INTRODUCTION

The presence of attached bacteria (sometimes referred to as biofilms) on food-contact surfaces has become a major concern to the food processing industry. Microorganisms attached to such surfaces may be a substantial source of product contamination (Zottola, 1991; Dunsmore et al., 1981), and therefore, potentially influence the safety and quality of processed food products. Several workers have investigated the development of biofilms on food processing surfaces (Zoltai et al., 1981; Schwach and Zottola, 1982; Stone and Zottola, 1985; Herald and Zottola 1988; Mafu et al., 1990a). Such research has given insight into the mechanisms by which microorganisms associate with surfaces.

Many authors have demonstrated that surface adhesion increases the resistance of microorganisms to sanitizers (LeChavallier et al., 1988; Mustapha and Liewen 1989; Frank and Koffi, 1990; Krysinski et al., 1992; Mosteller and Bishop, 1993). Although strides have been made to reduce the incidence of biofilms on surfaces, the complexity of biofilms has made their reduction or elimination extremely difficult. Furthermore, the response of a specific attached bacterium to a given sanitizer has sometimes been inconsistent, making the

study of sanitizer effectiveness against biofilms somewhat empirical.

For example, Mustapha and Liewen (1989) found that Listeria monocytogenes attached to stainless steel was as susceptible to treatment with a quaternary ammonium sanitizer as cells *in vitro*. Conversely, Mafu et al. (1990a) reported that L. monocytogenes adhering to polypropylene was highly resistant to a quaternary ammonium sanitizer. Such factors as bacterial specie or strain differences, type of attachment surface, sanitizer type, growth medium and temperature, experimental conditions, initial bacterial levels, or the interaction of any of these may explain the inconsistent results sometimes seen.

Little work has been conducted to determine the efficacy of sanitizers against bacteria attached to synthetic meat processing surfaces. Considering this fact, and the variation that has been acknowledged in sanitizer testing of biofilms, it was felt that the these materials (especially when populated with biofilms of pathogenic bacteria) warranted investigation relative to sanitizer testing.

The objectives of this research were to use: 1) scanning electron microscopy to determine the attachment nature of two potential meat pathogens, Salmonella typhimurium and L. monocytogenes, to synthetic meat processing surfaces and 2) impedance monitoring to quantitate the effectiveness of

selected sanitizers against surface biofilms of these bacteria.

MATERIALS AND METHODS

Processing Surfaces

New (unused) polyvinylchloride (PVC; Burrell Belting, Marietta, Ga.) and polyurethane (PU; Eagle Belting Co., Des Plains, Ill.) belting materials were acquired directly from a commercial poultry processing plant. High density polyethylene (HDPE; McMaster Inc., New Brunswick, N.J.) represented a meat cutting board surface. An arch die was used to cut discs from flat sections of PVC or HDPE and a utility knife was employed to slice strands of PU into small cylinders. The dimensions of each material were 2.17 mm (height) x 6.50 mm (diameter) for PVC, 8.70 mm (height) x 3.44 mm (diameter) for PU, and 1.61 mm (height) x 6.50 mm (diameter) for HDPE. These dimensions correlated to surface areas in cm^2 of 1.11 for PVC, 1.13 for PU, and 0.99 for HDPE.

Poultry Slurry Preparation

The poultry slurry was prepared by macerating (Stomacher Lab Blender 400, Tekmar Co., Cincinnati, Oh.) 25 g of ground dark turkey meat in 275 mL of distilled water for 3 min. This solution was then diluted further with 700 mL of distilled water and dispensed into milk dilution bottles in 100-mL portions. The slurry was sterilized by steaming at

100°C for 30 min on two consecutive days; intermittent incubation was 18-24 hr at 30°C (Carpenter, 1977) between steaming treatments. Fractional sterilization was conducted instead of autoclaving to minimize coagulation of protein caused by higher sterilization temperatures. The final pH range of the slurry was 6.0-6.4.

Bacterial Culture Preparation for Scanning Electron Microscopy and Surface Sanitizer Tests

Salmonella typhimurium ATCC 19585 and *L. monocytogenes* Scott A were maintained at 4°C and subcultured monthly on trypticase soy agar (BBL, Cookeysville, Md.) slants supplemented with 0.6% yeast extract (BBL, Cookeysville, Md.). Refrigerated cultures were activated in trypticase soy broth supplemented with 0.6% yeast extract (TSBY) by three consecutive transfers (18-24 hr, 37°C incubation at each transfer).

Soiling of Surfaces

Surface pieces were vigorously rinsed with distilled water and then autoclaved at 121°C for 15 min. PU and PVC surfaces were placed in sterile 250-mL flasks and immersed in 50 mL of sterile poultry slurry. HDPE surfaces were transferred to sterile 250-mL beakers, covered with polyester mesh (McMaster Inc.) baskets fitted to the interior of the

beakers, and immersed in 50 mL of sterile poultry slurry. The mesh kept floatable HDPE pieces submerged during soiling. Each vessel contained 36-52 pieces.

A 1-mL portion of an 18-24 hr TSBY culture (either *S. typhimurium* or *L. monocytogenes*) incubated at 37°C was added to each slurry. This gave a bacterial concentration of 7.0-7.5 log CFU/mL. The inoculated slurries were agitated at 100 rpm for 2 hr at 18°C in an environmental shaker (Model G-26, New Brunswick Scientific Co., Edison, N.J.) to soil surfaces as well as deposit bacteria onto pieces. After the 2-hr soiling, the poultry slurry was incubated an additional 22-28 hr at 37°C without agitation. This was done to promote proliferation of attached bacteria into surface biofilms.

Scanning Electron Microscopy

Surfaces were soiled as described above and rinsed by moderate vortexing in 0.01M phosphate buffered saline (PBS, pH 7.6) for 30 sec and/or by gentle agitation (twice) in 0.1 M sodium phosphate buffer (SPB, pH 7.2). Rinsed pieces were fixed for 30 min in a 2.5% glutaraldehyde solution prepared with SPB. The glutaraldehyde solution was used within 24 hr of preparation.

Following glutaraldehyde fixation, samples were rinsed again in SPB and dehydrated in a 25, 50, 70, 95, and 100% graded ethanol series for 10 min in each alcohol

concentration. The surfaces were further dehydrated in a critical point dryer (Ladd Research Industries, Burlington, Vt.), mounted on aluminum stubs, coated with conductive silver paint, and dried at 70°C for 15 min. Resulting specimens were sputter-coated (Hummer X, Anatech LTD, Alexandria, Va.) with 20 nm of gold and viewed with a Phillips model 505 (Holland) scanning electron microscope at 15-20 kV.

Sanitizers and Detergents

Sanitizer solutions were formulated by diluting stock liquids with sterile distilled water in chemically cleaned glass bottles. Final concentrations were 200 ppm for sodium hypochlorite (Gil Super Chlor, Gilmer Co., Harrisonburg Va.), 25 ppm for iodophor (Gil-O-Fact, Gilmer Co.), 200 ppm for quaternary ammonium compound with phosphoric acid, (Gil Acid, Gilmer Co.), and 185 ppm for peracetic acid (P3 Oxonia, Klenzade Inc., St. Paul, Mn.). A neutral detergent (P3-topax 41, Klenzade Inc.) and an alkaline detergent (P3-topax 47, Klenzade Inc.) were formulated according to the instructions of the manufacturer. Prepared solutions were refrigerated and used within 24 hr.

To ensure that sanitizers were at active concentrations, solutions were checked approximately every two weeks using titration kits (Ecolab, St. Paul, Mn.). If required,

solution concentrations were adjusted according to titration results.

Suspension Tests

The effectiveness of sanitizers against bacteria in vitro was determined by the Germicidal and Detergent Sanitizer Test (GDST; AOAC, 1984) briefly described in Section I. For a germicide to be considered effective by the GDST, a $\geq$5 log reduction (99.999%) in microbial numbers must be observed within 30 sec. The GDST is considered a standard analysis relative to the evaluation of germicidal efficacy.

Sanitizer Efficacy Against Contaminated Surfaces

Soiled surfaces (8-15 pieces) were transferred to 125 x 20 mm glass culture tubes containing 10 mL of PBS and moderately vortexed for 30 sec. Organisms remaining after this treatment were considered irreversibly or firmly attached to pieces. A portion of the soiled pieces were drained of excess moisture and retained as unwashed surfaces (not vortexed). These samples represented the total bacterial concentration (reversibly and irreversibly attached cells) on surfaces.

Rinsed surfaces were transferred to sterile plastic petri dishes (100 X 10 mm) and exposed to 30 mL of sanitizer for 0, 1, 2, 6, 10, 14, and 20 min. When testing HDPE,

perforated, sterile, plexiglass plates with handles were fitted to 10 x 100 mm plastic petri dish bottoms to keep free-floating pieces submerged in sanitizer solutions. Tests were conducted at 23-27°C.

Duplicate pieces (including unwashed and time 0) from each exposure interval were transferred to vessels containing 2 mL of neutralizing buffer (AOAC, 1984) modified with 0.2% agar. After at least 3 min in neutralizing buffer, surfaces were placed in module wells (bioMerieux Vitek, Inc., Hazelwood, Mo.) containing 1 mL of modified plate count broth (MPCB; Bishop et al., 1984) formulated with 0.2% agar. Semisolid neutralizing buffer and MPCB were used to keep free-floating HDPE pieces submerged during analyses.

Modules were incubated at 37°C in a Bactometer Model 128 (bioMerieux Vitek) to generate impedance detection time (IDT) curves for samples tested. IDTs were correlated to log CFU/mL using calibration curves created for each bacterium (Fig. 1 and 2). The curves were obtained by conducting pour-plate counts for a dilution range of 10^{-1}-10^{-8}. All experiments were repeated three to four times.

Cleaning and Sanitizing Tests

Soiled materials (S. typhimurium attached to PVC and PU) were moderately vortexed in PBS for 30 sec, placed in sterile 250-mL beakers, and submerged for 10 min in 50-ml of either a

neutral detergent or an alkaline detergent. Detergent solutions were at 50°C. The pieces were agitated at 200 rpm for the first min of detergent exposure, and allowed to rest during the remainder of the 10 min period. This treatment was done to measure the physical attributes of detergency against biofilms.

Following the detergent step, surfaces were gently rinsed with PBS and then treated with the above sanitizers at the previously discussed time intervals (up to 10 min). Distilled water controls were run in conjunction with detergent tests to factor out temperature effects. Samples were neutralized as mentioned previously.

Statistical Analysis

Statistical analyses were conducted using the General Linear Models procedure of the Number Cruncher Statistical System (NCSS, 1990). IDTs were converted to log CFU/mL using calibration equations (Fig. 1 and 2). A split-plot analysis of variance (ANOVA) was used to evaluate log CFU/cm^2 numbers resulting from division of log CFU/mL data by appropriate surface area measurements. When significance ($p<0.05$) was detected by the ANOVA procedure, least significant difference (LSD) was employed as the mean comparison test.

Time intervals with no detection of bacterial activity were assigned an IDT that correlated to 0 log CFU/mL. Those

values exceeding the maximum IDT, and thus the calibration range (indicating that there was at least one surviving bacterium, but quantification was not possible), were designated an IDT that correlated 0.03 log CFU/mL. To rule out module well contamination, survivors detected out of the calibration range were confirmed by typical colony appearance and triple sugar iron agar reaction (Difco, Detroit, Mi.).

RESULTS AND DISCUSSION

Scanning Electron Microscopy

Scanning electron micrographs of clean attachment surfaces were presented in Section II (Fig. 3A-D). At high magnifications, PVC appeared to be the smoothest surface, whereas PU and HDPE exhibited areas of rougher topography.

Following a 22-28 hr incubation period, the S. typhimurium biofilms on processing surfaces appeared as single adherent cells or as cell monolayers (Fig. 3-5); bacterial aggregates or microcolonies were seen occasionally. Overall, fibril synthesis by S. typhimurium was very extensive, and individual fibrils were quite thick in many instances. At times, two fibril types seem to exist on S. typhimurium cells. One type was long and thick and extended from cell to cell or from cell to soil debris (Fig. 3 and 4). The other type was thinner and shorter and seemed to anchor the organism to the surface (Fig. 3 and 4).

Similar to S. typhimurium, L. monocytogenes existed on surfaces as single adherent cells (Fig. 6). However, fibril production by L. monocytogenes was observed only in rare instances (e.g., Fig. 7). Previous studies have documented the occurrence of attachment fibrils on L. monocytogenes adhering to surfaces (Mustapha and Liewen, 1989; Mafu et al., 1990a). However, SEM photographs presented by these authors showed more extensive fibril production by L. monocytogenes.

It is assumed that bacterial attachment occurs in two phases (Zobell, 1943; Marshall et al., 1971; Fletcher and Floodgate, 1973; Zoltai et al., 1981). The first phase probably involves an instantaneous reversible attachment to surfaces. In a time-dependent second phase, microorganisms irreversibly produce polymeric fibrils to firmly (irreversibly) attach to surfaces. S. typhimurium and P. fragi adherence to synthetic meat processing surfaces could have occurred in a similar manner.

Irreversibly (firmly) attached bacteria generally cannot be removed from surfaces by rinsing (Marshall et al., 1971). In the present study, a number of organisms (especially L. monocytogenes) that remained on surfaces following vortexing did not exhibit extracellular fibrils. However, the bridging substance involved in anchoring these cells to surfaces could have been less mature (no obvious fibril formation).

Sanitizer Efficacy Against Bacterial Suspensions

All sanitizers were deemed effective according to GSDT guidelines. Test germicides reduced cell suspension levels of both S. typhimurium and L. monocytogenes more than 5 logs (99.999%) within 30 sec (results in Section I).

Sanitizer Efficacy Against Attached Bacteria

S. typhimurium bacterial levels on surfaces before and

after vortexing (rinsing) are illustrated in Fig. 8. The rinsing step in this study could be considered analogous to the prerinsing procedure in a sanitation scheme. Bacteria remaining after rinsing were deemed firmly (irreversibly) attached.

Unwashed S. typhimurium counts varied according to the attachment surface (Fig. 8). Nevertheless, following rinsing, numbers of attached salmonellae were approximately the same on both PVC and HDPE. Levels of salmonellae on PU after rinsing were almost 2 log CFU/cm^2 lower than those on either PVC or HDPE.

Following sanitizer treatment, all materials had surviving populations of S. typhimurium (Fig. 9-11). Peracetic acid completely destroyed the S. typhimurium biofilm on any surface after 1-2 min. It was significantly more effective ($p<0.05$) than other germicides against S. typhimurium adhering to PVC (Fig. 9). With chlorine, it was also more effective ($p<0.05$) than either the iodophor or quat-acid sanitizers against the S. typhimurium biofilm on HDPE (Fig. 11). However, there were no differences ($p>0.05$) among germicides in reducing levels of S. typhimurium attached to PU (Fig. 10). In addition, the interaction of sanitizer level with surface level was not significant ($p>0.05$) for S. typhimurium (results not shown).

When compared to attached S. typhimurium, a similar

trend was observed for the unwashed and rinsed surface concentrations of L. monocytogenes (Fig. 12). Nevertheless, initial levels of listeriae on either unwashed or rinsed materials were lower (0.5-1.0 log) than those recorded for S. typhimurium.

Following sanitizer treatment, survival of L. monocytogenes occurred to some degree on all surfaces. Chlorine appeared to be the least effective germicide against L. monocytogenes on PVC and HDPE (Fig. 13 and 14). With iodophor, it was less effective ($p<0.05$) against listeriae on HDPE than either peracetic acid or quaternary ammonium compound and acid (quat-acid) (Fig. 14). All sanitizers reduced the PU biofilm of L. monocytogenes to 0.5 logs or lower following 1 min of exposure (results not shown). Peracetic acid completely eliminated the attached listeriae on all surfaces after 1 min.

As observed for S. typhimurium, interaction of sanitizer level with surface level was not significant ($p>0.05$) for L. monocytogenes (results not shown).

Considering all surfaces and sanitizers, lower survival was noted for attached L. monocytogenes than for attached S. typhimurium. This could be due to the higher initial levels of S. typhimurium (time 0) and/or the extensive fibril production observed for this organism. Costerton et al. (1978) reported that extracellular polymeric fibrils around

cells (known as a glycocalyx) could protect microorganisms to some degree from environmental stresses and antimicrobial agents.

Distilled water (DW) and neutral detergent (ND) had little effect on the PVC biofilm of S. typhimurium. On the contrary, alkaline detergent (AD) caused a 5 log reduction in levels of firmly attached salmonellae (Fig. 15). A similar result was noted for the above solutions against S. typhimurium on PU; however, AD completely eliminated this biofilm after 10 min (Fig. 16).

Following the DW and ND treatments with sanitizer application generally eliminated attached S. typhimurium within 6 min (results not shown). After 1 min, complete destruction of salmonellae was observed with any sanitizer used in conjunction with AD.

Although sanitizers in the GDST were deemed effective in reducing suspensions of S. typhimurium and L. monocytogenes, attached bacteria, in some instances, were able to survive germicide treatment for up to 20 min. This finding of increased sanitizer resistance agrees with previous work testing germicides against bacteria attached to food processing surfaces (Mosely et al., 1976; LeChavallier et al., 1988; Mustapha and Liewen, 1989; Frank and Koffi, 1990; Krysinski et al., 1992; Mosteller and Bishop, 1993).

Kryzinski et al. (1992) found that the tolerance of L.

monocytogenes to sanitizers increased dramatically when this organism attached to stainless steel or synthetic surfaces. For some sanitizers, these authors observed <1 log reduction of listeriae after exposing surfaces to germicidal solutions for 10 min. Commonly applied sanitizers such as chlorine, iodophor, and quaternary ammonium compound were among the least effective types. Peracetic acid was found to be one of the more active germicides against L. monocytogenes attached to these surfaces.

Mosteller and Bishop (1993) reported that bacteria adhering to buna-rubber and teflon gasket surfaces were more resistant to a variety of sanitizers than those in suspension. The goal reduction (≥3 logs) of biofilm levels, in many instances, was not observed after 30 seconds of sanitizer exposure.

The inability ND to cause reduction in the S. typhimurium biofilm could be related to the extensive fibril production (more avid adhesion) by this bacterium in the attached state. It is assumed that the high pH of AD caused the large decreases in the S. typhimurium surface biofilms (Fig. 15 and 16).

Impedance microbiology was used in this study to determine bacterial concentrations of surfaces after sanitizer treatment. Mosteller and Bishop (1993) indicated that impedance monitoring was a reliable method since all

viable organisms on a surface could be enumerated without employing physical techniques such as scraping, vortexing, etc. However, potential shortcomings of this method were stated in Section II.

CONCLUSIONS

Peracetic acid was consistently the most effective sanitizer against the biofilms of S. typhimurium and L. monocytogenes. Other sanitizers varied in effectiveness according to the attached bacterium/surface. Peracetic acid usually eliminated biofilms on test surfaces within 1 min. When a detergent step was employed prior to sanitizer application, effective reductions in bacterial surface populations were obtained. The increased resistance of S. typhimurium versus L. monocytogenes to sanitizers was probably attributable to the extensive fibril production of S. typhimurium.

In terms of practical application, peracetic acid may be quite effective when only a pre-rinsing procedure (no detergent step) is carried out prior to sanitizing (e.g., mid-shift break). Nevertheless, applying an alkaline detergent to surfaces before treatment with any of the sanitizers analyzed in this study should effectively decrease biofilms of S. typhimurium and L. monocytogenes. Therefore, results from this study should aid meat and poultry

processors in enhancing the efficacy of their sanitation programs.

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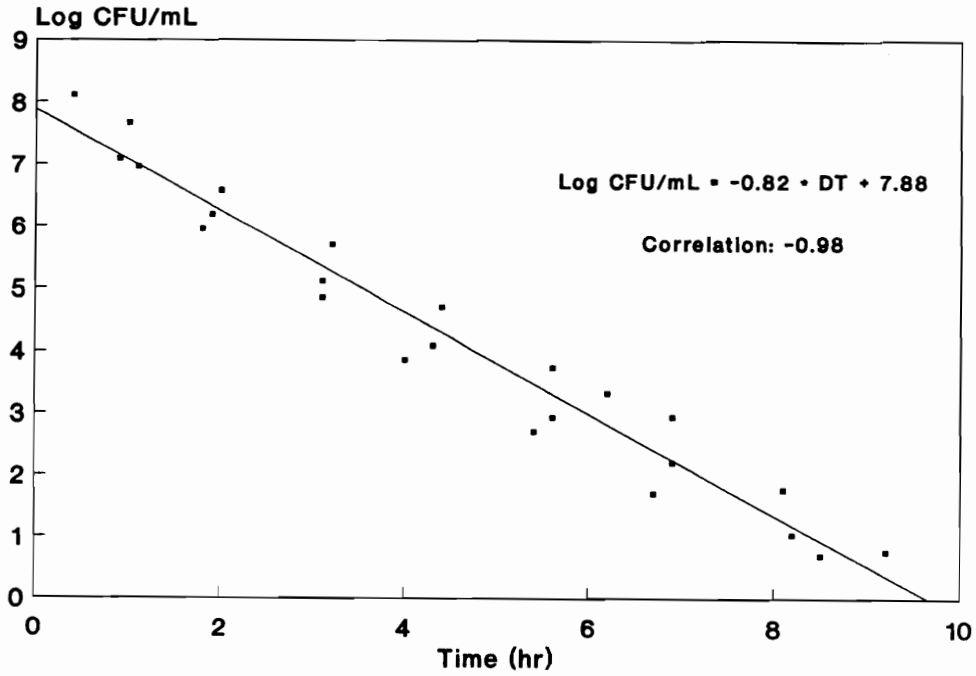


Figure 1. Calibration curve of impedance detection time (IDT) versus Log CFU/mL for *S. typhimurium*. Equation is Log CFU/mL = -0.82 * IDT + 7.88; Correlation = -0.98.

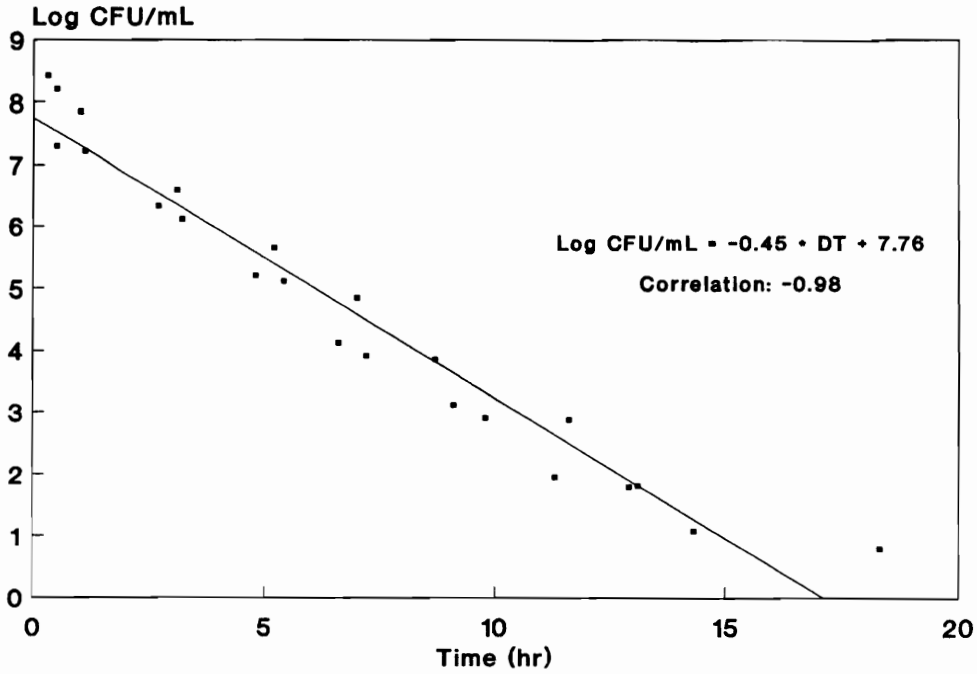


Figure 2. Calibration curve of impedance detection time (IDT) versus Log CFU/mL for L. monocytogenes. Equation is Log CFU/mL = -0.45 * IDT + 7.76; Correlation = -0.98.

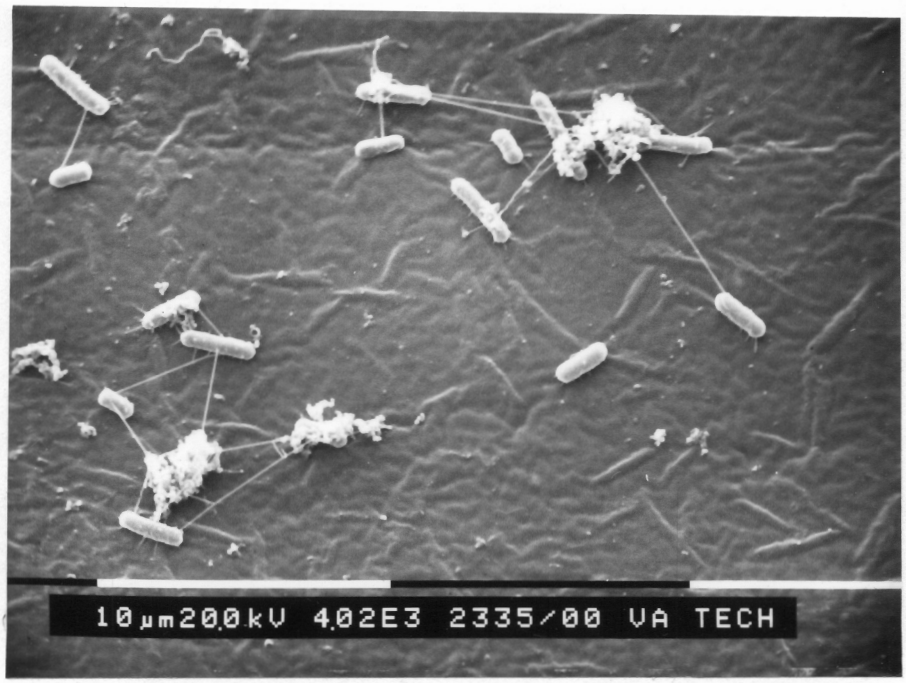


Figure 3. Scanning electron micrograph of S. typhimurium on polyvinyl chloride. Very long fibrils extend from cell to cell and from cells to the soil debris. Shorter fibers are seen reaching from cells to the attachment surface. Magnification is 4020X.

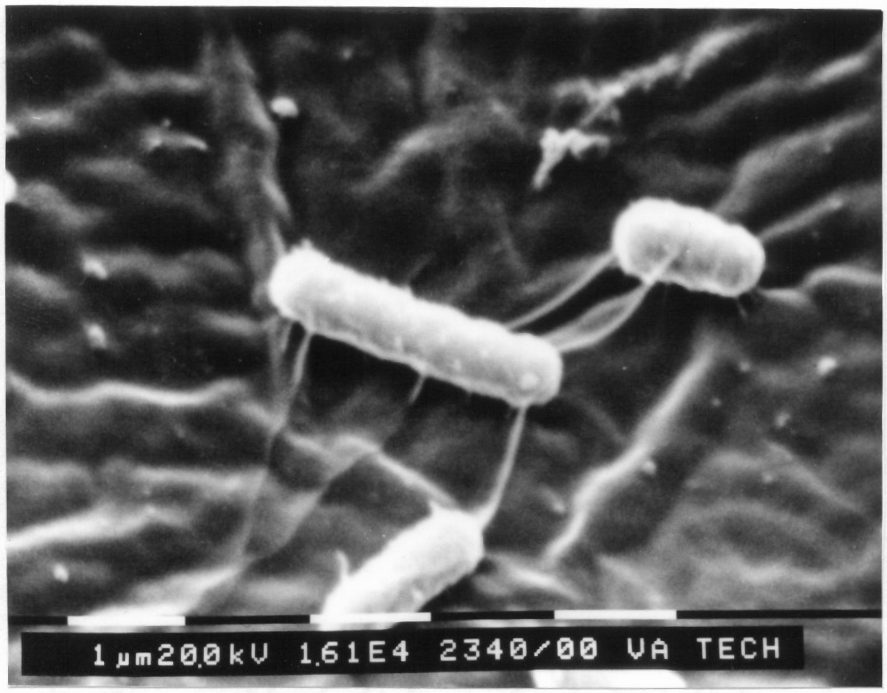


Figure 4. High magnification scanning electron micrograph of S. typhimurium on polyvinyl chloride. Magnification is 16,100X.

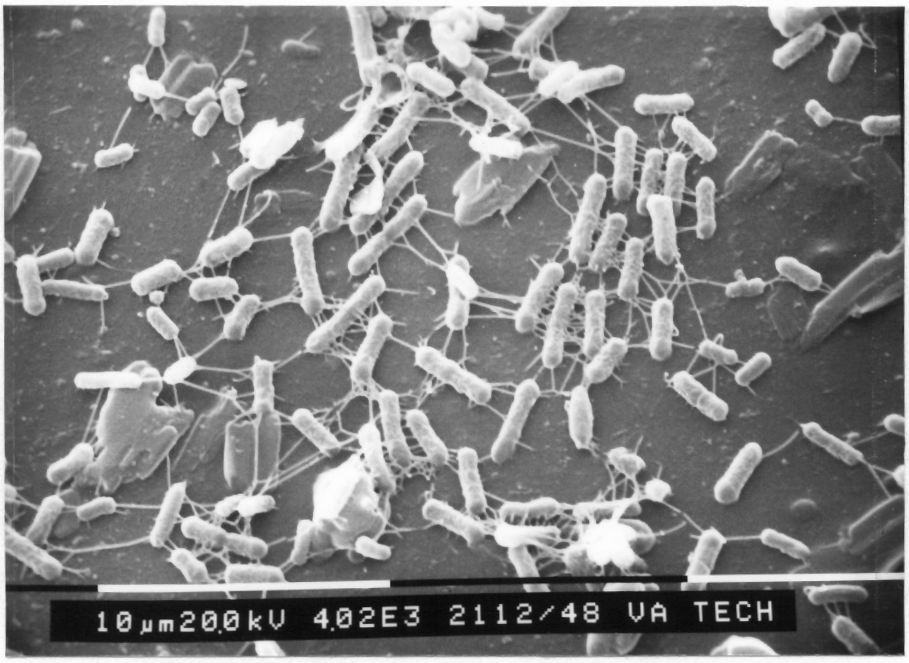
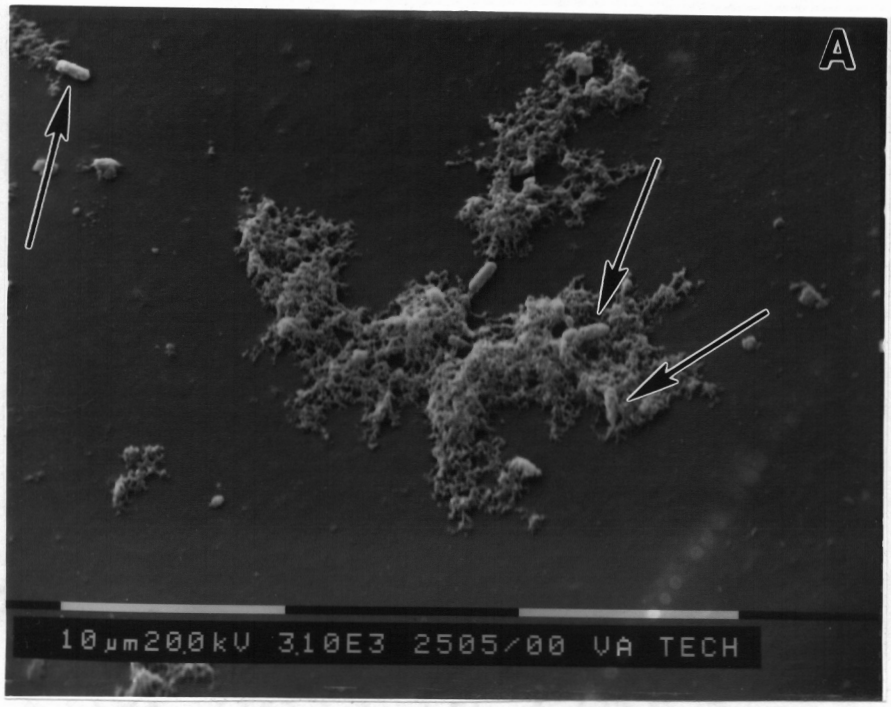


Figure 5. Scanning electron micrograph showing very extensive fibril production by S. typhimurium on polyurethane. Magnification is 4020X.



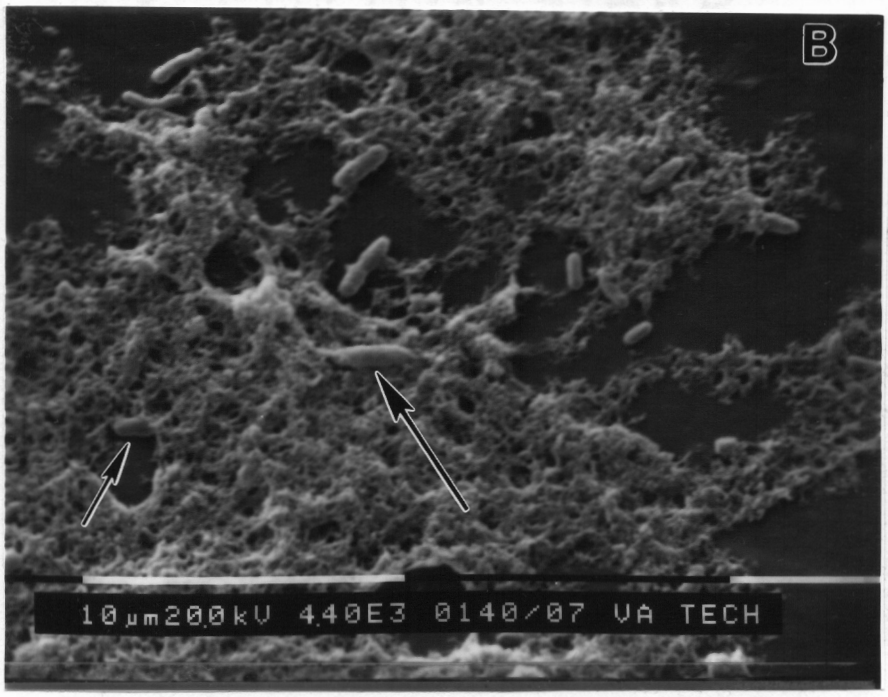


Figure 6A and 6B. Two scanning electron micrographs of L. monocytogenes on heavily soiled area of polyvinyl chloride. Magnification is 3100X for 6A and 4400X for 6B.

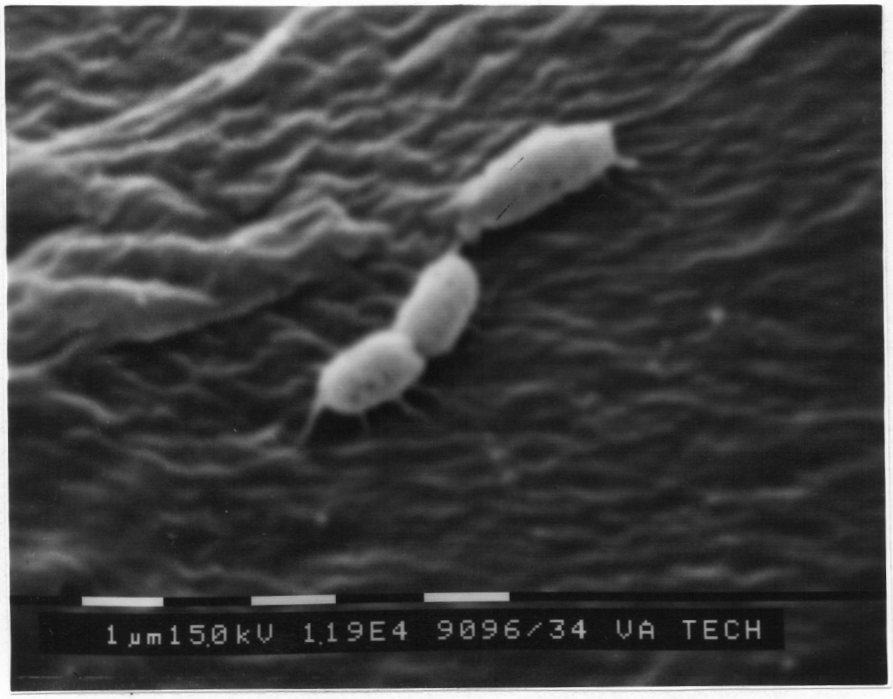


Figure 7. Rare observation of fibril production by L. monocytogenes. Magnification is 11,900X and material is high density polyethylene.

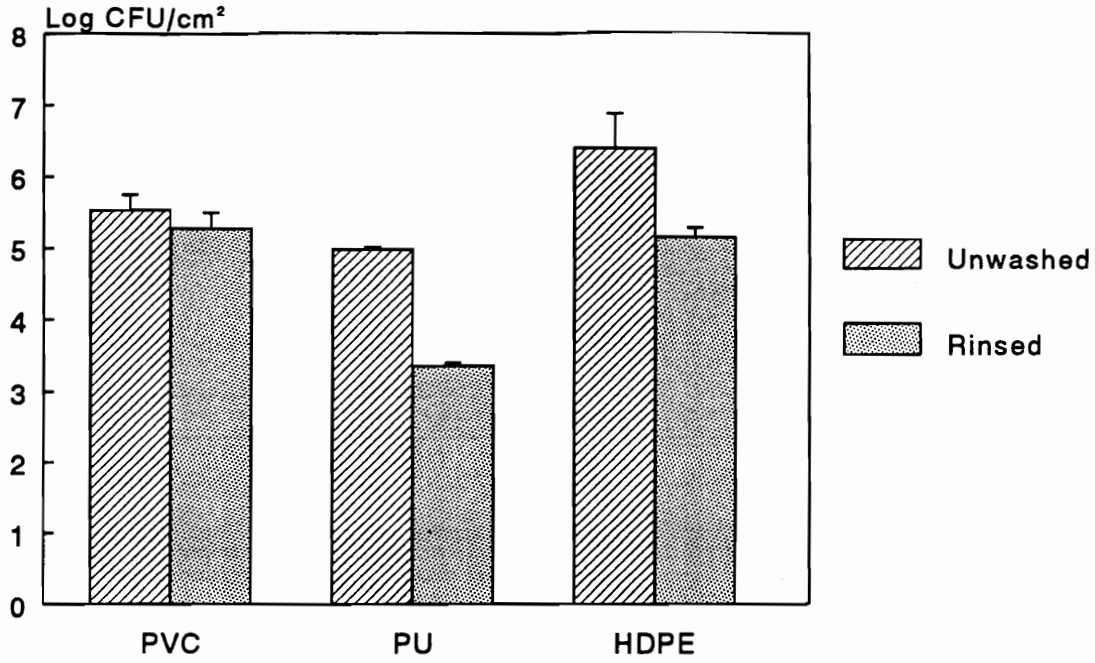


Figure 8. Levels of S. typhimurium on unwashed and rinsed surfaces: polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE). Rinsed surfaces were vortexed for 30 sec.

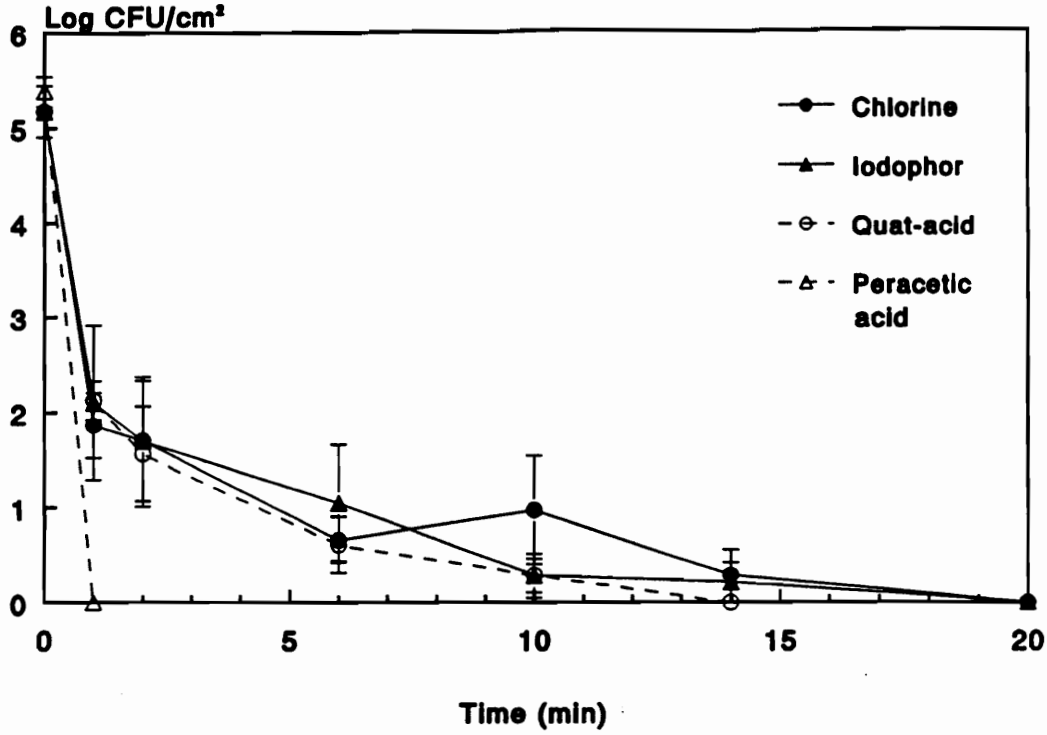


Figure 9. Sanitizer efficacy against S. typhimurium attached to polyvinyl chloride.

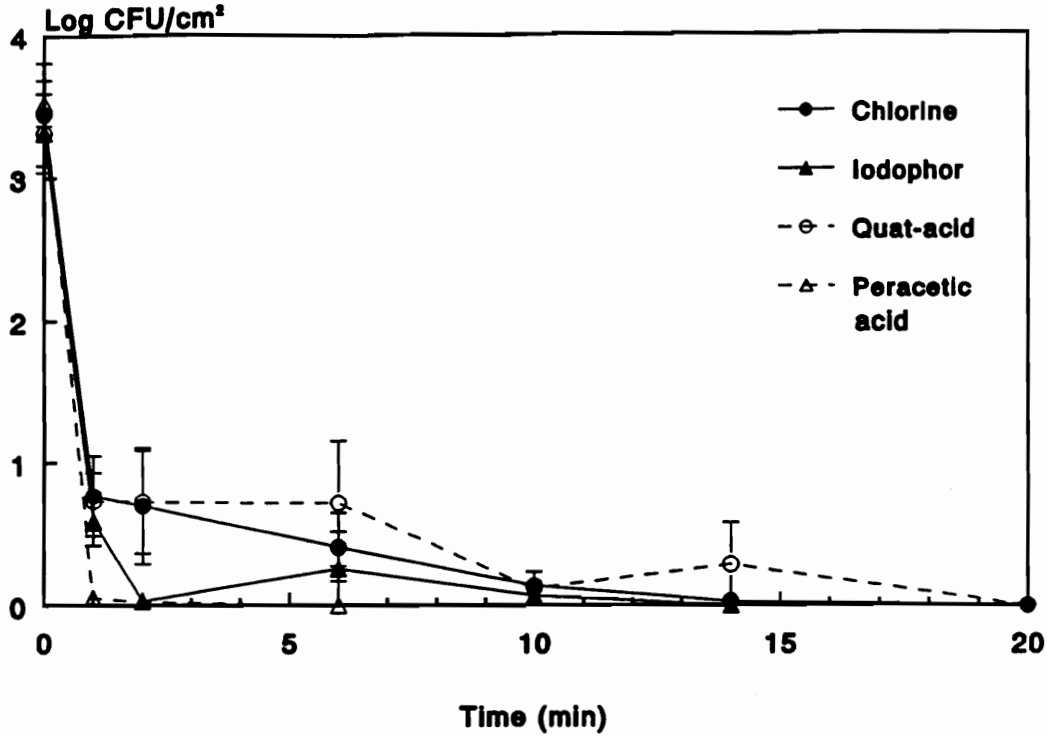


Figure 10. Sanitizer efficacy against S. typhimurium attached to polyurethane.

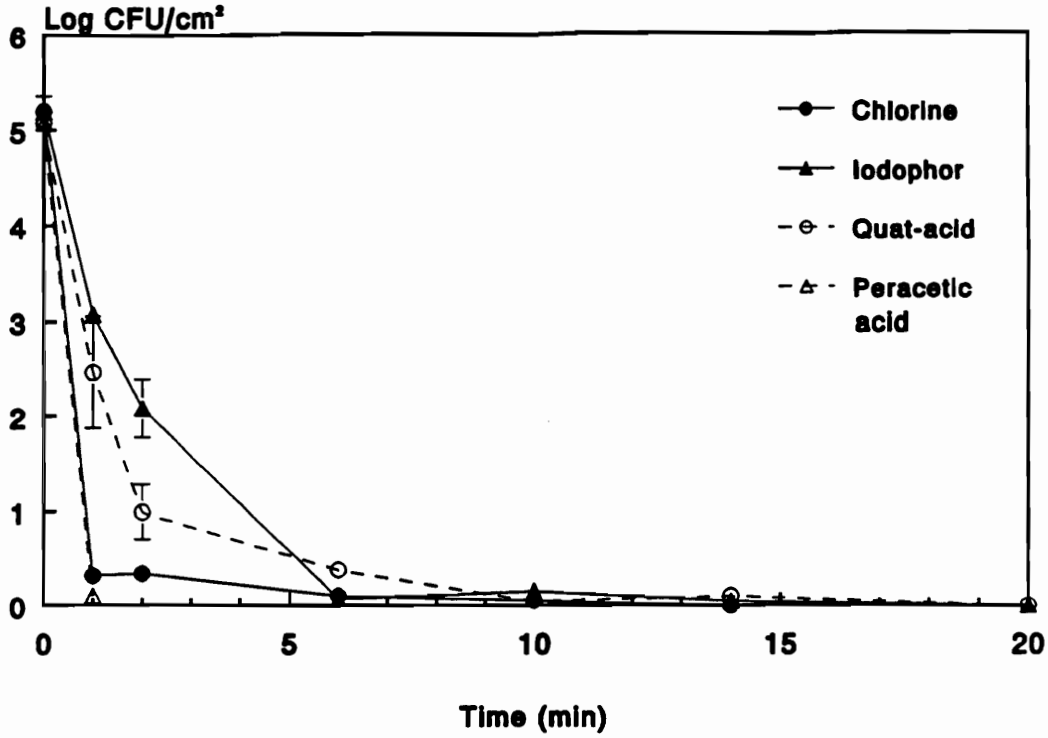


Figure 11. Sanitizer efficacy against S. typhimurium attached to high density polyethylene.

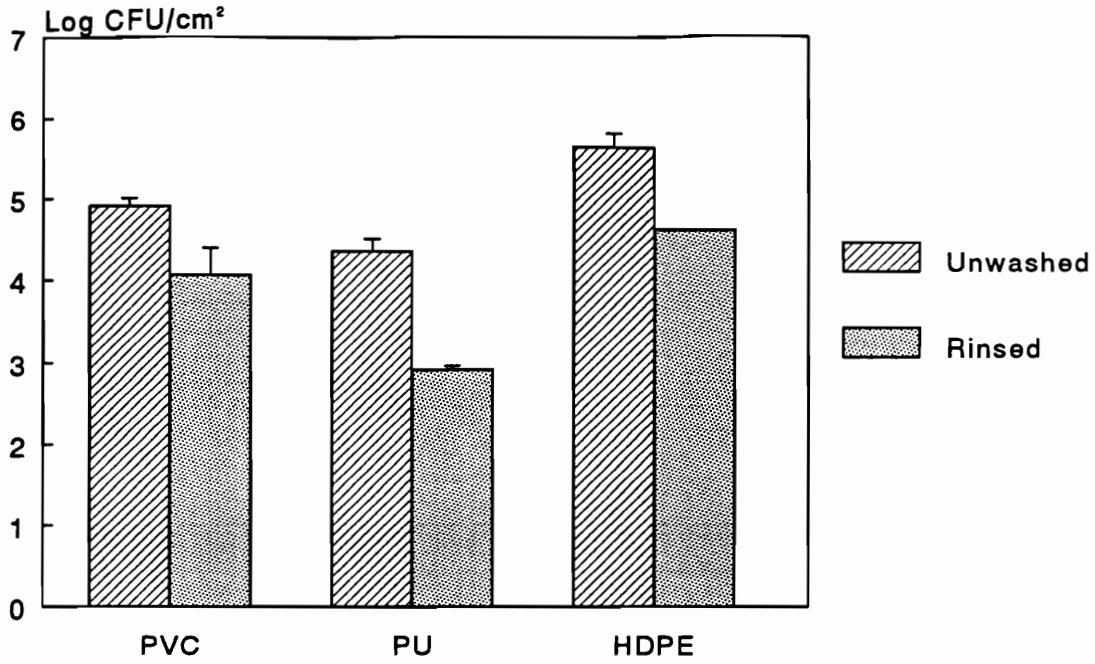


Figure 12. Levels of L. monocytogenes on unwashed and rinsed surfaces: polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE). Rinsed surfaces were vortexed for 30 sec.

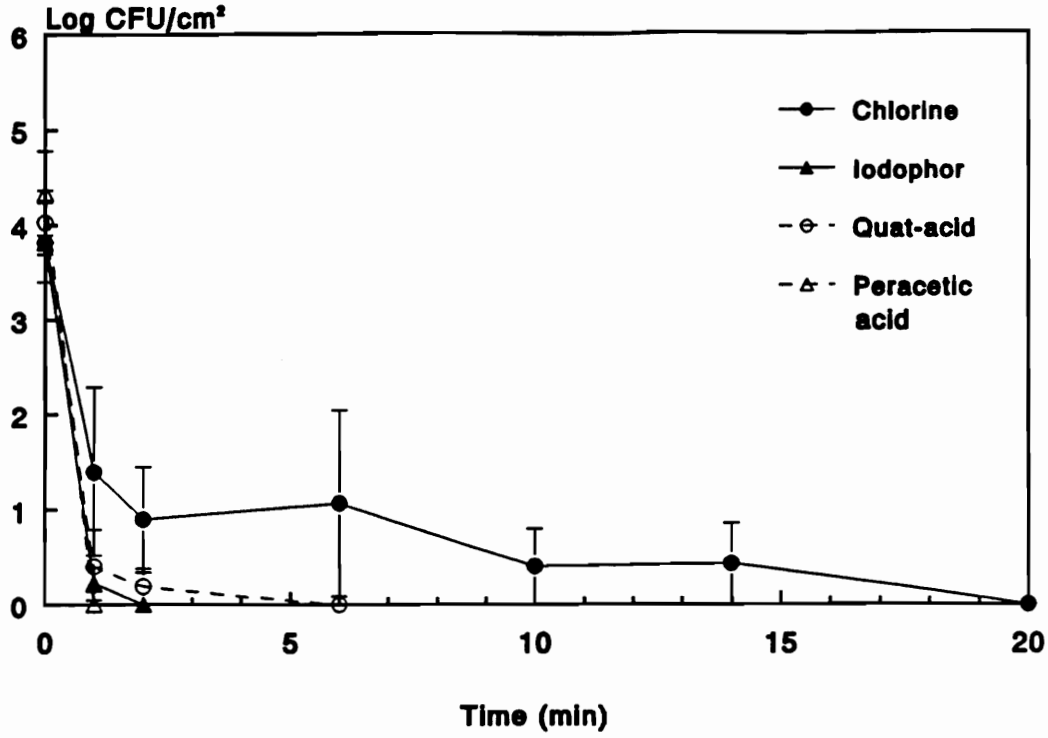


Figure 13. Sanitizer efficacy against L. monocytogenes attached to polyvinyl chloride.

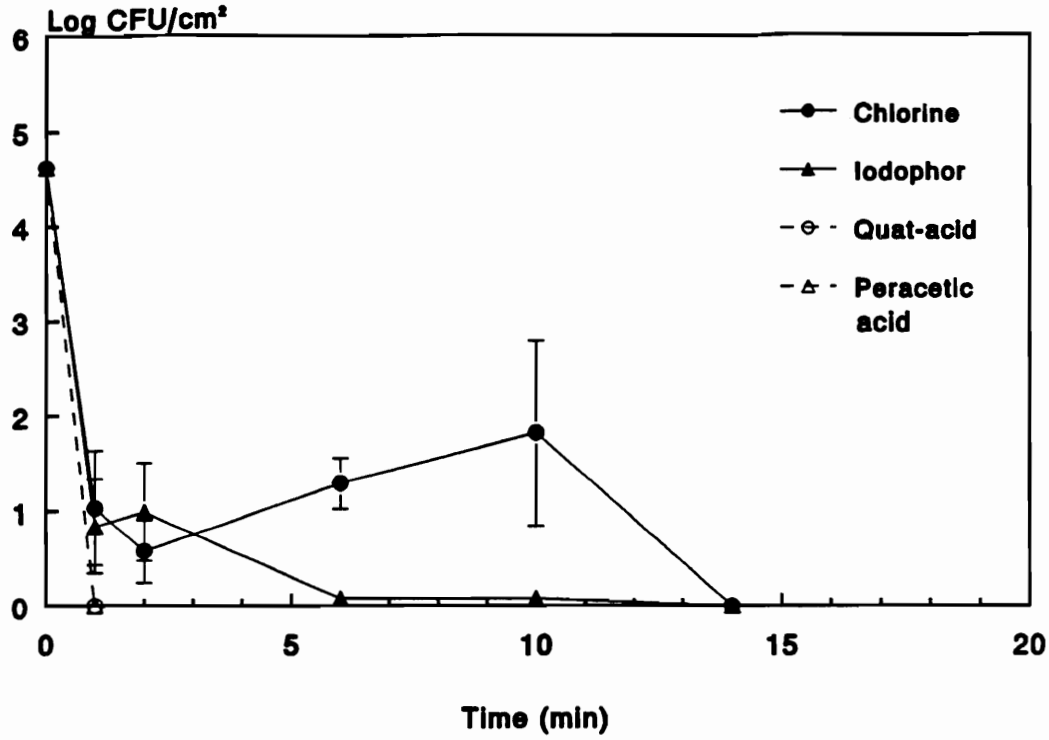


Figure 14. Sanitizer efficacy against L. monocytogenes attached to high density polyethylene

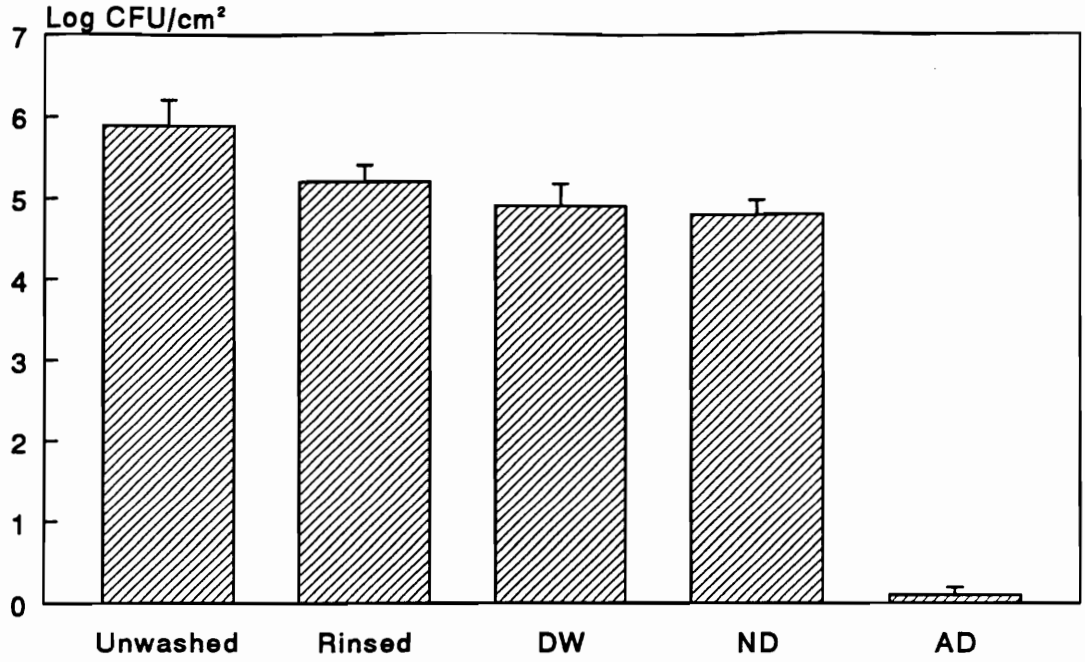


Figure 15. Detergent efficacy against S. typhimurium attached to polyvinyl chloride. Distilled water (DW), neutral detergent (ND), and alkaline detergent (AD) treatments were 10 min at 50°C.

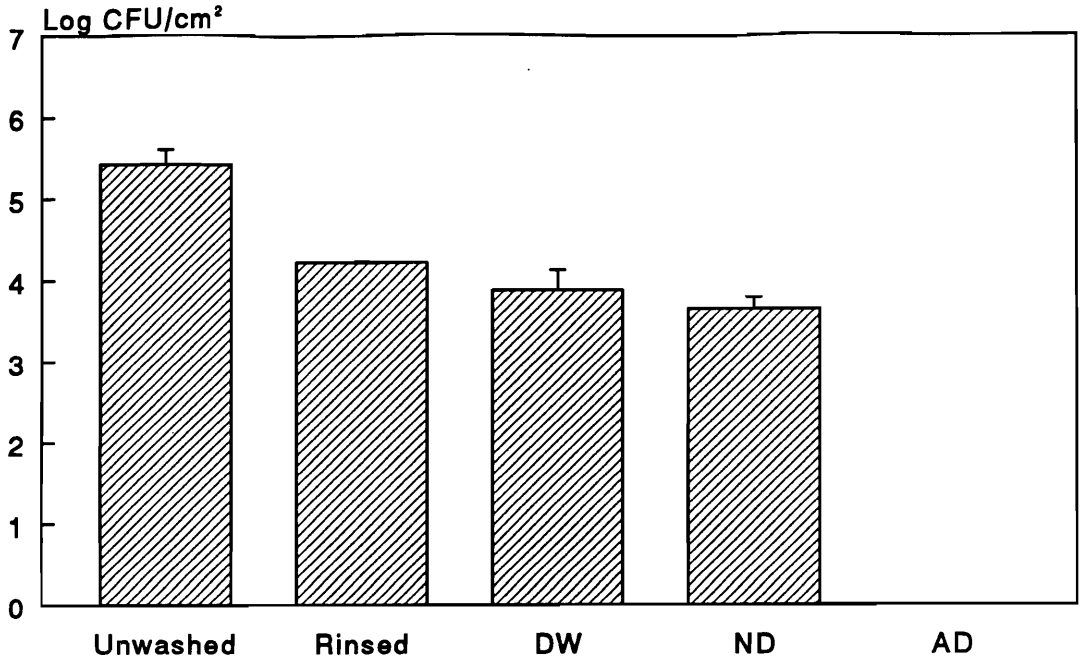


Figure 16. Detergent efficacy against S. typhimurium attached to polyurethane. Distilled water (DW), neutral detergent (ND), and alkaline detergent (AD) treatments were 10 min at 50°C.

SUMMARY

The GDST (a standard analysis) was employed in SECTION I to ascertain the efficacy of sanitizers against bacteria in suspension. All sanitizers {chlorine (200 ppm), iodophor (25 ppm), quaternary ammonium compound and acid (200 ppm), and peracetic acid (185 ppm)} reduced the levels of test bacteria (Shw. putrefaciens, P. fragi, S. typhimurium, and L. monocytogenes) >5 log CFU/mL. As a result, germicides were deemed acceptable according to GDST guidelines.

Efforts were made in subsequent experiments to better simulate *in situ* conditions of meat and poultry processing sanitation. Bacteria were allowed to attach to synthetic meat processing surfaces that included polyvinyl chloride (PVC), polyurethane (PU), and high density polyethylene (HDPE). SEM was employed to determine the manner in which organisms attached to or colonized surfaces. Contaminated surfaces were vortexed (rinsed) and then challenged with sanitizers, or detergents first and then sanitizers.

In SECTION II, Shw. putrefaciens and P. fragi (meat and poultry spoilage bacteria) were inoculated into poultry slurries containing processing surfaces. Test surfaces were agitated (100 rpm) for 2 hr at 18°C and then incubated for 22-28 hr at 26°C. According to SEM pictures, the 22-28 hr surface biofilms of Shw. putrefaciens could be characterized as single adherent cells or cell monolayers. Conversely, the

P. fragi biofilm could be described as occurring in cell aggregates or microcolonies on surfaces. Although test sanitizers were effective according to suspension test results, in many instances, attachment to surfaces increased bacterial resistance to germicidal agents (a 5 log reduction was not observed even after 1 min of chemical exposure). Peracetic acid, overall, was the most effective (fastest acting) sanitizer in reducing levels of attached Shw. putrefaciens and P. fragi. Whereas resistance to other germicides could be observed for up to 20 min, peracetic acid typically eliminated biofilm populations after 1 min. Treatment of surfaces with detergents (especially with alkaline detergent) prior to sanitizing led to effective reductions of attached bacteria.

In SECTION III, S. typhimurium, and L. monocytogenes (potential meat and poultry spoilage pathogens) were given the same experimental treatments as the test bacteria in SECTION II; however, the poultry slurry incubation temperature was 37°C. Attached S. typhimurium cells produced abundant extracellular fibril material; whereas, L. monocytogenes synthesized little of this substance. Similar to results observed in SECTION II, peracetic was found to be the most effective sanitizer, completely destroying biofilms of pathogens generally after 1 min. Detergent treatment (especially alkaline detergent) in conjunction with germicide

application led to more effective reductions of attached bacteria. The extensive fibril production by S. typhimurium may explain the greater overall sanitizer resistance of this bacterium as compared to other test bacteria. However, higher initial attachment numbers (time 0) could have been the reason for the greater resistance (higher survival levels) observed in some experiments

The results of this study further support the wisdom of incorporating an effective cleaning (detergent) step before applying a sanitizer solution. Additional work to ascertain why peracetic acid was more effective against bacterial biofilms would be of interest. The data of this research should aid meat and poultry processors in developing more effective sanitizer schemes to minimize bacterial contamination on synthetic meat processing surfaces.

VITA

The author of this dissertation, Wayne Payton Pruett, Jr., was born June 16, 1964 in Anniston, Alabama. He is the son of Mr. and Mrs. Wayne Payton Pruett, Sr. He completed his secondary education at Sullivan South High School in Kingsport, Tennessee.

The author received his Bachelor of Science degree in Microbiology with distinction from East Tennessee State University, Johnson City, Tennessee, on December 20, 1986. While at this institution, Payton served as Vice-President of Tri-Beta Biological Honor Society and as a dorm Vice-President and Representative of the Residential Hall Association. As a junior, he was selected Outstanding Microbiology Major, and as a senior, he was chosen Outstanding Health Sciences Major.

Payton married the former Angela Dawn Ward on December 29, 1987.

On June 4, 1990, Payton received his Master of Science degree in Food Science and Technology from Virginia Polytechnic Institute and State University and remained at the same institution for his doctorate. As a doctoral candidate, the author was selected as a 1991 Fellow of the Rapid Methods and Automation Workshop in Microbiology held at Kansas State University, Manhattan, Kansas.

The author is a member of the following honorary and

professional societies: Institute of Food Technologists; American Society of Microbiology; International Association of Milk, Food, and Environmental Sanitarians; Phi Sigma; Gamma Sigma Delta; Phi Kappa Phi; and Sigma Xi.